TICK-HOST-PATHOGEN INTERACTIONS

EDITED BY: Sarah Irène Bonnet, Ard Menzo Nijhof and Jose De La Fuente
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TICK-HOST-PATHOGEN INTERACTIONS

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Questing Dermacentor tick. Image: Mathilde Gondard.

Besides causing direct damage associated with blood feeding and in some cases through the excretion of toxins with their saliva, the main relevance of ticks lies in the wide variety of pathogens that they can transmit, including viruses, bacteria, protozoa and helminths. Owing to socioeconomic and environmental changes, tick distribution is changing with incursions of ticks and tick-borne diseases occurring in different regions of the world when the widespread deployment of chemical acaricides and repellents has led to the selection of resistance in multiple populations of ticks. New approaches that are environmentally sustainable and that provide broad protection against current and future tick-borne pathogens (TBPs) are thus urgently needed. Such development, however, requires improved understanding of factors resulting in vector competence and tick-host-pathogen interactions. This Research Topic provides an overview of known molecular tick-host-pathogen interactions for a number of TBPs and highlights how this knowledge can contribute to novel control and prevention strategies for tick-borne diseases.

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EDITORIAL
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Editorial: Tick-Host-Pathogen Interactions

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Editorial on the Research Topic

Tick-Host-Pathogen Interactions

Ticks are important vectors of pathogens affecting human and animal health around the world. They act as vectors of several pathogens causing diseases of major concern including Lyme borreliosis, anaplasmosis, rickettsiosis, ehrlichiosis, tularemia, and tick-borne encephalitis (TBE) in humans, and babesiosis, theileriosis, and anaplasmosis in livestock. Therefore, a One Health approach is important for the effective prevention and control of tick-borne diseases (TBDs). Current tick control strategies rely essentially on the use of chemical acaricides, but their widespread use in livestock is associated with the selection of resistant tick populations and environmental contamination. In addition, socioeconomic and environmental changes affect tick distribution, with global incursions of TBDs. New approaches that are environmentally sustainable and control the threat posed by tick-borne pathogens (TBPs) are therefore needed. Tick-host-pathogen interactions drive vector competence for transmitted pathogens, which together with behavioral and environmental factors affect vectorial capacity (de la Fuente et al.). Therefore, the understanding of tick biology and the interactions between ticks, their hosts, and TBPs is essential for the identification of drivers for TBDs. In this research topic, we have gathered original research, review, perspective and opinion papers from scientists who have common interests in unraveling tick biology and tick-host-pathogen interactions for the development of new interventions for the control and prevention of TBDs.

A recent landmark in tick research was the publication of the first draft tick genome of *Ixodes scapularis*, the main vector of *Borrelia burgdorferi* in North America (Gulia-Nuss et al., 2016). Papers on how the availability of this genome and other advances made in genomics could be used within research on tick-host-pathogen interactions to eventually improve global health were included within this research topic (de la Fuente et al.; Grabowski and Hill). Examples of how genomic data can be deployed to identify and characterize metabolic pathways was presented in original research papers investigating changes in tick cell metabolism in response to *Anaplasma phagocytophilum* infection (Cabezas-Cruz et al.; Cabezas-Cruz et al.).

A comprehensive overview of tick-pathogen interactions and vector competence for bacteria, viruses, and protozoa was presented in a review by de la Fuente et al. It is remarkable that many vector-borne pathogens use similar strategies, such as remodeling of the cytoskeleton and manipulation of the vector immune response, to facilitate infection, multiplication, and transmission. A review on tick-pathogen interactions was included for *Babesia* species (Antunes et al.), and interactions between tick-borne viruses with both ticks and hosts were outlined in two other reviews (Kazimirova et al.; Papa et al.). In this research topic, Adams et al. presented the first dual reporter system for *B. burgdorferi*, a highly

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versatile tool for investigating pathogen transcription and gene regulation, both *in vitro* and in infected ticks. Lesser known *Borrelia* species associated with relapsing fevers in humans and mainly transmitted by soft ticks of the *Ornithodoros* genus were reviewed by Talagrand-Reboul et al. while emerging tick-borne viruses such as severe fever with thrombocytopenia syndrome virus (SFTSV), and factors leading to their emergence were reviewed by Mansfield et al.. Pruneau et al. used a comparative transcriptome profile to characterize the interactions between host and virulent and attenuated strains of *Ehrlichia ruminantium*, the causative agent of heartwater, to identify genes involved in pathogen virulence.

When taken up with an infected blood meal, pathogens first encounter the microbiome residing within the tick gut. Although still largely unexplored, Bonnet et al. reviewed the current knowledge on the role of non-pathogenic microbes in the gut and other tick tissues in pathogen transmission and tick biology with implications for the control of TBDs. The transcriptomics response to infection with the bacterial pathogen *Rickettsia rickettsii*, the causal agent of Rocky Mountain Spotted Fever, in the midgut of two *Amblyomma* tick species with different susceptibility to infection was described by Martins et al. and led to the selection of tick transcripts that might be implicated in the vector competence. Once pathogens cross the midgut barrier, they encounter components of tick innate immunity in the tick hemocoel. How different pathogens manage to evade the tick's innate immune system was the subject of a review by Sonenshine and Macaluso, and the role of Toll signaling in ticks in response to virus infections was addressed in a perspective article (Johnson). Components of the innate immunity in *Ixodes ricinus* ticks, the main vector of *B. burgdorferi* s.l. in Europe, were examined in more detail in two original research papers (Honig Mondekova et al.; Urbanova et al.). A review from Blisnick et al. also emphasized the importance of protease inhibitors in both tick biology and tick-borne pathogen transmission, being involved in tick feeding process but also in tick innate immune defense. These studies highlighted the mechanisms used by transmitted pathogens to evade tick innate immunity for infection while ticks respond to limit infection and preserve fitness.

Tick salivary glands and tick saliva play a pivotal role during tick feeding and pathogen transmission, and they were therefore a subject of several reviews within this research topic (Chmelar et al.; Mans et al.; Simo et al.). In addition, five original research papers investigated the sialotranscriptome and sialome of *Amblyomma sculptum* ticks (Esteves et al.), the sialotranscriptomics response of *Rhipicephalus bursa* ticks infected with *Babesia ovis* (Antunes et al.), the effect of salivary cystatin OmC2 from *Ornithodoros moubata* saliva on the host immune response (Zavasnik-Bergant et al.), and differences in the saliva protein profile of *I. scapularis* and *Amblyomma americanum* ticks stimulated to feed on different hosts (Tirloni et al.). The latter study showed that the salivary protein profiles differed between ticks of the same species that fed on different animals, an observation which for instance may have implications for studies looking at tick salivary antigens as anti-tick vaccine candidates. A research paper by Rodrigues et al. presented the relationship between *Amblyomma variegatum* salivary composition and regulation of host defenses. In another study, Zavasnik-Bergant et al. demonstrated the effect of salivary cystatin OmC2 from *O. moubata* on the host immune response through interaction with Cathepsins S and C.

Knowledge of tick genes differentially regulated in response to feeding or infection can be used to identify candidate tick protective antigens for the prevention and control of tick infestations and/or pathogen transmission through vaccination. The identification of tick protective antigens could be approached using different screening platforms (de la Fuente et al., 2016). A vaccinomics approach was applied to *Ixodes* ticks infected with *A. phagocytophilum* in an original research paper by Contreras et al. to identify and characterize candidate tick protective antigens. The current status and future prospects of vaccines targeting metazoan parasites, including ticks, was reviewed in a paper by Stutzer et al.

Upon transmission by ticks, TBPs need to evade the host immune response. An original research paper described the transcriptional profile of cutaneous immune responses to *I. ricinus*-transmitted tick-borne encephalitis virus (TBEV) during early stages of pathogen transmission (Thangamani et al.). The role of two *A. phagocytophilum* proteins, MSP4 and HSP70, in interactions with host cells was shown in another study (Contreras et al.), and analysis of the host cell response of human HL60 cells to *A. phagocytophilum* infection showed an increase in transcripts with a high proportion of alternatively spliced transcript events, thus providing important new fields of research (Dumler et al.). Host responses to tick infestations and tick resistance in cattle were the subjects of two complementary reviews (Robbertse et al.; Tabor et al.). A network analysis presented by Estrada-Peña and de la Fuente quantified the interactions between *I. ricinus* and transmitted *B. burgdorferi sensu lato* (s.l.) bacteria in the Western Palaearctic. The results showed that contrary to the prevailing paradigm, complex communities of vertebrates, which have large distribution ranges, instead of a few dominant vertebrates, support both *I. ricinus* and *B. burgdorferi* s.l. Associations of *I. ricinus* with its hosts and environmental niches that impact pathogen circulation was further evaluated in another study (Estrada-Peña et al.). It was demonstrated that the diversity of hosts increases the niche available for ticks and promotes the circulation of transmitted-pathogens. These results suggested that TBPs might manipulate ticks to occupy sub-optimal environmental niches (Estrada-Peña et al.). This manipulation can take place on an epigenetic level and there are increasing evidences that pathogens can induce transcriptional changes in both their vertebrate and tick hosts, thereby facilitating propagation of the pathogen. The potential implications of these tick-pathogen interactions for tick ecology were further discussed in an opinion paper (Cabezas-Cruz et al.).

Last but not least, recent advances in the study of tick-host-pathogen interactions have also led to the discovery of a wide variety of tick bioactive molecules that may be a source of novel therapeutics (Murfin and Fikrig).

As editors of the "Tick-host-pathogen interactions" Research Topic, we would like to acknowledge all contributing authors

for providing insight into the exciting research that continues to improve our understanding of tick-host-pathogen interactions at molecular and ecological levels, and translation into new interventions for the control of TBDs.

AUTHOR CONTRIBUTIONS

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PERSPECTIVE
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Tick Genome Assembled: New Opportunities for Research on Tick-Host-Pathogen Interactions

José de la Fuente[1,2]*, *Robert M. Waterhouse*[3,4,5,6], *Daniel E. Sonenshine*[7], *R. Michael Roe*[8], *Jose M. Ribeiro*[9], *David B. Sattelle*[10] and *Catherine A. Hill*[11]

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As tick-borne diseases are on the rise, an international effort resulted in the sequence and assembly of the first genome of a tick vector. This result promotes research on comparative, functional and evolutionary genomics and the study of tick-host-pathogen interactions to improve human, animal and ecosystem health on a global scale.

Keywords: tick, *Ixodes scapularis*, genomics, proteomics, *Borrelia*, *Anaplasma*, evolution

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TICK-BORNE DISEASES: A GROWING BURDEN FOR HUMAN AND ANIMAL HEALTH WORLDWIDE

Ticks are obligate blood-feeding arthropod ectoparasites that are distributed worldwide and one of the most important vectors of pathogens affecting humans and animals (Jongejan and Uilenberg, 2004; de la Fuente et al., 2008). Globally, emerging and re-emerging tick-borne diseases exert an enormous impact on public health (Jones et al., 2008). Urbanization, exploitation of environmental resources and outdoor recreational activities increase human contact with ticks and the transmission of tick-borne pathogens (Gortazar et al., 2014). In addition, tick populations are expanding due to changes in climate (Estrada-Peña et al., 2014) and new tick-borne diseases are emerging (Kosoy et al., 2015; Kernif et al., 2016). However, despite the growing burden that tick-borne diseases represent for human and animal health worldwide, the pace of research in this area has been restricted by the lack of access to a completed tick genome. The recent description of the first tick genome is therefore timely and a spur to future research (Gulia-Nuss et al., 2016).

THE FIRST TICK GENOME SEQUENCED AND ASSEMBLED: RESULTS AND POSSIBILITIES

More recently, a global consortium of 93 scientists described the 2.1 Gbp nuclear genome of the black-legged tick, *Ixodes scapularis* (Say) (Gulia-Nuss et al., 2016). This tick species is a vector of pathogens that cause, among others, the emerging diseases Lyme disease [Lyme borreliosis is the most common tick-borne disease in Europe and the U.S. (Centers for Disease Control and Prevention (CDC), 2016; European Centre for Disease Prevention and Control (ECDC), 2016)], human granulocytic anaplasmosis (HGA), babesiosis and tick-borne encephalitis (TBE)

(Wormser et al., 2006). The genome of *I. scapularis* Wikel strain was sequenced in a joint effort by the Broad Institute of MIT and Harvard and The J. Craig Venter Institute (JCVI) and funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Annotation for this assembly was produced in a joint effort between JCVI and VectorBase (https://www.vectorbase.org/) with support from The Broad Institute (Gulia-Nuss et al., 2016).

The *I. scapularis* project proved challenging due to the large size and high repeat content of the genome. However, the results show the assembly and description of features associated with ∼57% of the genome. As the only assembly available for a tick, the *I. scapularis* genome constitutes an invaluable reference for comparative genomic analyses, including resolution of phylogenetic relationships within the diverse phylum Arthropoda. Analysis of the *I. scapularis* genome revealed new features that may be unique to this organism and with important implications for future research. Highlights include the identification of two new repeat elements, a large-scale gene duplication event that likely occurred ∼40 MYA coinciding with tick radiation, gene exon-intron structures more closely resembling that of an ancient protostome/deuterostome ancestor than of extant arthropods examined to date, an expansion of Kunitz domain proteins and other proteins implicated in tick blood feeding, possible remnants of a heme synthesis pathway contrasting with an expansion of heme carrier and storage proteins. Also identified were orthologs for at least 39 invertebrate neuropeptides and neuropeptide receptor genes that are believed or known to regulate tick diuresis, ecdysis, cuticle synthesis, blood feeding and reproduction. The genome contains one of the largest expansions of cytochrome P450 genes known for sequenced arthropods, suggesting potential for rapid development of acaricide resistance in ticks, and it will be important to explore the families of candidate acaricide targets uncovered by genome analyses, the de-orphanisation of which is underway.

The first genome-wide population genomics study suggested genetic variation between ticks from Lyme prevalent northern and mid-western states compared with southern states in the U.S., paving the way for identification of genes tied to vector competence (Gulia-Nuss et al., 2016). The *I. scapularis* genome sequence and annotation also contributed to the characterization of the transcriptome in related tick species such as *I. ricinus* (Genomic Resources Development Consortium et al., 2014; Kotsyfakis et al., 2015), the main vector for tick-borne pathogens of public health importance in Europe. Additional studies that explore the biology of *I. scapularis* and other tick species, and extend genome analyses were described based on the publication

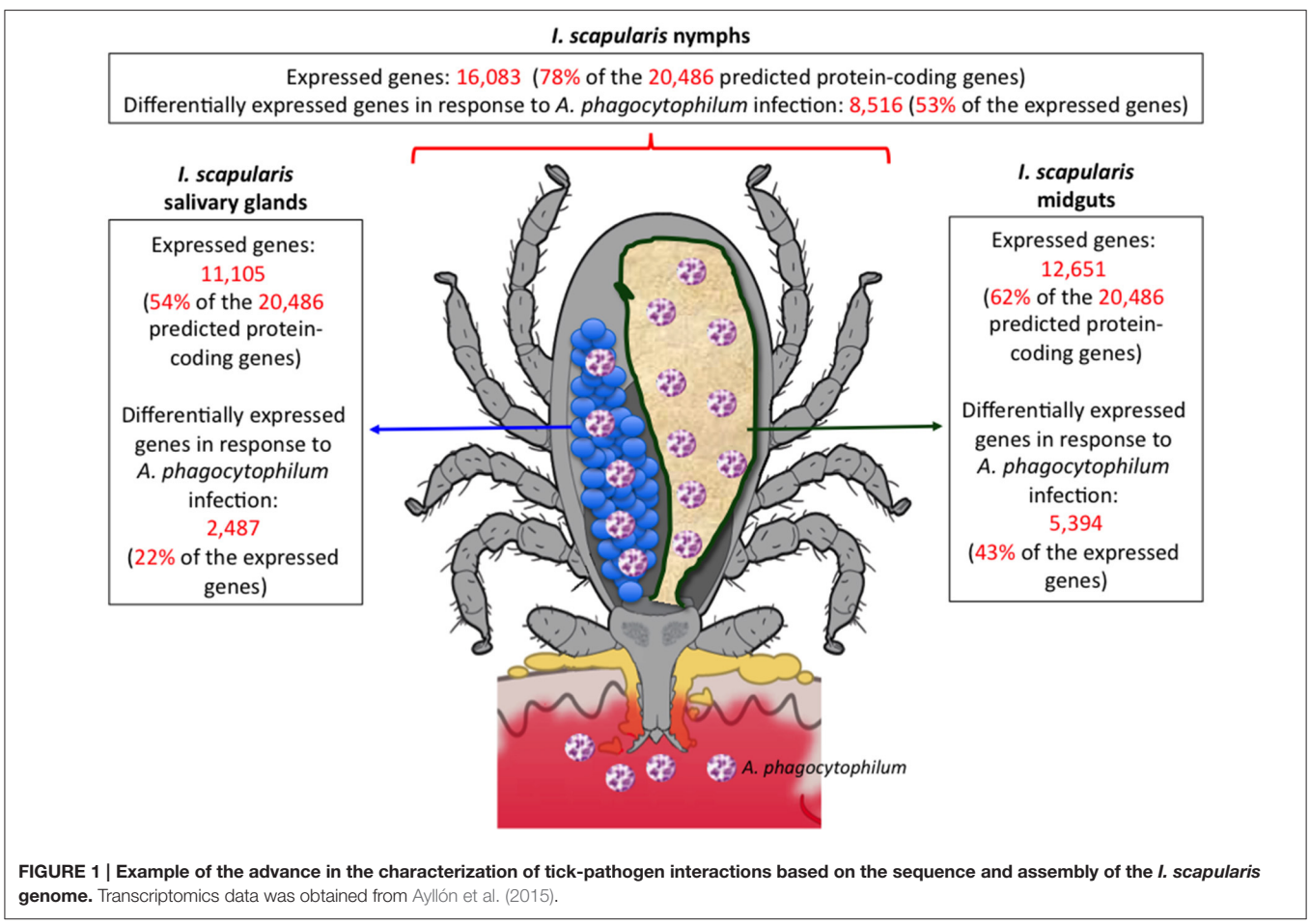

FIGURE 1 | Example of the advance in the characterization of tick-pathogen interactions based on the sequence and assembly of the *I. scapularis* genome. Transcriptomics data was obtained from Ayllón et al. (2015).

of the *I. scapularis* genome sequence (e.g., Cabezas-Cruz et al., 2016; Carr et al., 2016; Egekwu et al., 2016; Grabowski et al., 2016; Van Zee et al., 2016; Zhu et al., 2016). The *I. scapularis* genome also provides a key reference for comparative genomics with other Parasitiformes like the western orchard predatory mite (Hoy et al., 2016), as well as across Chelicerata where large genome sizes often make sequencing and assembly a very challenging undertaking.

Recent results on the characterization of tick-host and tick-pathogen interactions highlighted the impact of *I. scapularis* genome sequence and assembly on these studies (**Figure 1**). Transcriptomics, proteomics and metabolomics studies showed the tissue-specific tick response to infection with *Anaplasma phagocytophilum*, the causative agent of HGA (Ayllón et al., 2015; Villar et al., 2015; Alberdi et al., 2016). Complementary proteomics analyses also revealed proteins associated with transmission of *A. phagocytophilum* and the encephalitis-causing Langat virus (Grabowski et al., 2016; Gulia-Nuss et al., 2016). New advances in experimental approaches using omics technologies also boost our knowledge of the tick-host interface (Sojka et al., 2013; Schwarz et al., 2014; Chmelař et al., 2016a,b). Finally, the analysis of the evolution of tick-host-pathogen interactions suggested conflict and cooperation between hosts, vectors and pathogens (de la Fuente et al., 2016a).

CONCLUSIONS AND FUTURE DIRECTIONS

The features discovered in the *I. scapularis* genome provide insights into parasitic processes unique to ticks, including host "questing," prolonged feeding, cuticle synthesis, blood meal concentration, novel methods of hemoglobin digestion, heme detoxification, vitellogenesis, reproduction, oviposition, prolonged off-host survival and host-tick-pathogen interactions. The *I. scapularis* gene models will advance research on comparative and functional genomics, while the assembly and physical map will underpin much needed studies of tick genetics. Recent efforts addressed the need for additional tick genomic resources by focusing on tick species relevant for human and animal health (Guerrero et al., 2010; Cramaro et al., 2015). Advances in tick genomics have also facilitated the characterization of the impact of co-infections and microbiome composition on tick vector capacity (Narasimhan and Fikrig, 2015; Vayssier-Taussat et al., 2015). These results greatly improve our understanding of tick biology and will advance research on tick-host-pathogen interactions to develop effective and environmentally friendly measures to control ticks and the many pathogens and parasites they transmit (de la Fuente and Contreras, 2015; Benelli et al., 2016; Carr and Roe, 2016; de la Fuente et al., 2016b; Esteve-Gassent et al., 2016; Kuleš et al., 2016).

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All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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frontiers in Cellular and Infection Microbiology

REVIEW
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Tick-Pathogen Interactions and Vector Competence: Identification of Molecular Drivers for Tick-Borne Diseases

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Ticks and the pathogens they transmit constitute a growing burden for human and animal health worldwide. Vector competence is a component of vectorial capacity and depends on genetic determinants affecting the ability of a vector to transmit a pathogen. These determinants affect traits such as tick-host-pathogen and susceptibility to pathogen infection. Therefore, the elucidation of the mechanisms involved in tick-pathogen interactions that affect vector competence is essential for the identification of molecular drivers for tick-borne diseases. In this review, we provide a comprehensive overview of tick-pathogen molecular interactions for bacteria, viruses, and protozoa affecting human and animal health. Additionally, the impact of tick microbiome on these interactions was considered. Results show that different pathogens evolved similar strategies such as manipulation of the immune response to infect vectors and facilitate multiplication and transmission. Furthermore, some of these strategies may be used by pathogens to infect both tick and mammalian hosts. Identification of interactions that promote tick survival, spread, and pathogen transmission provides the opportunity to disrupt these interactions and lead to a reduction in tick burden and the prevalence of tick-borne diseases. Targeting some of the similar mechanisms used by the pathogens for infection and transmission by ticks may assist in development of preventative strategies against multiple tick-borne diseases.

Keywords: tick, *Anaplasma*, *flavivirus*, *Babesia*, *Borrelia*, microbiome, immunology, vaccine

INTRODUCTION

Ectoparasites that derive nutrition through blood feeding (haematophagy) are efficient vectors of disease. Ticks are haematophagous ectoparasites of vertebrates. Approximately 10% of the 900 currently known tick species are of significant medical or veterinary importance. Besides causing direct damage associated with blood feeding and in some cases through the excretion of toxins within their saliva, the main relevance of ticks lies in the wide variety of pathogens they can transmit, including bacteria, viruses, protozoa, and helminths (Jongejan and Uilenberg, 2004). The continuous exploitation of environmental resources and the increase in human outdoor activities, which have allowed for the contact with tick vectors normally present in the field, has promoted the emergence and resurgence of tick-borne pathogens (Jongejan and Uilenberg, 2004).

As previously discussed (Beerntsen et al., 2000), the terms "vectorial capacity" and "vector competence" are often used to describe the ability of an arthropod to serve as a disease vector. However, while vectorial capacity is influenced by behavioral and environmental determinants affecting variables such as vector density, longevity, and competence, vector competence is a component of vectorial capacity that depends on genetic factors affecting the ability of a vector to transmit a pathogen (Beerntsen et al., 2000, **Box 1**). These genetic determinants affect traits such as tick host preferences, duration of tick attachment, tick-host-pathogen and microbiome-pathogen interactions, and susceptibility to pathogen infection (Ramamoorthi et al., 2005; Hajdušek et al., 2013; Narasimhan et al., 2014; Nuttall, 2014; Rynkiewicz et al., 2015; Vayssier-Taussat et al., 2015). Therefore, the elucidation of the mechanisms involved in tick-pathogen interactions that affect vector competence is essential for the identification of molecular drivers for tick-borne diseases, and exposes paradigms for controlling and preventing these diseases.

Although our understanding of tick-pathogen interactions is still limited, advances in this field are facilitated by the increasing number of available genomic resources, including metabolomics, transcriptomics, and proteomics datasets of various ticks and tick-borne pathogens (TBPs) (Nene et al., 2004; Ayllón et al., 2015a; Cramaro et al., 2015; Kotsyfakis et al., 2015; Villar et al., 2015a; Gulia-Nuss et al., 2016; de Castro et al., 2016), and the recently published genome from *Ixodes scapularis*, a vector of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in North America (Gulia-Nuss et al., 2016). Together with tools such as tick cell lines and the widespread adaptation of RNA interference (RNAi) to study tick gene function (Bell-Sakyi et al., 2007; de la Fuente et al., 2007), this has opened exciting possibilities to identify determinants affecting tick vector competence.

Most studies of tick-pathogen interactions focus on certain pathogens (e.g., de la Fuente et al., 2016) or on certain aspects of these interactions (e.g., Hajdušek et al., 2013). However, for a better understanding of tick-pathogen molecular interactions and their role in vector competence, a comprehensive analysis involving major pathogens is crucial. In this review, we provide an overview of tick-pathogen molecular interactions for TBPs that constitute a growing burden for human and animal health (**Figure 1**). Additionally, the impact of tick microbiome on these interactions was considered to further contribute to the identification of molecular drivers affecting vector competence and the development of novel control and prevention strategies for tick-borne diseases.

BOX 1 | Important determinants influencing the acquisition, maintenance and transmission of pathogens by ticks.

Host range	Ticks with a wide host range such as *I. ricinus*, are naturally exposed to a greater variety of pathogens compared to ticks with a narrow host range such as *R. microplus* (Estrada-Peña et al., 2015).
Number of hosts	The potential transmission of pathogens could be limited when considering the host contact rate of 1- and 2- host ticks vs. 3-host ticks. This effect may however be partially annulled by the phenomenon of transovarial passage, when pathogens are passaged from the female to her eggs and offspring, which can subsequently infect new hosts. Argasid ticks of which the nymphs and adults take several blood meals, have a high host contact rate and could theoretically acquire or transmit pathogens from and to multiple hosts.
Midgut infection and escape barrier	The pathogen needs to pass through the midgut to reach the salivary glands and be transmitted with tick saliva, and for migration of some pathogens to the ovaries to allow transovarial pathogen passage. Mechanisms to pass the midgut infection barrier may depend on the presence and structure of specific surface receptors, such as TROSPA, to which OspA from *B. burgdorferi* adheres, allowing the spirochete to colonize the midgut (Pal et al., 2004).
Innate immune response	Pathogens need to overcome tick defense mechanisms, such as the phagocytosis of microbes by hemocytes, antimicrobial peptides and RNA interference, in order to be transmitted with tick saliva (Hajdušek et al., 2013).
Salivary gland infection and escape barrier	Pathogens must cross into the salivary glands for transmission with saliva during feeding, but little is known about the molecular mechanisms behind this entry. Once inside the salivary glands, the pathogen has to be released into the saliva stream to be transmitted. For example, *B. burgdorferi* uses tick salivary gland proteins to facilitate infection of the mammalian host (Ramamoorthi et al., 2005).
Pathogen strains	Differences between pathogen strains to infect and be transmitted by ticks have been widely reported (e.g., Kleiboeker et al., 1999; de la Fuente et al., 2001).
Tick microbiome-pathogen interactions	Microbiome play an essential role in various aspects of the arthropods life cycle and there is an increasing interest to elucidate arthropod-microbiome interactions. Perturbation of the microbiome caused changes in the integrity of the peritrophic membrane and may affect pathogen infection (Narasimhan et al., 2014).
Cross-Immunity interference	Competition between microorganisms within the tick may affect vector competence. Ticks infected with one *Rickettsia* species were for instance refractory to transovarial passage of a second *Rickettsia* species (Macaluso et al., 2002).
Abiotic factors	Abiotic factors such as temperature and relative humidity not only have a direct effect on tick development, questing activity and longevity, but temperature may also modulate pathogen development and survival in ticks (Shih et al., 1995; Estrada-Peña et al., 2011).

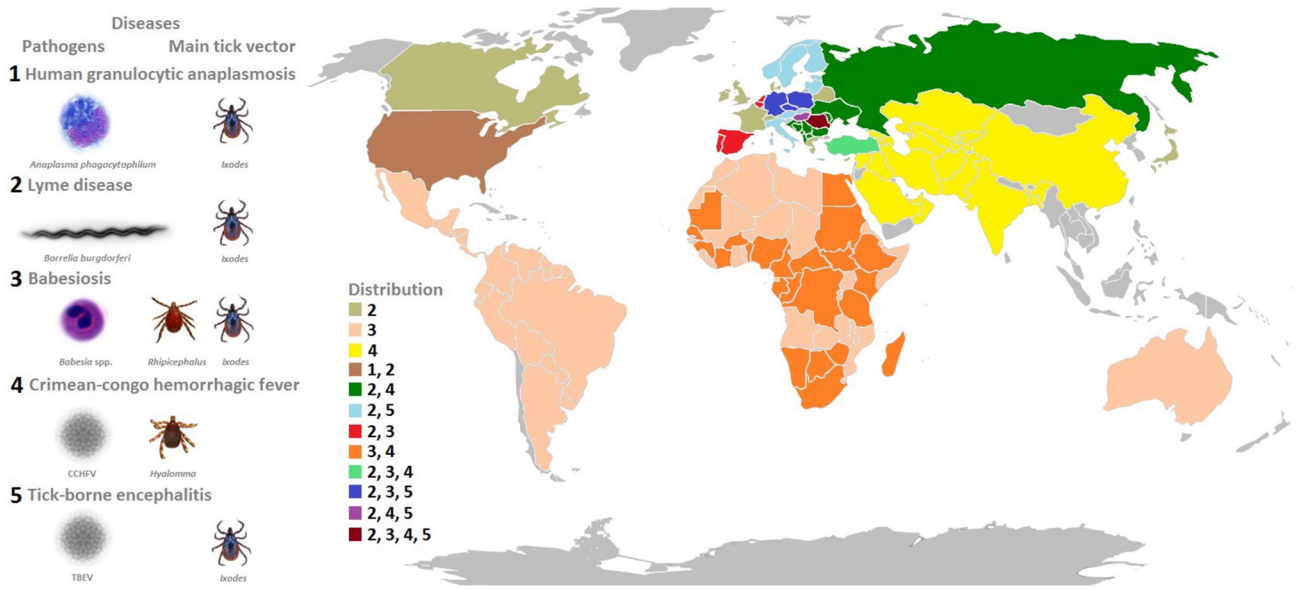

FIGURE 1 | Model organisms: tick-borne pathogens that constitute a growing burden for human and animal health. The pathogens covered in this review include bacteria (*A. phagocytophilum* and *B. burgdorferi*), viruses (Crimean-Congo hemorrhagic fever virus, tick-borne encephalitis virus), and protozoa (*Babesia* spp.) transmitted by hard ticks (Ixodidae). The most prevalent diseases caused by these pathogens, main tick vectors, and disease distribution worldwide is shown in the figure.

MODEL MICROORGANISMS

In this review, we used different tick-borne microorganisms including bacteria (*A. phagocytophilum* and *B. burgdorferi*), viruses (Crimean-Congo hemorrhagic fever virus, tick-borne encephalitis virus, and louping ill virus), and protozoa (*Babesia* spp.) to illustrate their impact on vector competence, behavior and transmission (**Figure 1**).

Bacteria

Anaplasma phagocytophilum is an obligate intracellular rickettsial pathogen vectored primarily by *Ixodes* spp. and causes human granulocytic anaplasmosis (HGA), equine, and canine granulocytic anaplasmosis, and tick-borne fever (TBF) (de la Fuente et al., 2008). In the vertebrate host, *A. phagocytophilum* infects neutrophils where the pathogen multiplies within a parasitophorous vacuole or morula (Ayllón et al., 2015a; Severo et al., 2015). In the absence of transovarial passage, ticks must acquire infection in each generation during a bloodmeal. *A. phagocytophilum* initially infects tick midgut cells and then subsequently develops in the salivary glands for transmission to susceptible hosts during tick feeding. Bacteria from the *B. burgdorferi* sensu lato complex are transmitted by Ixodid ticks and cause various symptoms associated with Lyme disease (Radolf et al., 2012). *B. burgdorferi* s.l. are acquired by larvae or nymphs from an infected host as they are not transovarially transmitted (Rollend et al., 2013). In the tick, spirochetes colonize the midgut and then traverse into the hemocoel and migrate to salivary glands for transmission during tick feeding (Pal et al., 2004; Ramamoorthi et al., 2005; Zhang L. et al., 2011; Coumou et al., 2016).

Viruses

Ticks transmit a range of viruses that are of significant public and veterinary health concern (**Table 1**). It is estimated that these viruses spend over 95% of their life cycle within the tick vector. Tick-borne encephalitis virus (TBEV) causes neurological disease in humans, whereas louping ill virus (LIV) causes neurological disease in sheep (Labuda and Nuttall, 2003). Ixodid ticks transmit these viruses to particular host species through a bite (Doherty and Reid, 1971; Mansfield et al., 2016). Crimean-Congo hemorrhagic fever virus (CCHFV) is transmitted to humans by the bite of infected ticks (*Hyalomma* spp. are the most competent vectors) or by direct contact with blood or tissues of viremic patients or animals, causing a disease characterized by fever, headache, myalgia, and hemorrhagic manifestations (Papa, 2010). If the appropriate receptors are present in the tick, following a blood meal TBEV and CCHFV enter vector host cells by endocytosis (Labuda and Nuttall, 2003; Simon et al., 2009; Garrison et al., 2013; Shtanko et al., 2014; Suda et al., 2016). These viruses replicate in the lining of the tick midgut where they disseminate to the hemolymph and subsequently infect different tissues reaching the highest titers in the salivary glands and reproductive organs to exit the cell via exocytosis (Dickson and Turell, 1992).

Protozoa

Babesia spp. are tick-borne Apicomplexan protozoans which invade vertebrate host erythrocytes, where all hemoparasite phases occur (Yokoyama et al., 2006; Chauvin et al., 2009; Florin-Christensen and Schnittger, 2009). *Babesia bovis* and *Babesia bigemina*, transmitted mainly by *Rhipicephalus microplus* and *Rhipicephalus annulatus*, are considered the most important

TABLE 1 | Viruses transmitted by ticks of medical or veterinary importance.

Virus (abbreviation)	Family/Genus	Principal vector	Species affected	Endemic presence
Alkhurma hemorrhagic fever virus (AHFV)	*Flaviviridae/Flavivirus*	*Ornithidoros savigny*	Humans	Saudi Arabia
African swine fever virus (ASFV)	*Asfarviridae/Asfivirus*	*Ornithodoros moubata*	Pigs	Africa
Colorado tick fever virus (CTFV)	*Reoviridae/Coltivirus*	*Dermacentor andersoni*	Humans	North America
Crimean-Congo haemorrhagic fever virus (CCHFV)	*Bunyaviridae/Nairovirus*	*Hyalomma* spp.	Humans	Africa/Asia/Southern Europe
Kyasanur Forrest virus (KFV)	*Flaviviridae/Flavivirus*	*Haemaphysalis spingera*	Humans	India
Louping ill virus (LIV)	*Flaviviridae/Flavivirus*	*Ixodes ricinus*	Sheep/Grouse	British Isles
Nairobi sheep disease virus (NSDV)	*Bunyaviridae/Nairovirus*	*Rhipicephalus appendiculatus*	Sheep	Africa
Omsk Hemorrhagic fever virus (OHFV)	*Flaviviridae/Flavivirus*	*Dermacentor reticulatus*	Humans	Asia
Powassan virus (POWV)	*Flaviviridae/Flavivirus*	*Ixodes cookei*	Humans	North America/Russia
Tick-borne encephalitis virus (TBEV)	*Flaviviridae/Flavivirus*	*I. ricinus/Ixodes persulcatus*	Humans	Europe/Asia

Table adapted from Labuda and Nuttall (2003) and Johnson et al. (2012).

species for their great economic impact on the cattle industry. Humans are accidental hosts, but human babesiosis caused by *Babesia microti* is now considered an emerging zoonosis as cases are increasing yearly (Schnittger et al., 2012). Ticks become infected with *Babesia* parasites when ingesting blood cells containing piroplasms, which develop into male and female gametes in the tick midgut (Uilenberg, 2006). The zygotes then multiply and invade numerous tick organs including the ovaries, which results in transovarial passage for some species such as *B. bovis* and *B. bigemina* but not *B. microti* (Uilenberg, 2006). When ticks attach to a new host, the sporozoites mature and the parasites are transmitted with tick saliva and infect red blood cells (Uilenberg, 2006).

BIOLOGICAL PROCESSES INVOLVED IN TICK-PATHOGEN INTERACTIONS

The objective of this paper is to review the information available on tick-pathogen molecular interactions and their role in vector competence. To address this objective, we discussed the main biological processes involved in tick-pathogen interactions. Additionally, the impact of tick microbiome on these interactions was considered. Although host-tick and host-pathogen molecular interactions also affect vector competence, this review focuses on tick-pathogen interactions for the identification of molecular drivers affecting vector competence that may result in the identification of tick-derived and pathogen-derived antigens for the development of novel control and prevention strategies for tick-borne diseases.

Role of Bacterial Proteins in Tick-Pathogen Interactions

Tick-pathogen protein-protein interactions play a crucial role during pathogen infection, persistence and transmission. The analysis of *A. phagocytophilum* proteins differentially represented during infection in ticks demonstrated that heat shock protein 70 (HSP70) and major surface protein 4 (MSP4) interact and bind to tick cells, thus playing a role in tick-pathogen interactions (Villar et al., 2015b). The type IV secretion system (T4SS) was proposed to be involved in the secretion of HSP70 and the MSP4 interaction with tick cells may induce the secretion of vesicles at the phagocytic cup to aid in adhesin secretion for rickettsial infection of tick cells (Villar et al., 2015b). Recent results have advanced our understanding of the molecular factors that are involved in the acquisition, persistence and transmission of *B. burgdorferi* in ticks (Rosa et al., 2005; Kung et al., 2013). An important protein involved in spirochete colonization of the tick midgut is the outer surface protein A (OspA), which binds to the tick receptor for OspA (TROSPA) (Pal et al., 2004). An *I. scapularis* dystroglycan like protein (ISDLP) as well as a tick receptor for the *B. burgdorferi* protein BBE31 (TRE31) help spirochetes traverse from the tick midgut into the hemocoel (Zhang L. et al., 2011; Coumou et al., 2016). *B. burgdorferi* outer surface protein C (OspC), produced when bacteria leave the tick midgut, binds to tick salivary protein 15 (Salp15) (Ramamoorthi et al., 2005), providing protection against mammalian antibody/complement-mediated immune response during bacterial transmission (Garg et al., 2006; Schuijt et al., 2011a). The TROSPA homolog in the *B. bigemina* vectors, *R. microplus,* and *R. annulatus* was proposed to be a putative receptor for *Babesia* ligands based on the decrease in infection after RNAi and vaccination experiments targeting this protein (Antunes et al., 2012; Merino et al., 2013). Flaviviruses and CCHFV enter vertebrate and vector host cells by attachment of viral envelope proteins to host receptors, which activates the actin-dependent clathrin-mediated endocytic pathway (Labuda and Nuttall, 2003; Simon et al., 2009; Garrison et al., 2013).

Tick Cytoskeleton

Intracellular bacteria induce cytoskeletal rearrangement to establish infection (Ireton, 2013). In *I. scapularis*, *A. phagocytophilum* remodels tick cytoskeleton by altering the ratio between monomeric globular G actin and filamentous F actin to facilitate infection through selective regulation of gene transcription in association with the RNA polymerase II and the TATA-binding protein (Sultana et al., 2010). In *I. scapularis* midgut cells, the up-regulation of Spectrin alpha chain or Alpha-fodrin in response to infection results in cytoskeleton remodeling that is used by *A. phagocytophilum* to facilitate infection (Ayllón et al., 2013, **Figure 2A**). Although not functionally characterized, a proteomics analysis in *I. ricinus* tick salivary glands showed

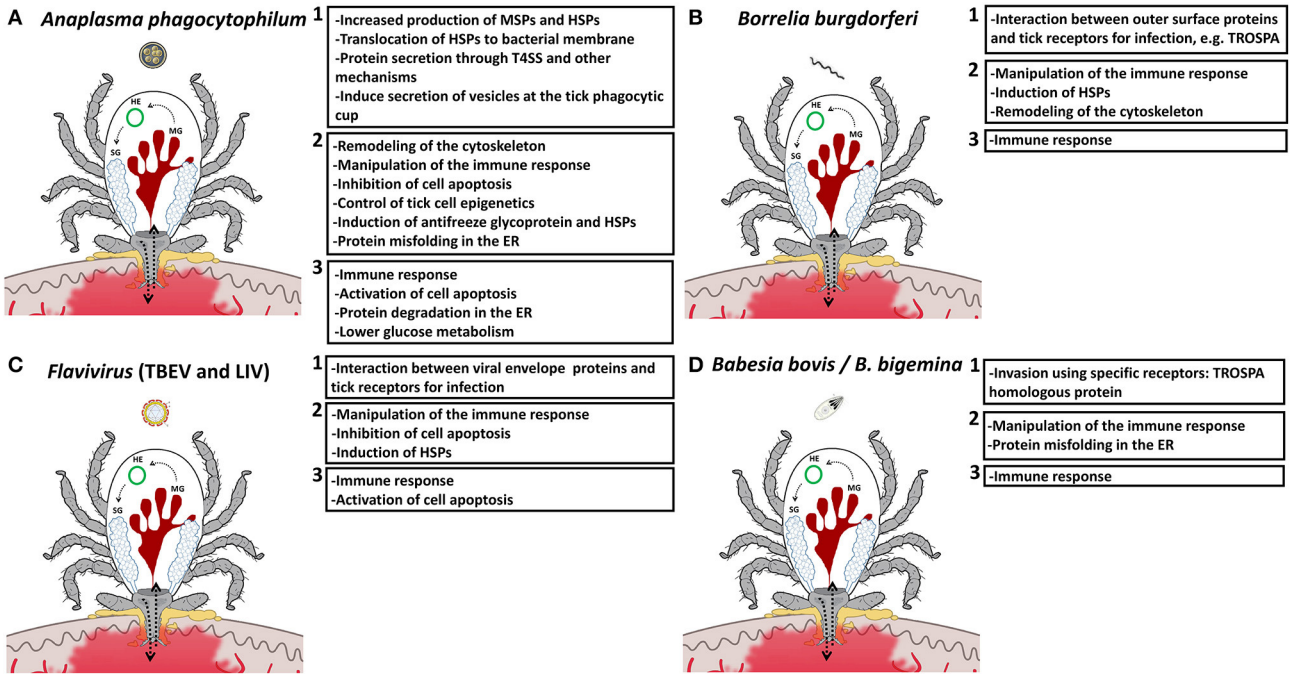

FIGURE 2 | Tick-pathogen molecular interactions. (A) *A. phagocytophilum* **(B)** *B. burgdorferi* s.l., **(C)** TBEV, and **(D)** *B. bovis/B. bigemina* activate mechanisms (panel 1) and manipulate tick protective responses and other biological processes in order to facilitate infection (panel 2), while ticks respond to limit pathogen infection and preserve feeding fitness and vector competence for survival of both ticks and pathogens (panel 3). MG, midgut; HE, hemocyte; SG, salivary gland; MSPs, major surface proteins; HSPs, heat shock proteins; ER, endoplasmic reticulum.

the under-representation of cytoskeleton proteins in response to *Borrelia* infection, suggesting that some *Borrelia* strains promote a cytoskeleton rearrangement in ticks (Cotté et al., 2014, **Figure 2B**).

Tick Cell Apoptosis

Apoptosis is an intrinsic immune defense mechanism in response to microbial infection that results in reduction of infected cells, but several pathogens have developed different strategies to inhibit cell apoptosis in order to enhance their infection, replication and survival (Ashida et al., 2011). Infection of tick salivary glands with *A. phagocytophilum* results in inhibition of the intrinsic apoptosis pathway through porin down-regulation, favoring bacterial infection (Ayllón et al., 2015a). Tick cells respond to infection via activation of the extrinsic apoptosis pathway, which limits *A. phagocytophilum* infection and promotes tick survival (Ayllón et al., 2015a). In tick midguts, *A. phagocytophilum* infection results in activation of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, which inhibits apoptosis and promotes pathogen infection (Ayllón et al., 2015a). The ISE6 cultured cells, derived from embryonic *I. scapularis*, have provided a model for tick hemocyte responses to pathogen infection. In this cell line, *A. phagocytophilum* infection promotes protein misfolding in the endoplasmic reticulum (ER), counteracting the tick cell response to infection. However, tick cells respond by activating protein targeting and degradation, which reduces ER stress and apoptosis, thus favoring *A. phagocytophilum* infection (Villar et al., 2015a). Additionally, *A. phagocytophilum* may benefit from the tick cells ability to limit pathogen infection through phosphoenolpyruvate carboxykinase (PEPCK) inhibition that results in lower glucose metabolism and the reduction in the availability of essential metabolites for bacterial growth, which leads to the inhibition of cell apoptosis that increases infection in tick cells (Villar et al., 2015a). These results show that the inhibition of tick cell apoptosis is a physiologically relevant mechanism used by *A. phagocytophilum* to facilitate infection and multiplication in both tick and vertebrate host cells (de la Fuente et al., 2016, **Figure 4**). Infection of *I. ricinus* cells with flaviviruses leads to the differential expression of a large number of genes involved in a variety of cellular functions, including up-regulation of genes such as *cytochrome c* associated with cellular stress and apoptosis (Mansfield et al., 2017). However, the lack of detection of *caspase* genes, and the up-regulation of genes that inhibit apoptosis (including *hsp70*) suggest that flavivirus infection inhibits tick cell apoptosis in order to promote cell survival during infection as previously shown for *A. phagocytophilum* (Ayllón et al., 2015a; Alberdi et al., 2016).

Tick Innate Immune Response

Tick vector competence is influenced by the ability of transmitted pathogens to evade tick innate immune response (Hajdušek et al., 2013). Several humoral and cell-mediated immune response pathways are involved in tick innate immunity, and play a

role in defense to *Anaplasma*, *Borrelia*, flavivirus, and *Babesia* infection or are manipulated by pathogens to facilitate infection (Turell, 2007; Hajdušek et al., 2013; Mansfield et al., 2017, **Figure 2**). With respect to the tick innate immune response, *A. phagocytophilum* subverts tick RNAi by mechanisms other than reduction of Tudor staphylococcal nuclease (Tudor-SN) levels to preserve a protein that is important for tick feeding (Ayllón et al., 2015b). In contrast, Subolesin (SUB), also involved in tick innate immune response for limiting pathogen infection (Naranjo et al., 2013; de la Fuente and Contreras, 2015), is not manipulated by *A. phagocytophilum*. SUB has been shown to be required for tick feeding and reproduction and for pathogen infection, and therefore the preservation of this protein is important for both tick and pathogen survival (de la Fuente and Contreras, 2015). In *I. scapularis*, the x-linked inhibitor of apoptosis protein (XIAP) interacts with the E2 conjugating enzyme Bendless affecting positive and negative regulators of the immune deficiency (IMD) pathway resulting in protection against infection by *A. phagocytophilum* (Severo et al., 2013).

After molting, tick nymphs attach and start feeding, displaying an altered midgut transcriptome when infected with *B. burgdorferi* (Rudenko et al., 2005). Some of the genes affected by infection include innate immune factors (defensin and thioredoxin peroxidase) that possibly limit tick *Borrelia* infection. Tick salivary protein 20 (Salp20) belongs to a protein family with complement-inhibitory activity that blocks the host alternative complement pathway and assists in *Borrelia* transmission (Hourcade et al., 2016). Tick salivary lectin pathway inhibitor (TSLPI) inhibits the human lectin complement pathway by interfering with the mannose binding lectin activity and enables transmission of *Borrelia* by protecting it from complement-mediated killing (Schuijt et al., 2011b; Wagemakers et al., 2016). Recently, Smith et al. (2016) showed that *I. scapularis* respond to interferon gamma acquired in the blood meal when parasitizing on *B. burgdorferi*-infected mice, leading to the up-regulation of the Rho-like GTPase and induction of antimicrobial peptides to inhibit pathogen infection.

Preliminary studies focusing on transcriptomic changes induced by TBEV infection of *I. scapularis* and *I. ricinus* cells have revealed the role of particular proteins within tick innate immune pathways that act to control infection (Weisheit et al., 2015). A similar approach has identified this response in tick cells infected with LIV and TBEV, with a range of transcripts being up and down-regulated (Weisheit et al., 2015; Mansfield et al., 2017). Flavivirus infection also induced transcripts associated with activation of innate immune pathways in tick cells, including JAK/STAT and Mitogen-activated protein kinase (MAPK) pathways (Mansfield et al., 2017), with additional up-regulation of genes with host resistance functions, including FK506 binding protein (FKBP) and the antiviral helicase Slh1 (Mansfield et al., 2017, **Figure 2C**). CCHFV is capable of evading the tick innate immune response. Following intracoelomic CCHFV inoculation, virus titers in male and female ticks are the same and infection rates and titers in salivary glands, ovaries, and testes increase upon blood feeding (Dickson and Turell, 1992). Therefore, viral replication in tissues associated with possible CCHFV transmission in infected ticks may be stimulated by attachment and feeding on susceptible hosts. This might reduce the stress induced by viral replication while ticks are waiting to find a vertebrate host, but increase the potential for viral transmission once the host is infested (Turell, 2007).

Using different methodologies, some molecules have been identified as being implicated in tick-*Babesia* interactions (Hajdušek et al., 2013). Genes involved in immunity, stress, and defense responses showed up-regulation in response to *B. bovis* infection (Heekin et al., 2012), while genes encoding for calreticulin, kunitz-type serine protease inhibitors and microplusin which exhibits antimicrobial activity, were differentially expressed in *B. bovis/B. bigemina* infected *Rhipicephalus* ticks (Rachinsky et al., 2007; Antunes et al., 2012; Heekin et al., 2013; Lu et al., 2016). Tick SUB (Almazán et al., 2005) was shown to be up-regulated in *B. microti* inoculated intrahemocoelically into *Rhipicephalus haemaphysaloides* (Lu et al., 2016) and *B. bigemina*-infected *R. microplus* (Merino et al., 2013) (**Figure 2D**). The putative role of SUB in *B. bigemina* infection in ticks was supported by showing a decrease in pathogen levels in ticks fed on cattle immunized with recombinant SUB (Merino et al., 2013).

Tick Cell Epigenetics

Intracellular pathogens manipulate the transcriptional programs of their host cells via epigenetic mechanisms, leading to stress, and inflammatory responses (Gómez-Díaz et al., 2012). Recently, *A. phagocytophilum* was shown to manipulate tick cell epigenetics to increase the levels of the histone modifying enzymes (HMEs), histone acetyltransferases (HATs; 300/CBP), and histone deacetylases (HDACs and Sirtuins) resulting in the inhibition of cell apoptosis to facilitate pathogen infection and multiplication (Cabezas-Cruz et al., 2016). The results of this study suggested that a compensatory mechanism might exist by which *A. phagocytophilum* differentially manipulates tick HMEs to regulate transcription and apoptosis in a tissue-specific manner to facilitate infection but preserving tick fitness to guarantee survival of both pathogens and ticks (Cabezas-Cruz et al., 2016). As previously discussed (Cabezas-Cruz et al., 2016), the mechanisms by which *A. phagocytophilum* affects tick cell epigenetics is unknown but effector proteins such as AnkA, secreted through T4SS or other secretion mechanisms probably control it (Garcia-Garcia et al., 2009a,b; Rennoll-Bankert et al., 2015). It has been previously demonstrated that *A. phagocytophilum* AnkA recruits host histone deacetylase 1 (HDAC1) and modifies neutrophils gene expression (Garcia-Garcia et al., 2009a,b; Rennoll-Bankert et al., 2015). Interestingly, the homolog of HDAC1 in *I. scapularis* was overrepresented upon *A. phagocytophilum* infection in tick salivary glands (Cabezas-Cruz et al., 2016). It remains to be tested whether *A. phagocytophilum* AnkA plays the same role in ticks as in vertebrate neutrophils.

Effect of Pathogen Infection on Tick Fitness

The characterization of *I. scapularis-A. phagocytophilum* molecular interactions revealed complex responses by both ticks and pathogens that were necessary for maintenance of tick health

while ensuring robust vector capacity (Ayllón et al., 2015a; Villar et al., 2015a; Gulia-Nuss et al., 2016; de la Fuente et al., 2016). Several lines of evidence suggest that tick-pathogen associations evolved to form "*intimate epigenetic relationships*" that have the potential to increase tick fitness (Cabezas-Cruz et al., 2017). At the tick-pathogen interface, *A. phagocytophilum* induces an antifreeze glycoprotein (IAFGP) and heat shock proteins (HSPs) to increase tick survival and feeding fitness (Neelakanta et al., 2010; Busby et al., 2012). Neelakanta et al. (2010) demonstrated that *I. scapularis* ticks infected with *A. phagocytophilum* show enhanced fitness against freezing injury due to the induced expression of IAFGP. They further showed that improved survival of infected ticks correlated with higher bacterial infection, therefore providing a direct link between pathogen infection and tick fitness in unfavorable ecological conditions. The fact that *Borrelia* and TBEV-infected ticks choose a higher questing height suggests that these pathogens help ticks to survive under dry conditions. In agreement with this hypothesis, *I. ricinus* infected by *B. burgdorferi* move less toward a humid environment and their survival is higher in highly desiccating conditions (Hermann and Gern, 2010; Herrmann and Gern, 2012). The tick histamine release factor (tHRF), up-regulated in *B. burgdorferi*-infected *I. scapularis* during feeding, facilitates tick engorgement and *B. burgdorferi* infection by increasing the blood flow to the tick-bite site and modulating vascular permeability (Dai et al., 2010).

TICK-MICROBIOME INTERACTIONS

The recent development of high-throughput next generation sequencing technologies has highlighted the complexity of the tick microbiome that includes both pathogens and potential symbionts (Vayssier-Taussat et al., 2015). It is readily apparent that interactions frequently occur among tick microbial communities, as relationships between microorganisms existing in one environment can be competitive, exclusive, facilitating, or absent, with many potential implications for human and animal health that remain to be elucidated (Ahantarig et al., 2013; Vayssier-Taussat et al., 2015). Both positive and negative associations have been reported for pathogens (Mather et al., 1987; de la Fuente et al., 2003). However, the role of tick endosymbionts in pathogen transmission has only been studied in a few selected bacterial and tick species.

Symbionts may confer crucial and diverse benefits to their hosts, playing nutritional roles, or affecting fitness, development, reproduction, defense against environmental stress, and immunity (Ahantarig et al., 2013). *Coxiella*-like endosymbionts are believed to be the most common vertically transmitted agents in hard ticks (Bernasconi et al., 2002; Lee et al., 2004; Clay et al., 2008; Bonnet et al., 2013; Cooper et al., 2013). In *Amblyomma americanum,* the removal of *Coxiella* symbionts following antibiotic treatment reduced tick offspring production and increased time to oviposition (Zhong et al., 2007). In *I. ricinus* (Lo et al., 2006; Sassera et al., 2006; Montagna et al., 2013), *Candidatus* Midichloria mitochondrii is an intra-mitochondrial bacterium that has also been detected in other tick genera (Harrus et al., 2011; Williams-Newkirk et al., 2012). It has been ascribed a possible helper role in tick molting processes (Zchori-Fein and Bourtzis, 2011, **Figure 3**). *Rickettsia*-like symbionts have also been reported to infect hard ticks from several genera (Baldridge et al., 2004; Clay et al., 2008; Liu et al., 2013). One study reported that *Rickettsia*-infected *Dermacentor variabilis* have slightly greater motility than uninfected ticks, indirectly influencing disease risk (Kagemann and Clay, 2013). *Francisella*-like symbionts have been reported in several hard tick genera (Venzal et al., 2008; Ivanov et al., 2011; Michelet et al., 2013), but their effect on tick fitness and biology remains unknown. Being able to manipulate host reproduction and then to affect vector populations, *Wolbachia* spp. have also been identified in several hard tick genera (Engelstadter and Hurst, 2007; Andreotti et al., 2011; Reis et al., 2011; Zhang X. et al.,

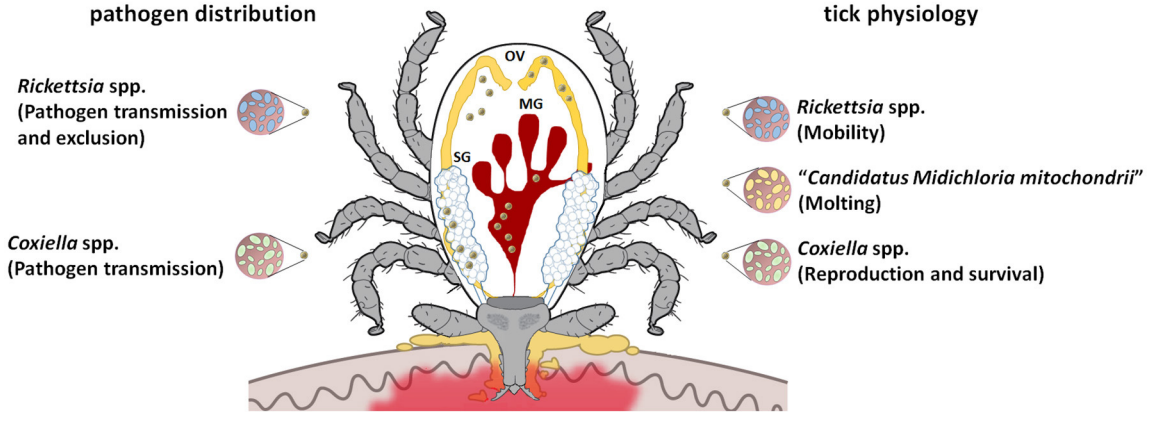

FIGURE 3 | Possible impact of tick microbiome on pathogen transmission. Tick microbiome may affect pathogen transmission either directly via nutrient competition or induced/reduced immunity, or indirectly by affecting tick populations (viability, reproduction) or fitness (affecting host-seeking success). MG, midgut; SG, salivary gland; OV, ovaries.

2011). Their role in pathogen transmission requires further attention, as reports suggest that this bacterium can protect some arthropods against microbial infections (Martinez et al., 2014). In *I. ricinus*, *Wolbachia pipientis* is known to be associated with the hymenoptera tick endoparasitoid *Ixodiphagus hookeri* (Plantard et al., 2012; Bohacsova et al., 2016), and *Arsenophonus* spp. symbionts (Dergousoff and Chilton, 2010). The latter, detected in several tick species (Clay et al., 2008; Dergousoff and Chilton, 2010; Reis et al., 2011), are responsible for sex-ratio distortion in arthropods, and some studies suggest that they can affect host-seeking success by decreasing tick motility in *A. americanum* and *D. variabilis* (Kagemann and Clay, 2013). Lastly, some *Spiroplasma* spp. detected in *Ixodes* spp. such as *Spiroplasma ixodetis* (Tully et al., 1995) may cause sex-ratio distortion in some insect species via male killing (Tabata et al., 2011).

Recently, Abraham et al. (2017) showed how *A. phagocytophilum* manipulates *I. scapularis* tick microbiota to promote infection. Firstly, they showed that IAFGP, apart from protecting ticks against cold injury (see above), has antimicrobial activity against biofilm-forming bacteria, particularly *Staphylococcus aureus* and *Enterococcus faecalis*. They further showed that by targeting biofilm-forming bacteria, *A. phagocytophilum* modifies the composition of gut microbiota and alters tick midguts permeability, which results in higher *A. phagocytophilum* infection in the vector (Abraham et al., 2017). Regarding the relationship between symbionts and pathogens, exclusion has been reported in Rickettsiales, which may be due to intra-family bacterial cross-immunity. Exclusion has been documented in *Dermacentor* ticks infected with *Rickettsia peacockii* or *Rickettsia montana* that limits *Rickettsia rickettsii* and *Rickettsia rhipicephali* distribution, respectively (Burgdorfer et al., 1981; Macaluso et al., 2002, **Figure 3**). It has also been reported that *I. scapularis* male ticks infected by a rickettsial endosymbiont had significantly lower rates of infection by *B. burgdorferi* than symbiont-free males, thus evidencing interactions among microbial species (Steiner et al., 2008). Further research showed that perturbation of the midgut microbiome in *I. scapularis* influences *B. burgdorferi* colonization of ticks through a transcriptional mechanism resulting in lower expression of peritrophin, which perturbs the integrity of the peritrophic matrix (Narasimhan et al., 2014). In *A. americanum,* the presence of *Coxiella*-related symbionts seems to influence *Ehrlichia chaffeensis* transmission (Klyachko et al., 2007), and infection with *Arsenophonus* appears to be negatively correlated with the frequency of *Rickettsia* sp. infection (Clay et al., 2008, **Figure 3**).

CONCLUSIONS AND FUTURE DIRECTIONS FOR THE CONTROL OF TICK-BORNE DISEASES

Over millions of years, arthropod vectors have co-evolved with a variety of microorganisms including bacteria, viruses, and protozoa to the point where they appear to co-exist with little impact on the vector (Beerntsen et al., 2000; Estrada-Peña et al., 2015; de la Fuente et al., 2015). These arthropods have become efficient vectors of pathogens to humans and other vertebrate hosts that are susceptible to infection and disease.

Present results show that different pathogens have developed similar strategies such as manipulation of the immune response to infect ticks and facilitate multiplication and transmission. Some of these strategies may be used by pathogens to infect both ticks and mammalian hosts (de la Fuente et al., 2016). Additionally, recent evidence demonstrates that the microbiome has an effect on tick fitness and pathogen infection and transmission, highlighting the importance of tick-microbiome interactions for vector competence. Overall, these results illustrate how pathogens activate mechanisms and manipulate tick protective responses and other biological processes in order to facilitate infection, while ticks respond to limit pathogen infection and preserve feeding fitness and vector competence for survival of both ticks and pathogens. However, how different molecular mechanisms make certain tick species suitable vectors for certain pathogens is still not fully characterized. The presence of tick receptors that are pathogen-specific affects vector competence for these pathogens, but other mechanisms are probably also involved in this process. Furthermore, the biological processes involved in tick-pathogen interactions are also affected in other arthropod vectors (**Box 2**).

The identification of the molecular drivers that promote tick survival, spread, and pathogen transmission provides the opportunity to disrupt these processes and lead to a reduction in tick burden and prevalence of tick-borne diseases. Targeting some of the similar mechanisms used by the pathogens for infection and transmission by ticks may be used to develop strategies against multiple tick-borne diseases. As shown for *B. burgdorferi* OspA (Gomes-Solecki, 2014), pathogen-derived proteins involved in interactions with tick cells and playing a role during infection provide targets for development of novel control strategies for pathogen infection and transmission. Similarly, tick-derived antigens such as SUB involved in different biological processes may be used to reduce vector infestations and pathogen infection in ticks feeding on immunized animals (de la Fuente and Contreras, 2015). One novel approach to control populations might be to target specific endosymbionts, which requires detailed knowledge of microbial communities and their impact on tick biology (Taylor et al., 2012). Finally, the surveillance of microbial populations in tick salivary glands may enable the early identification of pathogens likely to be transmitted to vertebrate host (Qiu et al., 2014). Overall, the combination of effective and early diagnostics along with tick vaccines and strategies such as harnessing genetics to improve livestock breeds, and the rational application of acaricides, antivirals and other therapeutic interventions will result in a more effective and environmentally friendly control of tick populations. In addition, transgenic or paratransgenic ticks and vertebrate host genetically modified to confer resistance to pathogen infection may be produced and combined with vaccine applications and other interventions (de la Fuente and Kocan, 2014).

BOX 2 | Are the biological processes involved in tick-pathogen interactions unique for ticks?

The answer to this question is that several of the processes involved in tick-pathogen interactions have also been identified in other vector-pathogen interactions (see for example, Beerntsen et al., 2000; Vlachou et al., 2005; Wang et al., 2010; Gómez-Díaz et al., 2012; Sabin et al., 2013; Ramphul et al., 2015; Eng et al., 2016; Shaw et al., 2017). For example, as described in ticks, receptor-ligand-like interactions mediate pathogen recognition and infection in mosquitoes (Beerntsen et al., 2000). Remodeling of the cytoskeleton seems to be a general mechanism for tick pathogen infection (Cotté et al., 2014; de la Fuente et al., 2016). Pathogens such as Dengue virus (DENV), West Nile virus (WNV), and *Plasmodium* parasites also affect mosquito cytoskeleton during infection (Vlachou et al., 2005; Wang et al., 2010). The finding that some pathogens manipulate tick immune response to facilitate infection has been also reported in mosquitoes infected with *Plasmodium falciparum* (Beerntsen et al., 2000). Similarly, the expression of immune response genes such as those involved in the JAK/STAT pathway may serve to limit bacterial and fungal proliferation in fruit fly and mosquitoes (Beerntsen et al., 2000). Apoptosis plays an important role in tick-pathogen interactions (de la Fuente et al., 2016). While inhibition of cell apoptosis by pathogens facilitates infection, host cell response may activate alternative apoptotic pathways to limit infection (de la Fuente et al., 2016). These findings have been also described in for example *Aedes aegypti* and *Anopheles gambiae* mosquitoes infected with DENV and *P. falciparum*, respectively (Ramphul et al., 2015; Eng et al., 2016). The control of tick cell epigenetics by *A. phagocytophilum* has been proposed as a mechanism used by the pathogen to facilitate infection and multiplication (Cabezas-Cruz et al., 2016). Similar mechanisms have been described to operate at the mosquito-*Plasmodium* interface (Gómez-Díaz et al., 2012).

However, the functional mechanisms by which these processes are affected at the vector-pathogen interface may vary between pathogen and vector species (**Figure 4**). The limited information available on the functional characterization of these processes in ticks and other arthropods limits the scope of the comparative analysis between different vectors. Nevertheless, recent results support that in some cases the protein function described in model insect species may be different in the evolutionarily distant ticks. Differences in vector competence may be genetically encoded by differences in the immune response pathways operating at each vector-pathogen interaction (Baxter et al., 2017). For example, Tudor-SN, a conserved component of the basic RNAi machinery with a variety of functions including immune response and gene regulation, is involved in defense against infection in *Drosophila* (Sabin et al., 2013) but not in ticks (Ayllón et al., 2015b). The IMD pathway is involved in protection against infection in arthropods, but recent results support the existence of two functionally distinct IMD circuits in insects and ticks (Shaw et al., 2017). Future comparative analyses between different vector species will provide additional information on the functional implication of the different biological processes in vector-pathogen interactions and vector competence (Gerold et al., 2017).

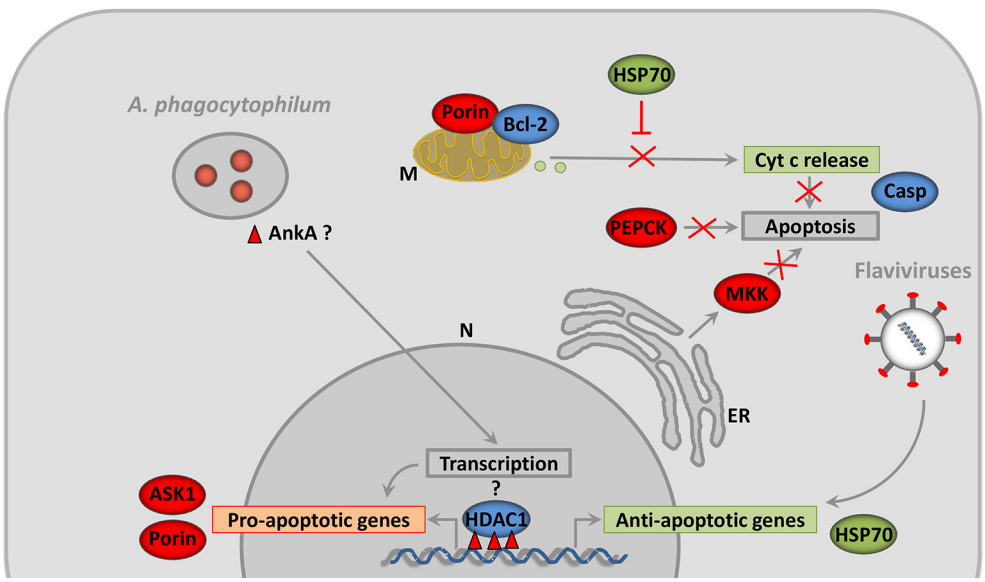

FIGURE 4 | Pathogens inhibit vector cell apoptosis by different mechanisms. After infection of tick salivary glands, *A. phagocytophilum* inhibit apoptosis by decreasing the expression of the pro-apoptotic genes coding for proteins such as ASK1 and Porin. Porin down-regulation is associated with the inhibition of mitochondrial Cyt c release (Ayllón et al., 2015a). In contrast, *A. phagocytophilum* infection does not affect Bcl-2 levels, probably because this protein but not Porin is essential for tick feeding (Ayllón et al., 2015a). *A. phagocytophilum* also induces ER stress in tick cells which play a role in reducing the levels of MKK that inhibits apoptosis (Villar et al., 2015a). Another interesting mechanism of *A. phagocytophilum* to inhibit apoptosis is the manipulation of glucose metabolism by reducing the levels of PEPCK (Villar et al., 2015a). The capacity of *A. phagocytophilum* to downregulate gene expression in neutrophils was associated with HDAC1 recruitment to the promoters of target genes by the ankyrin repeat protein AnkA (Garcia-Garcia et al., 2009a,b; Rennoll-Bankert et al., 2015). Tick HDAC1 is overrepresented in *A. phagocytophilum*-infected salivary glands and chemical inhibition of this protein decreases *A. phagocytophilum* burden in tick cells (Cabezas-Cruz et al., 2016). Infection of tick cells with flaviviruses results in the up-regulation of genes such as *hsp70* that inhibit apoptosis (Mansfield et al., 2017). N, Nucleus; M, Mitochondria; ER, Endoplasmic Reticulum; Cyt c, Cytochrome c; ASK1, Apoptosis signal-regulating kinase 1; MKK, Mitogen-activated Protein Kinase; HDAC1, Histone Deacetylase 1; AnkA, Ankyrin A; PEPCK, Phosphoenolpyruvate Carboxykinase; FOXO, Forkhead box O; Hid, Head involution defective; JNK, Jun amino-terminal kinases; Casp, caspases. The molecules and processes represented in green are up-regulated, while those represented in red are down-regulated in response to infection. The activity of the molecules represented in blue varies in response to infection.

AUTHOR CONTRIBUTIONS

JF, SA, SB, AD, AE, NJ, KM, AN, AP, NR, AF, ROMR conducted the literature research and wrote the paper. JF, AC, AP, SB, AN, NJ prepared the figures and tables. All authors provided critical review and revisions.

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Tick-Pathogen Ensembles: Do Molecular Interactions Lead Ecological Innovation?

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Ticks are arthropods distributed worldwide that constitute the most important vectors of diseases to animals, and second to mosquitoes regarding pathogens of public health importance. Ticks are remarkably plastic and can colonize diverse ecological niches of the planet, from tropics to polar areas (de la Fuente et al., 2008). In the last decade, the reports of tick-borne pathogens have increased sharply, motivating vigorous research programs that addressed major questions on the epidemiology of tick-borne diseases, vector-host-pathogen interactions, tick ecology, and tick genomics. Notably, the first tick genome was released this year (Gulia-Nuss et al., 2016), opening new possibilities to explore tick-host-pathogen interactions (de la Fuente et al., 2016a). In contrast, the evolutionary and ecological implications of tick-pathogen associations have received comparatively less attention. Herein, we hypothesized that tick-pathogen associations evolved to form "*intimate epigenetic relationships*" similar to those described for *Theileria* spp. and its vertebrate host (Cheeseman and Weitzman, 2015) in which the pathogen induces transcriptional reprogramming in infected ticks. This will ultimately favor pathogen propagation, but will also select for the most suitable ecological adaptations in the tick vector. These phenotypic and genetic changes may have the potential to be transmitted to the next generation of ticks. As a result, the ecological associations between tick, vertebrates, and pathogens would evolve to maximize pathogen circulation in these communities (Estrada-Peña et al., 2015, 2016).

Our hypothesis was based on the following evidences: (i) tick-borne pathogens induce transcriptional reprogramming in infected tick (Ayllón et al., 2015; Villar et al., 2015; Weisheit et al., 2015) and vertebrate cells (Lee et al., 2008; Bouquet et al., 2016); (ii) tick-borne pathogens produce and secrete effector proteins, nucleomodulins, which constitute a family of proteins produced by bacterial pathogens to control host transcription and other nuclear processes (Bierne and Cossart, 2012), that interact with host epigenetic machinery and induce transcriptional reprogramming (Garcia-Garcia et al., 2009a,b; Rennoll-Bankert et al., 2015; Sinclair et al., 2015; Lina et al., 2016), and (iii) tick-pathogen interactions increase tick fitness (Neelakanta et al., 2010; Belova et al., 2012; Herrmann and Gern, 2015; de la Fuente et al., 2016b).

TICK-BORNE PATHOGENS INDUCE TRANSCRIPTIONAL REPROGRAMMING IN HOST CELLS

Several studies using "omics" technologies have revealed that a common pattern in the infection by tick-borne pathogens is the transcriptional reprograming of the host cells. These pathogens include obligate intracellular bacterial such as *Anaplasma phagocytophilum* (Carlyon et al., 2002; Borjesson et al., 2005; Pedra et al., 2005; Sukumaran et al., 2005; Lee et al., 2008; Ayllón et al., 2015) and *Ehrlichia chaffeensis* (Miura and Rikihisa, 2009), the extracellular bacterial pathogen *Borrelia burgdorferi* (Bouquet et al., 2016) and viruses such as TBEV (Weisheit et al., 2015). This transcriptional reprograming not only affect gene expression but also impact protein abundance (Lin et al., 2011; Ayllón et al., 2015). Among the cellular components and processes affected in ticks by pathogen infection are the cytoskeleton, cell immunity, apoptosis, metabolism, and potentially the posttranslational modification of histone tails (Ayllón et al., 2015; Villar et al., 2015; Cabezas-Cruz et al., 2016). Notably, gene expression regulation by tick-borne pathogens occurs in a tissue-specific manner. For example, to establish an infection in ticks, *A. phagocytophilum* inhibits the apoptosis in infected midgut and salivary glands. However, in tick midgut, *A. phagocytophilum* inhibits the apoptosis by upregulating the Janus kinase (JAK)-signaling transducer activator of transcription (JAK-STAT) pathway, but in salivary glands this bacterium down-regulates the expression of porin, which results in the inhibition of cytochrome c release and the intrinsic apoptosis pathway (Ayllón et al., 2015; Alberdi et al., 2016). Taken together, these findings reveal that during evolution tick-borne pathogens have developed specific mechanisms to manipulate gene expression in host cells.

MOLECULAR MESSENGERS OF PATHOGEN MANIPULATION

To manipulate gene expression, pathogens activate signaling pathways or hijack the epigenetic machinery of host cells. Both mechanisms have been described during *A. phagocytophilum* infection in ticks. For example, *A. phagocytophilum* infection triggers expression of antimicrobial peptides in salivary glands that control bacterial load. The expression of this family of antimicrobial peptides is mediated by the activation of the JAK-STAT pathway (Liu et al., 2012). It has also been shown that *A. phagocytophilum* induces the activation of the PI3K signaling pathway leading to actin phosphorylation to increase the expression of the gene *salp16* coding for a tick salivary protein crucial for *A. phagocytophilum* survival (Sultana et al., 2010). However, while signaling pathways activation can explain the regulation of some genes, (Sultana et al., 2010; Liu et al., 2012), it does not explain the massive gene regulation induced by *A. phagocytophilum* infection in ticks (Ayllón et al., 2015). In fact, *A. phagocytophilum* induces the differential expression of 8,516 (from 16,083 gene transcripts identified), 5,394 (12,651) and 2,487 (11,105) genes in *Ixodes scapularis* tick nymphs, adult midguts, and salivary gland, respectively (Ayllón et al., 2015; de la Fuente et al., 2016a).

A. phagocytophilum produces a family of proteins called nucleomodulins that control host gene expression at the epigenetic level (Sinclair et al., 2015). In particular, the ankyrin repeat effector protein ankyrin A (AnkA) was reported to be secreted by *A. phagocytophilum* through the bacterial type IV secretion system (T4SS) in infected neutrophils (Garcia-Garcia et al., 2009a,b; Rennoll-Bankert et al., 2015). AnkA enters the granulocyte nucleus, binds stretches of AT-rich DNA and alters transcription of antimicrobial defense genes, including down-regulation of *CYBB*, which codes for a NADPH oxidase 2 (Nox2). This enzyme is involved in the production of reactive oxygen species (ROS), which is crucial in the neutrophil immune response against intracellular bacteria. To achieve this regulatory process, AnkA recruits host histone deacetylase 1 (HDAC1) and decreases histone H3 acetylation in infected cells (Garcia-Garcia et al., 2009a,b). This results in chromatin changes that down-regulate the expression of target genes (e.g., *CYBB*). Remarkably, 50 proteins were identified in the genome of *A. phagocytophilum* that may have a function similar to that of AnkA (Sinclair et al., 2015). In addition, genome wide evidence showed that AnkA not only binds to *CYBB* promoter regions, but broadly throughout all chromosomes and correlates with infection-induced differential gene expression (Dumler et al., 2016). Whether *A. phagocytophilum* AnkA is expressed during tick infection is not known. However, it was recently shown that *I. scapularis* has a homolog of the HDAC1 protein that is over-represented in salivary glands in response to *A. phagocytophilum* infection (Cabezas-Cruz et al., 2016). In addition, pharmacological inhibition of tick HDAC1 reduced the load of *A. phagocytophilum* in ISE6 tick cells (Cabezas-Cruz et al., 2016). This result suggests that *A. phagocytophilum* uses similar strategies to manipulate tick and vertebrate host cells (de la Fuente et al., 2016c). The role of *A. phagocytophilum* nucleomodulins during infection provides the molecular basis for specific and genome wide manipulation of host gene expression.

TICK-PATHOGEN INTERACTIONS INCREASE TICK FITNESS

Pathogens must overcome many barriers in order to establish an infection in the tick. Increasing tick fitness by pathogen infection so as to survive would be a *win-win strategy* (de la Fuente et al., 2016b). There are remarkable examples in which pathogens manipulate tick protective responses to facilitate infection but preserving tick feeding and vector capacity to guarantee the survival of both the pathogens and ticks. For example, Neelakanta et al. (2010) demonstrated that *I. scapularis* ticks infected with *A. phagocytophilum* show enhanced fitness against freezing injury due to the induced expression of a tick antifreeze glycoprotein. They further showed that improved survival of infected ticks correlated with higher bacterial infection, therefore providing a direct link between pathogen infection and tick fitness in unfavorable ecological conditions. *A. phagocytophilum* may also affect tick questing behavior by increasing the levels of Heat

Shock Proteins (HSP), which also prevent blood-feeding stress and desiccation at high temperatures (Busby et al., 2012; Villar et al., 2015). Tick questing behavior is essential to find new hosts and survive in nature. Similarly, *A. phagocytophilum* does not manipulate the levels of Subolesin, a protein involved in the tick innate immune response, because it affects infection, tick feeding, and reproduction (de la Fuente et al., 2016b). In contrast, Porin levels are down-regulated by *A. phagocytophilum* infection as a mechanism to inhibit apoptosis, but without affecting tick fitness (Ayllón et al., 2015; Alberdi et al., 2016; de la Fuente et al., 2016b). These results support that *A. phagocytophilum*-induced transcriptional reprogramming selectively manipulates the expression of tick genes that increase tick fitness and therefore pathogen circulation.

Although similar molecular mechanisms have not been described for *Borrelia* spp. and TBEV infections, they also appear to increase tick fitness (Herrmann and Gern, 2015). *Borrelia* and TBEV-infected *I. scapularis* and *I. persulcatus* ticks were found at higher questing heights when compared to uninfected ticks. Higher questing height increases the chances of a tick to find a larger host that could accommodate more ticks increasing their feeding possibilities, but at the same time exposes ticks to more desiccating conditions (Lefcort and Durden, 1996; Romashchenko et al., 2012). Low relative humidity is detrimental for ticks because they spend their energy reserves quicker than at higher relative humidity (Randolph and Storey, 1999). The fact that *Borrelia* and TBEV-infected ticks choose higher questing height suggests that these pathogens help ticks to survive under dry conditions. In agreement with this hypothesis, *I. ricinus* infected by *B. burgdorferi* move less toward a humid environment and their survival is higher in highly desiccating conditions (Herrmann and Gern, 2010, 2012).

TICK-BORNE PATHOGENS HAVE THE POTENTIAL TO LEAD ECOLOGICAL ADAPTATION DURING TICK EVOLUTION

It was generally assumed that DNA changes are the only way information can be passed from parents to the offspring, and that some phenotypic changes acquired during the life span cannot be transmitted to the following generations. Accumulating evidence, however, indicates that both genetic and epigenetic (defined as changes in gene expression due to processes that arise independent of changes in the underlying DNA sequence) have important effects on evolutionary outcomes (Danchin et al., 2011; Gómez-Díaz et al., 2012). While host physiology manipulation by pathogens is a widely accepted phenomenon, we lack evidence of the heritable character of host phenotypes induced by pathogens (Gómez-Díaz et al., 2012; Poulin and Maure, 2015). It was previously proposed that all trans-generational effects on host offspring phenotype that are induced by parasites must involve

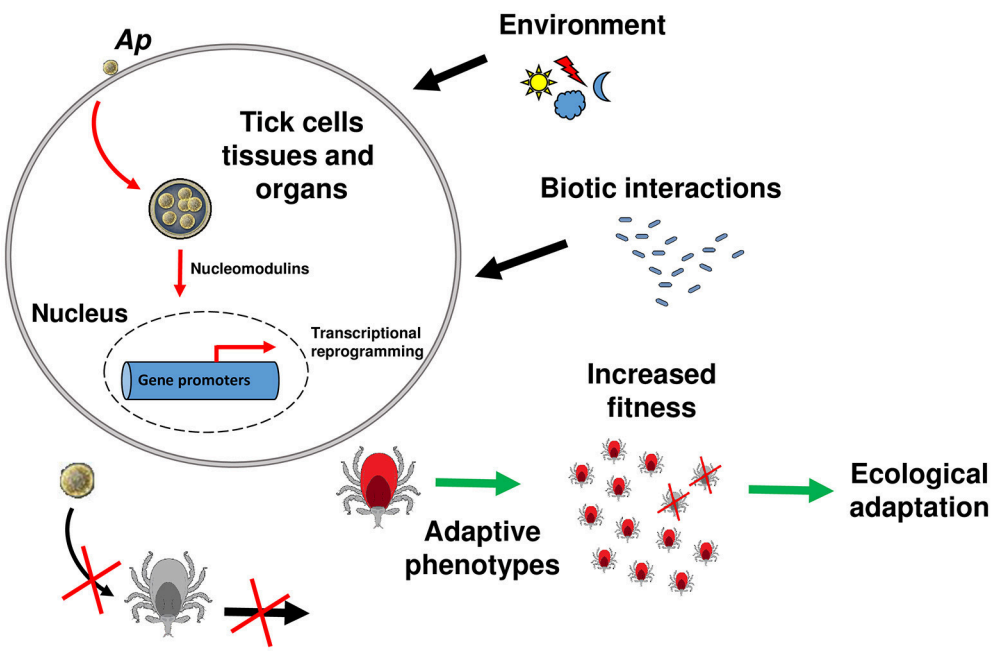

FIGURE 1 | Model of tick ecological adaptation induced by *A. phagocytophilum*. A model is proposed for the general mechanism of tick manipulation by tick-borne pathogens and induction of ecological adaptation. The intracellular bacterium *A. phagocytophilum* (*Ap*) is used as a model. Upon contact with the host membrane or once inside the parasitophorous vacuole, *A. phagocytophilum* secretes nucleomodulins that will enter the tick cell nucleus and recruit histone modifying enzymes (i.e., HDAC1) to modify the expression of target genes. Some of these genes are involved in traits that favor adaptive phenotypes (red ticks) to abiotic factors (e.g., environmental conditions) or biotic factors (e.g., interactions with microorganisms that may be harmful for the ticks). Histone tail modifications (deacetylation/acetylation, methylation/demethylation, etc) resulting from histone modifying enzymes recruitment, will be passed to the next generation. The ticks able to stablish this "*intimate epigenetic relationships*" (Cheeseman and Weitzman, 2015) with the pathogen will have higher fitness compared to the ticks that are not infected (gray ticks). During evolution, this process will lead to tick ecological adaptation and innovation.

a strong epigenetic component (Poulin and Thomas, 2008). Given the demonstrated propensity of tick-borne pathogens to modulate tick gene expression, to do so epigenetically, and the increase in tick fitness that can result, we propose that: pathogen-induced effects on tick phenotype have the potential to be transmitted across generations, therefore accelerating the ecological adaptation of ticks to natural environments (**Figure 1**). The tick antifreeze glycoprotein triggered by *A. phagocytophilum* infection in *I. scapularis* offers a good model to study this phenomenon (Neelakanta et al., 2010). Natural populations of *I. scapularis* in North America inhabit regions where the temperature reaches freezing conditions for much of the winter (Eisen et al., 2016). Ticks infected by *A. phagocytophilum* will be better adapted to cold temperatures in natural environments. It is reasonable to hypothesize that the up-regulation of the antifreeze glycoprotein expression is associated with specific histones or DNA modifications. Inheritance of these epigenetic modifications may transmit the cold-survival phenotype to tick offspring. If this phenotype is advantageous, it may be fixed in the tick population even in the absence of the initial stimulus (i.e., *A. phagocytophilum* infection).

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Serine Protease Inhibitors in Ticks: An Overview of Their Role in Tick Biology and Tick-Borne Pathogen Transmission

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New tick and tick-borne pathogen control approaches that are both environmentally sustainable and which provide broad protection are urgently needed. Their development, however, will rely on a greater understanding of tick biology, tick-pathogen, and tick-host interactions. The recent advances in new generation technologies to study genomes, transcriptomes, and proteomes has resulted in a plethora of tick biomacromolecular studies. Among these, many enzyme inhibitors have been described, notably serine protease inhibitors (SPIs), whose importance in various tick biological processes is only just beginning to be fully appreciated. Among the multiple active substances secreted during tick feeding, SPIs have been shown to be directly involved in regulation of inflammation, blood clotting, wound healing, vasoconstriction and the modulation of host defense mechanisms. In light of these activities, several SPIs were examined and were experimentally confirmed to facilitate tick pathogen transmission. In addition, to prevent coagulation of the ingested blood meal within the tick alimentary canal, SPIs are also involved in blood digestion and nutrient extraction from the meal. The presence of SPIs in tick hemocytes and their involvement in tick innate immune defenses have also been demonstrated, as well as their implication in hemolymph coagulation and egg development. Considering the involvement of SPIs in multiple crucial aspects of tick-host-pathogen interactions, as well as in various aspects of the tick parasitic lifestyle, these molecules represent highly suitable and attractive targets for the development of effective tick control strategies. Here we review the current knowledge regarding this class of inhibitors in tick biology and tick-borne pathogen transmission, and their potential as targets for future tick control trials.

Keywords: ticks, tick serine protease inhibitors, tick-borne pathogens, tick–host interactions, immune responses

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INTRODUCTION

Ticks are among the most common and important vectors of both human and animal pathogens worldwide including some parasites, bacteria and viruses (Dantas-Torres et al., 2012). These obligate hematophagous arthropods are divided into two main families; soft and hard ticks. Tick developmental stages include larval, nymphal and adult forms, all of which—for most species—require blood meals to complete development and enable reproduction. Compared to

other hematophagous arthropods, hard –or *Ixodidae*- tick feeding is a slow and complex process, taking several days until repletion, and thus necessitates extended control over the vertebrate host's immune response. Whereas the soft—or *Argasidae*—ticks usually complete a blood meal in less than 1 h (Sonenshine and Anderson, 2014). During this feeding process, all ticks inject saliva and absorb blood alternately. Blood cells are lysed in the midgut lumen and, in contrast to other hematophagous arthropods, further digestion of proteins and other blood molecules occurs intracellularly, taking place within midgut epithelial cells.

Current tick control strategies essentially rely on the use of chemical acaricides and repellents. Their widespread deployment however, has a profound environmental impact (Rajput et al., 2006; De Meneghi et al., 2016), and has led to the emergence of resistance in multiple tick species (Rosario-Cruz et al., 2009; Adakal et al., 2013). New environmentally sustainable approaches providing broader protection against current and future tick-borne pathogens (TBP) are thus urgently needed. To investigate new candidate pathways to fight the spread of these pathogens, a complete understanding of tick biology, tick-pathogen and tick-host interactions is essential. Since the beginning of the twenty first century the continual development of cutting-edge high-throughput methods has enabled the study of genomes, transcriptomes, and proteomes, thus facilitating many diverse biomolecular studies (Metzker, 2010). These tools have been essential in the discovery of specific tick biological gene products. The most studied tick species have been those that present significant human and/or livestock disease risk in the northern hemisphere: *Ixodes scapularis* in the USA (deer; black-legged tick); *Ixodes persulcatus* in Asia and Eastern Europe (Taiga tick); and *Ixodes ricinus* in western and central Europe (sheep tick). Additionally, the *Rhipicephalus (Boophilus) microplus* cattle tick that causes massive damage in Australia, Africa, Central America, and Asia has also been intensively studied.

Most studies have investigated specific tick organ transcriptomes under a variety of conditions, especially tick salivary glands (SGs) (Santos et al., 2004; Francischetti et al., 2005b; Ribeiro et al., 2006; Garcia et al., 2014; Liu et al., 2014) or midgut (Anderson et al., 2008; Chmelar et al., 2016), occasionally eggs or ovaries (Santos et al., 2004), and less frequently hemocytes (Santos et al., 2004; Kotsyfakis et al., 2015), body fat or synganglia (Bissinger et al., 2011; Egekwu et al., 2014). Several comprehensive protein catalogs describing protein diversity in various tick fluids such as saliva (Madden et al., 2004; Cotté et al., 2014; Radulovic et al., 2014; Tirloni et al., 2014a, 2015) or hemolymph (Gudderra et al., 2002; Stopforth et al., 2010), as well as in midgut during feeding (Schwarz et al., 2014; Oleaga et al., 2015), have been compiled, vital to understanding mechanisms implicated in different biological processes such as tick feeding or tick immunity.

Several studies also reported that TBP can influence gene and protein expression in tick, highlighting evidence of molecular interaction between pathogens and the vector (review in Liu and Bonnet, 2014). These studies focused on specific organs including SGs, midgut, ovaries, or on the whole tick during infections with several different pathogens, and reported differential expression of tick's genes links to pathogen transmission. TBP are imbibed by tick when feeding on a pathogen-infected vertebrate host and, once ingested, they directly or not -depending of the pathogen- escape the midgut and invade the SGs and the ovaries for vertically transmitted pathogens (see Liu and Bonnet, 2014). Then, for most TBP, transmission to a new host occurs via the saliva during blood feeding. During both their transmission and development into the vector, TBP undergo developmental transitions and migrations and suffer population losses, to which tick factors surely contribute. In addition, during the prolonged tick-host attachment period, many proteins injected into the host via tick saliva dampen host defenses, thereby creating a favorable environment for survival and propagation of TBP (Brossard and Wikel, 2004; Nuttall and Labuda, 2004; Ramamoorthi et al., 2005; Wikel, 2013).

Many enzyme activity inhibitors were described among the transcripts or proteins detected in these studies, including multiple protease inhibitors often belonging to serine protease inhibitor families. These inhibitors can vary in molecular weight from less than 10 kDa to almost 100 kDa, and can reversibly or irreversibly inhibit their targets via family-specific domains. Their global tissue expression suggests involvement in various important tick biological pathways, including innate immunity, hemolymph clotting formation, blood uptake, digestion, as well as oviposition and egg laying. In addition, tick serine protease inhibitors (tSPIs) also modulate vertebrate host responses during biting, act on hemostasis, immune responses, or angiogenesis. Their implications in these various processes suggest that tSPIs can indirectly influence tick pathogen transmission, and indeed some have been directly experimentally linked with TBP transmission. The aim of the present review is to summarize current knowledge concerning these tSPIs (detailed in **Table 1**), in order to highlight their role in tick biology, TBP transmission, and to identify putative targets which could contribute to effective tick and TBP control strategies.

Abbreviations: α2M, alpha 2-macroglobulin; APC, antigen presenting cells; aPTT, activated partial thromboplastin time; BPTI, bovine pancreatic trypsin inhibitor; BSAP, $BaSO4^{-}$ adsorbing protein; CAMs, chorioalantoic membranes; Efh, EF hand; FCT, fibrinogen clotting time; FRP, follistatin-related-protein; HBE, heparin binding exosite; HMWK, high molecular weight kininogen; hNE, human neutrophil elastase; hTFPI, human tissue factor pathway inhibitor; HuPK, human prekalikrein; HUVEC, human umbilical vein endothelial cells; LICI, limulus intracellular coagulation inhibitor; MA, methyl amine; PAR, proteinase activated receptor; PARP, poly (ADP-r-ribose) polymerase; PDGF, platelet-derived growth factor; PECAM-1, platelet-endothelial cell adhesion molecule-1; PPE, porcine pancreatic elastase; PT, prothrombin time; RCL, reactive center loop; RCT, recalcification time assay; RNAi, RNA interference; SGs, salivary glands; STAT 3, signal transducer and activator of transcription 3; STI, soybean trypsin inhibitor; TBP, tick-borne pathogens; TF, tissue factor; TGF-alpha, tissue growth factor alpha; TIL, trypsin inhibitor-like domain; tSPI, tick serine protease inhibitor; TT, thrombin clotting time; VEGF, vascular endothelial growth factor.

THE SERINE PROTEASE INHIBITOR FAMILY

Four groups of serine protease inhibitors have been identified in plants and animals, and can be classified into two main

TABLE 1 | Tick serine protease inhibitors implicated in both tick biology/physiology and modulation of vertebrate host responses to tick bite, classified according to their inhibitor group (Serpin, Macroglobuline, Kunitz, Kazal), and the corresponding tick species.

Inhibitor name	Molecular weight (kDa)	Inhibitor type	Tick species	Action	References
TICK IMMUNE SYSTEM FACTORS					
TAM	420	α2M	*O. moubata*	Tick immune defense	Kopacek et al., 2000
IrAM	440	α2M	*I. ricinus*	Antimicrobial activity	Buresova et al., 2009
BmCI	6.5	Kunitz	*R. (B.) microplus*	Antimicrobial activity	Lima et al., 2010
DvKPI	62	Kunitz	*D. variabilis*	Antimicrobial activity	Ceraul et al., 2008
Ixodidin	7.1	Trypsin Inhibitor Like (TIL)	*R. (B.) microplus*	Antimicrobial activity	Fogaça et al., 2006
BmSI 6-7	7.4, 7.3	Trypsin Inhibitor Like (TIL)	*R. (B.) microplus*	Antimicrobial activity and tissue preservation	Sasaki et al., 2008
BmTI-A	13.5	Kunitz	*R. (B.) microplus*	Probable antimicrobial activity	Tanaka et al., 1999
HEMOLYMPH CLOTTING FACTORS					
HLS 2	44	Serpin	*H. longicornis*	Hemolymph clot formation	Imamura et al., 2005
HLSG-1	37.7	Serpin	*H. longicornis*	Hemolymph clot formation	Mulenga et al., 2001
RAS 3-4	43.2, 53.9	Serpin	*R. appendiculatus*	Hemolymph clot formation	Mulenga et al., 2003b
BLOOD UPTAKE AND DIGESTION MODULATORS					
HLSG-2	31.2	Serpin	*H. longicornis*	Probable blood digestion helper	Mulenga et al., 2001
HlMKI	12	Kunitz	*H. longicornis*	Probable blood digestion helper	Miyoshi et al., 2010
HLS-1	41	Serpin	*H. longicornis*	Probable blood uptake and digestion helper	Sugino et al., 2003
HlChI	6.7	Kunitz	*H. longicornis*	Probable blood digestion helper	Alim et al., 2012
RAMSP 1-3	32.3, 51.2, 49.5	_	*R. appendiculatus*	Probable blood digestion helper	Mulenga et al., 2003a
RAS-1 and -2	41.9, 42.7	Serpin	*R. appendiculatus*	Probable blood digestion helper	Mulenga et al., 2003b
AAS19	43	Serpin	*A. americanum*	Probable blood digestion helper	Kim et al., 2015
RMS-3 -6 -9 -13 -15 -16 -17 -21 -22	40-55	Serpin	*R. (B.) microplus*	Probable blood digestion helper	Tirloni et al., 2014a,b; Rodriguez-Valle et al., 2015
BmTI-A	13.5	Kunitz	*R. (B.) microplus*	Probable blood digestion helper	Sasaki et al., 2004
BmTI-D	1.6	Kunitz	*R. (B.) microplus*	Probable blood digestion helper	Sasaki et al., 2004
AamS6	42	Serpin	*A. americanum*	Probable blood digestion helper	Chalaire et al., 2011; Mulenga et al., 2013
Ixophilin	54.4	Kunitz	*I. scapularis*	Probable blood digestion helper	Narasimhan et al., 2013
TICK DEVELOPMENT, OVIPOSITION, EGG LAYING, AND MOLTING FACTORS					
BmTIs	6.2-18.4	Kunitz	*R. (B.) microplus*	Tick egg production and development	Tanaka et al., 1999
RMS-3	40	Serpin	*R. (B.) microplus*	Tick reproduction egg production	Rodriguez-Valle et al., 2012
RMS-6	40	Serpin	*R. (B.) microplus*	Probable role in embryogenesis	Rodriguez-Valle et al., 2012
RMS-19	40.7	Serpin	*R. (B.) microplus*	Role in tick development	Rodriguez-Valle et al., 2012
RMS-20	31.1	Serpin	*R. (B.) microplus*	Role in tick development	Rodriguez-Valle et al., 2012
RMS-21	12.5	Serpin	*R. (B.) microplus*	Probable role in embryogenesis	Rodriguez-Valle et al., 2012
RMS-22	10.7	Serpin	*R. (B.) microplus*	Probable role in embryogenesis	Rodriguez-Valle et al., 2012
RmKK	16.7	kunitz	*R. (B.) microplus*	Probable protection of undesired egg proteolysis	Abreu et al., 2014
BmTI-6	33.8	kunitz	*R. (B.) microplus*	Regulation of egg production and proteases in eggs and larvae	Andreotti et al., 2001; Sasaki et al., 2004; Sasaki and Tanaka, 2008
RsTIs	8-18	kunitz	*R. (B.) microplus*	Regulation of egg production and proteases in eggs and larvae	Sant'Anna Azzolini et al., 2003
Tick FRP	32	Kazal	*H. longicornis*	Role in tick oviposition	Zhou et al., 2006
AAS19	43	Serpin	*A. americanum*	Role in tick oviposition	Kim et al., 2016
HOST-EXTRINSIC PATHWAY TICK INHIBITORS					
Ixolaris	15.7	Kunitz	*I. scapularis*	Blocks FVIIa/TF complex activity	Francischetti et al., 2002
Penthalaris	35	Kunitz	*I. scapularis*	Block FVIIa/TF complex activity	Francischetti et al., 2004

(Continued)

TABLE 1 | Continued

Inhibitor name	Molecular weight (kDa)	Inhibitor type	Tick species	Action	References
BSAP1	9.3	_	*O. savignyi*	Targets tissue factor (TF)	Ehebauer et al., 2002
BSAP2	9.1	_	*O. savignyi*	Targets tissue factor (TF)	Ehebauer et al., 2002
HOST-INTRINSIC PATHWAY TICK INHIBITORS					
IrCPI	9.7	Kunitz	*I. ricinus*	Blocks FXII, FXI, and kallikrein activation	Decrem et al., 2009
BmTI-A	13.5	Kunitz	*R. (B.) microplus*	Blocks plasmin, elastase, and plasma kallikrein	Tanaka et al., 1999
Rhipilin-2	22	Kunitz	*R. (B.) microplus*	Affects APTT test clotting time	Cao et al., 2013
Haemaphysalin	–	Kunitz	*H. longicornis*	Blocks kallikrein-kinin system activation	Kato et al., 2005a,b
DvKPI	62	Kunitz	*D. variabilis*	Affects APTT test clotting time	Ceraul et al., 2008
HOST-FX(a) FACTOR TICK INHIBITORS					
TAP	7	Kunitz	*O. moubata*	Blocks FXa activity	Waxman et al., 1990
FXa inhibitor	7	Kunitz	*O.savignyi*	Blocks FXa activity	Gaspar et al., 1996
	17	_	*H. truncatum*	Blocks FXa activity	Joubert et al., 1995
	15	_	*H. dromaderii*	Blocks FXa activity	Ibrahim et al., 2001b
	65	Serpin	*R. appendiculatus*	Blocks FXa activity	Limo et al., 1991
AAS19	43	Serpin	*A. americanum*	Blocks FXa and plasmin action	Kim et al., 2015, 2016
Amblyomin-X	13.5	Kunitz	*A. cajennense*	Blocks FVIIa/TF complex activity and prothombin conversion	Batista et al., 2010; Branco et al., 2016
HOST-THROMBIN INHIBITING TICK FACTOR					
BmAP	60	_	*R. (B.) microplus*	Thrombin inhibitor	Horn et al., 2000
Microphilin	1.7	_	*R. (B.) microplus*	Thrombin inhibitor	Ciprandi et al., 2006
BmGTI	26	_	*R. (B.) microplus*	Thrombin inhibitor	Ricci et al., 2007
RMS 15	48		*R. (B.) microplus*	Thrombin inhibitor	Rodriguez-Valle et al., 2015; Xu et al., 2016
Boophilin	13.9	Kunitz	*R. (B.) microplus*	Thrombin inhibitor	Macedo-Ribeiro et al., 2008; Assumpço et al., 2016
Ixin	–	_	*I. ricinus*	Thrombin inhibitor	Hoffmann et al., 1991
Iris	43	Serpin	*I. ricinus*	Thrombin inhibitor	Leboulle et al., 2002b
Madanin 1	67	_	*H. longicornis*	Thrombin inhibitor	Iwanaga et al., 2003
Madanin 2	71	_	*H. longicornis*	Thrombin inhibitor	Iwanaga et al., 2003
Chimadanin	7.4	_	*H. longicornis*	Thrombin inhibitor	Nakajima et al., 2006
HLS2	44	Serpin	*H. longicornis*	Weak thrombin inhibitor	Imamura et al., 2005
Hemalin	20	Kunitz	*H. longicornis*	Thrombin inhibitor	Liao et al., 2009
IxSc-1E1	45	Serpin	*I. scapularis*	Thrombin inhibitor	Ibelli et al., 2014
Americanin	12	_	*A. americanum*	Thrombin inhibitor	Zhu et al., 1997
Amblin	17.4	Kunitz	*A. hebraeum*	Thrombin inhibitor	Lai et al., 2004
Variegin	3.7	_	*A. variegatum*	Thrombin inhibitor	Kazimírová et al., 2002
Hyalomin 1-4	8.4, 8.5, 8.2, 7.4	_	*H. marginatum rufipes*	Thrombin inhibitor	Francischetti et al., 2011; Jablonka et al., 2015
NTI 1	3.4	_	*H. dromaderii*	Thrombin inhibitor	Ibrahim et al., 2001a
NTI 2	14.9	_	*H. dromaderii*	Thrombin inhibitor	Ibrahim et al., 2001a
Rhipilin-1	18	Kunitz	*R. haemaphysaloides*	Thrombin inhibitor	Gao et al., 2011
RHS-1	41.9	Serpin	*R. haemaphysaloides*	Thrombin inhibitor	Yu et al., 2013
RHS-2	42.7	Serpin	*R. haemaphysaloides*	Thrombin inhibitor	Yu et al., 2013
Calcaratin	14.5	_	*R. B. calcaratus*	Thrombin inhibitor	Motoyashiki et al., 2003
–		_	*I. holocyclus*	Thrombin inhibitor	Anastopoulos et al., 1991
Ornithodorin	12	Kunitz	*O. moubata*	Thrombin inhibitor	van de Locht et al., 1996
Savignin	12	_	*O. savignyi*	Thrombin inhibitor	Mans et al., 2002
Monobin	15	Kunitz	*A. monolakensis*	Thrombin inhibitor	Mans et al., 2008

(Continued)

TABLE 1 | Continued

Inhibitor name	Molecular weight (kDa)	Inhibitor type	Tick species	Action	References
HOST-IMMUNE SYSTEM MODULATION BY TICK FACTORS					
BmSI-7	7.3	_	*R. (B.) microplus*	Elastase inhibitor	Sasaki et al., 2008
Lopsins	43-44	Serpin	*A. americanum*	Probable anti-inflammatory action	Mulenga et al., 2007
AamS6	42	Serpin	*A. americanum*	Inhibits elastase, plasmin, and chymase	Chalaire et al., 2011; Syrovets et al., 2012
AAS19	43	Serpin	*A. americanum*	Inhibits plasmin	Syrovets et al., 2012; Kim et al., 2015, 2016
Iris	43	Serpin	*I. ricinus*	Inhibits elastase-like proteases and suppresses pro-inflammatory cytokine secretion	Leboulle et al., 2002a; Prevot et al., 2009
Ipis-1	41.7	Serpin	*I. persulcatus*	Modulates CD14+ cells activation	Toyomane et al., 2016
IRS-2	41.9	Serpin	*I. ricinus*	Modulates T cell differentiation, T17 cell maturation and inhibits chymase and cathepsin G	Chmelar et al., 2011
Tryptogalinin	10.3	Kunitz	*I. scapularis*	Inhibits elastase, tryptase, plasmin, matryptase	Payne and Kam, 2004; Dai et al., 2012; Valdés et al., 2013
Tdpi	11.1	Kunitz	*R. appendiculatus*	Inhibits plasmin and tryptase	Paesen et al., 1999, 2007
RMS-3	40	Serpin	*R. (B.) microplus*	Probable interaction/modulation of B cell action	Rodriguez-Valle et al., 2012
BmTI 2 -3	17,1, 3.1	Kunitz	*R. (B.) microplus*	Inflammatory response modulation	Sasaki et al., 2004
HOST ANGIOGENESIS MODULATION AND APOPTOSE INDUCTION BY TICK FACTORS					
BmCI	6.5	Kunitz	*R. (B.) microplus*	Pro-apoptotic role and inhibits cell proliferation	Lima et al., 2010
Haemangin	14.1	Kunitz	*I. scapularis*	Abolishes angiogenesis and neovascularization	Islam et al., 2009
BmTI-A	13.5	Kunitz	*R. (B.) microplus*	Inhibits plasma kallikrein, plasmin, and elastase, cell proliferation and migration	Soares et al., 2012, 2016
Amblyomin-X	13.5	Kunitz	*A. cajennense*	Tumor cell cycle alteration, proteasome inhibitor, caspase cascade activation, angiogenesis repressor	Chudzinski-Tavassi et al., 2010; Morais et al., 2016
ROLE IN TICK-BORNE PATHOGEN TRANSMISSION AND DEVELOPMENT					
BmTI-A	13.5	Kunitz	*R. (B.) microplus*	Limits *B. bovis* proliferation in ticks	Rachinsky et al., 2007
DvKPI	62	Kunitz	*D. variabilis*	Modulates Rickettsia development	Ceraul et al., 2008
IrSPI	12	Kunitz	*I. ricinus*	Modulates *Bartonella henselae* development	Liu et al., 2014
Ixophilin	54.4	Kunitz	*I. scapularis*	Probable role in *B. burgdorferi* development	Narasimhan et al., 2013
TICK MOLECULES TESTED IN VACCINE PROJECTS					
RAS-1	41.9	Serpin	*R. appendiculatus*	Impacts engorgement and tick viability	Imamura et al., 2006
RAS-2	42.7	Serpin	*R. appendiculatus*	Impacts engorgement and tick viability	Imamura et al., 2006
BmTIs	6.2-18.4	Kunitz	*R. (B.) microplus*	Impacts tick viability and engorged tick weight	Andreotti et al., 2002
HLS-1	41	Serpin	*H. longicornis*	Impacts tick viability and the developmental cycle	Sugino et al., 2003
RmLTI	46	Kunitz	*R. (B.) microplus*	Impacts egg eclosion, viability, and larval hatchability	Andreotti et al., 2012

categories: trapping inhibitors including the serpins and the α2 macroglobulines (α2M); and tight-binding inhibitors including the Kunitz or Kazal domain-containing proteins (**Figure 1**). Trapping inhibition results in proteolytic cleavage, whereas proteases bound to tight-binding inhibitors can be released undamaged, while the inhibitors are liberated in either native

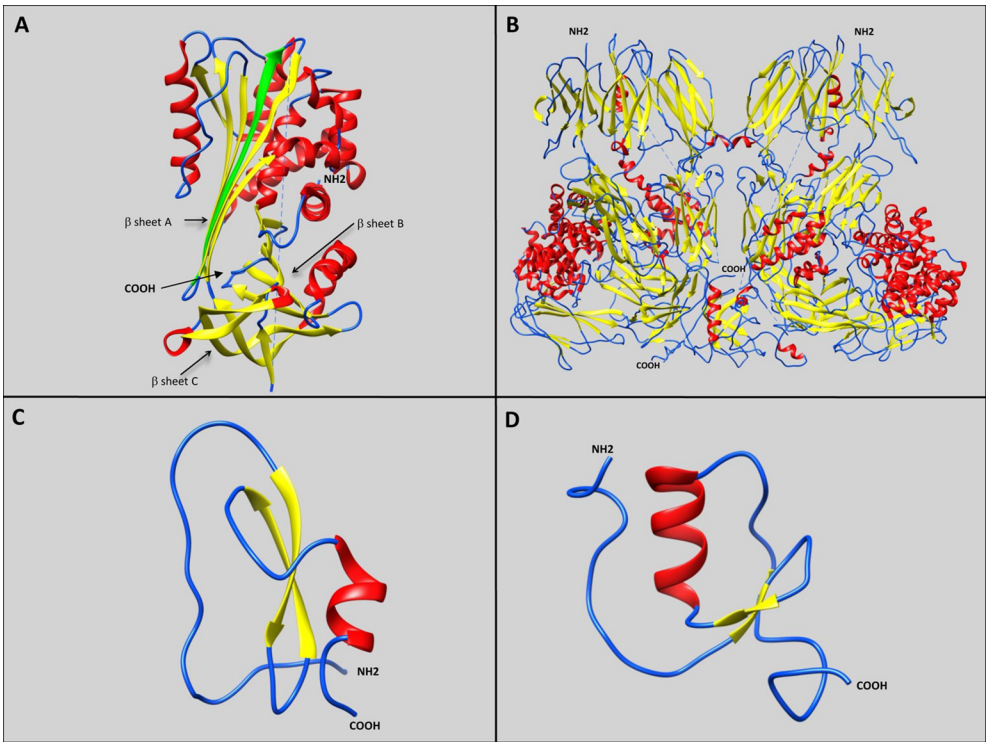

FIGURE 1 | Representative schematic diagrams of secondary structures of the four types of serine protease inhibitors: serpins, α2 macroglobulins, Kunitz-type inhibitors, and Kazal-type inhibitors. Loops, β-sheets, and α-helices are labeled with blue, yellow, and red, respectively. **(A)** IRS 2 serpin from the *I. ricinus* tick showing the three β-sheets A, B, and C (Chmelar et al., 2011; PDB accession number: 3NDA). The green arrow represents the additional strand (s4A) formed after proteolysis, resulting in Reactive Center Loop cleavage, and insertion of the amino-terminal portion into the A β-sheet. **(B)** Human α2 macroglobulin complement component 5 in native homodimer conformation (Fredslund et al., 2008; PDB accession number: 3CU7). **(C)** Kunitz-type inhibitor Tick-Derived Protease Inhibitor (TDPI) from *R. appendiculatus* harboring one Kunitz domain composed of β-sheets and an α-helix stabilized by three highly conserved disulphide bridges (Paesen et al., 2007; PDB accession number: 2UUX). **(D)** Kazal-type inhibitor Dipetalin from blood-sucking insect *Dipetalogaster maximus*. harboring one Kazal domain composed of one α-helix with adjacent ß-sheets and loop (Schlott et al., 2002; PDB accession number: 1KMA).

or cleaved forms. Target-protease interaction occurs via the reactive center loop (RCL), which demonstrates a range of different conformations and a high degree of conformational flexibility, with each inhibitor family displaying characteristic serine protease inhibitory mechanisms. Serine protease inhibitors can be classified into two functional groups based on their ability to inhibit either trypsin or chymotrypsin: inhibitors of trypsin-like proteases such as thrombin, or inhibitors of chymotrypsin-like proteases such as elastase.

Trapping Inhibitors

Serpins

Serpins form a large group of homologous proteins that appear to be ubiquitous in multicellular eukaryotes. They are composed of approximately 400 amino acids and are often glycosylated. Serpins fold into an NH_2-terminal helical domain and a COOH-terminal beta-sheet domain (**Figure 1A**). The serpin fold consisting of three beta-sheets (A-C) with eight or nine alpha-helical linkers and an exposed ∼20 residue RCL acts as bait for the protease target (Gubb et al., 2010). An unusual aspect of serpins is their native unstable fold, where the RCL is on top and the beta-sheet A is outward. Following proteolysis however, the RCL is cleaved, and the RCL amino-terminal portion inserts into the center of beta-sheet A to form an additional (fourth) strand (s4A), which effectively stabilizes the complex structure (Law et al., 2006). The protease is thus denatured and the serpin/protease complex is targeted for degradation (Huntington et al., 2000). This amino-terminal RCL insertion can either occur upon proteolytic cleavage, or spontaneously (Huntington, 2011). Hence serpins interact with their target via a "suicide-cleavage" mechanism, resulting in the formation of an inactive covalently linked serpin/protease complex. While the majority of serpins inhibit serine proteases, they can also bind to several others such as cysteine proteases, metalloproteases, caspases (Ray et al., 1992), papain-like cysteine proteases (Irving et al., 2002), as well as some non-protease ligands, such as collagen, DNA, or protein Z. Serpins have also been ascribed several additional roles, such as heparin or heparin sulfate co-factor (Khan et al., 2011), as well as rare non-inhibitory functions; as a hormone transporter (Pemberton et al., 1988), molecular chaperone (Nagata, 1996), or tumor suppressor (Zou et al., 1994).

α2-Macroglobulins

Members of α2-macroglobulin (α2M) group have been identified in a broad spectrum of vertebrate and invertebrate species and comprise the C3, C4, and C5 components of the vertebrate complement system (Sottrup-Jensen et al., 1985). α2Ms are considered as early-acting innate immunity components, similar

to opsonin, but their role in the proteolytic attack of invading pathogens remains hypothetical. Most α2Ms are tetramers assembled from pairwise subunits with disulfide-bridges, but monomeric and dimeric forms also exist, the latter more common in invertebrates (**Figure 1B**) (Starkey and Barrett, 1982). Interaction with targeted proteases is initiated by proteolytic cleavage at a defined motif characterized by an exposed and highly flexible 30–40 amino acid residue region (Sottrup-Jensen, 1989). The inhibitory activity of α2Ms is directly due to their thiol-ester bond, which can be abolished by small amines such as methylamine (Larsson and Bjork, 1984). This bait region with multiple cleavage sites inhibits a broad range of proteases including serine-, cysteine-, aspartic- and metallo-proteases (Sottrup-Jensen, 1989). In addition, α2Ms could play a role as hormone transporters (Peslova et al., 2009), and can counteract inhibition from other high molecular weight inhibitors by protecting protease active sites (Armstrong et al., 1985).

Tight-Binding Inhibitors

Kunitz/BPTI Inhibitors

Initially discovered at high concentrations in beans, Kunitz proteins are typically small proteins with a molecular weight close to or less than 20 kDa (Kunitz, 1945). The most well-studied inhibitor from this family is the bovine pancreatic trypsin inhibitor (BPTI) that gives the family its name (Creighton, 1975). Generally they contain between one to twelve Kunitz domains (Laskowski and Kato, 1980), and each domain encloses disulphide-rich α helices and ß-folds stabilized by three highly-conserved disulphide bridges, leading to a compact and stable molecule (Ranasinghe and McManus, 2013) (**Figure 1C**). Inhibitors of this kind possess a bait region inhibiting targeted proteases that precisely matches the enzyme's catalytic site, thus generating a particularly stable substrate/inhibitor complex (Ram et al., 1954). Through the RCL region, inhibitors block the serine protease active site with a tight non-covalent interaction without any conformational changes -similarly to enzyme-substrate Michaelis complex- forming a ß-sheet between the enzyme and its inhibitor (Ascenzi et al., 2003; Krowarsch et al., 2003; Chand et al., 2004). Despite opening of the bait ring region following proteolysis, the free cut ends tend to maintain the initial fold, so the hydrolysis reaction is likely to be reversible with an equilibrium between cleaved and uncleaved [inhibitor/protease] complexes (Laskowski and Qasim, 2000). The tight-binding exposed RCL loop of Kunitz/BPTI inhibitors is suited to a wide variety of protein folds suggesting a large range of possible protease targets. However, some Kunitz domain-containing proteins with RCL region substitutions have other functions such as ion channel blockers or snake toxins (Grzesiak et al., 2000).

Kazal Inhibitors

Initially identified in vertebrates, Kazal inhibitors have also been identified in several invertebrates (Rimphanitchayakit and Tassanakajon, 2010). They can carry from two to fifteen Kazal domains (Rawlings et al., 2004), which have between 40 and 60 amino acids of variable sequence, except for six well-conserved cysteine residues able to form three disulfide-linked sub-domains (Cerenius et al., 2010; Rimphanitchayakit and Tassanakajon, 2010). These domains comprise one α helix with three adjacent ß sheets and loops, that precisely fit the active sites of targeted proteases and block them stoichiometrically, resulting in a relatively stable protease/inhibitor complex (**Figure 1D**) (Laskowski and Kato, 1980). Although non-covalent binding occurs, Kazal inhibitor and protease association is tight, resulting in strong inhibition (Somprasong et al., 2006; Wang et al., 2009). Interactions between Kazal domains and proteases can occur via multiple different amino acids, thus influencing binding intensity and specificity. This enables the inhibition of several targets including trypsin, plasmin, porcine pancreatic elastase (PPE), human neutrophil elastase (hNE), chymotrypsin, proteinase K, or thrombin (Rimphanitchayakit and Tassanakajon, 2010).

ROLE OF SERINE PROTEASE INHIBITORS IN TICK BIOLOGY

Tick Immune System

It is well known that ticks possess innate immunity that also affects their vector competence (Hajdusek et al., 2013). Although all of the involved mechanisms have not yet been fully clarified, microbe phagocytosis by tick hemocytes seems to be coupled to a primitive complement-like system, a variety of antimicrobial peptides and possibly reactive oxygen species (Kopacek et al., 2010). Studies in both hard and soft ticks have implicated several tSPIs in immune responses against different microbes, mostly identified in hemolymph (**Figure 2**, **Table 2**).

The most studied tick species in this context is *R. (B.) microplus.* Firstly, **Ixodidin**, a tSPI discovered in hemocytes, was reported to have strong inhibitory activity against *Micrococcus luteus* and, to a lesser extent against *Escherichia coli* (Fogaça et al., 2006). In addition to this bacterial clearance role, Ixodidin also possesses inhibitory activity against chymotrypsin and elastase serine proteases (Fogaça et al., 2006). However, it remains unclear whether the antimicrobial activity is due to protease inhibition or directly through peptide effects on bacterial membranes. Two additional inhibitors have been identified from *R. (B.) microplus* eggs: **BmSI 6** and **BmSI 7** (*Boophilus microplus* subtilisin inhibitors), that target Pr1 proteases from *Metarhizium anisopliae*, a fungus used as a biological insecticide (Sasaki et al., 2008). These Pr1 proteases induce host cuticle degradation, enabling hyphae penetration to obtain nutrition (Leger et al., 1987). Only *BmSI 7*—which shares several similarities with Ixodidin—has been well characterized. It harbors disulphide bonds and a trypsin inhibitor-like cysteine-rich domain (TIL), and as it is expressed in adult ovaries, midgut, SGs, hemocytes, body fat, and larvae, it could be involved in tick defense mechanisms, and/or in avoiding tick tissue degradation (Sasaki et al., 2008). Random sequencing of a tick body fat cDNA library enabled the discovery of, **BmCI** (*Boophilus microplus* chymotrypsin inhibitor), a chymotrypsin inhibitor that belongs to the Kunitz/BPTI inhibitor family (Lima et al., 2010).

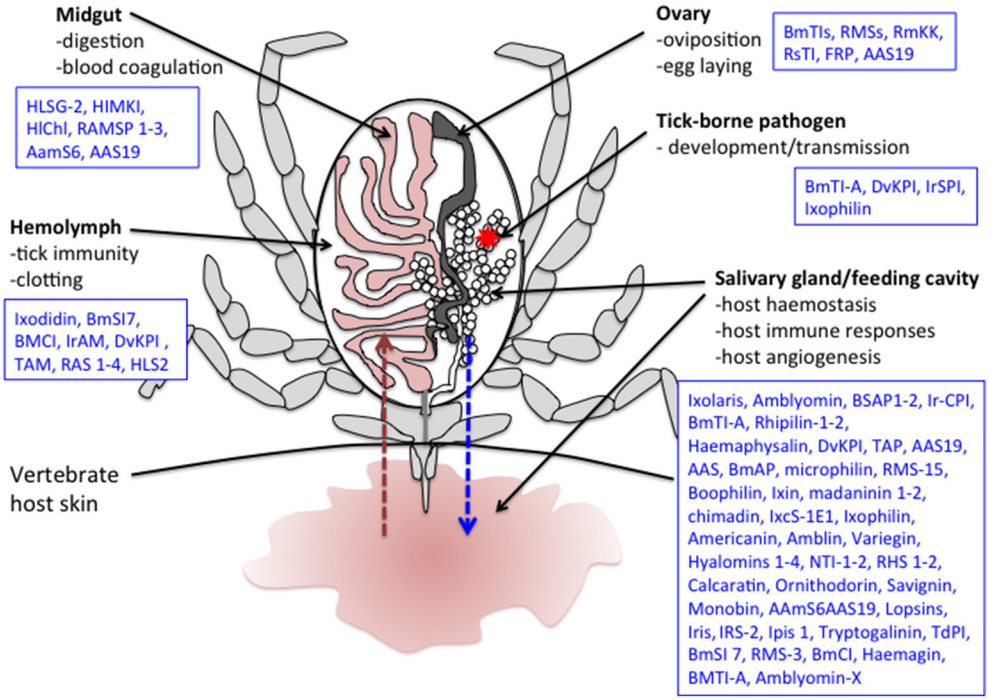

FIGURE 2 | Schematic representation of a tick during feeding. Several tick serine protease inhibitors identified in different tick species and implicated in both tick biology/physiology and modulation of the vertebrate host responses to tick bite are also indicated. The red arrow represents blood absorption. The blue arrow represents saliva injection. The red star corresponds to a tick-borne pathogen. Note that only half of the digestive tract and a single salivary gland and ovary are represented here. *Boophilus microplus* subtilisin inhibitors (BmSI); *Boophilus microplus* Chymotrypsin inhibitor (BmCI); *Ixodes ricinus* alpha macroglobuline (IrAM); *Dermacentor variabilis* Kunitz protease inhibitor (DvKPI); Tick α-Macroglobulin (TAM); *Boophilus microplus* trypsin inhibitor (BmTIs); *R. appendiculatus* Serpins (RAS); *H. longicornis* serine proteinase genes (HLSG); *Haemaphysalis longicornis* midgut Kunitz-type inhibitor (HlMKI); *Haemaphysalis longicornis* serpin (HLS); *Haemaphysalis longicornis* chymotrypsin inhibition (HlChl); *R. appendiculatus* Midgut Serine proteinases (RAMSP); *R.B. microplus* Serpin (RMS); *Amblyomma americanum* serpin 6 (AamS6); *Amblyomma americanum* serpin 19 (AAS19); *R.B. microplus* Kunitz kallikrein inhibitor (RmKK); *I. ricinus* contact phase inhibitor (IrCPI); Tick Anticoagulant Protein (TAP); *Boophilus microplus* Anticoagulant Protein (BmAP); *B. microplus* gut thrombin inhibitor (*BmGTI*); Nymph thrombin inhibitor (NTI); *Rhipicephalus haemaphysaloides* serpin (RHS); *I. ricinus* immunosuppressive (Iris); *I. ricinus* serpin (IRS); *Ixodes persulcatus* immunosuppressive (Ipis); *I. ricinus* serine protease inhibitor (IrSPI); *R. microplus* Larval Trypsin Inhibitor (RmLTI).

BmCI strongly and specifically inhibits chymotrypsin, and also hNE with reduced specificity. *BmCI* gene expression analysis demonstrated higher expression in hemocytes and ovaries than in SGs and body fat. Following infection with *M. anisopliae*, increased BmCI expression was only observed in tick hemocytes clearly suggesting a role in the tick defense system (Lima et al., 2010). Finally, among BmTIs (*Boophilus microplus* trypsin inhibitors) identified in both larvae and eggs (Tanaka et al., 1999), a double Kunitz-containing inhibitor, **BmTI-A**, had increased transcript levels in ovaries following *Babesia bovis* infection (normally transmitted transovarially in ticks), also suggesting a probable role in the tick immune system (Rachinsky et al., 2007).

IrAM (*I. ricinus* alpha macroglobuline), was identified in the hemolymph of *I. ricinus* as a α2M composed of two non-covalently linked subunits (Buresova et al., 2009). Alternative splicing occurring during IrAM synthesis generates seven bait variants, increasing the spectrum of targeted proteases. IrAM transcripts are detected throughout all tick developmental stages. Gene expression was higher in SGs from partially engorged females than in hemocytes or ovaries, even though IrAM protein is only detected in tick hemocytes. RNAi experiments revealed that IrAM is not involved in tick fitness, mortality, or fecundity (Buresova et al., 2009). However, IrAM enhanced phagocytosis and elimination of the bacteria *Chryseobacterium indologenes* due to its active thioester bonds, which was not observed with other bacteria such as *B. burgdorferi* (Buresová et al., 2006; Buresova et al., 2009). IrAM likely interacts with the major *C. indologenes* virulence factor, a metalloprotease, suggesting a role in the tick immune system during phagocytosis of metalloprotease-producing bacteria (Buresova et al., 2009).

Following the discovery of over-expressed genes in *Dermacentor variabilis* tick body fat and midgut in response to *Rickettsia montanensis* infection (Ceraul et al., 2007), several Kunitz/BPTI inhibitors were identified including **DvKPI** (*Dermacentor variabilis* Kunitz protease inhibitor with five Kunitz domains) (Ceraul et al., 2008). DvKPI was up-regulated six-fold in infected ticks that had fed for three days. These results suggested that DvKPI is involved in tick responses to Rickettsia, avoiding massive colonization which would be detrimental to ticks.

TABLE 2 | Antimicrobial activities of serine protease inhibitors of ticks (tSPI).

Targeted microbe	tSPI	Tick species	References
Micrococcus luteus	Ixodidin	*R. (B.) microplus*	Fogaça et al., 2006
Escherichia coli	Ixodidin	*R. (B.) microplus*	Fogaça et al., 2006
Metarhizium anisopliae	BmSI 6, BmSI 7	*R. (B.) microplus*	Sasaki et al., 2008
	BmCI		Lima et al., 2010
Babesia bovis	BmTI-A	*R. (B.) microplus*	Rachinsky et al., 2007
Chryseobacterium indologenes	IrAM	*I. ricinus*	Buresova et al., 2009
Rickettsia montanensis	DvKPI	*D. variabilis*	Ceraul et al., 2007
Bartonella henselae	IrSPI	*I. ricinus*	Liu et al., 2014
Borrelia burgdorferi	Ixophilin	*I. scapularis*	Narasimhan et al., 2013

Several tSPIs have also been identified in *Ornithodoros moubata* soft tick hemolymph, including **TAM**, (tick α-macroglobulin), the second most abundant protein after vitellogenin (Kopacek et al., 2000). TAM is a tetrameric glycosylated protein that displays similar structural features present on IrAM, and exerts inhibitory activity against both trypsin and thermolysin (Kopacek et al., 2000). Comparing the conserved cysteine motifs between human and limulus α2Ms enables the prediction of disulfide bridge patterns which explain the atypical molecular arrangement of the four TAM bait region variants, likely arising from alternate splicing (Saravanan et al., 2003). While TAM was initially detected in tick hemocytes, significant up-regulation has also been reported in SGs and gut after 1 day of feeding (Saravanan et al., 2003). TAM is believed to be involved in tick defense systems, but an additional role as an anti-coagulant has also been postulated (Keller et al., 1993; van de Locht et al., 1996).

Hemolymph Clotting Formation

As for vertebrates, effective clotting is critical in ticks to limit hemolymph loss and to inhibit pathogens from entering into the tick through the wound. Little is known about the proteins involved in tick hemolymph clotting but several tSPIs have been implicated in this defense system due to homology with known proteins (**Figure 2**).

Four *Rhipicephalus appendiculatus* tick serpins have been identified as potential clotting enzymes involved in the hemolymph coagulation cascade: *R. appendiculatus* serpin-1, -2, -3, and -4 (**RAS-1, -2, -3, and -4**) (Mulenga et al., 2003b). All exhibit similarities ranging from 25 to 30% with limulus intracellular coagulation inhibitor type 1 (LICI-1) from the Japanese horseshoe crab *Tachypleus tridendatus.* RAS-3 harbors also amino acids similarities with LICI-2 and is comparably expressed in all tick organs, while RAS-1, -2 and -4 expression was stronger in SGs than in other tick organs (Mulenga et al., 2003b).

HLS2, a serpin from *Haemaphysalis longicornis*, contains an RCL with high sequence similarities to both vertebrate and invertebrate serpins, and may interact with both chymotrypsin and thrombin (Imamura et al., 2005). HLS2 also demonstrates similar molecular features to RAS-3 and LICI, and its mRNA has only been detected in the hemolymph of partially or fully engorged nymphs and females, suggesting a possible role in endogenous hemolymph circulation. In the same tick species, the serpin **HLSG-1** harbors high similarity (32–44%) to Japanese and mangrove horseshoe crab clotting factor C precursors, mouse manan-binding lectin serine protease 1, and rat/mouse hepsin proteins (Mulenga et al., 2001). In partially fed ticks HLSG-1 transcripts were weakly expressed in the midgut and strongly detected in the SGs.

Blood Uptake and Digestion Modulation

As strict haematophagous acari, ticks require blood meals to complete their development and reproduction. These arthropods are pool-feeders and create haemorrhagic pools from which they collect nutritive fluids while biting, and interestingly, some female hard ticks can imbibe enough blood to increase in size by as much as 100 times (Sonenshine and Anderson, 2014). This unique feeding method implies the existence of very effective blood uptake and digestion mechanisms, in which several tSPIs have now been implicated (**Figure 2**).

In 2001, Mulenga et al. identified **HLSG-2** in *H. longicornis* ticks, a serpin with chymotrypsin-like protease selectivity (Mulenga et al., 2001). HLSG-2 transcripts were only detected in partially fed ticks and expression increased in parallel with feeding duration. HLSG-2 expression was strong in midgut and weak in salivary glands, suggesting probable links with blood uptake and digestion processes (Mulenga et al., 2001). In the same tick species, another serpin-1 (**HLS-1**) harboring similarities to the *I. ricinus* SG serpins RAS-2 and RAS-1 (Sugino et al., 2003) was identified. Taking into account additional sequence homologies with other anticoagulation factors, its specific expression in midgut of partially fed ticks, as well as the fact that clotting time can be delayed by HLS-1 in a dose-dependent manner, all indicate that HLS-1 has a probable role in tick blood meal uptake as well as maintaining blood fluidity in the midgut (Sugino et al., 2003). Then in 2010, Miyoshi et al. identified a Kunitz-type tSPI exclusively expressed in the midgut of adult ticks named **HlMKI** (*Haemaphysalis longicornis* midgut Kunitz-type inhibitor) (Miyoshi et al., 2010). Immunofluorescent analysis demonstrated that HlMKI likely interacts with HlSP, a hemolytic serine protease expressed in the tick midgut, and that both proteins harbored similar expression patterns with a 72 h peak during feeding (Miyoshi et al., 2007). HlMKI displayed inhibitory activity against the HlSP protein, and against chymotrypsin and elastase to a lesser extent. Altogether these results suggested that HlMKI can regulate blood digestion in tick midgut via HlSP modulation (Miyoshi et al., 2010). Lastly, another Kunitz-type tSPI was identified in *H. longicornis* hemocytes, and was named **HlChI** because of its chymotrypsin inhibitory profile (Alim et al., 2012). HlChI has strong chymotrypsin inhibitory activity but low trypsin inhibitory activity. The *HlChI*

gene is expressed in larvae, nymphs, and adults during all feeding phases, and transient up-regulation has been clearly detected from feeding initiation to repletion. During feeding, HlChI is mainly localized in hemocytes, though low expression levels were also detected in midgut, salivary glands, ovaries, and the epidermis (Alim et al., 2012). Several HlChI-RNAi-injected ticks died 48 h after feeding, while others ingested significantly smaller and slower blood meals, laid fewer eggs, and demonstrated lower larvae conversion (Alim et al., 2012). During feeding, HlChI expression in hemocytes peaks at 96 h, coinciding with low proteolytic activity and a low homeostasis level maintained by a few principal inhibitors (Franta et al., 2010; Alim et al., 2012). Thus HlChI is likely an indirect but essential actor in both vital blood feeding and tick reproduction processes.

In *R. appendiculatus* ticks, three serine protease inhibitors of chymotrypsin or trypsin and named **RAMSP 1-3** (*R. appendiculatus* midgut serine proteinases 1-3) were identified, and are likely involved in feeding processes or blood digestion (Mulenga et al., 2003a). RAMSP-1 and -2 transcripts have only been detected in partially fed ticks, while RAMSP-3 mRNA was detected in both unfed and partially fed ticks, with stronger signals in the latter. RAMSPs expression is not restricted to the midgut because RAMSP-1 is equivalently expressed in all tick organs, while RAMSP-2 is weakly expressed in both SGs and midgut, and RAMSP-3 is more weakly expressed in SGs than midgut (Mulenga et al., 2003a). Additionally, all three RAMSPs are expressed in tick carcasses (whole tick without SGs and midgut), suggesting widespread distribution of these inhibitors in other tick tissues (Mulenga et al., 2003a). In terms of **RAS** serpins, RAS-1 and RAS-2 mRNAs can be detected at all life stages, as well as in both sexes, with positive detection in 4-day partially fed and fully engorged ticks (Mulenga et al., 2003b). This suggests gene expression both during and after feeding, although they were not expressed in saliva, likely because they do not contain signal peptide sequences (Mulenga et al., 2003b). Indeed, RAS-1 and RAS-2 might be associated with feeding in both SGs and the midgut by modulating blood uptake and digestion. Confirmation occurred when significantly fewer fully engorged nymphs were counted when ticks were fed on vaccinated compared to non-vaccinated cattle (Imamura et al., 2006).

Then in 2014, NGS technologies enabled the identification of 22 **RMS** serpins (RMS-1 to RMS-22) from *R. (B.) microplus* (Tirloni et al., 2014a,b), among which 18 full-length coding sequences were identified (Rodriguez-Valle et al., 2015). While serpin consensus patterns were conserved, these 18 members of the RMS family showed high amino acid sequence variability suggesting a broad spectrum of targeted serine proteases. Transcription levels of RMS-13, -15, -16 in SGs, or RMS-6, -7, -9, -17 in both SGs and the midgut, suggested that they play a role in the blood meal process, either in uptake or during digestion (Rodriguez-Valle et al., 2015). Functionally, RMS-3 strongly inhibits chymotrypsin and elastase, but only weakly trypsin and thrombin. RMS-15 is a strong thrombin inhibitor and RMS-6 a chymotrypsin inhibitor. Finally while RMS-21 and -22 are not secreted, they were detected in the midgut and SGs suggesting a probable role in proteolysis activity during blood digestion (Rodriguez-Valle et al., 2015). Among the previously mentioned *R. (B.) microplus* BMTIs, the Kunitz inhibitors **BmTi A** and **BmTI D** were also believed to play an important role in feeding by inhibiting human prekallikrein (HuPK) implicated in the coagulation cascade (Sasaki et al., 2004), thus facilitating blood fluid uptake.

The serpin **AamS6** was identified in the *Amblyomma americanum* tick (Mulenga et al., 2013). Both AamS6 mRNA and protein are strongly expressed in the SGs and midgut in unfed ticks, as well as during the first 72 h of feeding, before fading at 96 h, suggesting injection into the bite site and a role in tick anchorage to the host (Chalaire et al., 2011). As expected according to its serpin-like sequences, AamS6 interacted clearly and selectively with trypsin, chymotrypsin, elastase, and chymase, but also surprisingly with papain-like cysteine proteases, indicating that it is a cross-class protease inhibitor (Mulenga et al., 2013). In addition, AamS6 seems to transiently interact with fibrin-lysing plasmin. Despite only *in vitro* data providing the proof for plasmin-AamS6 interaction, it was hypothesized that this inhibitor could sustain blood flow to the tick feeding site and prevent clot formation (Mulenga et al., 2013). In this series of experiments, AamS6 only delayed recalcification time (RCT, the time to plasma clotting once calcium ions and blood-clotting co-factor(s) are reintroduced to citrate plasma), and did not inhibit any of the three coagulation pathways (extrinsic, intrinsic, and common, **Figure 3**). Platelet aggregation inhibition was also reported, that, when combined with previous results, bears out AamS6's involvement in inhibiting blood coagulation both in the midgut and via saliva secretion (Mulenga et al., 2013). Later, an *A. americanum* transcriptomic study identified a novel serpin, **AAS19**, which has the most fully conserved RCL among the ixodid ticks (Kim et al., 2015; Porter et al., 2015). The presence of the tripeptide Arg-Gly-Asp which constitutes a "RGD" motif, in the AAS19 sequence suggests a potential relationship between integrin GPIIb-IIIa and AAS19 during platelet aggregation (Nurden, 2014). Additionally, as AAS19 is abundantly and mostly expressed in the midgut at 96 h during feeding, it likely ensures that host blood doesn't clot during feeding (Porter et al., 2015). AAS19 demonstrates inhibitory activity against plasmin, FXa, and FXIa, and at a lower efficacy rate against FXIIa, FIIa (activated thrombin), FIXa, and tryptase, demonstrating its broad activity spectrum. In addition, the three coagulation pathways have delayed clotting in the presence of AAS19, likely due to weak thrombin inhibition. Altogether these results support AAS19 involvement in blood digestion (Kim et al., 2015).

Finally, **Ixophilin** was identified as a Kunitz thrombin inhibitor secreted in the midgut of *I. scapularis* and which shares homology with Hemalin and Boophilin (see below) (Narasimhan et al., 2013). Ixophilin was preferentially expressed in adult and nymphal midgut and was induced upon feeding, consistent with a potential role in preventing blood clotting in the midgut. In addition, Ixophilin mice immunization experiments demonstrated that ixophilin was necessary for efficient engorgement (Narasimhan et al., 2013).

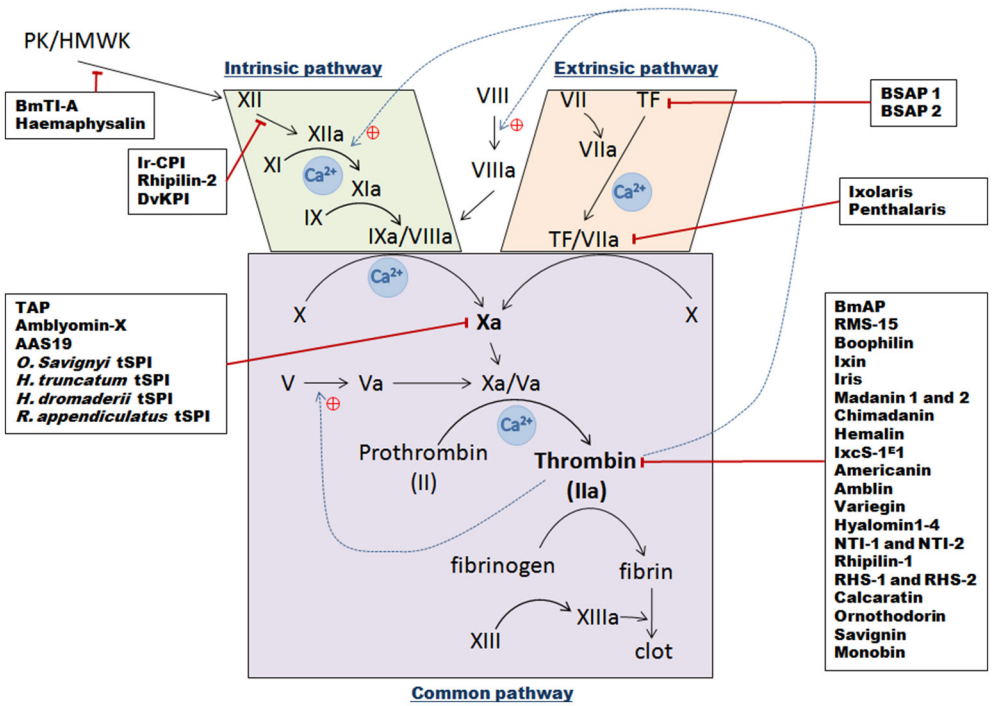

FIGURE 3 | Schematic overview of the three vertebrate blood coagulation cascade pathways, with indicated tick serine protease inhibitors and their targets. The intrinsic pathway is activated following a trauma and when blood comes into contact with subepithelial cells [contact phase with XII, Prekallikrein (PK), and High Molecular Weight Kininogen molecules (HMWK)], which then results in successive activation of factors XII, XI, and IX. The IXa/VIIIa complex then activates transformation of factor X into factor Xa. The extrinsic pathway is initiated following contact between Tissue Factor (TF) from vessels and circulating factor VII. After injury, they form a complex and catalyze the activation of factor X into factor Xa. Once activated by either pathway, factor Xa complexes with its cofactor Va to form the pro-thrombinase complex, which can then convert prothrombin into thrombin. Thrombin then transforms fibrinogen into fibrin to create a plug. Black arrows represent direct activation, dotted blue lines show positive thrombin feedback, red lines correspond to tSPI inhibition targets.

Tick Development, Oviposition, Egg Laying

Tick oviposition and egg laying are essential aspects of the tick life cycle determining tick population expansion. These processes are regulated by many proteins, including tSPIs (**Figure 2**). Inhibitors may be endogenous to certain organs and, for ovaries, it appears that some proteins—including tSPIs—can be captured from the midgut by receptor-mediated endocytosis followed by incorporation into the eggs (Tufail and Takeda, 2009).

Several tSPIs named **BmTIs,** with a similar target spectrum (trypsin, plasmin, and HuPK), have been discovered in the eggs and larvae of *R. (B.) microplus* (Tanaka et al., 1999). However, differing concentrations and inhibitor specificity changes have been reported between egg and larval stages (Andreotti et al., 2001; Sasaki et al., 2004). Andreotti et al. showed a 69.7 and 71.3% reduction in both engorged tick number and egg weight respectively when female ticks were fed on BmTIs-immunized cattle, confirming a crucial role for BmTIs in egg production and development (Andreotti et al., 2002). **BmTI-6**, a Kunitz tSPI identified in ovaries, was also expressed in tick body fat and demonstrated specific inhibitory activity against trypsin and trypsin-like proteases, such as plasmin (Sasaki and Tanaka, 2008). As for other BmTIs, BmTI-6 is suspected to play a role in controlling endogenous proteases in the egg and larval stages (Andreotti et al., 2001; Sasaki et al., 2004).

The **RMS-3** serpin is expressed in the SGs of semi-engorged females, but lower expression levels were also observed in the midgut and ovaries of *R. (B.) microplus* (Rodriguez-Valle et al., 2012). *In vitro* feeding assays showed that both egg weight and larval transformation rates were reduced in female ticks pre-fed on anti-RMS-3 sheep serum, thus implicating RMS-3 in reproduction and egg development (Rodriguez-Valle et al., 2012). An additional Kunitz serine protease inhibitor with trypsin and kallikrein inhibitory activities named **RmKK** was recently discovered in *R. (B.) microplus* eggs (Abreu et al., 2014). Although the native protein was obtained from eggs, the RmKK transcript was only detected in the midgut, suggesting possible midgut expression and subsequent transport to the ovaries and egg incorporation. Interestingly, kallikrein inhibitors were thought to protect against undesired egg proteolysis (Willadsen and Riding, 1980). Lastly, four other serpins were also recently implicated in tick embryogenesis regulation or vitellogenesis: **RMS-19** and **RMS-20**, which are expressed in all tissues and at all stages, **RMS-6** which is only detected in ovaries, and **RMS-21 and -22** which are only detected in eggs (Rodriguez-Valle et al., 2015).

Studies on the dog tick *Rhipicephalus sanguineus* identified a group of proteins belonging to the Kunitz/BPTI tSPI family, called **RsTIs** in larval stages (Sant'Anna Azzolini et al., 2003). Three of which—**RsTIQ2, RsTIQ7** and **RsTIS5**—inhibit trypsin,

neutrophil elastase, and human plasmin. RsTIQ2, and to a lesser extent RsTIQ7, also inhibit HuPK (Sant'Anna Azzolini et al., 2003). Because RsTIs are similar in structure and inhibitory activity to previously described tSPIs from *R. (B.) microplus,* a similar role in egg production has been hypothesized.

A human follistatin-related-protein (**FRP**) homolog was also identified in *H. longicornis* ticks, and is implicated in tick oviposition (Zhou et al., 2006). This protein harbors three distinct domains, a follistatin-like domain, a Kazal domain and two EFh calcium–binding motifs. Polyclonal antibodies revealed FRP presence in tick salivary glands, midgut, body fat, hemocytes, and a strong expression in ovaries (Zhou et al., 2006). RNAi experiments silencing FRP in adult rabbit-fed ticks as well as the use of anti-FRP antibodies showed significant negative effects on tick oviposition while no differences were observed in feeding duration, engorgement weight, and survival (Zhou et al., 2006).

Finally, when the aforementioned *A. americanum* **AAS19** gene was RNAi silenced, ticks imbibed much less blood and presented curious body deformities compared to controls, likely due to deficiencies in hemostasis regulation (Kim et al., 2016). In addition, ticks that fed on rAAS19-immunized rabbits took smaller blood meals and detached prematurely. Following a second round of infestation on these rabbits, ticks also failed to lay eggs, suggesting an important role for AAS19 in both tick homeostasis and reproduction (Kim et al., 2016).

ROLES OF SERINE PROTEASE INHIBITORS IN MODULATING VERTEBRATE HOST RESPONSES

The long feeding period of ticks necessitates extended control over the vertebrate host's haemostasis and immunity. During this feeding process, ticks alternatively inject saliva into and then absorb fluids from the bite wound. To enable the feeding process and avoid tick rejection, several salivary components are thought to control host responses, including several tSPIs (**Figure 2**).

Anti-Hemostatic Effects of Tick Serine Protease Inhibitors

Hemostasis in vertebrates is a tightly regulated process to avoid blood leakage following injury (Aird, 2003). Many biochemical mechanisms are involved in blood clot formation with a central enzymatic cascade that can be activated by three different pathways (**Figure 3**). When the vessel epithelium is damaged, tissue factors (TF) expressed by epithelium cells initiate clot cascades, leading to activation of extrinsic blood coagulation pathways. Displaying TF triggers factor VII activation thus forming TF/VIIa complexes which then activate central factor X. The second intrinsic pathway is activated by contact factors—XII, prekallikrein (PK) and high molecular weight kininogen (HMWK)—that cause successive activation of intermediate factors (factors XI, IX), and IXa/VIIIa complex formation, which also activates factor X. Thus intrinsic and extrinsic coagulation pathways merge in order to activate Factor X to Xa. Then in the "common pathway," activated factor Xa interacts with its co-factor Va and forms the pro-thrombinase complex. This complex then processes pro-thrombin (factor II) into thrombin (factor IIa) that converts fibrinogen into fibrin which then polymerizes to constitute a clot. Ticks prevent clot formation in the host, in the micro hematoma at the bite site, but also, in tick mouthparts and midgut. They inject numerous proteins via their saliva into the blood bowl, mostly tSPIs that affect serine proteases or their zymogens (Factors II, VII, IX, X, XI, XII, and prekallikrein) implicated in clot formation (Tatchell, 1969; Ribeiro et al., 1985). As anticoagulant factors, these inhibitors are essential in allowing ticks to take their blood meal, and concomitantly ingest and transmit pathogens. In addition, during the long feeding period, the blood in the tick midgut is maintained in a fluid state until repletion. To retain blood fluidity, anticoagulants are secreted from the tick midgut epithelium to the midgut lumen. Coagulation inhibitors secreted into the midgut mostly inhibit thrombin whereas anticoagulants secreted from SGs mainly inhibit FXa.

Extrinsic Pathway Tick Inhibitors

I. scapularis was intensively studied in order to understand tick saliva anticoagulant properties, leading to the discovery of multiple anticoagulant molecules, the first being **Ixolaris** from the SGs (Francischetti et al., 2002). This inhibitor, similar to human hTFPI (tissue factor pathway inhibitor), contains two Kunitz domains. Ixolaris, like hTFPI, either blocks FVIIa/TF complex activity through direct interaction with the active site, or sterically via the formation of a tight complex [FVIIa/TF/Ixolaris/FX(a)], resulting in effective extrinsic pathway inhibition (Francischetti et al., 2002). Ixolaris, reaching high concentrations only in the feeding cavity *in vivo*, behaves as a fast ligand of the FX and FXa heparin binding exosite (HBE)—a site distinct from the FXa reactive site—presumably via its second atypical Kunitz domain (Monteiro et al., 2005). The prothrombinase complex could be resistant to physiological concentrations of Ixolaris due to competition with its prothrombin substrate, as reported for hTFPI (Mast and Broze, 1996). Ixolaris' mechanism of action was then proposed to be competitive and concentration dependent (Monteiro et al., 2005). Further analyses of an SG cDNA library enabled the discovery of another Kunitz domain protein implicated in coagulation inhibition, **Penthalaris** (Francischetti et al., 2004). This inhibitor harbors five tandem Kunitz domains and like Ixolaris, inhibits FVIIa/TF-induced FX activation at high concentrations by binding to the HBE of both FX and FXa.

Finally, two *Ornithodoros savignyi* saliva anti-coagulants have been identified: **BSAP1** and **BSAP2** (Ehebauer et al., 2002). These proteins do not inhibit the intrinsic pathway, whereas the extrinsic pathway appeared to have delayed clot formation. Therefore, these proteins may only target TF, as FVII does not seem to be inhibited. It may be the first time a direct TF-interacting protein has been reported, because until now the majority of known extrinsic pathway inhibitors target other factors forming complexes with TF.

Intrinsic Pathway Inhibitors

The Kunitz protein **Ir-CPI** was identified in *I. ricinus* SGs and binds to contact phase factors, FXII, FXI, and kallikrein

(Decrem et al., 2009). *In vitro* experiments showed that Ir-CPI considerably prolonged aPTT, without modifying the extrinsic pathway, whereas experiments on both venous and arterial thrombus formation in animal models showed that Ir-CPI mainly inhibits clot propagation and thrombin generation (Decrem et al., 2009). Like Ixolaris, Ir-CPI does not block amidolytic activity of targeted proteases by binding to the catalytic site as is usual for Kunitz-type inhibitors, but likely acts by binding to an exosite, thereby preventing enzyme activity with high affinity steric hindrance (Laskowski and Kato, 1980).

Among the BmTIs from *R. (B.) microplus* tick eggs and larvae, **BmTI-A** strongly inhibits trypsin, hNE, plasmin and HuPK (Tanaka et al., 1999). It was initially described with two Kunitz domains, the first implicated in trypsin and HuPK inhibition and the second inhibiting hNE (Tanaka et al., 1999; Guerrero et al., 2005). Subsequently, a further five Kunitz-BPTI domains were identified (Soares et al., 2016). BmTI-A transcripts are mainly expressed in the tick midgut, and are weakly expressed in SGs and ovaries (Tanaka et al., 1999). It is possible that the inhibitor is transferred from ovaries to the larval stage where it could be important for controlling blood coagulation, inflammation, and angiogenesis during the larval feeding process, by inhibiting plasma kallikrein, neutrophil elastase, and plasmin (Tanaka et al., 1999; Soares et al., 2016).

Another Kunitz-type tSPI with a unique Kunitz domain named **Rhipilin-2** was identified in *Rhipicephalus hemaphysaloides*, and is highly similar to members of the TFPI mammalian protein family (Cao et al., 2013). Rhipilin-2 does not prolong recalcification time in PT assays, but increases coagulation time of citrated rabbit plasma in aPTT assays. It inhibits approximately 60% of trypsin activity but does not inhibit thrombin. Rhipilin-2 gene expression was only detected in SGs and the midgut of fed ticks (Cao et al., 2013). One-day-fed ticks presented the highest expression that gradually faded until total engorgement, suggesting probable injection via saliva into the host during feeding to restrict blood clotting at the wound site (Cao et al., 2013).

Haemaphysalin, a Kunitz-type inhibitor from the hard tick *H. longicornis* also inhibits intrinsic coagulation pathways by blocking kallikrein-kinin system activation (Kato et al., 2005a,b). Its acts via its two Kunitz domains, and does not affect the amidolytic activities of intrinsic coagulation factors. Direct binding assays demonstrated binding of the COOH-terminal domain to both high molecular weight kininogen (HMWK) and factor XII (Kato et al., 2005a,b). The COOH-terminal domain may then inhibit factor XII and HMWK association on the cell surface, and hence inhibits kallikrein-kinin system activation by interfering with prekallikrein and factor XII reciprocal activation. Zn^{2+} ions appear to be involved in interactions between haemaphysalin and its targets, suggesting that these cations induce conformational changes which enable haemaphysalin's inhibitory effect (Kato et al., 2005a,b).

Previously mentioned studies of **DvKPI** highlighted its ability to inhibit the coagulation cascade as revealed by both delayed aPTT assays and robust antitrypsin activity. Although DvKPI expression was detected in both body fat and SGs, the highest expression was in the midgut and which increased upon feeding, demonstrating that its anticoagulant activity in the midgut is essential (Ceraul et al., 2008).

FX(a) Factor Inhibitors

Tick anticoagulant protein **TAP**, the first tSPI to specifically inhibit FX(a) factor was identified in the soft tick *Ornithodoros moubata* as a Kunitz inhibitor (Waxman et al., 1990). Whereas Kunitz inhibitors are generally highly basic, TAP is acidic, and was classified as a slow, tight-binding inhibitor, because it requires at least 15-min pre-incubation for maximal FX(a) inhibition.

Amblyomin-X was initially discovered following SG transcriptome sequencing of the *Amblyomma cajennense* Cayenne tick (Batista et al., 2010). This protein harbors one Kunitz-type domain, and is observed in monomeric, dimeric, trimeric, and tetrameric conformations. Amblyomin-X inhibits FXa in a non-competitive manner but also increases PT and aPTT, suggesting an effect on prothrombin conversion (Batista et al., 2010; Branco et al., 2016). Four hypotheses have been proposed to explain the FXa inhibitory mechanism of Amblyomin-X. Firstly, as Amblyomin-X shares amino acid sequence similarities with TAP, it could bind FXA as a slow, tight-binding inhibitor. The second is that the strongly negatively charged COOH-terminal domain of Amblyomin-X could bind to positively charged FXa exosites. Thirdly, an additional binding of the Amblyomin-X NH_2-terminus to the FXa active site could exist. Lastly, Lys30 from the characteristic Kunitz domain loop could bind to the FXa active site (Batista et al., 2010).

The aforementioned **AAS19** serpin from *A. americanum* also appears to be injected into the host during feeding, and enhances feeding success by inhibiting trypsin-like proteases including Fxa, hemostasis, and host immune-defenses (Kim et al., 2015, 2016).

Lastly, and despite not being fully characterized, several other FX(a) inhibitors from different tick species have been reported. Amongst these is an SG protein from the soft tick *O. savignyi*, with an approximate 7 kDa molecular weight and six cysteine residues suggesting a single Kunitz domain. FXa inhibition appears specific, although thrombin was also very weakly inhibited (Gaspar et al., 1996). In *Hyalomma truncatum*, the bont-legged tick, several SG proteins inhibiting both extrinsic and intrinsic coagulation pathways were detected (Joubert et al., 1995). Of these, one 17 kDa nameless protein possesses Factor Xa inhibitory activity and was only identified in females pre-fed for 5–7 days, suggesting involvement in tick feeding process. This FXa inhibition appeared to be non-competitive, in contrast to TAP from *O. moubata,* but similar to another 15 kDa tSPI identified in nymphs of the camel tick, *Hyalomma dromedarii*. This last tSPI totally inhibits FXa but only partially inhibits thrombin activity (30% inhibition). Extremely efficient FXa inhibition could be explained by the presence of two binding sites on the inhibitor (Ibrahim et al., 2001b). Finally, a 65 kDa FXa inhibitor from *R. appendiculatus* was also isolated from SG extracts (Limo et al., 1991). No complexes between FXa and this inhibitor were identified, and it was established that inhibition may occur via exosite binding.

Thrombin Inhibitors

Crude saliva of *R.* (*B.) microplus* was initially investigated because of its effective bovine plasma coagulation inhibiting properties, and subsequently several thrombin inhibitors were identified. **BmAP** was described as a non-tight binding thrombin inhibitor, possibly dimerized, and which interacts with both thrombin active sites and subsites (Horn et al., 2000). Two **microphilin** isoforms were then discovered, and which are the smallest non-tight binding thrombin inhibitors identified thus far (Ciprandi et al., 2006). Microphilin only interacts with thrombin at exosite I, the crucial site for both fibrinogen and platelet thrombin receptor interactions. **BmGTI**, from the *R. (B.) microplus* midgut, was reported to inhibit fibrinogen cleavage by thrombin (Ricci et al., 2007), via interaction with thrombin's positively charged exosite I (Monteiro, 2005). The serpin **RMS-15** was identified as the highest affinity thrombin inhibitor (Rodriguez-Valle et al., 2015; Xu et al., 2016). RCT assays showed that plasma clotting time is delayed in the presence of RMS-15, in a dose-dependent manner. The elevated RMS-15 IgG titres found in bovine sera after prolonged exposure to tick infestation, and the presence of RMS-15 transcript in SGs and midgut (Rodriguez-Valle et al., 2015), suggest a likely secretion of this serpin into the tick-feeding site, which then acts on host coagulation (Xu et al., 2016). Lastly, a Kunitz-type serine-protease inhibitor harboring two Kunitz domains was isolated from the tick midgut and named **Boophilin** (Macedo-Ribeiro et al., 2008). Boophilin greatly increases TT (Assumpção et al., 2016), significantly affects PT, and increases aPTT, albeit weakly (Macedo-Ribeiro et al., 2008). Boophilin displayed partial effects on plasma kallikrein and weak inhibitory effects on FVIIa, showing probable, but limited, implication in both extrinsic and intrinsic pathways. In addition, boophilin can block amidolytic activity of other trypsin-like serine proteases, most notably trypsin and plasmin (Macedo-Ribeiro et al., 2008). Boophilin is a strong thrombin inhibitor forming a stable thrombin/boophilin complex in a non-canonical manner (Macedo-Ribeiro et al., 2008). The proximal interaction between boophilin and meizothrombin (MzT) suggests that boophilin may not only target circulating thrombin but also the MzT intermediate. When boophilin and MzT are complexed, the boophilin NH_2 terminus remains effectively available and able to bind proximal coagulation factors including FXa.

At the end of the nineteenth century it was demonstrated that *I. ricinus* saliva also contains anticoagulant molecules (Sabbatini, 1899). **Ixin,** a specific thrombin inhibitor, was isolated from adult saliva in 1991 (Hoffmann et al., 1991), and the serpin **Iris—**for "*I. ricinus* immunosuppressor"—was identified in 2002 (Leboulle et al., 2002b). Iris expression is induced in SGs while ticks feed, peaking at day four, coinciding with the period when the *I. ricinus* female ingests the most amount of blood. Iris demonstrates dose-dependent FXa inhibition and inhibits close to 30% of thrombin. Its inhibitory activity arises from the RCL domain where the P1 residue plays a key role, which was confirmed with Iris structural model interactions (Prevot, 2006). Iris hampers fibrinolysis by inhibiting both tissue plasminogen activator (t-PA) and elastase released by leukocytes. It also acts as a hypo-fibrinolytic factor by targeting serine proteases, especially elastase-like proteins, and appears to prevent platelet adhesion via a mechanism independent of its enzyme inhibitory activity (Prevot, 2006). However, even if Iris inhibits several serine proteases in coagulation pathways, it does not appear to be a powerful anticoagulant (Prevot, 2006).

Madanin 1 and 2 have also been identified from SGs of *H. longicornis,* as inhibiting both the intrinsic and extrinsic coagulation pathways (Iwanaga et al., 2003). Madanin proteins do not exhibit any sequence similarities with any other previously identified proteins, do harbor a signal peptide sequence, and interact with thrombin, but not with factor Xa (Iwanaga et al., 2003). Thrombin inhibition by madanins probably involves competitive binding to thrombin's fibrinogen-binding site (anion-binding exosite 1), and not via binding to the active site. They inhibit blood coagulation at an early stage by inhibiting thrombin activation of factors V and VIII, and thus likely contribute considerably to tick blood feeding success (Iwanaga et al., 2003). Another thrombin inhibitor named **Chimadanin** was identified in *H. longicornis* SGs with very weak expression. It was also detected in the hemolymph during nymphal and adult stages from the third day of blood feeding and declines until extinction on the 6th day (Nakajima et al., 2006). Lastly, **Hemalin** with two Kunitz domains, was identified in larval, nymphal, and adult stages, and exhibited highest expression levels during the rapid blood meal sucking period during all tick life stages (Liao et al., 2009). In addition to its role as a thrombin inhibitor, Hemalin also inhibits trypsin activity. Initially discovered in the midgut, Hemalin is also expressed in major tissues of the female tick including SGs, ovaries, hemolymph, and body fat.

In 2014, Ibelli et al. reported the presence of a new tSPI from *I. scapularis* saliva belonging to the serpin family, **IxcS-1E1** (Ibelli et al., 2014). IxcS-1E1 transcript was detected both in SGs and in the midgut of ticks with a dichotomous temporal expression pattern. In SGs, expression is up-regulated from the 24 first hours of feeding, while midgut expression was down-regulated in response to feeding activity (Ibelli et al., 2014). During feeding, IxcS-1E1 is injected into the host and likely prevents platelet aggregation, as it extends clotting time both in aPTT and PT *in vitro* assays. It inhibits thrombin and trypsin activities by forming stable complexes, and also probably inhibits cathepsin G and factor Xa enzymatic activities. Therefore IxcS-1E1 appears to be one of the most important *I. scapularis* saliva proteins mediating tick evasion from the host's hemostatic defense system (Ibelli et al., 2014).

In the *Amblyomma* genus, the **Americanin** protein was isolated from the SGs of *A. americanum*, and was shown to be a slow reversible tight-binding-type thrombin inhibitor (Zhu et al., 1997). Similar to Ixolaris and boophilin, **Amblin** was isolated from the synganglia of engorged *A. hebraeum* females, from where the protein is exported into the hemolymph where it can also be detected (Lai et al., 2004). Without a signal peptide and composed of two Kunitz-like domains, amblin specifically inhibits the thrombin enzyme via an unknown inhibitory mechanism. Crude SG extracts from *A. variegatum,* the tropical bont tick, also exhibited potent anticoagulant activity in the three TT, PT, and aPTT coagulation assays, thus inhibiting at least one factor implicated in the two coagulation pathways (Koh et al., 2007). The TT assay demonstrated the most significant results,

indicating that the major targeted factor is thrombin. **Variegin** was then identified as a protein without any similarities to other tSPIs (Kazimírová et al., 2002). Nevertheless, its NH_2-terminal sequence appears to be a fast competitive tight-binding inhibitor of thrombin. Following HPLC purification, another inhibitor with anti-thrombin effects on human blood platelets and with hirudin-like activity, was also identified from the saliva of *A. variegatum*, but has not been further characterized (Kazimírová et al., 2002).

Hard tick *Hyalomma marginatum rufipes* SG transcriptome investigations unearthed four peptide-encoding sequences, named **hyalomins-1-4**, that showed weak similarity to madanin 1 and 2 from *H. longicornis* (Francischetti et al., 2011). The central core of these polypeptides contains a weakly conserved acidic region that forms a putative serine protease cleavage site. Hyalomin-1 appears to be a specific thrombin inhibitor and noncompetitively binds to its active site, as well as an exosite I, thus significantly extending fibrin clot formation time in whole plasma (Jablonka et al., 2015). It also blocks thrombin activation of coagulation factor XI and factor V. In addition, hyalomin-1 also impedes platelet aggregation by inhibiting thrombin activation of platelet proteinase activated receptor (PAR).

Two thrombin inhibitors from *H. dromedarii* nymphs were also described and named **NTI-1** and **NTI-2** (Ibrahim et al., 2001a). Inhibition assays revealed that NTI-1 inhibits 13% of FXa activity and 65% of thrombin activity, whereas NTI-2 inhibits 100% of FXa activity and 58% of thrombin activity, but with higher affinity for this enzyme than NTI-1. In addition, thrombin inhibition by NTI-1 and NTI-2 is non-competitive and competitive, respectively.

Rhipilin-1, identified in the hard tick *Rhipicephalus haemaphysaloides,* shares a similar conformation—a unique Kunitz domain—with other thrombin inhibitors such as boophillin, amblin, and hemalin (Gao et al., 2011). The protein, tested on rabbit citrated plasma, has the capacity to extend both RCT and aPTT in a dose-dependent manner. Both tick attachment and engorgement processes require *rhipilin-1* expression, which is consistent with specific gene expression only in fed ticks (Gao et al., 2011). Two other thrombin inhibiting serpins were also identified in this tick species, **serpin-1** (**RHS-1**) and **serpin-2** (**RHS-2**) (Yu et al., 2013). They share similarities with other tSPIs described in this review including: RHS-1 with lopsins 1 and 2; and RHS-2 with lopsin 7 and RAS-2. Functional characterization indicated that RHS-1 is expressed in SGs of fed ticks and secreted at the tick-host interface during feeding, whereas RHS-2 is only expressed in fed tick midgut (Yu et al., 2013). Inhibition assays showed that both RHS-1 and RHS-2 mostly inhibit chymotrypsin but they also have thrombin inhibitory activity, with maximum inhibition rates of 65.5 and 20%, respectively (Yu et al., 2013). For both, weak FXa inhibition was observed, and neither trypsin nor elastase was inhibited. Anticoagulation assays revealed that aPTT was prolonged in the presence of RHS-1 but not RHS-2. RNAi assays implicated both proteins in tick attachment and engorgement rates, but not in repletion time or average body weight, confirming the role of RHS-1 and RHS-2 in tick blood feeding by blocking blood coagulation (Yu et al., 2013).

Calcaratin was identified from *Rhipicephalus* (*Boophilus) calcaratus* and was able to delay coagulation time in all tests (aPTT, TT, and FCT), via an unknown thrombin inhibition mechanism (Motoyashiki et al., 2003).

Concerning soft ticks, ornithodorin was described from *O. moubata* as a thrombin inhibitor harboring two Kunitz domains (van de Locht et al., 1996). Interactions with thrombin implicate its active and exosite: the NH_2-terminal domain appears to be responsible for the majority of thrombin interaction with two van-der-Waals contacts and hydrogen bonds, whereas the COOH-terminal domain has mostly electrostatic interactions with thrombin (Klingler and Friedrich, 1997). An ornithodorin ortholog was identified in *O. savignyi* SGs, named savignin (Mans et al., 2002). Its NH_2-terminal region seems to be implicated in binding to the thrombin active site, whereas the COOH-terminal domain helix binds to the fibrinogen-recognition exosite domain. Savignin was then described as a slow competitive tight-binding inhibitor that binds to thrombin's fibrinogen-binding exosite for inhibition. It carries a signal peptide substantiating likely secretion during blood feeding (Mans et al., 2002). Lastly, the **monobin**, a slow specific tight-binding thrombin inhibitor, and an ornithodorin and savignin ortholog belonging to the Kunitz family, was also identified in *Argas monolakensis* (Mans et al., 2008). These three tSPIs harbor a non-canonical mechanism of action as inhibition results when their NH_2-terminal residues are inserted into the enzyme active site instead of their active Kunitz loops (van de Locht et al., 1996).

Lastly, studies on salivary gland extracts from *Ixodes holocyclus,* the Australian paralysis tick, showed that thrombin was the main enzyme targeted by salivary anticoagulant molecules, with an uncharacterized mechanism (Anastopoulos et al., 1991).

Vertebrate Host-Immune Modulation by Tick Serine Protease Inhibitors

Because ticks are blood-feeding arthropods requiring hours to weeks to complete their blood meal, they have developed several mechanisms to evade host rejection during this long feeding period (Ribeiro, 1995; Francischetti et al., 2009; Kazimirova and Stibraniova, 2013; Kotal et al., 2015). Their saliva injected into the wound site carries many components able to manipulate vertebrate host responses, including anti-inflammatory compounds as well as immunomodulators acting on both innate and acquired immunity, and among which are included several tSPIs.

In *A. americanum,* the aforementioned anticoagulants **AamS6** and **AAS19** can also target plasmin, known for its role in pro-inflammatory cytokine release, monocyte and dendritic cell chemotaxis, neutrophil attraction, tissue remodeling, and wound healing, suggesting involvement in the regulation of monocyte, macrophage, and dendritic cell functions, and in inflammatory responses (Syrovets et al., 2012). In addition, 17 different serpins called **lopsins** (L1-L17) were found to be expressed in various organs such as SGs, midguts, ovaries, and the carcasses of partially fed *A. americanum* ticks (Mulenga et al., 2007). Sequence analysis revealed the presence of glycosaminoglycan

binding sites on all lopsins, similar to several other proteins involved in the modulation of blood coagulation, inflammatory responses, or immune cell migration (Munoz and Linhardt, 2004). However, further investigations are required to identify their inhibitory targets and to decrypt mechanisms governing lopsins' likely immunomodulatory activities.

Iris from *I. ricinus* is also a powerful immunosuppressive molecule (Leboulle et al., 2002a). It is secreted at the tick-bite site and strongly inhibits elastase-like proteases (leukocyte elastase and pancreatic elastase) with rapid kinetics, thus repressing host inflammation (Prevot, 2006). Iris regulates innate immune mechanisms by suppressing T lymphocyte proliferation and inducing Th2-type immune responses with increased IL-4 and by inhibiting typical Th1 molecule production (IL2, IFN-γ) (Leboulle et al., 2002a). **IRS-2**, another serpin exhibiting specific anti-chymotrypsin activity, was identified from *I. ricinus* SGs (Chmelar et al., 2011). In SGs, IRS-2 is highly expressed at 2 days with maximum expression 6 days after attachment, suggesting a role in the early stage of feeding. IRS-2 inhibits both tissue swelling and neutrophil migration into inflamed tissue, modulates T cell differentiation, and decreases IL-6 production at both protein and mRNA levels in spleen dendritic cells activated by *B. burgdorferi* (Chmelar et al., 2011). In addition, by decreasing STAT-3 signaling molecule phosphorylation, IRS-2 impairs Th17 cell development (Páleníková et al., 2015). IRS-2 is also the only known serpin that targets both cathepsin G and chymase. Both of these proteases are secreted following neutrophil (cathepsin G) and mast cell (chymase) activation, and are involved in a huge range of physiological processes associated with the acute inflammatory response, particularly in crosstalk between neutrophils and platelets (Zarbock et al., 2007). IRS-2 also affects thrombin-induced platelet aggregation, thus likely playing multiple roles in inflammation and hemostasis particularly through the modulation of PAR activation (Chmelar et al., 2011).

The serpin **Ipis-1**, which shares 95.5% sequence identity with Iris, was isolated from SGs of fed *Ixodes persulcatus* and may be associated with immunomodulatory effects on both innate and acquired immune responses (Toyomane et al., 2016). Ipis-1 may directly interact with and inhibit T cells and $CD14^+$ cells (mainly macrophages, neutrophils, and dendritic cells), with an as yet unidentified inhibitory mechanism. Similarly, Ipis-1 affects cytokine and chemokine activity via currently unknown mechanisms (Toyomane et al., 2016).

Based on previous *I. scapularis* sialome exploration (Ribeiro et al., 2006), a salivary Kunitz inhibitor with unusual structure was characterized and named **tryptogalinin** (Dai et al., 2012; Valdés et al., 2013). Tryptogalinin has a broad spectrum (potentially explained by the presence of three intrinsic disordered regions) and a high affinity for serine proteases playing an important role in inflammation and host immune responses (Heutinck et al., 2010). In fact, tryptogalinin inhibits trypsin, α-chymotrypsin, plasmin, matriptase, elastase, and especially human skin β tryptase found in mast cells (Valdés et al., 2013). Mast cell β-tryptase has a key role in host-inflammatory responses by stimulating chemoattractant release, such as IL-8, and by inducing IL-1β mRNA expression (Caughey, 2007). Tryptogalinin causes excitation of sensory neurons and has anti-inflammatory activity by repressing tryptase-PAR type 2 activation which normally mobilizes intracellular calcium stores to increase intracellular Ca^{2+} concentrations (Payne and Kam, 2004).

The glycosylated Kunitz inhibitor, **TdPI** (tick-derived protease inhibitor), was isolated from SGs of *R. appendiculatus* adult females (Paesen et al., 2007). *TdPI* is only expressed during the four first hours of feeding and manipulates host-immune defenses during the tick feeding process. TdPI potently inhibits trypsin and moderately affects human plasmin and human tryptase activity (Paesen et al., 2007). Mast cells and eosinophils initiate and/or amplify inflammation at the tick feeding site, mostly via the production and degranulation of several pro-inflammatory molecules such as histamine and tryptase. Accordingly, TdPI transcription coincides with that of RaHBPs (*R. appendiculatus* Histamine-Binding Proteins) known to sequester histamine in ticks (Paesen et al., 1999), thus highlighting a probable role for TdPI as a human tryptase inhibitor via complex formation (Paesen et al., 2007). In addition, TdPI may bind to tryptase inside mast cells, and may suppress its autocatalytic activation step (Sakai et al., 1996).

In *R. (B.) microplus,* **BmSI 7** inhibitor regulates bovine neutrophil elastase pro-inflammation activity, due to its strong elastase inhibitory activity. Thus by reducing inflammation and/or avoiding tick tissue degradation, it enables the tick to thwart the host immune system (Sasaki et al., 2008). Similarly, **RMS-3** is likely secreted into tick saliva during feeding, and carries a B-Lymphocyte epitope, highlighting a probable role in host-immune response modulation (Rodriguez-Valle et al., 2012). Finally, 12 further proteins have been extracted from *R. (B.) microplus* larvae, initially **BmTI A-F**, then **BmTI 1-7**, among which only **BmTI 2** and **BmTI 3** were further characterized as follows (Sasaki et al., 2004). Both BmTIs were classified into the BPTI-Kunitz family as they are suspected to have two Kunitz domains. Both inhibit trypsin and hNE, BmTI 2 demonstrating greater trypsin inhibition, and BmTI 3 stronger hNE inhibition, suggesting a role inhibiting the host's inflammatory response (Sasaki et al., 2004).

Host Angiogenesis and Apoptosis Induction

While ticks are biting, they must control and limit wound neovascularization and cell proliferation responses to enable blood meal uptake. Thus, they inject salivary proteins to favor apoptosis and slow down neovascularization as reported for *I. scapularis* (Francischetti et al., 2005a), and interestingly, tSPIs have also been implicated in this role.

Significant similarities are shared between **BmCI** from *R.(B.) microplus* (Lima et al., 2010) and dendrotoxins. Dendrotoxins are non-inhibitory Kunitz proteins able to block different ion channels (Na^+, K^+, Ca^+) involved in both cell proliferation (Lang et al., 2005) and apoptosis (Nutt et al., 2002). BmCI appears to be highly cytotoxic and causes fibroblast cell death via pro-apoptotic activity, without affecting cell cycle integrity, likely by Ca^+ channel activity regulation (Lima et al., 2010).

Haemangin was identified as a salivary Kunitz inhibitor from *H. lonigicornis* carrying one single Kunitz domain, which is up-regulated during blood feeding (Islam et al., 2009). It strongly inhibits trypsin and chymotrypsin, poorly inhibits elastase, and is able to efficiently stimulate degradation of both heavy and light plasmin chains during fibrinolysis, therefore strongly supporting plasmin-dependent fibrinolysis inhibition (Islam et al., 2009). Haemangin also inhibits chick aortic explant angiogenesis; human umbilical vein endothelial cell (HUVEC) differentiation, proliferation, and tube formation; and chick ChorioAlantoic Membrane (CAMs) neovascularization, demonstrating that it can impede with both pre-existing vessel angiogenesis and neovascularization (Islam et al., 2009). Haemangin also significantly induces apoptosis in HUVECs (Nagata, 2000), and affects wound healing in an artificially wounded HUVEC monolayer (Islam et al., 2009). Anti-haemangin RNAi experiments showed that ticks completely failed to make blood pools by 72 h and achieved significantly diminished engorgement by 144 h, while control ticks become engorged by 120 h, with the simultaneous increase of neovascularization in knockdown ticks (Islam et al., 2009). High-throughput studies also indicated that Haemangin can utilize multiple intracellular signaling pathways to negatively regulate angiogenesis, and angiogenesis-dependent wound healing (Islam et al., 2009). Interestingly, it was noticed that soft ticks—which are fast blood-feeders compared to hard tick—lack Haemangin homologs, thereby highlighting the importance of these molecules during the long blood feeding processes of hard ticks (Francischetti et al., 2005a).

BmTI-A from *R. (B.) microplus* also strongly inhibits neovascularization, and prevents new vessel formation *in vitro* by inhibiting plasma kallikrein, plasmin, and elastase (Soares et al., 2016). In addition, BmTI-A inhibits endothelial cell viability and proliferation through kallikrein inhibition (Lang et al., 2005), leading to an absence of bradykinin release, which normally stimulates cell growth and survival (Andoh et al., 2010). The inhibition of plasma kallikrein and plasmin by BmTI-A prevents angiogenic growth factor release during tick infestation, thereby averting cell adhesion mechanisms (Soares et al., 2016). Neutrophil elastase degrades a broad spectrum of extracellular matrix and cell surface proteins, and the release of growth factors such as TGF-β, PDGF, and VEGF, induces cell proliferation and migration. By inhibiting neutrophil elastase, BmTI-A could also inhibit angiogenesis (Wada et al., 2007). BmTI-A is also believed to have an inhibitory action on both vessel formation and wound healing, thus enabling *R. (B.) microplus* to continue feeding (Soares et al., 2012).

Finally, the anticoagulant **Amblyomin-X** from *A. cajennense* can also act as a proteasome inhibitor (Chudzinski-Tavassi et al., 2010). Amblyomin-X demonstrates cytotoxicity in different tumor cells lines, but not in healthy human fibroblast cells (Chudzinski-Tavassi et al., 2010; Morais et al., 2016). In human tumor cells, Amblyomin-X alters the expression of several genes related to the cell cycle (related to the G0/G1 phase, as supported by observed G0/G1 alterations), causes endoplasmic reticulum stress marker accumulation, slightly modulates intracellular calcium concentration [$Ca2^{+}$], causes mitochondrial dysfunction, cytochrome C release, poly (ADP-ribose) polymerase (PARP) cleavage, and activates the caspase cascade (Chudzinski-Tavassi et al., 2010; Morais et al., 2016). Additional *in vitro* assays on endothelial cells showed that Amblyomin-X delays the cell cycle, inhibits cell proliferation and adhesion, tube formation, and membrane expression of the platelet-endothelial cell adhesion molecule-1 (PECAM-1) (Drewes et al., 2012). *In vivo*, Amblyomin-X reduces tumoral mass in a murine melanoma model, decreases the number of metastatic events (Chudzinski-Tavassi et al., 2010), and also inhibits VEGF-A-induced angiogenesis in the dorsal subcutaneous tissue in mice (Drewes et al., 2012). Similar effects were observed in chicken chorioallantoic membrane (CAM). These investigations confirm the anti-tumorigenic and anti-angiogenic properties of Amblyomin-X.

SERINE PROTEASE INHIBITORS INVOLVED IN TICK-BORNE PATHOGEN DEVELOPMENT AND TRANSMISSION

During tick feeding, all the above-mentioned molecules modulating host immune responses and homeostasis create an environment conducive to pathogen transmission and host infection (Brossard and Wikel, 2004; Nuttall and Labuda, 2004; Ramamoorthi et al., 2005; Wikel, 2013). Several tick molecules directly implicated in pathogen transmission have also been identified. They either facilitate pathogen development in the tick vector, or enhance pathogen transmission to the vertebrate host (Liu and Bonnet, 2014). Among them, a few tSPIs have been shown to affect pathogen development and/or transmission (**Figure 2**, **Table 2**) but, in all cases, the precise mechanisms involved remain unknown.

Two-dimensional electrophoresis of total proteins from fully engorged *R. (B.) microplus* adults revealed **BmTI-A** up-regulation in *B. bovis*-infected ticks. This differential expression suggests a putative role for this protein in the tick's immune system, to either limit the potentially detrimental proliferation of the parasite in the vector, or as a molecule required for parasite development and/or colonization of tick tissues (Rachinsky et al., 2007). It is noticeable that the expression of BmTI-A in tick ovaries is coherent with the vertically transmission that occurs for this parasite from the female to the next generation.

DvKPI from *D. variabilis* reduces burden of the obligate intracellular bacteria *R. montanensis* at 24 h post-infection in L929 mouse fibroblasts *in vitro* (Ceraul et al., 2008), and associations between the inhibitor and the bacteria have been reported, thus it was hypothesized that bacteria may express trypsin-like proteases on their outer surface (Ceraul et al., 2011). Alternatively, DvKPI may be implicated in recruiting host factors, such as plasminogen activation system factors facilitating midgut epithelium transmigration, as has been demonstrated for *B. burgdorferi* (Coleman et al., 1997). Therefore DvKPI might be involved in both the tick immune system and in *Rickettsia* development (Walker et al., 1984).

In *I. ricinus,* a comparison of *Bartonella henselae*-infected or non-infected SG transcriptomes led to the discovery of **IrSPI**

(*Ixodes ricinus* serine protease inhibitor), a Kunitz inhibitor with the highest expression following bacterial infection (Liu et al., 2014). It was demonstrated that *B. henselae*, a facultative intracellular bacterium responsible for cat-scratch disease, can be transmitted by *I. ricinus* through the injection of saliva (Cotté et al., 2008). RNAi experiments showed that when *IrSPI* expression was significantly knocked down, both the weight of engorged ticks and *B. henselae* SG load were significantly decreased, suggesting IrSPI involvement in blood feeding, as well as in SG bacterial development (Liu et al., 2014).

Lastly, Lyme disease caused by bacteria from the *Borrelia* genus is unquestionably the predominant concern for the Northern latitude (Dantas-Torres et al., 2012) and several studies concern their transmission by tick from the *Ixodes* genus. **Ixophilin**-immunized mouse assays showed that despite no effects on *Borrelia burgdorferi* burden in *I. scapularis* tick midgut, significantly increased *B. burgdorferi* burden in the skin, heart, and bladder of immunized mice was observed, suggesting a probable role in *B. burgdorferi* transmission to and/or development in the host (Narasimhan et al., 2013).

APPLICATIONS IN TICK CONTROL

To date, acaricides are mainly used to control ticks. But their use has several detrimental effects, including a negative impact on the environment and non-targeted species, as well as an associated rise in resistant tick strains. Thus new effective control measures are urgently required, such as anti-tick vaccines. Vaccines that target important molecules implicated in tick feeding processes and physiology could decrease tick populations and limit transmission of a vast number of pathogens. This is the manner in which the only two commercially-available anti-tick vaccines—based on an *R. (B.) microplus* midgut protein—are believed to function (Kemp et al., 1989). By targeting tick molecules implicated in pathogen establishment, development and transmission, vaccines could also completely impair pathogen transmission.

Several tSPIs have been investigated as possible targets to create anti-tick vaccines. First, a combination of **RAS-1 and -2** *R. appendiculatus* serpins was tested on cattle (Imamura et al., 2006). Results showed that the cumulative number of engorged nymphs and adults fed on vaccinated cattle was significantly lower compared to controls, tick mortality rates were significantly higher, and eggs masses from females were lower. But for ticks that did feed, feeding duration as well as engorgement weight did not differ between the two tick groups.

BmTIs from *R. (B.) microplus* have also been used in cattle immunization assays. A reduction in the total number of engorged ticks was observed (72.8%), as well as reduced engorged female weight in vaccinated animals comparing to controls (Andreotti et al., 2002).

The Serpin-1 **(HLS-1)** from *H. longicornis* was also evaluated as a vaccine candidate against tick infestation (Sugino et al., 2003). No differences in feeding duration or engorgement weight between vaccinated rabbits and controls groups were observed, but a significant increase in tick mortality rates was reported for ticks fed on vaccinated animals. As rabbit anti-tick immunity compromised tick physiology of both nymphs and adults, these promising results support HLS-1 as a vaccine cocktail component, along with other previously characterized antigens (Mulenga et al., 1999; Tsuda et al., 2001).

Another trypsin inhibitor from *R. (B). microplus* was recently identified due to its homology with BmTI. Named **RmLTI**—for *R. (B.) microplus* larval trypsin inhibitor—it belongs to the Kunitz inhibitor family (Guerrero et al., 2005; Andreotti et al., 2012). RmLTI's role is completely unknown, but due to encouraging results reported for BmTIs, RmLTI's potential as a vaccine antigen was also tested in cattle. Vaccination with recombinant RmLTI showed that the average weight of engorged ticks was significantly lower after feeding on vaccinated cattle for up to 9 days after vaccination, but then remained equivalent from days 9–13, probably due to declining antibody levels (Andreotti et al., 2012). However, ticks detaching from RmLTI-immunized cattle still appeared to be affected at day 13, and while no differences were observed in egg laying, significant effects were observed on egg viability, eclosion rate, as well as larval hatchability (Andreotti et al., 2012).

CONCLUSION

The goal of this review was to comprehensively describe the varied roles of tSPIs in both tick physiology and vertebrate host response modulation following tick bite, emphasizing their vital roles in tick-host-pathogen interactions. tSPIs are thus involved in essential processes such as tick's innate immune system and hemolymph clotting. In addition, some tSPIs expressed in ovaries and eggs are essential for tick development, oviposition and egg laying. Some ovary-expressed inhibitors are also utilized by eggs to avoid self-proteolysis and protection against foreign micro-organisms. Ticks have been obligatory blood feeding arthropods for more than 90 million years, as such, they've developed and adapted appropriate molecules to facilitate their extremely efficient feeding processes. Several tSPIs are implicated in blood uptake and digestion in the tick midgut, SGs, and at the wound feeding pool on the host. In addition to immunomodulation and angiogenesis suppression, most tSPIs inhibit blood coagulation, often via FXa and thrombin targeting, enabling effective blood intake. Some tSPIs have also been reported to be involved in direct pathogen establishment and/or transmission, and also by creating opportune conditions to facilitate pathogen transmission from ticks to hosts. Such a wide spectrum of actions ensures that tSPIs are attractive and promising target candidates in anti-tick vaccine strategies to block tick feeding and/or TBP transmission.

AUTHOR CONTRIBUTIONS

AB and SB conducted the literature research and prepared the figures and tables. SB, AB, and TF wrote the paper, provided critical review, and revisions.

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Host Distribution Does Not Limit the Range of the Tick *Ixodes ricinus* but Impacts the Circulation of Transmitted Pathogens

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Ticks, pathogens, and vertebrates interact in a background of environmental features that regulate the densities of ticks and vertebrates, affecting their contact rates and thence the circulation of the pathogens. Regional scale studies are invaluable sources of information about the regulation of these interactions, but a large-scale analysis of the interaction of communities of ticks, hosts, and the environment has been never modeled. This study builds on network analysis, satellite-derived climate and vegetation, and environmental modeling, quantifying the interactions between the tick *Ixodes ricinus* and the transmitted bacteria of the complex *Borrelia burgdorferi* s.l. in the Western Palaearctic. We derived the rates of contact of the tick with 162 species of vertebrates recorded as hosts, and the relative importance of each vertebrate in the circulation of the pathogen. We compiled more than 11 millions of pairs of coordinates of the vertebrates, deriving distribution models of each species and the relative faunal composition in the target territory. The results of the modeling of the distribution of the tick and its hosts, weighted by their importance in the circulation of *Borrelia* captured the spatial patterns of interactions that allow the circulation of the pathogen. Results indicate that both *I. ricinus* and *B. burgdorferi* s.l. are supported in the Western Palaearctic by complex communities of vertebrates, which have large distribution ranges. This high functional redundancy results in the pervasiveness of *B. burgdorferi* s.l., which depends on the gradient of contributions of the large community of vertebrates, instead of relying on a few dominant vertebrates, which was the prevailing paradigm. Most prominent reservoirs of the pathogen are distributed in specific regions of the environmental niche. However, literally dozens of potential reservoirs can colonize many other environmental regions, marginally but efficiently contributing to the circulation of the pathogen. These results consistently point to the need of evaluating the beta-diversity of the community of vertebrates acting as reservoirs of the pathogen to better know the interactions with the vector. They also demonstrate why the pathogen is so resilient to perturbations in the composition of the reservoirs.

Keywords: tick-borne pathogens, communities, habitat overlap, functional redundancy

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INTRODUCTION

The classic procedure to evaluate the potential distribution ranges of ticks has been the capture of their abiotic ecological relationships, with a main attention on climate, using more or less coherent series of records of the focal species and different modeling strategies. Modeling approaches may include variables other than climate, such as categories of the vegetation (which are qualitative proxies for climate), topological features (like slope or altitude) and several indexes derived from the habitat fragmentation and its connectivity (Estrada-Peña, 2003a,b; Brownstein et al., 2005; Li et al., 2012). Other than some advances in producing consistent datasets of climate features and general reviews about the most common gaps in modeling procedures (Estrada-Peña et al., 2013, 2015a), the approach of environmental modeling for ticks and the transmitted pathogens has not experienced significant improvements. The use of habitat fragmentation as an index impacting the resilience of the metapopulation of ticks provided a framework to test the effects of the hosts habitat corridors, and thus its contribution for supporting foci of tick-borne pathogens (Estrada-Peña, 2003a,b). However, field data have proven to be difficult to obtain for verifying this hypothesis (Linard et al., 2007; Lambin et al., 2010; Li et al., 2014; Pérez et al., 2016). Detailed predictions of how individual host species densities will change with fragmentation are not yet possible, despite their importance in the ecology of tick-borne pathogens (Kikpatrick et al., 2017).

In Europe, research has been focused on the tick *Ixodes ricinus*, because of its central role in the transmission of pathogens affecting human health (Medlock et al., 2013). Interest exists to understand the factors shaping tick local densities or the factors regulating the foci of pathogens that the tick transmits. It has been shown that different factors influence the acquisition, maintenance and transmission of pathogens by ticks (de la Fuente et al., 2017). Initial studies demonstrated that the distribution of the tick could be adequately sketched from the main environmental variables in large territories, but also pointed out that the prediction of abundance at regional scales is complex (Vanwambeke et al., 2016). Biotic relationships between a species of tick and its hosts are rarely considered in evaluating the presence and/or abundance of the tick in a territory. Ticks with a wide range of hosts have generalist feeding habits and feed on a large number of vertebrate species. Therefore, it is implicitly assumed that if the environmental factors are suitable, the tick could colonize an area without further evaluation of the biotic component represented by the hosts. This has yet to be empirically proven for most generalist species of ticks, but it is known that *Hyalomma marginatum* has biotic restrictions for colonization in a relatively large area in southern France because of the low densities of large animals necessary to feed the adults (Vial et al., 2016).

Feeding rates of ticks on different vertebrates result from both the availability of the hosts, and their suitability as hosts for the tick. These diverse relationships between hosts and ticks have an impact on the circulation of tick-borne pathogens. Each species of vertebrate has a different capacity to support the circulation of some pathogens. This is well studied for the bacteria of the group *Borrelia burgdorferi* s.l., transmitted by ticks of the complex *Ixodes ricinus*, for which evidence indicates that interactions between the pathogen and reservoirs result in the circulation of combinations of pathogen species according to the availability of prevailing reservoirs for the tick (Kurtenbach et al., 2002; Margos et al., 2011). Studies on *Ixodes scapularis*, which is the vector of the pathogen in parts of USA, demonstrated that, even if several host species are available for feeding the ticks, the carrying capacity of each host is different, some of them feeding a large number of ticks, some other allowing small fractions of the tick population to feed on them (LoGiudice et al., 2003). Further studies demonstrated the differential ability of different host species to feed ticks (Eisen et al., 2004; Castro and Wright, 2007; Barbour et al., 2015). Studies on *I. ricinus* have focused on the durability of the enzootic cycles of *B. burgdorferi*, which depend on the density and abundance of the various vertebrate reservoirs (Hofmeester et al., 2016).

A complex pattern emerges, driven by the abundance of the tick and the faunal composition of vertebrates, serving as hosts for the tick and/or reservoirs for the pathogen, which are not necessarily the same species. Environmental variables shape the distribution of *I. ricinus* and its vertebrate hosts, while the later act as filters of the foci of tick-transmitted pathogens, supporting variable feeding success of the tick and allowing or blocking the circulation of the genospecies of *B. burgdorferi* s.l. These interactions result from (i) the presence/absence and abundance of different host species for the tick, (ii) the degree of spatial overlap between the host species and the tick, (iii) the seasonality of the partners (allowing the temporal overlap and contact for efficient transmission of the pathogen), and (iv) the reservoir capacity of the set of hosts available to feed the ticks. The geographical distribution of *B. burgdorferi* s.l. in questing ticks is therefore a function of the densities of different host species, their capacity to feed ticks and their ability to transmit the bacteria to those ticks.

A comprehensive review of the uncertainties regarding the dynamics of *B. burgdorferi* s.l. has been recently published (Kikpatrick et al., 2017). These authors explicitly stated the need of predicting the nymphal infection prevalence from vector-host-pathogen interactions which "requires data on the fraction of larval ticks that feed on each host species, the fraction of hosts of each species that are infected, and the reservoir competence of these hosts for transmitting *Borrelia* spirochetes" (Kikpatrick et al., 2017). Because the intrinsic difficulties to obtain these estimates from field studies and extrapolate to different sites and time periods, a holistic approach is necessary, connecting the parts in a common framework.

We previously produced a coherent set of records of the tick *I. ricinus* in its Western Palaearctic distribution area, and its recorded hosts (162 species, more than 11 millions of geo-referenced records) and demonstrated that the methodological approach termed network analysis is adequate to examine the biotic relationships between ticks, hosts, and pathogens (Estrada-Peña et al., 2015b). This study was aimed to examine how the environmental variables impact the availability of hosts for the tick in the Western Palaearctic shaping the circulation of the pathogens of the group *B. burgdorferi* s.l. The results provided the

first large scale predictive assessment of the contribution of 162 species of vertebrates to the circulation of *B. burgdorferi* s.l. in the target territory identifying critical portions of the environmental niche for these interactions to take place. The results in the environmental niche where explicitly translated to its spatial counterpart for examining territories where the presence of the tick and the circulation of the pathogens could be restricted by an insufficient availability of vertebrates.

MATERIALS AND METHODS

Background

The study addresses the predicted interactions between the tick *I. ricinus* and a set of 162 species of vertebrates, which are either hosts for the tick or reservoirs for *B. burgdorferi* s.l., which is transmitted by the tick. The aim is to establish the portions of the niche were interactions are high and foci persist. All the data were previously compiled, reported, and assessed for reliability (Estrada-Peña and de la Fuente, 2016). The complete set of data has been already published (i.e., Estrada-Peña et al., 2013; Estrada-Peña and de la Fuente, 2016) further updated for this study, and is available in a public repository (http://datadryad.org/resource/doi:10.5061/dryad.2h3f2). In this study we aimed to (i) capture the suitability of the environment for the tick and each species of the vertebrate in the Western Palaearctic, (ii) compute an index of habitat overlap of the tick with each species of vertebrate, (iii) weight the index before according to the importance of each species of vertebrate for either the tick or the pathogen, (iv) project the results in the environmental niche, and (v) to project the results in the spatial (geographical) niche. The results must to be interpreted as the predicted circulation of the pathogen in either the dimensions of the niche or its spatial translation.

Set of Distribution Data of *I. ricinus* and Its Hosts

Data on *I. ricinus* were compiled from literature references published since the year 1990. Data reporting either questing ticks or feeding on hosts were included. In the first case, only data including reliable coordinates or an unambiguous locality name (which was later resolved to coordinates) were considered. In the second, only data mentioning the species of hosts were further processed if adequately geo-referenced.

We downloaded the data of the recorded distribution of the vertebrates reported as hosts or reservoirs from the Global Biodiversity Information Facility (GBIF: http://gbif.org). The purpose of the compilation is to obtain the largest available source of recorded distributions of every vertebrate involved in the circulation of the pathogens.

Choice of Environmental Variables and Environmental Modeling

A number of reports have argued for the use of predictors that are ecologically relevant to the target species (Glass et al., 1995; Guerra et al., 2002). In this sense, Araújo and Guisan (2006) stated that the "use of automated solutions to predictor selection... should not be seen as a substitution for preselecting sound eco-physiological predictors based on deep knowledge of the bio-geographical and ecological theory." We already expressed our concerns about the reliability of interpolated variables in the building of predictive models (Estrada-Peña et al., 2016) and satellite-derived information seems to be far more robust than interpolated measures of climate, which otherwise retain its value to explain the weather conditions in a given interval of time. We adhered to published protocols (Estrada-Peña and de la Fuente, 2016) to obtain a time series of MODIS-derived satellite data regarding land surface temperature (LSTD) and the Normalized Difference Vegetation Index (NDVI) which is a measure of the photosynthetic activity of the vegetation. It has been reported that variables derived from these two basic measurements of the environment are able to capture a large fraction of the factors driving the distribution of organisms (Estrada-Peña et al., 2015a). We used data at 16-days intervals spanning the period 2001–2015, which were subjected to a Fourier transformation (or harmonic regression). After the transformation, the average, maximum and minimum LSTD and NDVI were used for modeling purposes.

Probabilities of occurrence of both the tick and the hosts were produced using correlative modeling. We calculated the expected environmental suitability for the tick and the hosts using the pairs of coordinates for the recorded distribution of every organisms (as reported by Estrada-Peña and de la Fuente, 2016; and available in http://datadryad.org/resource/doi:10.5061/dryad.2h3f2). We independently modeled the distribution of each species using the niche modeling algorithm MaxEnt integrated in the package dismo for R (Hijmans et al., 2017). This modeling algorithm demonstrated robust performance when presence-only data is available. Models were developed with lineal and quadratic features, with a maximum of 10,000 background points, 10 replicates per species modeled, and 70% of points for training purposes, using cross-validation to compare the resulting models. The regularization multiplier was set to 1. Each model was replicated 100 times using the cross-validation function in MaxEnt to partition the data into replicate folds.

Modeling of the Biotic Interactions between the Tick and Hosts

The procedure above produces an estimation of the probable distribution of each organism. However, biotic interactions between the tick and the hosts are not taken into account. It is therefore necessary not only to evaluate the amount of habitat overlap between the tick and each host, but also to weight that ratio by the estimated interaction between the partners. It has been reported that the networks theory can provide light to evaluate these relationships (Estrada-Peña et al., 2015b). The basic tenets of network theory evaluate interactions between "nodes" (i.e., organisms) using the number of times such relationship has been recorded. It has been proposed (Estrada-Peña and de la Fuente, 2016) that a Centrality-Weighted habitat overlap (CWho) index is a simple definition of (i) the habitat overlap between the tick and each species of hosts, and (ii) the number of recorded interactions that weights the crude value of habitat overlapping. The readers are referred to the previous

publication for the complete derivation and rationale of the index.

Results from the CWho must to be interpreted as "expected probabilities of interactions" between the tick, and the host(s). These interactions are derived from the habitat overlap between tick and host(s) weighted by the relative importance of each vertebrate as host(s) for the tick or as reservoir(s) for the pathogen. Thus, high values of the CWho mean for high predicted interactions resulting in a large circulation of the pathogen. Low values of CWho may result from a low habitat overlap of the tick and host(s) or from the low importance of a vertebrate for either the tick or the pathogen. The methodology introduced allows the expression of these values in either the environmental or the spatial dimensions. This immediately provides an estimation of these predicted interactions in the climate or spatial gradients.

Spatial Processing and Plotting on the Environmental Niche

The environmental niche is a gradient of n dimensions that equal the number of variables used for its definition. To obtain a tractable framework without losing reliability it is necessary to divide the gradient of the niche into categories. We used two multifactorial methods to (i) reduce the number of dimensions and divide the territory in "categories of niche" using a Principal Components Analysis (PCA) approach, and (ii) associate the results of the predictive modeling of each species of hosts with the environmental using a Canonical Correspondence Analysis (CCA). The first method produces "categories of niche" and the second associates the CWho to these categories. In this application, PCA takes the 6-dimensions environmental niche and produces a number of categories using recursive rules. Each category is thus composed by the portions of the niche gradient that are more similar between them than with the other, always considering the 6 dimensions. **Figure 1** shows the spatial distribution of the average LSTD and NDVI in the target territory, and how the combination of the six variables reduced by PCA aggregate into sites.

The use of the sites emanating from a PCA has the immediate application of associating the interactions between organisms with the different sites in a simple two-axes chart. This results in the mapping of the CWho along the reduced environmental niche. We choose CCA as the ordination technique to derive biologically scaled responses along environmental gradients: this results in the association between "sites" (which represent the zonation obtained from the environmental gradients) and the "organisms" (which are the hosts of *I. ricinus* or reservoirs for *B. burgdorferi s.l.*). Since its original development (ter Braak, 1986) CCA has been already used to describe a variety of ecological communities, together with the factors that shape them, and to display the relationships between survey points and environmental variables prevailing at the collections points (Dumbrell et al., 2010; Legendre et al., 2011). The method has been adequately tested under several conditions and proved to be enough robust and confident (Gittins, 2012; ter Braak, 2014).

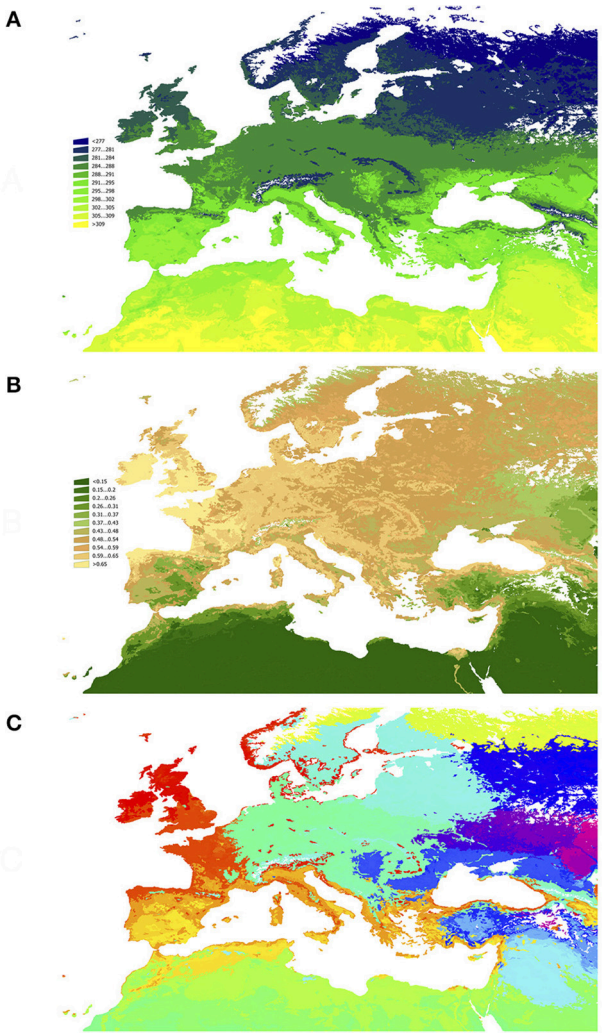

FIGURE 1 | A map of the target territory with the gradient of land surface temperature (**A**, in Kelvin), and of NDVI (**B**, unit less), and **(C)** the categories of the territory (sites) according to the gradients in **(A,B)**, after disaggregation using a Principal Components Analysis (sites colored consecutively from blue to yellow).

RESULTS

The Categories of Climate and the Environmental Suitability for Hosts

The aggregation of the territory along the environmental gradients of LSTD and NDVI produced a total of 340 sites (**Figures 1A–C**). Each correlatively colored region in **Figure 1C** represents a portion of the territory that has a statistically significant different combination of both LSTD and NDVI. **Figure 2** shows the distribution of all the sites in the environmental space of the average LSTD × NDVI, with the values of CWho. The plot shows each site correlatively numbered: the position of each site corresponds to its values of LSTD (X axis) and NDVI (Y axis). The size, color and transparency of each site correspond to the predicted interactions, collectively for all

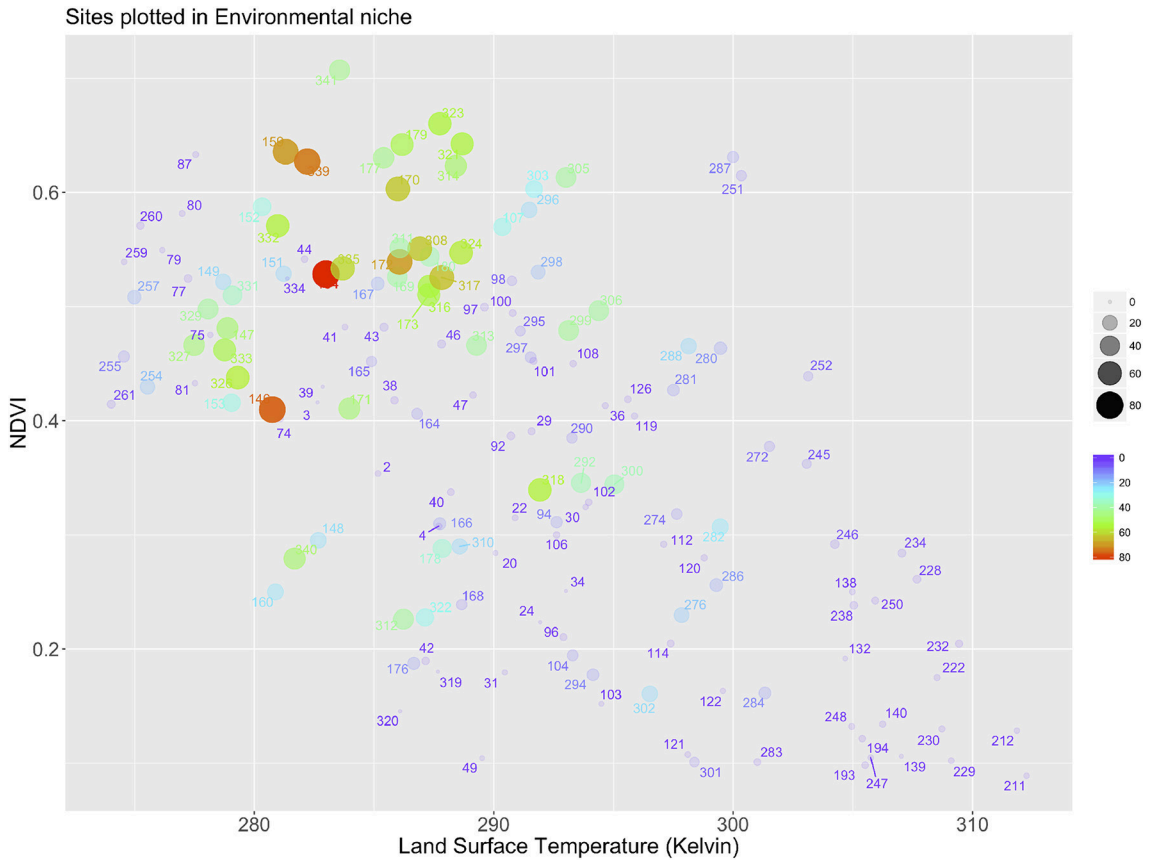

FIGURE 2 | The plot of the sites in Western Palaearctic, along the two axes delimiting land surface temperature and the NDVI. Each dot corresponds to one of the sites produced by aggregation of the gradients of the environmental variables after a Principal Components Analysis. Each dot represents the position of one site according to its values of average of LST and NDVI. The color, the size and the transparency (to improve readability) of each dot is proportional to the Centrality-Weighted habitat overlap of the complete set of hosts.

vertebrates. Relatively high values of CWho exist in large regions of the environmental niche. A cluster of sites with the highest CWho appears at colder (280–290 K) and wet (NDVI = 0.4–0.7) portions of the niche, with isolated spots of high CWho in the warmer and drier portions of the niche. However, CWho clearly decreases in the warmest and driest part of the environmental gradient.

The Interactions in the Reduced Environmental Space

Figure 3 plots the sites according to the reduction of the 6 environmental variables after a PCA, using the color and the size of the symbols to display the average LSTD and NDVI of each site, respectively. The axis X is mainly driven by the temperature, with colder sites at the right of the ordination and warmer sites at the left. The values of NDVI have smaller importance in this ordination, and variability follows mainly the axis Y. This plot of sites associates immediately with the plot of the CWho, as displayed in the **Figure 4**. To improve the readability of the chart we labeled only the 23 most prominent species of vertebrates for the circulation of *B. burgdorferi* s.l. A clear patterns appears: interactions with most prominent reservoirs of *B. burgdorferi* are associated with a variable range of temperature (appearing along most of the range of the X axis) but in the portions of the chart corresponding with medium and high values of NDVI. *Apodemus agrarius* is however separated of that gradient and interactions associate with cold portions of the niche and medium values of NDVI. The remaining 133 species of hosts are restricted to portions of higher LSTD and lower values of NDVI (**Figure 4**).

The Interactions in the Geographic Space

The CWho was translated from the environmental gradient into the geographic space to capture the spatial gradient of variability in the interactions. These results were summarized in **Figures 5**–**7**, including the expected interactions with two species of Rodentia: Muridae (*Apodemus sylvaticus* and *A. flavicollis*) and two species of Passeriformes: Turdidae (*Turdus merula* and *T. phylomelos*) (**Figures 5A–D**), the expected interactions with Aves: Passeriformes (**Figure 6**), and the same values for Mammalia: Rodentia (**Figure 7**). The results clearly indicated that large portions of the territory are suitable resulting in a potential circulation of the pathogen in a wide territory.

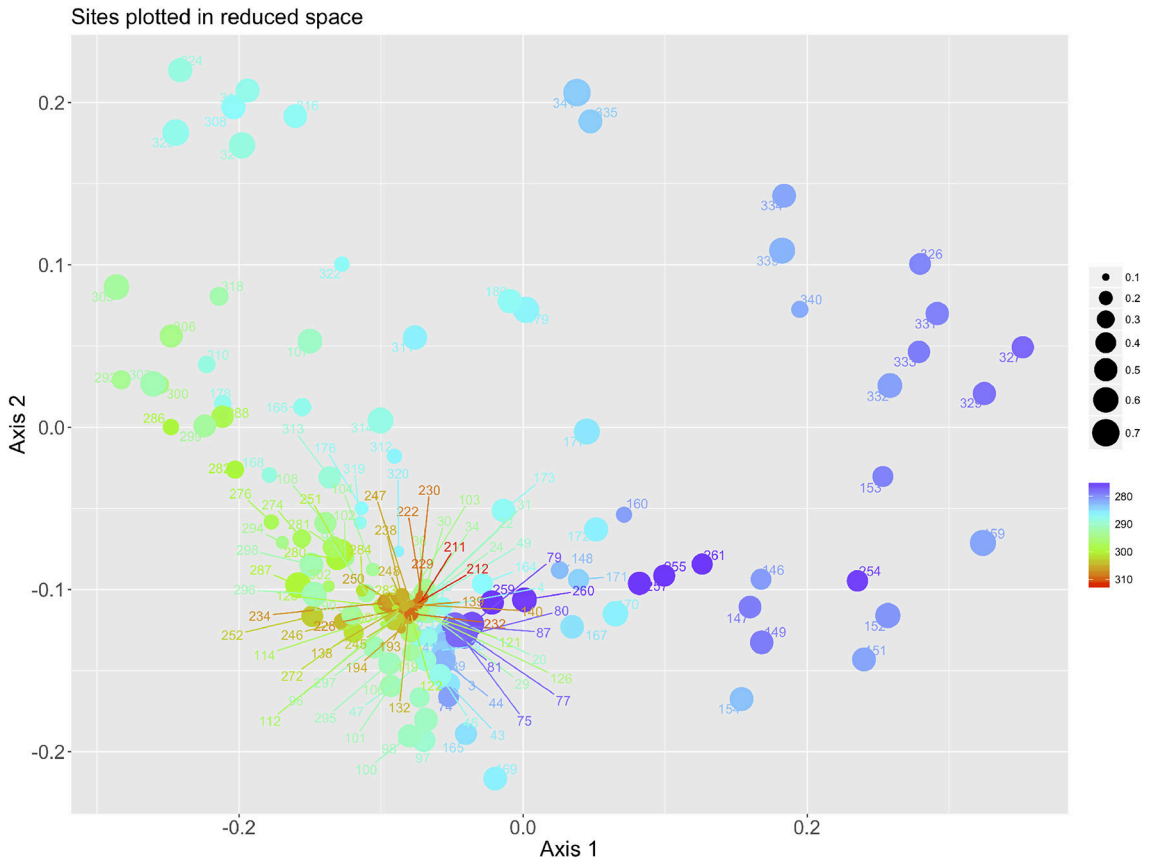

FIGURE 3 | The plot of the sites of Western Palaearctic, along the two axes obtained after a Canonical Correspondence Analysis of the 6 variables of LST and NDVI. Each dot corresponds to one of the sites produced by aggregation in categories along the gradients of the environmental variables and represents the position of one site in the reduced niche. The color is proportional to the temperature range (in Kelvin), the size is proportional to the NDVI (unit less).

DISCUSSION

This study showed that *B. burgdorferi* s.l. persists in the Western Palaearctic because of a redundancy of the interactions vertebrates that are both suitable hosts for the tick vector and adequate reservoirs of the pathogen. Even if the top reservoirs are absent in a territory, the wide availability of vertebrates considered secondary reservoirs contribute to the persistence of the pathogen. The role of biotic interactions has been classically discussed as one of the factors driving the distribution of ticks but its importance for the circulation of pathogens has been neglected. We specifically focused this study on *I. ricinus* and *B. burgdorferi* s.l. because its impact on human health (i.e., Medlock et al., 2013; Jahfari et al., 2014; Biernat et al., 2016; Radzijevskaja et al., 2016). *Ixodes ricinus* is one of the species of ticks that transmits *B. burgdorferi* s.l. and feeds on a wide spectrum of hosts, with a variable feeding success because their innate resistance to the tick (Pérez et al., 2016; van Duijvendijk et al., 2016; Van Oosten et al., 2016). These tick-vertebrate contact rates are primarily governed by the prevailing climate, which shapes the abundance of the hosts and the tick (Estrada-Peña et al., 2014) further modulated by a complex set of factors including the immune system plus morphological and physiological traits of the vertebrates (Barbour et al., 2015; Hofmeester et al., 2016). Many studies have demonstrated that the relative faunal composition of vertebrates is a key factor driving the community of this pathogen (Rudenko et al., 2014) and recent reports addressed meta-analyses of the relative importance of the reservoirs in the maintenance of active foci (i.e., Barbour et al., 2015; van Duijvendijk et al., 2015; Kikpatrick et al., 2017). However, a predictive approach for modeling Lyme borreliosis utilizing *separately* the abiotic interactions of the tick and the environment, the contact rates between ticks and vertebrates, and the reservoir capacity of the vertebrates, is unreliable (Estrada-Peña and de la Fuente, 2016). A holistic approach is necessary for understanding the patterns of circulation of the pathogen (Franke et al., 2013; Schotthoefer and Frost, 2015).

We formulated a theoretical background derived from a large dataset of interactions between ticks, pathogens and vertebrate reservoirs, demonstrating that ecological relationships between partners could be mapped in the environmental niche to extract relevant epidemiological information (Estrada-Peña and de la Fuente, 2016). The method has a strict mathematical foundation and can evaluate the relative importance of each vertebrate

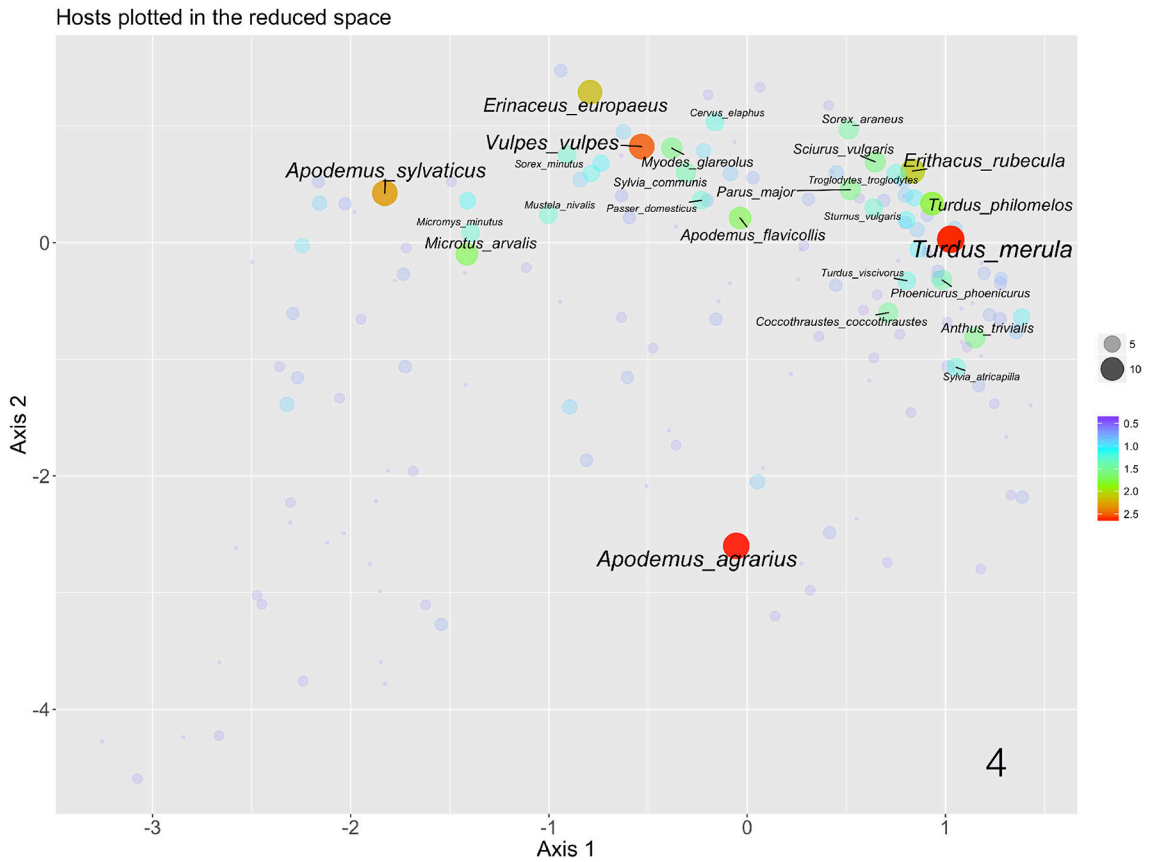

FIGURE 4 | The plot of the species of vertebrates involved in the support of *I. ricinus* and *B. burgdorferi* s.l. in Western Palaearctic, along the two axes obtained after a Canonical Correspondence Analysis of the 6 variables of LST and NDVI. Each dot corresponds to a species of vertebrate and represents its position in the reduced niche (to be compared with the values of LST and NDVI as shown in the previous chart). The color and the size are proportional to the Centrality-Weighted habitat overlap. Only the top 23 species of vertebrates in the range of importance for the circulation of *B. burgdorferi* s.l. are labeled.

species supporting the circulation of a tick-transmitted pathogen. We herein demonstrated that (i) the plotting on the reduced space is suitable for understanding the relationships between vertebrates and their niches, (ii) the use of a CCA provides a coherent framework to demonstrate these links, (iii) *I. ricinus* does not depend on a few hosts to colonize different portions of the environmental niche, since literally every portion of that niche is filled with a large array of hosts, and (iv) prevailing weather shapes the patterns of circulation of the complex *B. burgdorferi* s.l. supporting a large redundancy of the vertebrates allowing permanent foci. This conclusion is of special interest since local or regional analyses of the reservoir capacity of the vertebrates could not be extrapolated to other regions where the pathogen circulates. Our study has explicitly restricted the analyses to the environmental niche, with only a partial translation of the results to the spatial (geographical) domain as a proof of concept. The extrapolation of results to the geographical dimensions is of special complexity giving the genetic diversity and competition events among the reservoirs established at each site, which has a direct impact on the circulation of the pathogen (Becker et al., 2016). This results in a complex circumstance in which risk assessment in the space is necessary for human health managers, but for which our level of knowledge is far from complete.

The results showed that the wide circulation of *B. burgdorferi* s.l. in Western Palaearctic arises from a large number of vertebrate species covering literally every portion of the niche available for the tick vector. Everywhere *I. ricinus* exists, a considerable variety of vertebrates exist, amplifying the transmission of the pathogen. Therefore, the circulation of *B. burgdorferi* s.l. is based on the concept of functional redundancy, something that escaped to meta-analyses. Since the niche produces an obvious gradient of suitability (and therefore abundance) of the main reservoirs, variable prevalence of infected ticks with different species of *Borrelia* should be expected. This fact produces the already reported regional differences in the prevalent *Borrelia* spp. (van Duijvendijk et al., 2015; Pérez et al., 2016; Ruyts et al., 2016). It is necessary to stress that the circulation of *B. burgdorferi* s.l. has been never tested in the context of the complete community of vertebrates, most extensive data coming from areas in the United States (i.e., LoGiudice et al., 2003). This is of

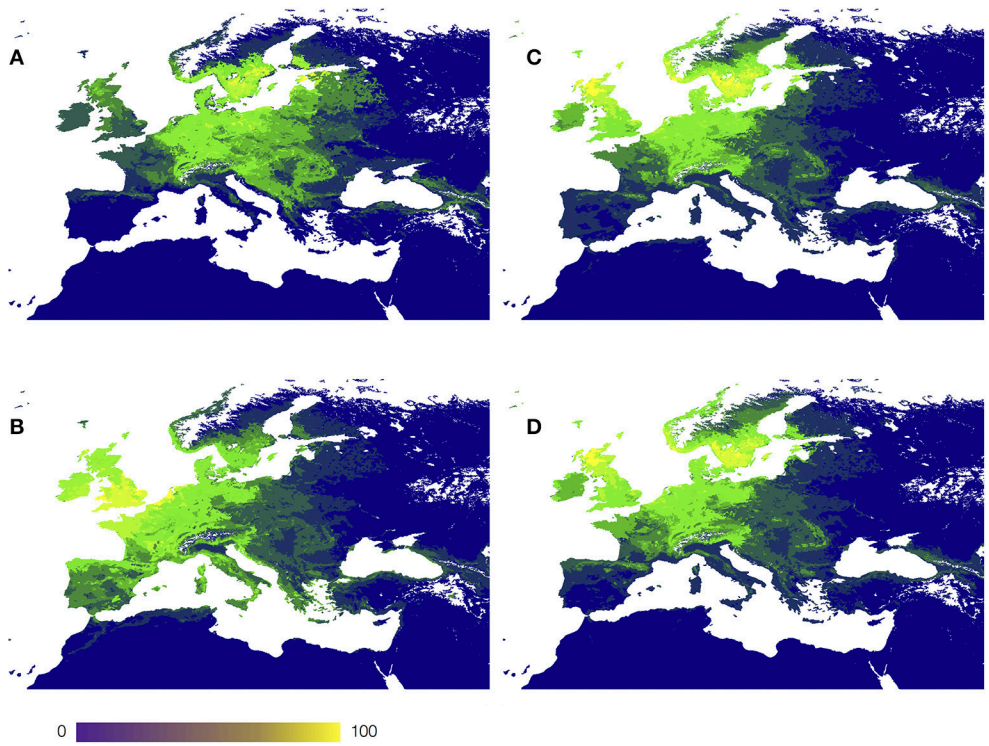

FIGURE 5 | The plot of the Centrality-Weighted habitat overlap for *B. burgdorferi* s.l. in Western Palaearctic with four selected species of vertebrates, rescaled to the range 0–100 for comparability. **(A)** *Apodemus agrarius*; **(B)** *Apodemus sylvaticus*; **(C)** *Turdus merula*; **(D)** *Turdus philomelos*.

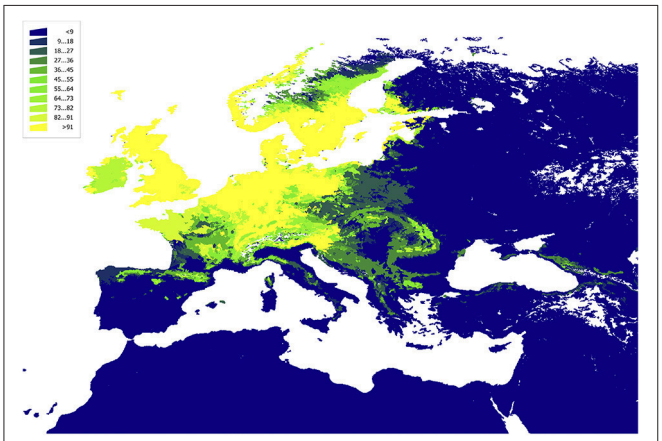

FIGURE 6 | The plot of the Centrality-Weighted habitat overlap for *B. burgdorferi* s.l. in Western Palaearctic with Aves, Passeriformes, rescaled to the range 0–100 for comparability.

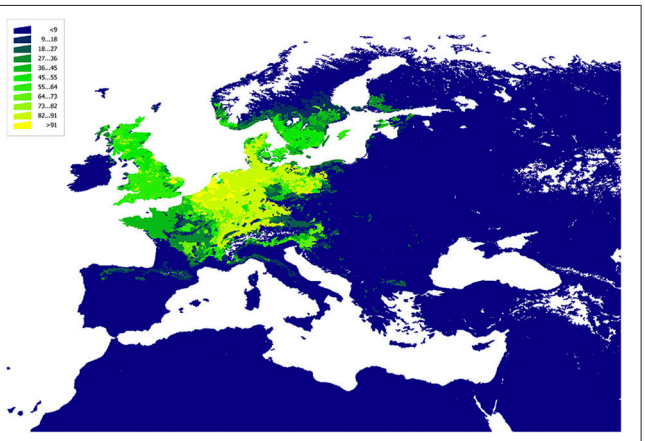

FIGURE 7 | The plot of the Centrality-Weighted habitat overlap for *B. burgdorferi* s.l. in Western Palaearctic for Rodentia, rescaled to the range 0–100 for comparability.

significance because in the absence of a vertebrate, other(s) could assume a different role as hosts for the tick, which may have different capacities for supporting the circulation of the pathogen. Available reports and meta-analyses refer commonly to the most easily trapped subset of vertebrates, since some others may be especially difficult to trap or constitute endangered species. These procedures would under-represent some vertebrates, potentially resulting in a distortion of the perception of the tick-vertebrate-pathogen system. This "context view" would be desirable to further contribute to the understanding of any tick-pathogen system (Hofmeester et al., 2016).

The results of this study stressed the descriptive abilities of a network of interactions between ticks and vertebrates for

supporting foci of a tick-borne pathogen on large portions of the abiotic niche. This conclusion challenges the classic dogma of only a few vertebrates supporting the stability of the circulation of *B. burgodrferi* s.l. (States et al., 2014; Hofmeester et al., 2016). Our results demonstrated that the generalist feeding habits of the tick and the large availability of the reservoirs provide the substratum where the pathogen largely circulates. Environmental forces that affect niche overlap between the tick and the vertebrates shape the gradient of transmission; it is unrealistic to observe these transmission forces under the scope of a reduced set of reservoirs. Therefore, one of our main conclusion is that reservoir competence analyses obtained from regional studies, which are invaluable to understand local circulation rates of the pathogen, are meaningless if extrapolated to different regions. Most of the niche where the tick survives is filled with a high diversity of vertebrates, allowing the circulation of the pathogen at variable rates according to the genetic composition of the prevailing fauna. Their contributions, together, largely support the wide circulation of the pathogen in the target territory. We explicitly recommend the exploration of the environmental niche to characterize the circumstance under which a tick-borne pathogen can be prevalent, before exploring the spatial relationships of its distribution.

This framework could be applied to other species of ticks, or, perhaps of most importance, to integrate the current knowledge of the ecological relationships between the species of ticks transmitting the different genospecies of *B. burgdorferi* s.l. in the world. This would provide an overview of the large amount of data so far reported from regional surveys. Quantitative data about reservoir abilities of each vertebrate as well as explicit considerations of their abilities to support the feeding of ticks should be included in further improvements of this framework.

AUTHOR CONTRIBUTIONS

Both AE-P and JdlF designed the experiments, contributed to the development of the study, and wrote the paper.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Anaplasma phagocytophilum Infection Subverts Carbohydrate Metabolic Pathways in the Tick Vector, *Ixodes scapularis*

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The obligate intracellular pathogen, *Anaplasma phagocytophilum*, is the causative agent of human, equine, and canine granulocytic anaplasmosis and tick-borne fever (TBF) in ruminants. *A. phagocytophilum* has become an emerging tick-borne pathogen in the United States, Europe, Africa, and Asia, with increasing numbers of infected people and animals every year. It has been recognized that intracellular pathogens manipulate host cell metabolic pathways to increase infection and transmission in both vertebrate and invertebrate hosts. However, our current knowledge on how *A. phagocytophilum* affect these processes in the tick vector, *Ixodes scapularis* is limited. In this study, a genome-wide search for components of major carbohydrate metabolic pathways was performed in *I. scapularis* ticks for which the genome was recently published. The enzymes involved in the seven major carbohydrate metabolic pathways glycolysis, gluconeogenesis, pentose phosphate, tricarboxylic acid cycle (TCA), glyceroneogenesis, and mitochondrial oxidative phosphorylation and β-oxidation were identified. Then, the available transcriptomics and proteomics data was used to characterize the mRNA and protein levels of *I. scapularis* major carbohydrate metabolic pathway components in response to *A. phagocytophilum* infection of tick tissues and cultured cells. The results showed that major carbohydrate metabolic pathways are conserved in ticks. *A. phagocytophilum* infection inhibits gluconeogenesis and mitochondrial metabolism, but increases the expression of glycolytic genes. A model was proposed to explain how *A. phagocytophilum* could simultaneously control tick cell glucose metabolism and cytoskeleton organization, which may be achieved in part by up-regulating and stabilizing hypoxia inducible factor 1 alpha in a hypoxia-independent manner. The present work provides a more comprehensive view of the major carbohydrate metabolic pathways involved in the response to *A. phagocytophilum* infection in ticks, and provides the basis for further studies to develop novel strategies for the control of granulocytic anaplasmosis.

Keywords: proteomics, transcriptomics, glucose metabolism, *Ixodes scapularis*, *Anaplasma phagocytophilum*

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INTRODUCTION

Anaplasma phagocytophilum (Rickettsiales: Anaplasmataceae) is an obligate intracellular bacterium mainly transmitted by *Ixodes* spp. ticks. This emerging pathogen has been reported in the United States, Europe, Africa, and Asia (de la Fuente et al., 2008; Stuen et al., 2013; Kocan et al., 2015), causing human granulocytic anaplasmosis (HGA), equine and canine granulocytic anaplasmosis and tick-borne fever (TBF) of ruminants (de la Fuente et al., 2008; Stuen et al., 2013; Kocan et al., 2015).

The development of *A. phagocytophilum* is complex and coordinated with the tick feeding cycle. Infection and multiplication in ticks occurs first in midgut cells during blood feeding, and then subsequently in other tissues including hemocytes and salivary glands from where transmission occurs to susceptible hosts (Kocan et al., 2015). To establish infection, *A. phagocytophilum* affect mechanisms that appear to be common to ticks and vertebrate hosts (de la Fuente et al., 2016a). These mechanisms include but are not limited to remodeling of the cytoskeleton, inhibition of cell apoptosis, manipulation of the immune response, and modification of cell epigenetics and metabolism (de la Fuente et al., 2016a).

Recently, transcriptomics, proteomics and metabolomics analyses of infected *I. scapularis* ISE6 cells showed that *A. phagocytophilum* infection affects glucose metabolic pathways (Villar et al., 2015). These results suggested that *A. phagocytophilum* manipulate carbohydrate metabolism to facilitate infection and multiplication in tick cells. However, the mechanisms used by *A. phagocytophilum* for the manipulation of carbohydrate metabolic pathways have not been fully characterized.

To better characterize the mechanisms used by *A. phagocytophilum* to manipulate carbohydrate metabolic pathways during infection of tick cells, the dynamics of the carbohydrate metabolism was characterized in the tick vector, *I. scapularis* in response to pathogen infection. First, the composition of major carbohydrate metabolic pathways was annotated using the recently published genome of *I. scapularis* (Gulia-Nuss et al., 2016). Then, previously published transcriptomics and proteomics data (Ayllón et al., 2015; Villar et al., 2015) was used to characterize the mRNA and protein levels of carbohydrate metabolic pathway components in response to *A. phagocytophilum* infection of *I. scapularis* nymphs, female midguts and salivary glands, and ISE6 cultured tick cells. Finally, functional studies were conducted in ISE6 tick cells to provide additional support for the role of these components during pathogen infection. These results expanded our knowledge of the different pathways affected by *A. phagocytophilum* infection in ticks, and provided new potential targets for the development of therapeutic and prevention strategies for the control of granulocytic anaplasmosis and other tick-borne diseases.

MATERIALS AND METHODS

Annotation of the Major Carbohydrate Metabolic Pathway Components in the *I. scapularis* Genome

The *I. scapularis* genome (Gulia-Nuss et al., 2016) was searched with the specific names of genes encoding for enzymes involved in the major carbohydrate metabolic pathways, glycolysis, gluconeogenesis, pentose phosphate, tricarboxylic acid cycle (TCA), glyceroneogenesis, and mitochondrial oxidative phosphorylation and β-oxidation. When records were not obtained using specific enzyme names, then the *I. scapularis* genome was searched with the Blastp tool from the Basic Local Alignment Search Tool (BLAST) using the human ortholog as "query" (Altschul et al., 1990; Madden et al., 1996). The sequences with the lowest *E*-value were selected. The conserved domains of identified protein sequences were classified using the protein families database Pfam (Finn et al., 2014). The *I. scapularis* orthologs found in the genome were double-checked by searching the *Homo sapiens* genome database using as queries the tick homologs identified in the previous step.

Characterization of the *I. scapularis* mRNA and Protein Levels in Response to *A. phagocytophilum* Infection

The quantitative transcriptomics and proteomics data for uninfected and *A. phagocytophilum*-infected *I. scapularis* nymphs, female midguts and salivary glands, and ISE6 cultured cells were obtained from previously published results (Ayllón et al., 2015; Villar et al., 2015) and deposited at the Dryad repository database, NCBI's Gene Expression Omnibus database and ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002181 and doi: 10.6019/PXD002181. For transcriptomics and proteomics analysis in *I. scapularis* nymphs, female midguts and salivary glands, the procedures were described in Ayllón et al. (2015). Briefly, nymphs and adult female *I. scapularis* were infected with *A. phagocytophilum* by feeding on a sheep inoculated intravenously with $\sim 1 \times 10^7$ *A. phagocytophilum* (NY18 isolate)-infected HL-60 cells (90–100% infected cells). In this model, over 85% of ticks become infected with *A. phagocytophilum* in nymphs, midguts and salivary glands. Ticks (200 nymphs and 100 female adults) were removed from the sheep 7 days after infestation, held in the humidity chamber for 4 days and dissected for DNA, RNA, and protein extraction from whole internal tissues (nymphs) or midguts and salivary glands (adult females). Adult midguts and salivary glands were washed in PBS after collection to remove hemolymphs-related cells. Uninfected ticks were prepared in a similar way but feeding on an uninfected sheep. Two independent samples were collected and processed for each tick developmental stage and tissue. After RNA sequencing on an Illumina Hiseq 2000, TopHat was used to align the reads to the *I. scapularis* (assembly JCVI_ISG_i3_1.0; http://www.ncbi.nlm.nih.gov/

nuccore/NZ_ABJB000000000) reference genome. Raw counts per gene were estimated by the Python script HTSeq count [http://www-huber.embl.de/users/anders/HTSeq/] using the reference genome. The raw counts per gene were used by DEGseq to estimate differential expression at $P < 0.05$. For peptide identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using iTRAQ labeled peptides, all spectra were analyzed with Proteome Discoverer (version 1.4.0.29, Thermo Fisher Scientific) using a Uniprot database (http://www.uniprot.org) containing all sequences from Ixodida, Anaplasmataceae and Ruminantia. Peptide identification was validated using the probability ratio method and false discovery rate (FDR) was calculated using inverted databases and the refined method with an additional filtering for precursor mass tolerance of 12 ppm. Only peptides with a confidence of at least 95% were used to quantify the relative abundance of each peptide. Outliers at the scan and peptide levels and significant protein-abundance changes were detected from the *z*-values (the standardized variable used by the model that expresses the quantitative values in units of standard deviation) by using a FDR threshold of 5%. Results were the mean of two replicates. For transcriptomics and proteomics analysis in tick cells, the *I. scapularis* embryo-derived tick cell line ISE6, provided by Ulrike Munderloh, University of Minnesota, USA, was cultured in L-15B300 medium (Munderloh et al., 1999), except that the osmotic pressure was lowered by the addition of one-fourth sterile water by volume. The ISE6 cells were first inoculated with *A. phagocytophilum* (human NY18 isolate; Asanovich et al., 1997)-infected HL-60 cells and maintained until infection was established and routinely passaged. Uninfected and infected cultures ($N = 3$ independent cultures with $\sim 10^7$ cells each) were sampled at 7 days post-infection (dpi; percent infected cells 71–77%; Ave $\pm$ *SD*, 74 $\pm$ 3). Transcriptomics data was obtained as described above for nymphs and adult female ticks. Two biological replicates were used for each of uninfected and infected tick cells and genes differentially expressed in response to *A. phagocytophilum* infection were selected with $P \leq 0.05$. The proteomics analysis followed the same pipeline describe above in ticks and the MS/MS raw files generated with Xcalibur (version 2.1, Thermo Fisher Scientific) were searched against a compiled database containing all sequences from Ixodida and Anaplasmataceae (http://www.uniprot.org). Three biological replicates were used for each of uninfected and infected tick cells. For the quantitative analysis of tick proteins, after discarding *Anaplasma* proteins in infected cells, the total number of peptide-spectrum matches (PSMs) for each tick protein were normalized against the total number of PSMs in tick cells and compared between control and infected cells by Chi2-test ($P \leq 0.05$). Although the percent of infected ticks and cultured cells was determined as described above, the bacterial load on these samples was not considered in the analysis.

The identified genes in the carbohydrate metabolic pathways were searched against the transcriptomics and proteomics data to characterize their mRNA and protein levels in response to *A. phagocytophilum* infection.

Tertiary Structure Modeling and Optimization

The tertiary structures of the partial protein sequences of *I. scapularis* hypoxia-inducible factor 1 alpha HIF-1α (XP_002414889) and HIF-1β (XP_002416629) proteins, which are the DNA binding N-terminus domains, were modeled using the Swiss-Model server (Biasini et al., 2014). The tertiary models were optimized using the Protein Preparation Wizard (Li et al., 2007) in the Schrödinger's Maestro software package. The Protein Preparation Wizard clusters at the highest degree of hydrogen bonding in equilibrium. Monte Carlo orientations are performed (100,000) for each cluster. The optimized structure is based on electrostatic and geometric scoring functions. Any remaining steric clashes were eliminated by minimization of the entire system with the default settings in the Schrodinger's Maestro package. The ternary structure that includes the hypoxia response element (HRE) was constructed via superimposition by using the Swiss-Model server used the mouse HIF-1α, HIF-1β, and the HRE (Dalei et al., 2015) as a template for both *I. scapularis* hypoxia-inducible protein sequences. As a final optimization step, the ternary structure (HIF-1α/HIF-1β/HRE) was processed using the normal mode ready-made script in the Metropolis Monte Carlo-based Protein Energy Landscape Exploration (PELE) server (Borrelli et al., 2005). The PELE software implements an anisotropic network model (Atilgan et al., 2001) for perturbations of the alpha-carbon backbone causing structural conformational changes. The PELE server can be accessed at https://pele.bsc.es/.

Immunofluorescence Assay in *I. scapularis* Midguts and Salivary Glands

Female ticks fed on *A. phagocytophilum*-infected and uninfected sheep and fixed with 4% paraformaldehyde in 0.2 M sodium cacodylate buffer were embedded in paraffin and used to prepare sections on glass slides as previously described (Ayllón et al., 2015). The paraffin was removed from the sections through two washes in xylene and the sections were hydrated by successive 5 min washes with a graded series of 100, 96, and 65% ethanol and finally with distilled water. Next, the slides were treated with Proteinase K (Dako, Barcelona, Spain) for 7 min, washed with 0,1% PBS-Tween 20 (Sigma-Aldrich, St. Louis, MI, USA) and blocked with 2% bovine serum albumin (BSA; Sigma-Aldrich) in PBS-Tween 20 during 1 h at room temperature. The slides were then incubated overnight at 4°C with mouse anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibodies (ab50567; Abcam, Cambridge, UK) diluted 1:100 in 2% BSA/PBS-Tween 20. Preimmune serum was used as control. After 3 washes with PBS-Tween 20, the slides were incubated for 1 h with rabbit anti-mouse IgG conjugated with FITC (Sigma-Aldrich) diluted 1:160 in 2% BSA/PBS-Tween 20. Finally, after two washes with PBS the slides were mounted on ProLong Diamond Antifade Mountant with DAPI reagent (Thermo Scientific, Madrid, Spain). The sections were examined using a Zeiss LSM 800 with Airyscan (Carl Zeiss, Oberkochen, Germany).

Infected and Uninfected Cultured ISE6 Tick Cells

The *I. scapularis* embryo-derived tick cell line ISE6 were cultured as described above, infected with *A. phagocytophilum* (human NY18 isolate; Asanovich et al., 1997) or mock-infected and maintained according to Munderloh et al. (1999).

Pharmacological Studies in Cultured ISE6 Tick Cells

A. phagocytophilum-infected ISE6 cells were left untreated or treated for 24 or 48 h with 5 mM 2-Deoxy-D-Glucose (ab142242, Abcam) to inhibit glycolysis (Wang et al., 2011), 100 μM LY294002 (ab120243, Abcam) to inhibit the phosphatidylinositol 3-kinase (PI3K; Sultana et al., 2010), 100 nM Chetomin (ab144222, Abcam) to inhibit the activity of HIF-1α (Misra et al., 2012) or 0.5 mM Deferoxamine mesylate (Sigma-Aldrich) to activate HIF-1α (Choi et al., 2004). After treatment, cells were harvested for the preparation of whole cell lysates to determine HIF-1α activity and for DNA extraction. Tick cell lysates were prepared by adding to cell pellets the RIPA lysis buffer (Thermo Scientific) supplemented with a protease inhibitor cocktail (cOmplete Mini, EDTA-free, Roche, Sigma-Aldrich). HIF-1α activity was determined in the cell lysate supernatants with the HIF-1 alpha Transcription Factor Assay Kit (ab133104, Abcam) following manufacturer's recommendations. *A. phagocytophilum* DNA levels were characterized by *msp4* real-time PCR normalizing against tick *16S rDNA* as described previously (Ayllón et al., 2013). Optical density values (O.D. 450 nm) for HIF-1α activity and normalized Ct values for *A. phagocytophilum* DNA levels were compared between treated and untreated control cells by Student's *t*-test with unequal variance ($P = 0.05$; $N = 4$ biological replicates).

RESULTS

Major Carbohydrate Metabolic Pathways Described in Other Organisms Are Present in *I. scapularis* and Are Affected by *A. phagocytophilum* Infection

Seven major pathways involved in carbohydrate metabolism were selected for characterization (**Table 1**). A total of 79 genes coding for the proteins involved in glycolysis, gluconeogenesis, pentose phosphate pathway (PPP), glyceroneogenesis, TCA, mitochondrial oxidative phosphorylation (OXPHOS) and β-oxidation were identified in the *I. scapularis* genome (**Table 1**). Based on these results, a model for glucose metabolism in ticks was proposed (**Figure 1**). At least in humans, pyruvate carboxylase (PC) catalyzes the irreversible carboxylation of pyruvate to form oxaloacetate, which is then transformed in phosphoenolpyruvate by the cytoplasmic enzyme phosphoenolpyruvate carboxykinase (PEPCK-C; Berg et al., 2002). However, the PC orthologue was not identified in the *I. scapularis* genome.

The carbohydrate metabolic response to *A. phagocytophilum* infection was then characterized using the quantitative transcriptomics and proteomics data generated from uninfected and *A. phagocytophilum*-infected *I. scapularis* ticks and ISE6 cultured cells (Ayllón et al., 2015; Villar et al., 2015). Most of the identified carbohydrate metabolism genes were differentially regulated in response to *A. phagocytophilum* infection in at least one of the analyzed tick tissues (**Figure 2**). Twenty-eight (35%), 23 (29%), 62 (78%), and 64 (81%) carbohydrate metabolism components were identified in both transcriptome and proteome of ISE6 cells, nymphs, adult female midguts, and salivary glands, respectively (**Figure 2**). Of these genes, 59 (75%), 14 (18%), 60 (76%), and 41 (52%) were up-regulated, while 20 (25%), 65 (82%), 16 (20%), and 35 (44%) were down-regulated in response to infection in ISE6 cells, nymphs, adult female midguts, and salivary glands, respectively (**Figure 2**). Many of the carbohydrate metabolism proteins were not identified by mass spectrometry (**Figure 2**), but the results showed similar differential regulation at the mRNA and protein levels for 50% of the identified proteins.

Glucose transporters, and mainly facilitative glucose transporters (GLUT) are an important component of the glycolytic pathway because they transport glucose from the extracellular space to the cellular cytoplasm, which makes the glucose accessible to hexokinase (HXK; Augustin, 2010; Li et al., 2015). We found 11 putative glucose transporters in the genome of *I. scapularis*, 2 members (*sglt1* and *sglt2*) of the sodium-glucose linked transporter family (SGLT), and 9 members (2 isoforms of *glut1*, *glut3*, 4 isoforms of *glut8*, *glut10,* and *glut12*) of the GLUT family. In response to *A. phagocytophilum* infection, *glut1B*, *glut3, glut8C, glut10, glut12,* and *sglt1* genes were up-regulated in tick midguts, and the GLUT1A and SGLT2 proteins were over-represented in infected midguts when compared to uninfected controls (**Figure 3**).

As in previous experiments (Villar et al., 2015), these results supported a role for carbohydrate metabolism during *A. phagocytophilum* infection in *I. scapularis*, and suggested tissue-specific differences in response to infection.

A. phagocytophilum Infection Activates the Glycolysis Pathway, but Reduces Gluconeogenesis and the TCA Cycle in Tick Midguts

Based on these results, the putative glucose metabolic pathways affected by *A. phagocytophilum* infection in different *I. scapularis* tissues and ISE6 cells were proposed (**Figures 4**, **5**). The results showed that the genes involved in glycolysis were all up-regulated in tick midguts after *A. phagocytophilum* infection, except for phosphofructokinase (*pfk*) and *hxk* genes that did not change in response to infection (**Figure 2**). The up-regulation of glycolytic genes correlated with protein over-representation in tick midguts, except for the cofactor-independent phosphoglycerate mutase (*ipgm*) and pyruvate kinase (*pk*) that despite gene up-regulation in infected midguts, protein was under-represented in response to infection (**Figure 2**). However, since iPGM catalyzes a reversible reaction, it is not a site of major regulatory mechanisms for the glycolytic pathway. In contrast, HXK and PFK proteins, which catalyze irreversible steps of glucose glycolysis and therefore constitute major regulatory

TABLE 1 | Annotation of carbohydrate metabolic enzymes identified in the *I. scapularis* genome.

Enzymes	Abbreviation	Genome accession	GenBank ID	Length (amino acids)
GLYCOLYSIS (GLY)				
Hexokinase	HXK	ISCW012387	EEC16047	454
Phosphoglucose isomerase	PGI	ISCW014868	EEC19698	543
Phosphofructokinase	PFK	ISCW014412	EEC18711	55
Fructose-bisphosphate aldolase A	ALDA	ISCW011371	EEC14101	364
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	ISCW018700	EEC07171	334
Phosphoglycerate kinase 1	PGK1	ISCW015616	EEC01763	415
Phosphoglycerate mutase (cofactor-independent)	iPGM	ISCW020443	EEC12432	510
Enolase	ENOL	ISCW017666	EEC06821	199
Pyruvate kinase	PK	ISCW020197	EEC12307	538
GLUCONEOGENESIS (GLN)				
Glucose 6-phosphatase (1)	G6Pase (1)	ISCW017459	EEC03691	356
Glucose 6-phosphatase (2)	G6Pase (2)	ISCW018612	EEC07273	282
Fructose-1,6-bisphosphatase	FBP	ISCW005292	EEC07486	338
Phosphoenolpyruvate carboxykinase mitochondrial (1)	PEPCK-M (1)	ISCW001902	EEC03297	503
Phosphoenolpyruvate carboxykinase mitochondrial (2)	PEPCK-M (2)	ISCW000524	EEC01808	288
Phosphoenolpyruvate carboxykinase 1 cytoplasmatic	PEPCK-C	ISCW001905	EEC03300	165
Phosphoenolpyruvate carboxykinase	PEPCK	ISCW008211	EEC10279	192
Pyruvate carboxylase	PC	Not found	Not found	...
PENTOSE PHOSPHATE PATHWAY (PPP)				
Glucose 6-phosphate dehydrogenase	G6PD	ISCW010043	EEC13253	523
6-phosphogluconolactonase	PGLS	ISCW002068	EEC04321	101
6-phosphogluconate dehydrogenase	6PGD	ISCW010101	EEC12854	507
Ribose 5-Phosphate Isomerase	RPI	ISCW012092	EEC16597	172
Ribulose 5-Phosphate 3-Epimerase	RPPE	ISCW001125	EEC01731	236
Transketolase	TKT	Not found	Not found	...
Transaldolase	TALDO	ISCW023506	EEC20008	219
TRICARBOXYLIC ACID CYCLE (TCA CYCLE)				
Pyruvate dehydrogenase E1	PDE1	ISCW019126	EEC09108	393
Citrate synthase	CS	ISCW009586	EEC13404	471
Aconitase	ACON	ISCW010818	EEC15463	65
Isocitrate dehydrogenase 3 α subunit	IDH3A	ISCW004116	EEC06101	362
Isocitrate dehydrogenase 3 β subunit	IDH3B	ISCW018585	EEC07698	207
Isocitrate dehydrogenase 3 γ-subunit (I)	IDH3G1	ISCW022614	EEC16834	132
Isocitrate dehydrogenase 3 γ-subunit (II)	IDH3G2	ISCW017423	EEC04192	365
2-oxoglutarate dehydrogenase E1	OXOE1	ISCW021407	EEC14367	831
GTP/ATP-specific Succinyl-CoA synthetase—α subunit	B/A-SCS-α	ISCW020686	EEC11198	217
ATP-specific Succinyl-CoA synthetase—β subunit	A-SCS-β	ISCW011750	EEC14163	425
GTP-specific Succinyl-CoA synthetase—β subunit	G-SCS-β	ISCW015999	EEC00562	422
Succinate dehydrogenase flavoprotein subunit	SDHA	ISCW000555	EEC02042	608
Succinate dehydrogenase iron-sulfur subunit	SDHB	ISCW018067	EEC06202	286
Succinate dehydrogenase cytochrome b560 subunit	SDHC	ISCW020744	EEC12476	132
Succinate dehydrogenase cytochrome b small subunit	SDHD	ISCW012012	EEC15988	208
Fumarase hydratase mitochondrial	FH	ISCW020593	EEC13182	481
Malate dehydrogenase 1 cytoplasmatic	MDH1	ISCW007624	EEC09194	302
Malate dehydrogenase 2 mitochondrial	MDH2	ISCW003528	EEC05916	340
REGULATORY PROTEINS OF TCA CYCLE				
Pyruvate dehydrogenase kinase	PDK	ISCW013649	EEC19175	344
Pyruvate dehyrogenase phosphatase catalytic subunit 1	PDPC1	ISCW014874	EEC19704	401

(Continued)

TABLE 1 | Continued

Enzymes	Abbreviation	Genome accession	GenBank ID	Length (amino acids)
OXIDATIVE PHOSPHORYLATION (OXPHOS) COMPLEX I (CORE SUBUNITS)				
NADH dehydrogenase (ubiquinone) iron-sulfur protein 7	NDUS7	ISCW013042	EEC17810	162
NADH dehydrogenase (ubiquinone) iron-sulfur protein 8	NDUS8	ISCW010554	EEC13775	210
NADH dehydrogenase (ubiquinone) flavoprotein 2	NDUV2	ISCW014272	EEC18480	244
NADH dehydrogenase (ubiquinone) iron-sulfur protein 3	NDUS3	ISCW012366	EEC17298	268
NADH dehydrogenase (ubiquinone) iron-sulfur protein 2	NDUS2	ISCW017643	EEC06293	462
NADH dehydrogenase (ubiquinone) flavoprotein 1	NDUV1	ISCW005985	EEC08296	477
NADH-ubiquinone oxidoreductase 75 kDa subunit	NDUS1	ISCW003299	EEC03949	729
NADH-ubiquinone oxidoreductase chain 1	ND1	ISCW016073	EEC02134	339
NADH-ubiquinone oxidoreductase chain 2	ND2	Not found	Not found	...
NADH-ubiquinone oxidoreductase chain 3	ND3	Not found	Not found	...
NADH-ubiquinone oxidoreductase chain 4	ND4	Not found	Not found	...
NADH-ubiquinone oxidoreductase chain 4L	ND4L	Not found	Not found	...
NADH-ubiquinone oxidoreductase chain 5	ND5	ISCW016735	EEC02838	385
NADH-ubiquinone oxidoreductase chain 6	ND6	Not found	Not found	...
OXPHOS COMPLEX II				
Succinate dehydrogenase flavoprotein subunit	SDHA	ISCW000555	EEC02042	608
Succinate dehydrogenase iron-sulfur subunit	SDHB	ISCW018067	EEC06202	286
Succinate dehydrogenase cytochrome b560 subunit	SDHC	ISCW020744	EEC12476	132
Succinate dehydrogenase cytochrome b small subunit	SDHD	ISCW012012	EEC15988	208
OXPHOS COMPLEX III				
Cytochrome b-c1 complex subunit 1	QCR1	Not found	Not found	...
Cytochrome b-c1 complex subunit 2	QCR2	Not found	Not found	...
Cytochrome b	CYTB	ISCW016730	EEC02834	385
Cytochrome c1	CYTC1	ISCW016731	EEC02835	251
Cytochrome b-c1 complex subunit Rieske	RIESKE	ISCW014071	EEC19414	229
Cytochrome b-c1 complex subunit 6	QCR6	ISCW017835	EEC05133	80
Cytochrome b-c1 complex subunit 7	QCR7	ISCW012532	EEC17642	81
Cytochrome b-c1 complex subunit 8	QCR8	ISCW019560	EEC09147	82
Cytochrome b-c1 complex subunit 9	QCR9	ISCW008376	EEC10144	59
Cytochrome b-c1 complex subunit 10	QCR10	Not found	Not found	...
Cytochrome b-c1 complex subunit 11	QCR11	Not found	Not found	...
OXPHOS COMPLEX IV				
Cytochrome c oxidase subunit 1	COX1	Not found	ADO64507	276
Cytochrome c oxidase subunit 2	COX2	ISCW016021	EEC02091	244
Cytochrome c oxidase subunit 3	COX3	ISCW020757	EEC11360	278
Cytochrome c oxidase subunit 4	COX4	Not found	AAY66918	179
Cytochrome c oxidase subunit 5A	COX5A	ISCW008682	EEC12772	153
Cytochrome c oxidase subunit 5B	COX5B	ISCW019526	EEC10693	124
Cytochrome c oxidase subunit 6A	COX6A	ISCW019235	EEC08541	111
Cytochrome c oxidase subunit 6B	COX6B	ISCW015139	EEC20473	82
Cytochrome c oxidase subunit 6C	COX6C	ISCW016559	EEC02407	76
Cytochrome c oxidase subunit 7	COX7	Not found	Not found	...
Cytochrome c oxidase subunit 8	COX8	ISCW024928	EEC20083	68
OXPHOS COMPLEX V (ATP SYNTHASE: F1-ATP SYNTHASE STRUCTURE/F0-ATP SYNTHASE STRUCTURE)				
ATP synthase subunit α 1 (F1)	ATPSA1	ISCW011988	EEC16574	555
ATP synthase subunit α 2 (F1)	ATPSA2	ISCW019217	EEC08626	355
ATP synthase subunit β (F1)	ATPSB	ISCW012509	EEC17118	563
ATP synthase subunit γ (F1)	ATPSG	ISCW023477	EEC19169	314
ATP synthase subunit δ (F1)	ATPSD	ISCW018418	EEC06183	168

(Continued)

TABLE 1 | Continued

Enzymes	Abbreviation	Genome accession	GenBank ID	Length (amino acids)
ATP synthase subunit ϵ (F1)	ATPSE	ISCW022265	EEC17228	55
ATP synthase subunit A (F0)	ATPA	Not found	Not found	...
ATP synthase subunit B (F0)	ATPB	ISCW012060	EEC15991	264
ATP synthase subunit C (F0)	ATPC	ISCW010495	EEC14551	152
β-OXIDATION				
Acyl-CoA dehydrogenase family member 9	ACAD9	ISCW010038	EEC13248	433
Short-chain specific acyl-CoA dehydrogenase (1)	SCAD1	ISCW013995	EEC19715	76
Short-chain specific acyl-CoA dehydrogenase (2)	SCAD2	ISCW002863	EEC02614	410
Medium-chain acyl-CoA dehydrogenase	MCAD	ISCW001274	EEC01138	275
Very long-chain acyl-CoA dehydrogenase	VLCAD	ISCW024772	EEC16420	254
Enoyl-CoA hydratase	ECHD	ISCW012475	EEC16250	290
3-hydroxyacyl CoA dehydrogenase	3HCD	ISCW014460	EEC18745	310
3-ketoacyl-CoA thiolase (Thiolase I)	THIOL	ISCW022661	EEC17467	406
GLYCERONEOGENESIS				
Triosephosphate isomerase	TPI	ISCW008869	EEC13429	247
Glycerol-3-phosphate dehydrogenase cytoplasmic	GPDHc	ISCW005254	EEC07468	340

steps of this pathway, were over-represented and did not change, respectively in response to infection of tick midguts (**Figure 4**). The expression of the p53 target TP53-inducible glycolysis and apoptosis regulator (*tigar*, ISCW020485), which was recently shown to inhibit glycolysis (Bensaad et al., 2006), was down-regulated in tick nymphs, midguts, salivary glands, and ISE6 cells. The over-representation of all glycolytic enzymes correlated with up-regulation of their respective genes, suggesting transcriptional regulation. These data suggested that *A. phagocytophilum* infection activates glucose uptake and degradation in *I. scapularis* midguts. In agreement with an increase in glucose catabolism, the levels of glucose were significantly reduced (0.51 ± 0.06 vs. 0.31 ± 0.05, $p < 0.005$) in infected ISE6 cells when compared to uninfected controls (Villar et al., 2015).

PK catalyzes the final irreversible step of glycolysis, the transfer of a phosphate group from phosphoenolpyruvate to ADP, yielding one molecule of pyruvate and one molecule of adenosine 5′-triphosphate (ATP; Li et al., 2015). This gene was up-regulated, but protein was under-represented in response to infection in tick midguts (**Figures 2**, **4**). Due to the regulatory role of PK, low levels of this enzyme may hamper the flux of pyruvate toward the mitochondrial matrix, decreasing the activity of TCA cycle. PK generates pyruvate, which is transported to the mitochondrial matrix by mitochondrial pyruvate carriers 1 and 2 (MPC1 and MPC2). The homologs for *mpc1* and *mpc2* genes were identified in *I. scapularis* and were up-regulated in tick midguts, but down-regulated in salivary glands in response to *A. phagocytophilum* infection (**Figure 3**). In the mitochondrial matrix, pyruvate is decarboxylated by pyruvate dehydrogenase E1 (PDE1; Berg et al., 2002). The decarboxylation of pyruvate is the first step of a series of enzymatic reactions that transform pyruvate into acetyl coenzyme A (acetyl-Coa), which is oxidized to CO_2 and water in the TCA cycle (Berg et al., 2002). PDE1 is an important regulator of the TCA cycle (Berg et al., 2002). Pyruvate dehydrogenase kinase 1 (PDK1) inhibits, while pyruvate dehydrogenase phosphatase catalytic subunit 1 (PDPC1) activates PDE1 (Berg et al., 2002). We found that in the midguts of infected ticks, PDE1 was under-represented and the genes *pdk1* and *pdpc1* were up-regulated and down-regulated, respectively in response to infection (**Figure 2**). This result strongly suggested that *A. phagocytophilum* infection inhibited the TCA cycle in *I. scapularis* midguts. In agreement with this suggestion, several TCA cycle enzymes such as citrate synthase (CS), aconitase (ACON), isocitrate dehydrogenase [subunits α (IDH3A) and γ (IDH3G1)], 2-oxoglutarate dehydrogenase E1 (OXOE1), succinate dehydrogenase (flavoprotein subunit (SDHA), and iron-sulfur subunit (SDHB) were under-represented in midguts from infected ticks when compared to uninfected controls (**Figures 2**, **4**).

The NADH generated by the TCA cycle is fed into the OXPHOS pathway to produce ATP (Li et al., 2015). The mRNA and protein levels of components of the OXPHOS complex I–IV present in *I. scapularis* were examined. Higher mRNA and protein levels were found for most of the respiratory components in infected tick midguts when compared to uninfected controls (**Figure 2**). This result suggested that ticks might have a regulatory mechanism to maintain the levels of ATP in response to the inhibition of the TCA cycle.

Finally, we found that although most genes were up-regulated, all proteins of gluconeogenesis (synthesis of glucose from non-carbohydrate precursors) were under-represented in tick midguts, except for fructose-1,6-bisphosphatase (FBP; **Figure 2**). FBP catalyzes an irreversible reaction in the gluconeogenesis, the conversion of fructose 1,6-bisphosphate into fructose 6-phosphate, and therefore it is a regulatory step of this anabolic pathway. However, the enzyme glucose

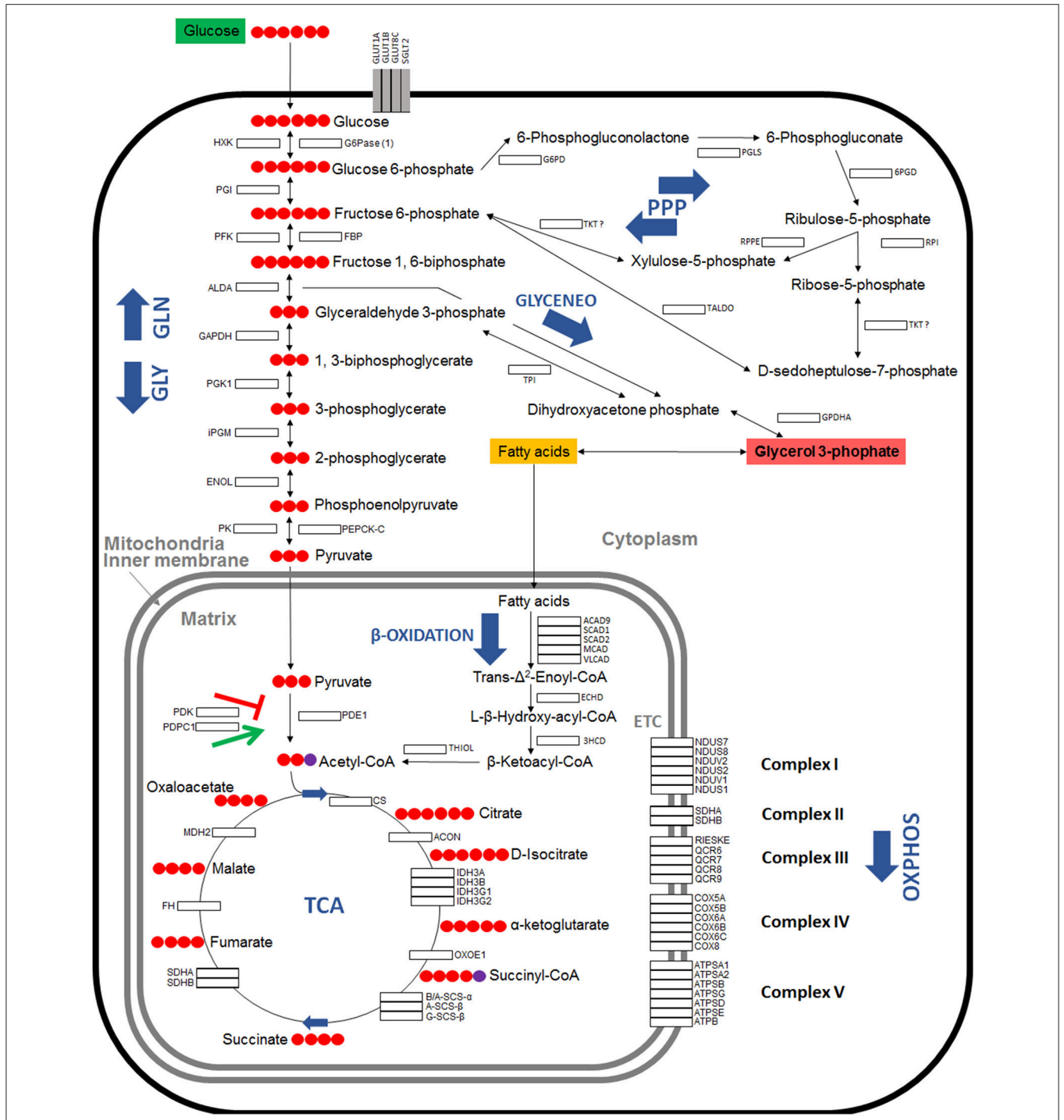

FIGURE 1 | Model of carbohydrate metabolism in *I. scapularis*. The main enzymes involved in Glycolysis (GLY), Gluconeogenesis (GLN), Pentose phosphate pathway (PPP), Tricarboxylic acid cycle (TCA), Oxidative phosphorylation (OXPHOS) complex I to V, β-Oxidation and Glyceroneogenesis (GLYCENEO) and present in the genome of *I. scapularis* (**Table 1**) are shown. The names and number of carbon molecules (Red circles) of the metabolic intermediates of these metabolic pathways are also shown. The names of the enzymes were abbreviated for the different pathways as GLY: Hexokinase (HXK), Phosphoglucose isomerase (PGI), Phosphofructokinase (PFK), Fructose-bisphosphate aldolase A (ALDA), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Phosphoglycerate kinase 1 (PGK1), Phosphoglycerate mutase (cofactor-independent) (iPGM), Enolase (ENOL), Pyruvate kinase (PK); GLN: Glucose 6-phosphatase (1) (G6Pase (1)), Fructose-1,6-bisphosphatase (FBP), Phosphoenolpyruvate carboxykinase mitochondrial (1) (PEPCK-M (1)), Phosphoenolpyruvate carboxykinase mitochondrial (2) (PEPCK-M (2)), Phosphoenolpyruvate carboxykinase 1 cytoplasmatic (PEPCK-C), Pyruvate carboxylase (PC); PPP: Glucose 6-phosphate dehydrogenase (G6PD), 6-phosphogluconolactonase (PGLS), 6-phosphogluconate dehydrogenase (6PGD), Ribose 5-Phosphate Isomerase (RPI), Ribulose 5-Phosphate 3-Epimerase (RPPE), Transketolase (TKT), Transaldolase (TALDO); β-Oxidation: Acyl-CoA dehydrogenase family member 9 (ACAD9), Short-chain specific acyl-CoA dehydrogenase

(Continued)

FIGURE 1 | Continued
(1) (SCAD1), Short-chain specific acyl-CoA dehydrogenase (2) (SCAD2), Medium-chain acyl-CoA dehydrogenase (MCAD), Very long-chain acyl-CoA dehydrogenase (VLCAD), Enoyl-CoA hydratase (ECHD), 3-hydroxyacyl CoA dehydrogenase (3HCD), 3-ketoacyl-CoA thiolase (Thiolase I) (THIOL); GLYCENEO: Triosephosphate isomerase (TPI), Glycerol-3-phosphate dehydrogenase cytoplasmic (GPDHc); OXPHOS: Complex I: NADH dehydrogenases (NDUS7, NDUS8, NDUV2, NDUS3, NDUS2 and NDUV1), NADH-ubiquinone oxidoreductases (NDUS1, ND1 and ND5); Complex II: Succinate dehydrogenases (SDHA, SDHB, SDHC, SDHD); Complex III: Cytochrome b and b-c1 complexe subunits (QCR1, QCR2, CYTB, RIESKE, QCR6, QCR7, QCR8, QCR9, QCR10, QCR11), Cytochrome c1 (CYTC1); Complex IV: Cytochrome c oxidase subunits (COX1, COX2, COX3, COX5A, COX5B, COX6A, COX6B, COX6C, COX8); Complex V: ATP synthase subunits (ATPSA1, ATPSA2, ATPSB, ATPSG, ATPSD, ATPSE, ATPB, ATPC).

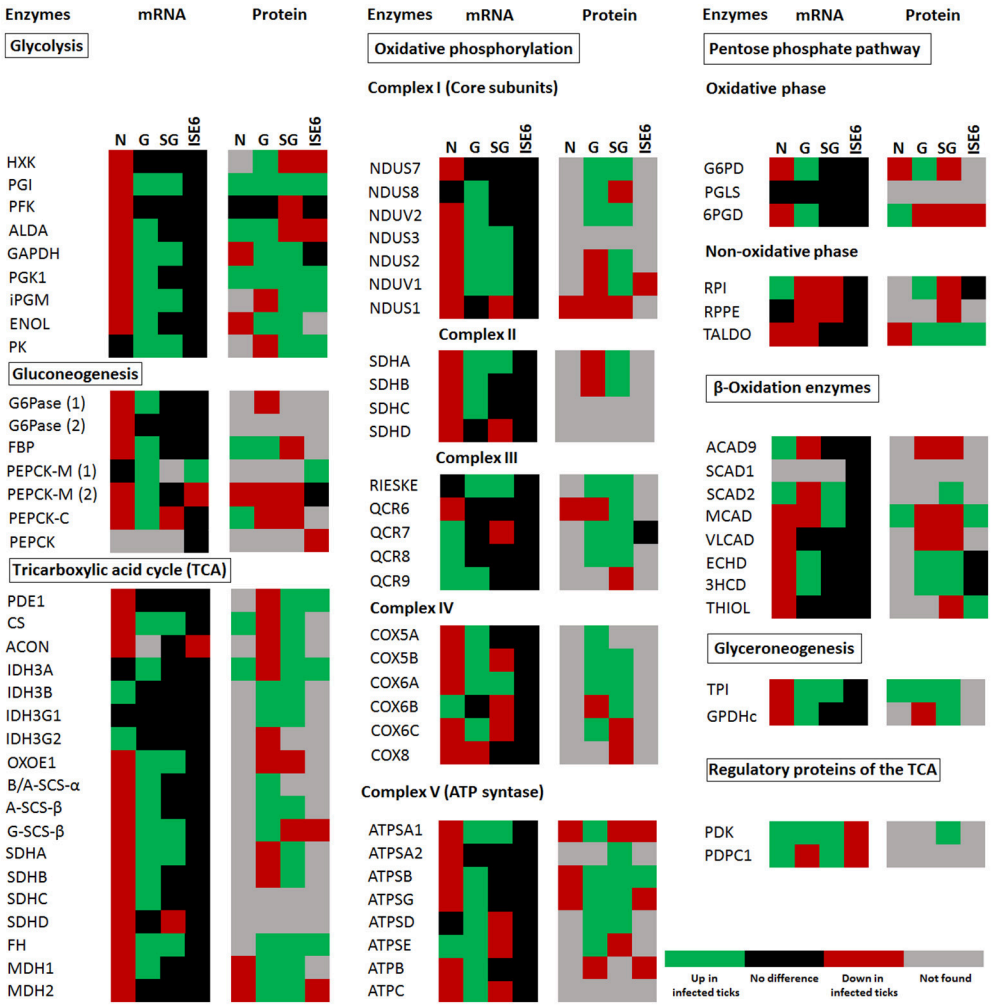

FIGURE 2 | *I. scapularis* carbohydrate metabolism enzymes mRNA and protein levels in response to *A. phagocytophilum* infection. Comparison of carbohydrate metabolism enzymes mRNA and protein levels in *I. scapularis* nymphs (N), female midguts (G), female salivary glands (SG), and ISE6 cells (ISE6) in response to *A. phagocytophilum* infection. Transcriptomics and proteomics data were obtained from previously published datasets available on the Dryad repository database, NCBI's Gene Expression Omnibus database and ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002181 and doi: 10.6019/PXD002181 (Ayllón et al., 2015; Villar et al., 2015). Name of enzymes are abbreviated as in **Table 1** and **Figure 1**.

6-phosphatase that catalyzes the last step of gluconeogenesis, the transformation of glucose 6-phosphate into free glucose was under-represented. These results suggested that midgut cells in *A. phagocytophilum*-infected ticks tend to keep glucose 6-phosphate within the cells for other metabolic processes (e.g., glycogen synthesis).

HIF-1 Components Are Up-Regulated in *I. scapularis* Infected with *A. phagocytophilum*

HIF-1 is a heterodimeric transcription factor consisting of a constitutively expressed β-subunit (HIF-1β) and an oxygen-regulated α-subunit (HIF-1α; Pagé et al., 2002; Déry et al., 2005;

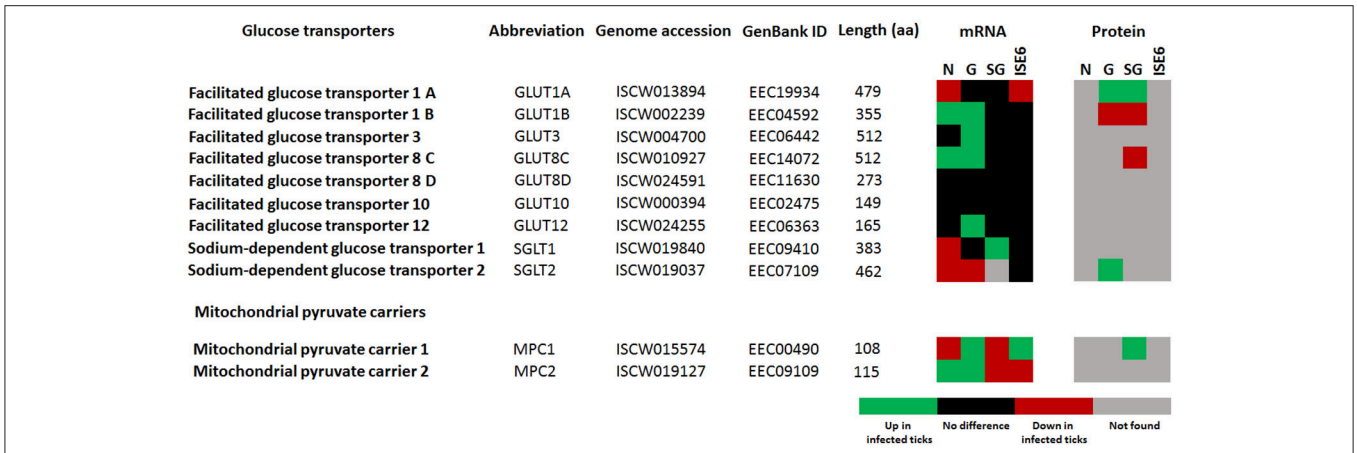

FIGURE 3 | *I. scapularis* glucose transporters mRNA and protein levels in response to *A. phagocytophilum* infection. Comparison of glucose transporters mRNA and protein levels in *I. scapularis* nymphs (N), female midguts (G), female salivary glands (SG) and ISE6 cells (ISE6) in response to *A. phagocytophilum* infection. Transcriptomics and proteomics data were obtained from previously published datasets available on the Dryad repository database, NCBI' Gene Expression Omnibus database and ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002181 and doi: 10.6019/PXD002181 (Ayllón et al., 2015; Villar et al., 2015).

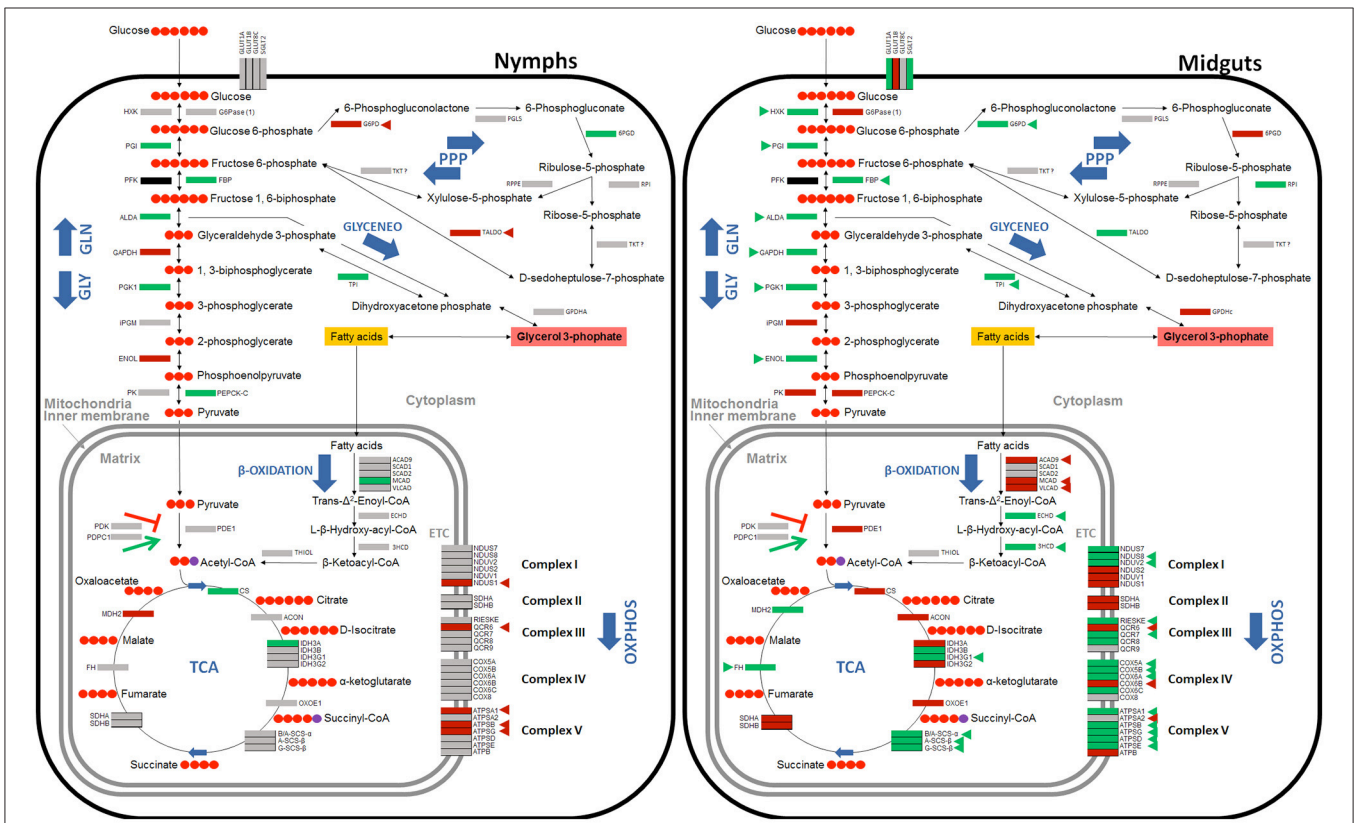

FIGURE 4 | Carbohydrate metabolic pathways in response to *A. phagocytophilum* infection in *I. scapularis* nymphs and female midguts. Protein representation of enzymes of the *I. scapularis* carbohydrate metabolic pathways in response to *A. phagocytophilum* infection is shown. Similar changes in mRNA and protein levels are highlighted (triangles). Code: green, up-regulated/over-represented; red, down-regulated/under-represented.

Ziello et al., 2007; Badeaux and Shi, 2013). The gene encoding for HIF-1α (ISCW023657), which is a major transcriptional activator of glycolytic genes (Hu et al., 2006), was significantly up-regulated in tick midguts, but not in nymphs, salivary glands or ISE6 cells in response to *A. phagocytophilum* infection (Ayllón et al., 2015; Villar et al., 2015). The expression of the gene

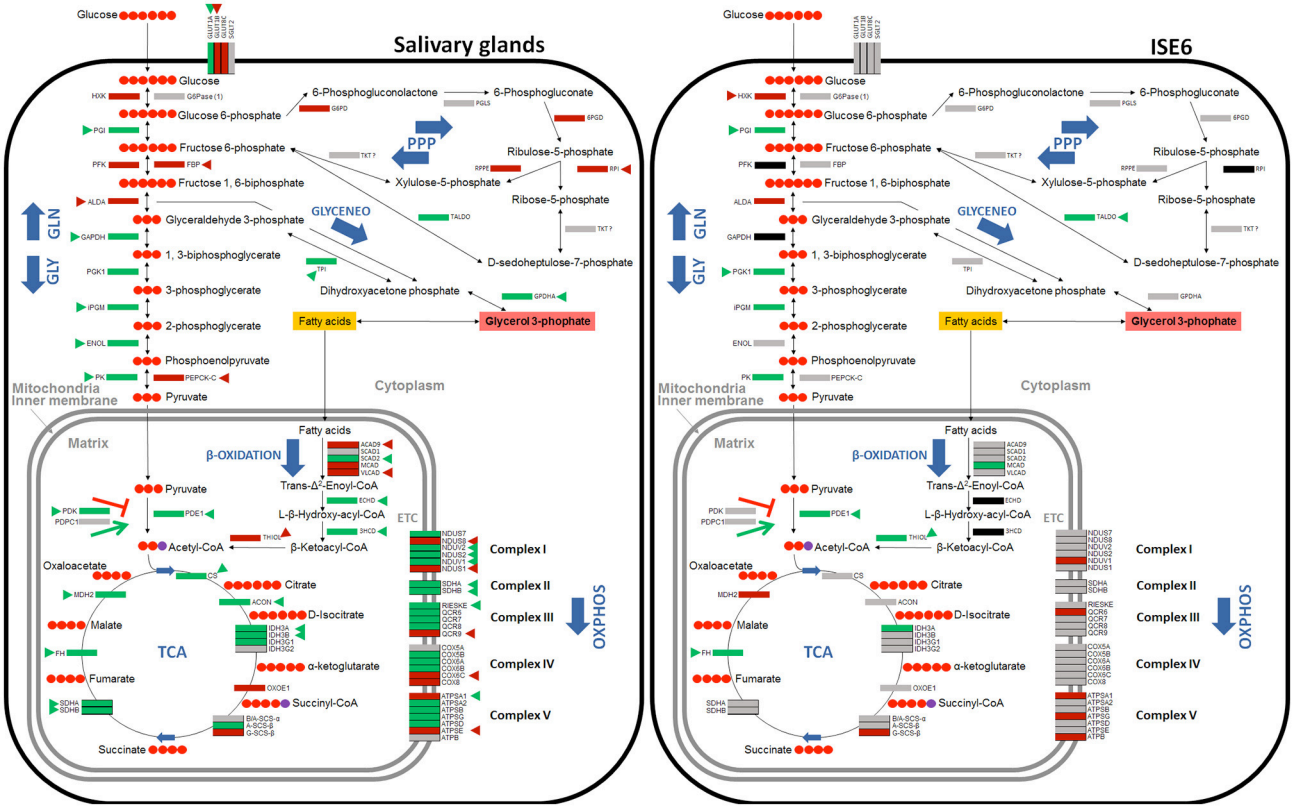

FIGURE 5 | Carbohydrate metabolic pathways in response to *A. phagocytophilum* infection in *I. scapularis* salivary glands and ISE6 cells. Protein representation of enzymes of the *I. scapularis* carbohydrate metabolic pathways in response to *A. phagocytophilum* infection is shown. Similar changes in mRNA and protein levels are highlighted (triangles). Code: green, up-regulated/over-represented; red, down-regulated/under-represented.

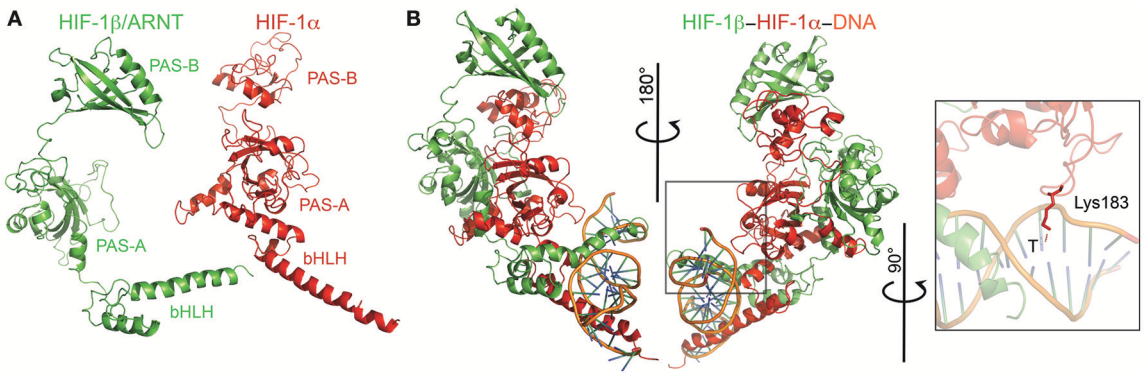

FIGURE 6 | Modeling of the HIF-1α-HIF-1β-HRE complex. (A) HIF-1α (red) and HIF-1β (green) monomers with their respective domains labeled. **(B)** Ternary complex of both monomers plus the hypoxia response element (HRE; orange). The inset shows the conserved Lys residue that interacts with the thymine (T) of HRE.

encoding for the HIF-1β (ISCW023999), which is a molecular partner of HIF-1α, was up-regulated in midguts from infected ticks, but was down-regulated in nymphs and did not change in salivary glands and ISE6 cells in response to infection (Ayllón et al., 2015; Villar et al., 2015).

Both *I. scapularis* HIF-1α and HIF-1β (a.k.a, aryl hydrocarbon receptor nuclear translocator or ARNT) possess the conserved basic helix-loop-helix (bHLH) domain and two PAS domains (**Figures 6A,B**). The bHLH domain of HIF-1α and HIF-1β were highly conserved with identical interacting residues as those previously identified (Dalei et al., 2015). The PAS-B domain of HIF-1α was disordered due to its low conservation and the presence of small insertions and deletions when compared to the mouse HIF-1α (Dalei et al., 2015). The PAS-B of HIF-1α

interacts with both the PAS domain of HIF-1β, thereby making it difficult to determine interacting residues. One key feature of HIF-1α is the presence of two residues lysine (Lys) and glycine (Gly), which interact with the nucleotides of hypoxia response element (HRE; Dalei et al., 2015). The *I. scapularis* HIF-1α had the conserved Lys, but showed an asparagine (Asn) to Gly substitution (**Figure 6**).

The PI3K/Akt Pathway Is Present in *I. scapularis* and Regulated in Response to *A. phagocytophilum* Infection

It has been demonstrated that a number of non-hypoxic stimuli are highly capable of inducing the expression of *hif-1* (Pagé et al., 2002; Déry et al., 2005). The PI3K pathway and its downstream effectors, mTOR and p70S6 kinase (p70S6K), may induce a hypoxia-independent increase in HIF-1α levels (Déry et al., 2005). In addition, diacylglycerol (DAG)-sensitive protein kinases C (PKC) were shown to up-regulate *hif-1α* gene expression in a hypoxia-independent manner (Pagé et al., 2002; Déry et al., 2005). Infection with *A. phagocytophilum* activates PI3K in *I. scapularis* (Sultana et al., 2010). Therefore, we characterized the mRNA and protein levels of tick PI3K components in response to *A. phagocytophilum* infection.

Most PI3K-mTOR pathway components found in other organisms (Pilot-Storck et al., 2010) were identified in the *I. scapularis* genome (**Table 2**). The tick PI3K components were differentially regulated in response to *A. phagocytophilum* infection (**Figure 7**). The *p70S6K* and PI3K components (*p85α* and *p110α*) were up-regulated in tick midguts and salivary glands in response to infection. Additionally, p70S6K and p85α proteins were over-represented in midguts from infected ticks when compared to uninfected controls (**Figure 7**). Oxygen-independent activation of *hif-1α* may involve PI3K components, p70S6K and mTOR (Déry et al., 2005). However, we did not find an mTOR ortholog in the *I. scapularis* genome. DAG-sensitive PKCs were also suggested to play a role in oxygen-independent activation of *hif-1α* (Pagé et al., 2002). Protein PKCα was found to be over-represented in tick midguts and salivary glands, while the *PKCε* gene was up-regulated in tick midguts in response to infection.

Functional Studies Support a Role for Carbohydrate Metabolism during *A. phagocytophilum* Infection of Tick Cells

Functional studies were focused on glycolysis by targeting the process at different levels (**Figures 8A,B**).

TABLE 2 | Annotation of PI3K-mTOR pathway components identified in the *I. scapularis* genome.

Proteins	Abbreviation	Genome accession	GenBank ID	Length (amino acids)
RAC-alpha serine/threonine-protein kinase	AKT2	ISCW018284	EEC06033	371
Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit α	p110α	ISCW014229	EEC18977	617
Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit β	p110β	ISCW010469	EEC14480	491
Phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing α (1)	PI3K-C2α1	ISCW022811	EEC17358	1638
Phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing α (2)	PI3K-C2α2	ISCW004026	EEC06376	1142
Phosphatidylinositol 3-kinase regulatory subunit α	p85α	ISCW011707	EEC13970	427
3- phosphoinositide-dependent protein kinase 1	PDK1	ISCW021115	EEC15400	521
Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1	SHIP1	ISCW000015	EEC00572	535
Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase PTEN	PTEN	ISCW021211	EEC15363	363
Arf-GAP with GTPase, ANK repeat and PH domain-containing protein	PIKE	ISCW014072	EEC18950	558
Glycogen synthase kinase 3 β (2)	GSK3β2	ISCW009451	EEC11886	588
Hamartin	TSC1	ISCW023319	EEC19233	874
Tuberin	TSC2	ISCW012320	EEC16560	429
Serine/threonine kinase 11 (1)	LKB1/STK11A	ISCW024898	EEC19357	170
Serine/threonine kinase 11 (2)	LKB1/STK11B	ISCW015294	EEC20092	293
AMP-activated protein kinase α	AMPKα	ISCW020637	EEC13103	510
AMP-activated protein kinase β	AMPKβ	ISCW021036	EEC15614	183
AMP-activated protein kinase γ	AMPKγ	ISCW010164	EEC13342	154
Ras homolog enriched in brain	RHEB	ISCW018020	EEC05182	182
Regulatory-associated protein of mTOR	RAPTOR	ISCW018753	EEC07385	995
G protein β subunit-like	GβL	ISCW002765	EEC03813	324
Rapamycin-insensitive companion of mTOR	RICTOR	ISCW018420	EEC06185	987
Phosphatidylinositol 3-kinase catalytic subunit type 3	Vps34	ISCW008679	EEC12769	864
Serine/threonine-protein kinase VPS15	Vps15	ISCW017661	EEC06819	1351
Pleckstrin homology domain-containing, family O member 1	CKIP1	ISCW021605	EEC15223	359
Casein kinase 2 subunit α	CK2α	ISCW007259	EEC10594	436
Casein kinase 2 subunit β	CK2β	ISCW014459	EEC18744	222
Ribosomal protein S6 kinase beta-1 (p70s6 kinase)	S6K1/p70s6K	ISCW017390	EEC03348	344

A. phagocytophilum-infected ISE6 cells were left untreated or treated for 24 or 48 h with 2-Deoxy-D-Glucose to inhibit glycolysis (Wang et al., 2011), LY294002 to inhibit the PI3K (Sultana et al., 2010), Chetomin to inhibit the activity of HIF-1α (Misra et al., 2012) or Deferoxamine mesylate to activate HIF-1α (**Figures 8A,B**). Both HIF-1α (Ayllón et al., 2015; Villar et al., 2015), which is a major transcriptional activator of glycolytic genes (Hu et al., 2006), and most PI3K components (**Figure 7**) were affected in response to *A. phagocytophilum* infection. The results showed that LY294002 was the only compound active in ISE6 tick cells (**Figure 8A**). The lack of effect of chetomin and Deferoxamine mesylate on tick HIF-1α activity may be due to structural differences between tick and mammalian HIF-1α. The treatment of tick cells with LY294002 for 48 h resulted in lower HIF-1α activity when compared to untreated control cells (**Figure 8A**). Treatment with LY294002 has been shown in *I. scapularis* and other organisms to inhibit the PI3K pathway (Sultana et al., 2010), which is involved in the induction of HIF-1α to activate the glycolysis (Déry et al., 2005). Therefore, the results obtained in tick cells supported a role for PI3K in HIF-1α induction in tick cells. The decrease in *A. phagocytophilum* infection after LY294002 treatment for 24 and 48 h (**Figure 8B**) provided additional support to these results. As expected from the lack of effect on HIF-1α activity after incubation of tick cells with the other compounds, treatment did not affect *A. phagocytophilum* infection in these cells. Additionally, the immunofluorescence assay of GAPDH, which was over-represented in *I. scapularis* midguts and salivary glands in response to *A. phagocytophilum* infection (**Figures 4**, **5**), corroborated the results of the proteomics analysis in tick salivary glands (**Figure 8C**).

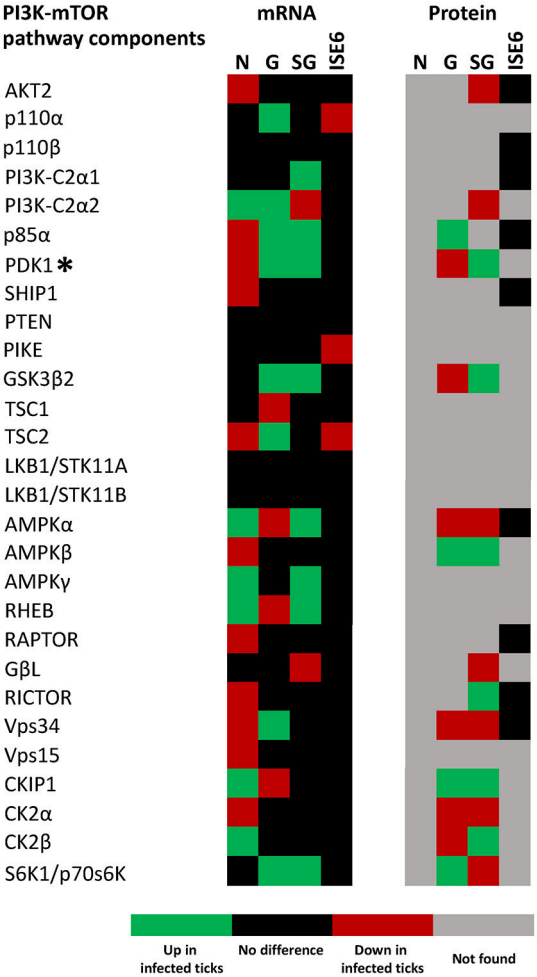

FIGURE 7 | *I. scapularis* PI3K-mTOR pathway components mRNA and protein levels in response to *A. phagocytophilum* infection. Comparison of PI3K-mTOR pathway components mRNA and protein levels in *I. scapularis* nymphs (N), female midguts (G), female salivary glands (SG) and ISE6 cells (ISE6) in response to *A. phagocytophilum* infection. Transcriptomics and proteomics data were obtained from previously published datasets available on the Dryad repository database, NCBI's Gene Expression Omnibus database and ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002181 and doi: 10.6019/PXD002181 (Ayllón et al., 2015; Villar et al., 2015). Name of enzymes are abbreviated as in **Table 2**. (*) In this figure, PDK1 stands for 3- phosphoinositide-dependent protein kinase 1, and not Pyruvate dehydrogenase kinase 1, which is also abbreviated as PDK1.

DISCUSSION

Recently, Villar et al. (2015) showed modifications in tick cell glucose metabolism during *A. phagocytophilum* infection. The results evidenced that infection affected the glucose metabolic pathway in tick cells through phosphoenolpyruvate carboxykinase (PEPCK) inhibition leading to decreased gluconeogenesis, which also results in the inhibition of cell apoptosis that increases pathogen infection of tick cells (Villar et al., 2015). Furthermore, these results provided evidence that other carbohydrate metabolic pathways are also affected by pathogen infection. Therefore, the objective of this study was the characterization of the dynamics of major carbohydrate pathways during *A. phagocytophilum* infection of *I. scapularis*.

Cellular glycolysis converts glucose to pyruvate, which enters the mitochondria where it is converted into acetyl-CoA, and is metabolized via the TCA cycle yielding reducing equivalents that are used for OXPHOS to generate ATP (Eisenreich et al., 2013, 2015). TCA cycle can be also fueled by the acetyl-CoA produced via degradation of fatty acids after β-Oxidation (Eisenreich et al., 2013). Alternatively, glucose can be used to produce Ribose 5-phosphate, a precursor for the synthesis of nucleotides through the PPP (Berg et al., 2002; **Figure 1**). In addition, glyceroneogenesis uses glycerol 3-phophate, obtained from the reduction of dihydroxyacetone phosphate (glycolytic intermediate) to synthetize fatty acids, and therefore is a major link between carbohydrate metabolism and lipid metabolism (Berg et al., 2002; **Figure 1**).

In this study, orthologs for 79 components of major carbohydrate metabolic pathways were identified in *I. scapularis*, and their role was characterized in response to *A. phagocytophilum* infection. The analysis was focused on seven pathways including glycolysis, gluconeogenesis, PPP, glyceroneogenesis, β-Oxidation, TCA cycle, and OXPHOS. The results showed that genes involved in glycolysis

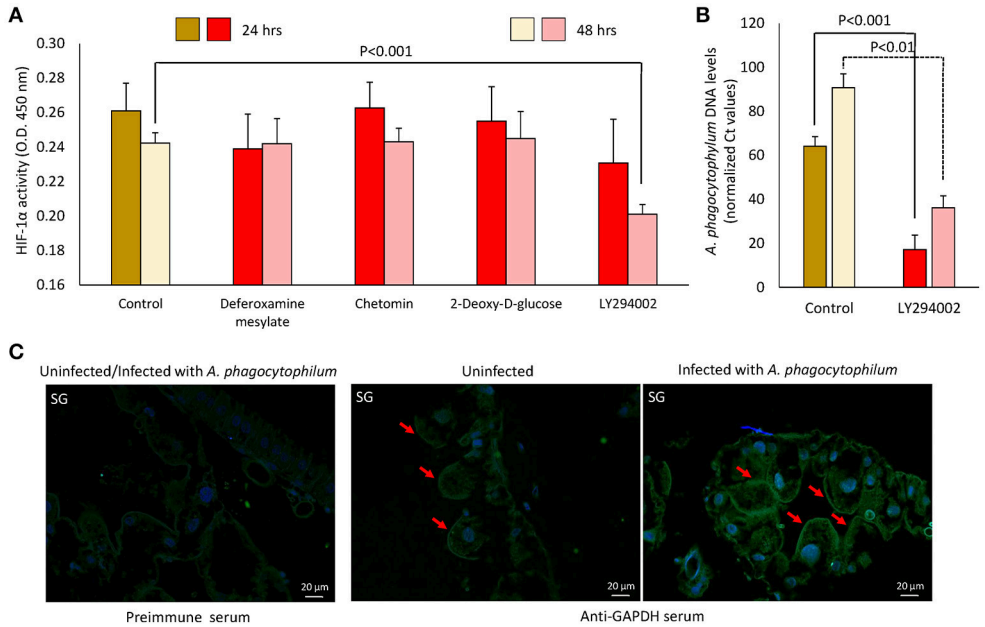

FIGURE 8 | Functional analysis supports a role for glycolysis during *A. phagocytophilum* infection of tick cells. (A) *A. phagocytophilum*-infected ISE6 cells were left untreated (Control) or treated for 24 or 48 h with 2-Deoxy-D-Glucose to inhibit glycolysis, LY294002 to inhibit the PI3K, Chetomin to inhibit the activity of HIF-1α or Deferoxamine mesylate to activate HIF-1α. HIF-1α activity was determined in cell lysates, and O.D. 450 nm values were shown as average + *SD* and compared between treated and untreated control cells by Student's *t*-test with unequal variance ($P = 0.05$; $N = 4$ biological replicates). **(B)** *A. phagocytophilum* DNA levels were characterized in LY294002-treated and untreated control cells by *msp4* real-time PCR normalizing against tick *16S rDNA*. Normalized Ct values were compared between treated and untreated control cells by Student's *t*-test with unequal variance ($P < 0.01$; $N = 4$ biological replicates). **(C)** Representative images of imunofluorescence analysis of uninfected and *A. phagocytophilum*-infected adult female *I. scapularis* salivary gland acini (SG). Tick tissues were stained with preimmune control serum or mouse anti-GAPDH monoclonal antibodies (green, FITC) or DAPI (blue) and superimposed (FITC+DAPI). Bars, 20 μm. Red arrows illustrate the positive staining for GAPDH in tick SG.

were up-regulated in *I. scapularis* ticks infected with *A. phagocytophilum*. In contrast, PEPCK, which is the enzyme that catalyzes the first step of gluconeogenesis (after this step the molecules are "committed" to the pathway and will ultimately end up in the pathway's final product) was under-represented (**Figure 2**). However, in addition to low levels of PEPCK, low TCA cycle activity might be also necessary to control gluconeogenesis (Burgess et al., 2007). Interestingly, we found that several enzymes and intermediates such as succinate of the TCA cycle are also under-represented in infected tick ISE6 cells and midguts (**Figures 4**, **5**; Villar et al., 2015). These results strongly suggested that upon *A. phagocytophilum* infection, glycolysis is enhanced, TCA cycle inhibited and in agreement with our previous results (Villar et al., 2015), gluconeogenesis is inhibited. These findings supported the idea that *A. phagocytophilum* infection might be an energy-demanding process for the tick cells and that the pathogen may benefit from glycolytic intermediates.

Intracellular bacteria trigger diverse host metabolic responses (Eisenreich et al., 2013, 2015). Numerous transcriptome studies have identified some of these metabolic responses as unspecific (triggered by extracellular/intracellular and pathogenic/non-pathogenic bacteria) and are therefore called "core host responses" (Boldrick et al., 2002). These "core host responses" are modulated in different ways by virulence mechanisms of different bacteria species (Boldrick et al., 2002). For example, *in vivo* studies in mice showed that the facultative intracellular pathogen *Mycobacterium tuberculosis* induces a reduction in the levels of glucose and the TCA cycle intermediates oxaloacetate and fumarate, but also an increase in lactate and succinate concentration (Shin et al., 2011; Somashekar et al., 2011). In *Listeria monocytogenes*, Lecuit et al. (2007) found that the transcription of most glycolytic genes was enhanced after infection in mice, particularly *hxk II*. The upregulation of glycolytic genes was linked to enhanced *hif-1α* expression and downregulation of the gluconeogenic gene *fbp* (Lecuit et al., 2007). The above examples illustrate commonalities among the host metabolic responses to *M. tuberculosis*, *L. monocytogenes* and *A. phagocytophilum* infection.

Based on the results of this study, a model was proposed in which *A. phagocytophilum* induces transcriptional activation of *hif-1α* through PI3K (p85α, p110α, and p70S6K) and PKC to activate the glycolytic pathway and inhibit the TCA cycle in infected ticks (**Figure 9**). This model was partially supported by functional studies using a PI3K inhibitor to monitor the effect on HIF-1α activity and pathogen infection (**Figures 8A,B**). In normoxia, HIF-1α is rapidly degraded. Therefore, *A. phagocytophilum* may have additional mechanisms to stabilize HIF-1α at normal oxygen concentrations. The increase in the levels of heat shock proteins HSP70 and HSP90

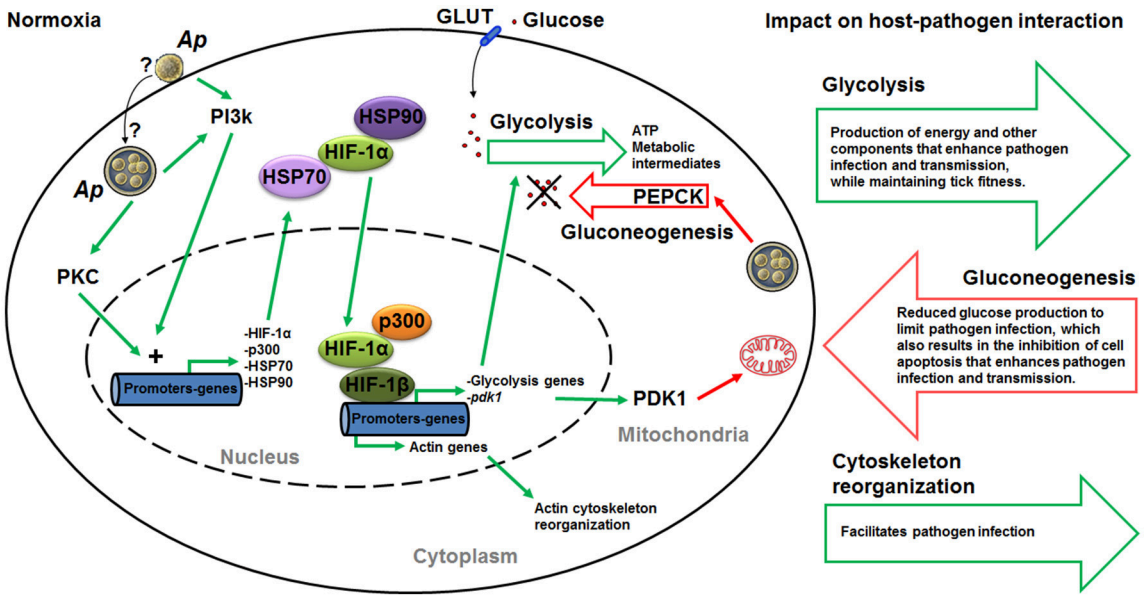

FIGURE 9 | Mechanistic model of carbohydrate metabolism manipulation by *A. phagocytophilum*. We propose a mechanism by which *A. phagocytophilum* enhances glycolysis through HIF activation under normoxic conditions. Upon contact with the host membrane or once inside the parasitophorous vacuole, *A. phagocytophilum* induces the activation of PI3K and PKC that ultimately induce the expression of heat shock proteins HSP70 and HSP90, the acetyltransferase p300 and *hif-1α*. In normoxia, HSP70 and HSP90 stabilize HIF-1α, making possible the recruitment of p300 and HIF-1α to form a complex with HIF-1β in the nucleus. This complex activates the expression of HIF-1α target genes, which include glycolytic genes and *pdk1*. This mechanism will result in the increase in glycolysis and inhibition of gluconeogenesis. Furthermore, HIF activation can also leads to actin cytoskeleton reorganization. Therefore, by activating the HIF system *A. phagocytophilum* may regulate at the same time the tick cell carbohydrate metabolism and cytoskeleton organization. Green and red lines indicate induction/activation and inhibition, respectively. Question marks indicate that we do not know whether *A. phagocytophilum* induces the activation of PI3K and PKC upon contact with the host membrane or once inside the parasitophorous vacuole. Ap, *A. phagocytophilum*; PI3K, phosphatidylinositol 3-kinase pathway; diacylglycerol (DAG)-sensitive protein kinases C (PKC); HIF, hypoxia-inducible factor; PDK1, pyruvate dehydrogenase kinase 1; GLUT, facilitative glucose transporters; PEPCK, phosphoenolpyruvate carboxykinase.

may contribute to the stabilization of HIF-1α in normoxia (Zhou et al., 2004). Previously, we showed that HSP70 and HSP90 were over-represented in *A. phagocytophilum*-infected ISE6 tick cells when compared to uninfected cells (Villar et al., 2015). Furthermore, mobilization of p300 at HIF target genes may be an additional requirement of HIF-mediated transcriptional activation (Badeaux and Shi, 2013). In agreement with this model, recently we showed that *A. phagocytophilum* induces the production of p300 to inhibit cell apoptosis and increase bacterial multiplication in tick cells (Cabezas-Cruz et al., 2016). By inhibiting the TCA cycle in host cells, *A. phagocytophilum* may inhibit the entrance of glutamine and glutamate to the TCA cycle via α-ketoglutarate, which is one of the intermediates in the TCA cycle. This process may increase the cytoplasmic concentration of glutamine and glutamate that can then be used by *A. phagocytophilum*. The TCA cycle of *A. phagocytophilum* is incomplete (because one gene for isocitrate dehydrogenase is missing from the genome) and requires the exogenous acquisition of glutamine and glutamate (Huang et al., 2007), which the pathogen may obtain from the host cell cytoplasm. *A. phagocytophilum* has the enzyme that converts glutamine to glutamate and glutamate can be then transformed to α-ketoglutarate that may fuel the bacterial TCA cycle.

Interestingly, it was recently shown that inhibition of prolyl hydroxylases, which are involved in the regulation of HIF-1α activity, induces HIF-1α-dependent cytoskeletal remodeling in endothelial cells (Weidemann et al., 2013). Therefore, it is possible that *A. phagocytophilum* activates HIF-1α through PI3K to regulate simultaneously the carbohydrate metabolism and cytoskeleton organization to facilitate infection and multiplication in tick cells (**Figure 9**).

The comparison of metabolic pathways between selected Anaplasmataceae showed that *Rickettsia prowazekii*, *Ehrlichia chaffeensis*, *Neorickettsia sennetsu*, *Wolbachia pipientis*, and *A. phagocytophilum* might not be able to actively carry out glycolysis. Therefore, glycolytic metabolic intermediates produced by host cells may be necessary for the development of these bacteria. Only those glycolysis enzymes necessary to produce glyceraldehyde-3-phosphate and dihydroxyacetone phosphate from phosphoenolpyruvate are present in the genome of these *Rickettsia* spp. (Dunning Hotopp et al., 2006). Therefore, phosphoenolpyruvate may be one of the glycolytic intermediates that *A. phagocytophilum* hijacks from the host cell cytoplasm. Additionally, glycerol-3-phosphate, which is a product of glyceroneogenesis, was proposed as another metabolite taken from the host by *Rickettsia* spp. (Dunning Hotopp et al., 2006). In agreement with this proposed model,

the glyceroneogenesis enzymes triosephosphate isomerase (TPI) and glycerol-3-phosphate dehydrogenase cytoplasmic (GPDHc) were over-represented in infected tick nymphs, midguts and/or salivary glands when compared to uninfected controls.

The failure to identify *I. scapularis* orthologs for some genes may be due to the absence of these pathway components in ticks or the fact that only ∼57% of the genome have been sequenced and assembled for this species (de la Fuente et al., 2016b; Gulia-Nuss et al., 2016). These results were similar to those obtained before for other genes and proteins in response to *A. phagocytophilum* infection, showing tissue-specific differences in the response to pathogen infection (Ayllón et al., 2015). As previously discussed, these results suggested that differences between mRNA and protein levels could be due to delay between mRNA and protein accumulation which requires sampling at different time points and/or the role for post-transcriptional and post-translational modifications in the tick tissue-specific response to *A. phagocytophilum* infection (Ayllón et al., 2015; Villar et al., 2015; Cabezas-Cruz et al., 2016).

CONCLUSIONS

These results support that major carbohydrate metabolic pathways are conserved in ticks. *A. phagocytophilum* infection has a major impact on the regulation of carbohydrate metabolic pathways in tick cells. As a result of the studies reported here, a mechanism was proposed by which this pathogen might induce the expression and stabilization of HIF-1α to increase glycolysis, suppress TCA cycle to reduce gluconeogenesis, and regulate cytoskeleton organization (**Figure 9**). This may be achieved by a coordinated action of PI3K/PKC pathway, for induction of *hif-1α* expression, and HSP70/90 and p300, for HIF-1α stabilization. The increase in glycolysis results in the production of energy and other components that enhance pathogen infection and transmission while preserving tick fitness. The reduction in gluconeogenesis may be a cell response to limit pathogen infection, but also results in the inhibition of cell apoptosis to enhance pathogen infection and transmission. These mechanisms provided additional support for the co-evolution of tick-pathogen interactions that can produce both conflict and cooperation between them (de la Fuente et al., 2016c). The identification of these mechanisms provided additional evidences to support that *A. phagocytophilum* uses similar strategies to infect vertebrate hosts and ticks (de la Fuente et al., 2016a), therefore suggesting the possibility of developing strategies for a more effective control of *A. phagocytophilum* and its associated diseases by targeting similar mechanisms in both vertebrate hosts and tick vectors.

AUTHOR CONTRIBUTIONS

AC and JF conceived the study. PA performed the experiments. AC, PA, JV, MV, and JF performed data analyses. AC and JF wrote the paper, and other co-authors made additional suggestions and approved the manuscript.

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Ixodes scapularis Tick Cells Control *Anaplasma phagocytophilum* Infection by Increasing the Synthesis of Phosphoenolpyruvate from Tyrosine

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The obligate intracellular pathogen, *Anaplasma phagocytophilum*, is the causative agent of life-threatening diseases in humans and animals. *A. phagocytophilum* is an emerging tick-borne pathogen in the United States, Europe, Africa and Asia, with increasing numbers of infected people and animals every year. It is increasingly recognized that intracellular pathogens modify host cell metabolic pathways to increase infection and transmission in both vertebrate and invertebrate hosts. Recent reports have shown that amino acids are central to the host–pathogen metabolic interaction. In this study, a genome-wide search for components of amino acid metabolic pathways was performed in *Ixodes scapularis*, the main tick vector of *A. phagocytophilum* in the United States, for which the genome was recently published. The enzymes involved in the synthesis and degradation pathways of the twenty amino acids were identified. Then, the available transcriptomics and proteomics data was used to characterize the mRNA and protein levels of *I. scapularis* amino acid metabolic pathway components in response to *A. phagocytophilum* infection of tick tissues and ISE6 tick cells. Our analysis was focused on the interplay between carbohydrate and amino acid metabolism during *A. phagocytophilum* infection in ISE6 cells. The results showed that tick cells increase the synthesis of phosphoenolpyruvate (PEP) from tyrosine to control *A. phagocytophilum* infection. Metabolic pathway analysis suggested that this is achieved by (i) increasing the transcript and protein levels of mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M), (ii) shunting tyrosine into the tricarboxylic acid (TCA) cycle to increase fumarate and oxaloacetate which will be converted into PEP by PEPCK-M, and (iii) blocking all the pathways that use PEP downstream gluconeogenesis (i.e., *de novo* serine synthesis pathway (SSP), glyceroneogenesis and gluconeogenesis). While sequestering host PEP may be critical for this bacterium because it cannot actively carry out glycolysis to produce PEP, excess of this metabolite may be toxic for

A. phagocytophilum. The present work provides a more comprehensive view of the major amino acid metabolic pathways involved in the response to pathogen infection in ticks, and provides the basis for further studies to develop novel strategies for the control of granulocytic anaplasmosis.

Keywords: proteomics, transcriptomics, phosphoenolpyruvate, glycerol- 3-phosphate, *Ixodes scapularis*, *Anaplasma phagocytophilum*

INTRODUCTION

Anaplasma phagocytophilum (Rickettsiales: Anaplasmataceae) is an obligate intracellular bacterium that produces life-threatening disease in humans and animals (Kocan et al., 2015). This pathogen is mainly transmitted by *Ixodes* spp. ticks in the United States, Europe, Africa and Asia (de la Fuente et al., 2008; Kocan et al., 2015). *A. phagocytophilum* infects vertebrate host granulocytes, and tick midgut, hemocytes and salivary glands (de la Fuente et al., 2008; Stuen et al., 2013; Kocan et al., 2015). The life cycle of *A. phagocytophilum* includes two morphological forms, the dense and reticulated cells, which are the infective and replicative stages of this bacterium, respectively (Stuen et al., 2013; Kocan et al., 2015). *A. phagocytophilum* has a very small genome (approximately 1.47 Mb) with a reduced number of effector proteins (Dunning et al., 2006; Sinclair et al., 2014, 2015). Therefore, as an evolutionary adaptation to its multi-host life style, this pathogen uses similar strategies to manipulate host cells and facilitate infection in vertebrates and ticks (Stuen et al., 2013; de la Fuente et al., 2016a). These mechanisms include but are not limited to remodeling of the cytoskeleton, inhibition of cell apoptosis, manipulation of the immune response, and modification of cell epigenetics and metabolism (Cabezas-Cruz et al., 2016, 2017a,b; de la Fuente et al., 2016a).

Host metabolism manipulation by bacteria has deep evolutionary roots. Not only pathogens have been shown to manipulate host metabolism, but commensal bacteria can also induce dramatic changes in host physiology (and even behavior) by affecting host metabolism (Olive and Sassetti, 2016; Leitão-Gonçalves et al., 2017). Thus, host-bacteria associations might be based on both transient and lasting metabolic cooperation and competition (Husnik et al., 2013; Zhang and Rubin, 2013). By transiently exploiting host metabolism, bacterial pathogens often fall within the first category (Zhang and Rubin, 2013; Olive and Sassetti, 2016). There is evidence that bacterial pathogens rely on host amino acid metabolism and in response the host "starves" the pathogen by "denying" the required amino acids (Zhang and Rubin, 2013). For example, it has been shown that *Mycobacterium tuberculosis* as well as *Salmonella* amino acid auxotroph strains are attenuated *in vivo* (O'Callaghan et al., 1988; Hondalus et al., 2000). Pathogens respond to this host-mediated amino acid starvation in different ways: (i) by differentiating to a viable but non-replicating form (e.g., *Chlamydia trachomatis*), (ii) by constitutively synthesizing their own amino acids (e.g., *M. tuberculosis*) and (iii) by manipulating host cell machinery to make amino acids available to the bacteria (e.g., *Legionella pneumophila*) (Zhang and Rubin, 2013). In the case of vector-borne bacteria, such as *A. phagocytophilum*, a solely-competition-based mechanism does not explain how this vector-pathogen ensemble is kept during evolution. Therefore, alternative models of vector-pathogen interaction, where both partners benefit from the association, should be explored (de la Fuente et al., 2016b; Cabezas-Cruz et al., 2017c).

Out of twenty amino acids, the *A. phagocytophilum* genome encodes only for the enzymes responsible of proline, glutamine, glycine and aspartate biosynthesis (Dunning et al., 2006). Like other *Rickettsia* spp., this intracellular pathogen cannot actively carry out glycolysis (Dunning et al., 2006). The glycolysis enzymes present are reduced to those that produce glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (DHAP) from phosphoenolpyruvate (PEP). This fact suggests that *A. phagocytophilum* may hijack some glycolytic intermediates produced by the host to complement its limited metabolic capacity. Recently, transcriptomics, proteomics and metabolomics analyses of infected *I. scapularis* ISE6 cells showed that *A. phagocytophilum* infection affects amino acid and carbohydrate metabolic pathways (Villar et al., 2015; Cabezas-Cruz et al., 2017a). These results suggested that *A. phagocytophilum* subverts amino acid and carbohydrate metabolism to facilitate infection and multiplication in tick cells. This evidence led us to the hypothesis that *A. phagocytophilum* infection subverts amino acid and carbohydrate metabolism simultaneously to increase the levels of and hijack PEP, which is the glycolytic intermediate with the highest-energy phosphate bond found in living organisms (Berg et al., 2002).

To test this hypothesis, the metabolism of the 20 amino acids was characterized in the tick vector *I. scapularis* in response to *A. phagocytophilum* infection. Firstly, the composition of the 20 amino acid metabolic pathways was annotated using the recently published genome of *I. scapularis* (de la Fuente et al., 2016c; Gulia-Nuss et al., 2016). Then, previously published transcriptomics and proteomics data (Ayllón et al., 2015; Villar et al., 2015) was used to characterize the mRNA and protein levels of amino acid metabolic pathway components in response to *A. phagocytophilum* infection of *I. scapularis* nymphs, female midguts and salivary glands, and ISE6 cells. Metabolic pathways analysis combined with quantitative metabolomics suggested a mechanism by which *A. phagocytophilum* infection increases the intracellular concentration of PEP which in turn control bacterial burden. The results also showed that the increase in PEP levels is achieved by using tyrosine as a carbon source via tricarboxylic acid (TCA) cycle. These results expanded our knowledge of the different pathways affected by *A. phagocytophilum* infection in ticks, and provided new potential targets for the development of therapeutic and prevention strategies for the control of human granulocytic anaplasmosis and other diseases caused by *Rickettsia* spp. that may use similar mechanisms for infection of the tick vector.

MATERIALS AND METHODS

Annotation of the Amino Acid Metabolic Pathway Components in the *I. scapularis* Genome

The *I. scapularis* genome (Gulia-Nuss et al., 2016) was searched with the specific names of genes encoding for enzymes involved in the 20 amino acid metabolic pathways. When records were not obtained using specific enzyme names, then the *I. scapularis* genome was searched with the Blastp tool from the Basic Local Alignment Search Tool (BLAST) using the human ortholog as "query" (Altschul et al., 1990; Madden et al., 1996). The sequences with the lowest *E*-value were selected. The conserved domains of identified protein sequences were classified using the protein families database Pfam (Finn et al., 2014). The *I. scapularis* orthologs found in the genome were double-checked by searching the National Center for Biotechnology Information (NCBI) databases using as queries the tick homologs identified in the previous step and excluding "*I. scapularis*" genome database from the search.

Characterization of the *I. scapularis* mRNA and Protein Levels in Response to *A. phagocytophilum* Infection

The quantitative transcriptomics and proteomics data for uninfected and *A. phagocytophilum*-infected *I. scapularis* nymphs, female midguts and salivary glands, and ISE6 cultured cells were obtained from previously published results (Ayllón et al., 2015; Villar et al., 2015) and deposited at the Dryad repository database, NCBI's Gene Expression Omnibus database and ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002181 and doi: 10.6019/PXD002181. The identified genes in the amino acid metabolic pathways were searched against the transcriptomics and proteomics data to characterize their mRNA and protein levels in response to *A. phagocytophilum* infection.

Ixodes scapularis ISE6 Cells

The *I. scapularis* embryo-derived tick cell line ISE6, provided by Ulrike Munderloh, University of Minnesota, USA, was cultured in L-15B300 medium as described previously (Munderloh et al., 1994), except that the osmotic pressure was lowered by the addition of one-fourth sterile water by volume. The ISE6 cells were first inoculated with *A. phagocytophilum* (human NY18 isolate)-infected HL-60 cells (de la Fuente et al., 2005) and maintained according to Munderloh et al. (1999). Pathogen manipulation and disposal of residuals were performed following biosafety level-2 (BSL2) laboratory procedures.

Determination of Glycerol-3-Phosphate and Phosphoenolpyruvate Levels

ISE6 tick cells (approximately 5×10^5 cells/well) were inoculated with *A. phagocytophilum* NY18 then sampled at 7 days post-infection (dpi, % infected cells > 70%). Uninfected cells were included as controls. Harvested cells were used to determine the concentration of glycerol 3-phosphate (G-3P) and PEP using the glycerol 3-phosphate colorimetric assay Kit (Sigma Cat. No. MAK207) or the PEP colorimetric/fluorometric assay kit (Sigma Cat. No. MAK102) respectively, following manufacturer's protocols. G-3P and PEP levels (ng/μl) were compared between untreated and treated cells by Student's *t*-test with unequal variance ($P < 0.05$; $N = 4$ biological replicates).

Pharmacological Studies in Cultured Tick Cells

Uninfected ISE6 tick cells were treated for 6 h with 1 μM Nitisinone (Sigma Cat. No. SML0269) to inhibit the activity of hydroxyphenylpyruvate dioxygenase (HPPD). Then, they were infected with *A. phagocytophilum* NY18. Cells were harvested at 24 and 72 h and used for Annexin V-FITC staining to detect cell apoptosis (see below), for DNA extraction to quantify the levels of with *A. phagocytophilum* (see below) and to determine the levels of PEP as described above. All treatments were done in quadruplicate. Uninfected and infected untreated cells were used as controls.

Determination of *A. phagocytophilum* Burden by Real-Time PCR

Anaplasma phagocytophilum NY18 DNA levels were characterized by *msp4* real-time PCR normalizing against tick ribosomal protein S4 (*rps4*) as described previously (Alberdi et al., 2015). Normalized Ct values were compared between untreated and treated cells by Student's *t*-test with unequal variance ($P < 0.05$; $N = 4$).

Annexin V-FITC Staining to Detect Cell Apoptosis after Experimental Infection with *A. phagocytophilum*

Approximately 5×10^5–1×10^6 uninfected and *A. phagocytophilum*-infected ISE6 tick cells were collected after different treatments. Apoptosis was measured by flow cytometry using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Immunostep, Salamanca, Spain) following the manufacturers protocols. The technique detects changes in phospholipid symmetry analyzed by measuring Annexin V (labeled with FITC) binding to phosphatidylserine, which is exposed in the external surface of the cell membrane in apoptotic cells. Cells were stained simultaneously with the non-vital dye propidium iodide (PI) allowing the discrimination of intact cells (Annexin V-FITC negative, PI negative) and early apoptotic cells (Annexin V-FITC positive, PI negative). All samples were analyzed on a FAC-Scalibur flow cytometer equipped with CellQuest Pro software (BD Bio-Sciences, Madrid, Spain). The viable cell population was gated according to forward-scatter and side-scatter parameters. The percentage of apoptotic cells was determined by flow cytometry after Annexin V-FITC and PI labeling and compared between treated and untreated uninfected cells by Student's *t*-test with unequal variance ($P < 0.05$; $N = 4$).

RESULTS

Major Amino Acid Metabolic Pathways Described in Model Organisms Are Present in *I. scapularis* and Are Affected by *A. phagocytophilum* Infection

The major synthesis and degradation pathways of the 20 amino acids were selected for characterization. A total of 72 genes coding for the proteins involved in the metabolism of the major 20 amino acids were identified in the *I. scapularis* genome (**Table 1**). Based on these results, models for amino acid synthesis of nonessential amino acids and degradation in ticks were proposed (**Figures 1**, **2**). Despite being hematophagous ectoparasites that ingest large amounts of a protein-rich diet, the tick genome contains all genes coding for enzymes responsible for major amino acid metabolic pathways found in animal models (Berg et al., 2002). An exception was the enzyme cysteine sulfinic acid decarboxylase (CSAD), involved in cysteine metabolism, and for which no orthologue was found in the *I. scapularis* genome. At least in humans, cysteine sulfinate can be transformed to hypotaurine by CSAD. Hypotaurine is oxidased nonenzymatically to taurine. Searches in sequence databases of other tick species did not produce a CSAD orthologue.

There is a major crosstalk between carbohydrate and amino acid metabolism. For example, the α-ketoacids, α-ketoglutarate, oxaloacetate (OAA), and pyruvate, can be converted into amino acids in one step through the addition of an amino group (Berg et al., 2002). In this group of amino acids are alanine, aspartate and glutamate that can be synthesized from pyruvate, OAA and α-ketoglutarate, respectively. All enzymes required for these transformations were found in the *I. scapularis* genome (**Figure 1**). Aspartate can also be formed by deamination of asparagine catalyzed by asparaginase (ASPG). As displayed in **Figure 1**, glutamate and aspartate can be transformed into glutamine and asparagine in amidation reactions catalyzed by glutamine synthetase (GS) and asparagine synthetase (AsnRS), respectively. Apart from glutamine, glutamate is the precursor of two other nonessential amino acids: proline and arginine. The enzyme Δ-1-pyrroline-5-carboxylate synthetase (P5CS) converts glutamate to glutamate γ-semialdehyde, an intermediate in the biosynthesis of proline (**Figure 1**), ornithine and arginine (**Figure 2**). Although the metabolism of ornithine and citruline was not considered in this study, these amino acids were included whenever they form part of the metabolism of the 20 amino acids. Another major contributor to amino acid synthesis is the glycolytic intermediate 3-phosphoglycerate (3-PGA), which is the precursor of serine, cysteine, and glycine (**Figure 1**). Finally, tyrosine can be synthesized from the essential amino acid phenylalanine by action of the enzyme phenylalanine hydroxylase (PAH). The reaction catalyzed by PAH requires a cofactor, tetrahydrobiopterin, which is maintained in the reduced state by the NADH-dependent enzyme, dihydropteridine reductase (DHPR). Both enzymes were found in the *I. scapularis* genome.

In comparison to synthesis, amino acid degradation is carried out by far more complicated reactions requiring a larger number of steps (**Figure 2**). However, likewise synthesis, amino acid degradation is tightly connected to carbohydrate metabolism. Thus, depending on their metabolic fate, amino acids are classified in glucogenic (aspartate, asparagine, alanine, glycine, cysteine, serine, arginine, histidine, proline, glutamine, glutamate, methionine, and valine), ketogenic (lysine and leucine) and those that are both glucogenic and ketogenic (tyrosine, phenylalanine, threonine, tryptophan and isoleucine). Glucogenic amino acids can be transformed into intermediates of the TCA cycle or glucose metabolism for the synthesis of glucose through gluconeogenesis (Owen et al., 2002). Ketogenic amino acids can be degraded into ketone bodies and acetyl-CoA. **Figure 2** shows that the *I. scapularis* genome contains the enzymes that allows a full degradation of both glucogenic and ketogenic amino acids.

The amino acid metabolism response to *A. phagocytophilum* infection was characterized using the quantitative transcriptomics and proteomics data generated from uninfected and *A. phagocytophilum*-infected *I. scapularis* ticks and ISE6 cultured cells (Ayllón et al., 2015; Villar et al., 2015). As in previous reports for other biological processes (Ayllón et al., 2015; Villar et al., 2015; Cabezas-Cruz et al., 2016, 2017a,b), most of the identified amino acid metabolism genes were differentially regulated in response to *A. phagocytophilum* infection in at least one of the analyzed tick samples (**Figures 3**, **4**). Thirty-one (43%), 12 (16%), 32 (44%), and 58 (80%) amino acid metabolism components were identified in both transcriptome and proteome of ISE6 cells, nymphs, adult female midguts and salivary glands, respectively (**Figures 3**, **4**). The proteomics results showed that various proteins were not identified in one or several samples (**Figures 3**, **4**), suggesting low protein levels in these cells or tissues. However, the levels of several proteins changed in response to infection (**Figures 3**, **4**). Considering the protein levels to provide an indicator of the effect of *A. phagocytophilum* infection on tick amino acid metabolic pathways, the results showed a global decrease in serine, glycine, glutamine and tyrosine synthesis enzymes in nymphs, and adult midguts and salivary glands (**Figure 3**). Histidine, lysine and arginine degradation enzymes were underrepresented in salivary glands (**Figure 4**). On the contrary, leucine, isoleucine and valine degradation enzymes were overrepresented in most tick tissues and ISE6 cells (**Figure 4**). These results supported the presence of tissue-specific differences in the tick cell response to infection (Sunyakumthorn et al., 2013; Ayllón et al., 2015; Villar et al., 2015; Cabezas-Cruz et al., 2016, 2017a,b). The datasets used in this analysis on the tick transcriptomics and proteomics response to *A. phagocytophilum* infection have been validated before in several studies (Ayllón et al., 2015; Villar et al., 2015; Cabezas-Cruz et al., 2016, 2017a,b).

Anaplasma phagocytophilum Infection Redirects Tick Metabolism toward Production of Phosphoenolpyruvate

We previously showed that *A. phagocytophilum* infection activates the expression of glycolytic genes by a HIF-1α-mediated mechanism (Cabezas-Cruz et al., 2017a). Interestingly,

TABLE 1 | Annotation of amino acid metabolism enzymes identified in the genome of *Ixodes scapularis*.

Amino acid/Name	Abbreviation	Genome accession	Uniprot
ALANINE			
Alanine transaminase 1	ALT1	ISCW013629	B7QLI8
Alanine transaminase 2	ALT2	ISCW019179	B7PLP0
CYSTEINE			
Cystathionine beta-synthase	CBS	ISCW003755	B7PF92
Cysteine dioxygenase 1	CDO1	ISCW008991	B7PY67
Cysteine desulfurase	NFS1	ISCW004705	B7PGB2
Cystathionase (Cystationine gamma Lyase)	CTH	ISCW007691	B7PU74
Methionine adenosyltransferase	MAT	ISCW004274	B7PJM6
Adenosylhomocysteinase 1	AHCY1	ISCW015944	B7P4Y1
Adenosylhomocysteinase 2	AHCY2	ISCW016595	B7PCR2
Sulfite oxidase	SUOX	ISCW004861	B7PGM8
ASPARTIC ACID (ASPARTATE)			
Aspartate aminotransferase	AST	ISCW014718	B7QLB0
GOT2 aspartate aminotransferase (mitochondrial isoenzyme)	GOT2	ISCW020516	B7Q1F6
Asparaginase	ASPG	ISCW022371	B7QGF3
GLUTAMIC ACID (GLUTAMATE)			
Glutamate synthase	GLUS	ISCW005873	B7PNR9
Glutamate dehydrogenase	GDH	ISCW000393	B7P4E1
PHENYLALANINE			
Phenylalanine hydroxylase	PAH	ISCW006510	B7PLQ7
GLYCINE			
Aminomethyltransferase	AMT [GCC, T-subunit]	ISCW001092	B7P6X5
Glycine dehydrogenase	GLDC [GCC, P-subunit]	ISCW006033	B7PR45
Dihydrolipoamide dehydrogenase	DLD [GCC, L-subunit]	ISCW001462	B7P465
Glycine cleavage system H protein	GCSH [GCC, H-subunit]	ISCW002099	B7P916
Glycine/Serine hydroxymethyltransferase	SHMT	ISCW004246	B7PGD5
Alanine-glyoxylate aminotransferase 2	AGXT2	ISCW020823	B7Q1E3
HISTIDINE			
Urocanate hydratase 1	UROC1	ISCW021677	B7Q456
Histidine ammonia-lyase	HAL	ISCW012913	B7QBL3
Imidazolone propionase	AMDHD1	ISCW012713	B7QD45
Formiminotransferase cyclodeaminase	FTCD	ISCW010907	B7Q6E5
ISOLEUCINE, LEUCINE, VALINE			
Branched-chain amino acid aminotransferase	BCAT	ISCW015784	B7P5K8
Branched-chain α-keto acid decarboxylase	(BCKDHA) [BCKDC, E1]	ISCW018615	B7PL04
Lipoamide acyltransferase	(DBT) [BCKDC, E2]	ISCW018104	B7PEH7
Dihydrolipoamide dehydrogenase	(DLD) [BCKDC, E3]	ISCW001462	B7P465
short/branched-chain acyl-CoA dehydrogenase	(SBCAD) [Only for Isoleucine]	ISCW002863	B7P7P2
Isovaleryl-CoA dehydrogenase	(IVD) [Only for Leucine]	ISCW000209	B7P6T5
Isobutyryl-CoA dehydrogenase	(IBD) [Only for Valine]	ISCW003911	B7PJR8
LYSINE			
α-aminoadipic semialdehyde synthase	AASS	ISCW008489	B7PT93
METHIONINE			
Propionyl-CoA carboxylase beta chain	PCCB [VOMIT]	ISCW010503	B7Q9C9

(Continued)

TABLE 1 | Continued

Amino acid/Name	Abbreviation	Genome accession	Uniprot
Methylmalonyl-CoA epimerase	MCEE [VOMIT]	ISCW003889	B7PF37
Methylmalonyl-CoA mutase	MUT [VOMIT]	ISCW020522	B7Q1G1
Methionine synthase	MS	ISCW020738	B7Q2S9
ASPARAGINE			
Asparagine synthetase	AsnRS	ISCW004505	B7PH83
PROLINE			
Delta-1-pyrroline-5-carboxylate synthetase	P5CS	ISCW005278	B7PPZ5
Proline dehydrogenase	PRODH	ISCW000787	B7P4N1
Ornithine aminotransferase 1	OAT1	ISCW024076	B7P713
Ornithine aminotransferase 2	OAT2	ISCW007128	B7PUI7
Ornithine aminotransferase 3	OAT3	ISCW023390	B7QHR2
Pyrroline-5-carboxylate reductase A	PYCR1A	ISCW021802	B7Q8R2
Pyrroline-5-carboxylate reductase B	PYCR1B	ISCW020302	B7Q351
GLUTAMINE			
Glutamine synthetase	GS	ISCW018771	B7PNU5
Glutaminase	GLS	ISCW020515	B7Q1F5
ARGININE			
Argininosuccinate synthase	ASS	ISCW017094	B7PA12
Delta-1-pyrroline-5-carboxylate dehydrogenase	P5CDH	ISCW015982	B7P0M7
Argininosuccinate lyase	ASL	ISCW009296	B7Q096
Arginase	ARG	ISCW014441	B7QIY4
Nitric oxide synthase	NOS	ISCW018074	B7PGS0
SERINE			
3-phosphoglycerate dehydrogenase	PHGDH	ISCW015967	B7P3M8
Phosphoserine aminotransferase	PSAT1	ISCW020198	B7Q0D6
Phosphoserine phosphatase	PSP	ISCW003612	B7PHY5
THREONINE			
Threonine dehydrogenase	TDH	ISCW020011	B7PZ94
L-serine dehydratase/L-threonine deaminase	SDS	ISCW005673	B7PN84
2-amino-3-ketobutyrate coenzyme A ligase	GCAT	ISCW015295	B7QMM1
TRYPTOPHAN			
Tryptophan 2,3-dioxygenase	TDO	ISCW024183	B7PBJ2
Kynurenine formamidase	AFMID	ISCW007881	B7PSM5
Kynurenine-oxoglutarate transaminase 3 A	KYAT3-A	ISCW012664	B7QG90
Kynurenine-oxoglutarate transaminase 3 B	KYAT3-B	ISCW012663	B7QG89
Kynurenine 3-monooxygenase	KMO	ISCW011595	B7Q540
Kynureninase A	KYNU-A	ISCW012597	B7QAC8
Kynureninase B	KYNU-B	ISCW005441	B7PL61
3-hydroxyanthranilate 3,4-dioxygenase	HAAO	ISCW007038	B7PU50
TYROSINE			
Dihydropteridine reductase	DHPR	ISCW022552	B7QAP3
Tyrosine aminotransferase	TAT	ISCW014463	B7QLV1
Hydroxyphenylpyruvate dioxygenase	HPPD	ISCW018238	B7PHP6
Homogentisate 1,2-dioxygenase	HGD	ISCW004334	B7PK05
Maleylacetoacetate isomerase	GSTZ1	ISCW023198	B7QMI2
Fumarylacetoacetate hydrolase	FAH	ISCW020196	B7Q0D4

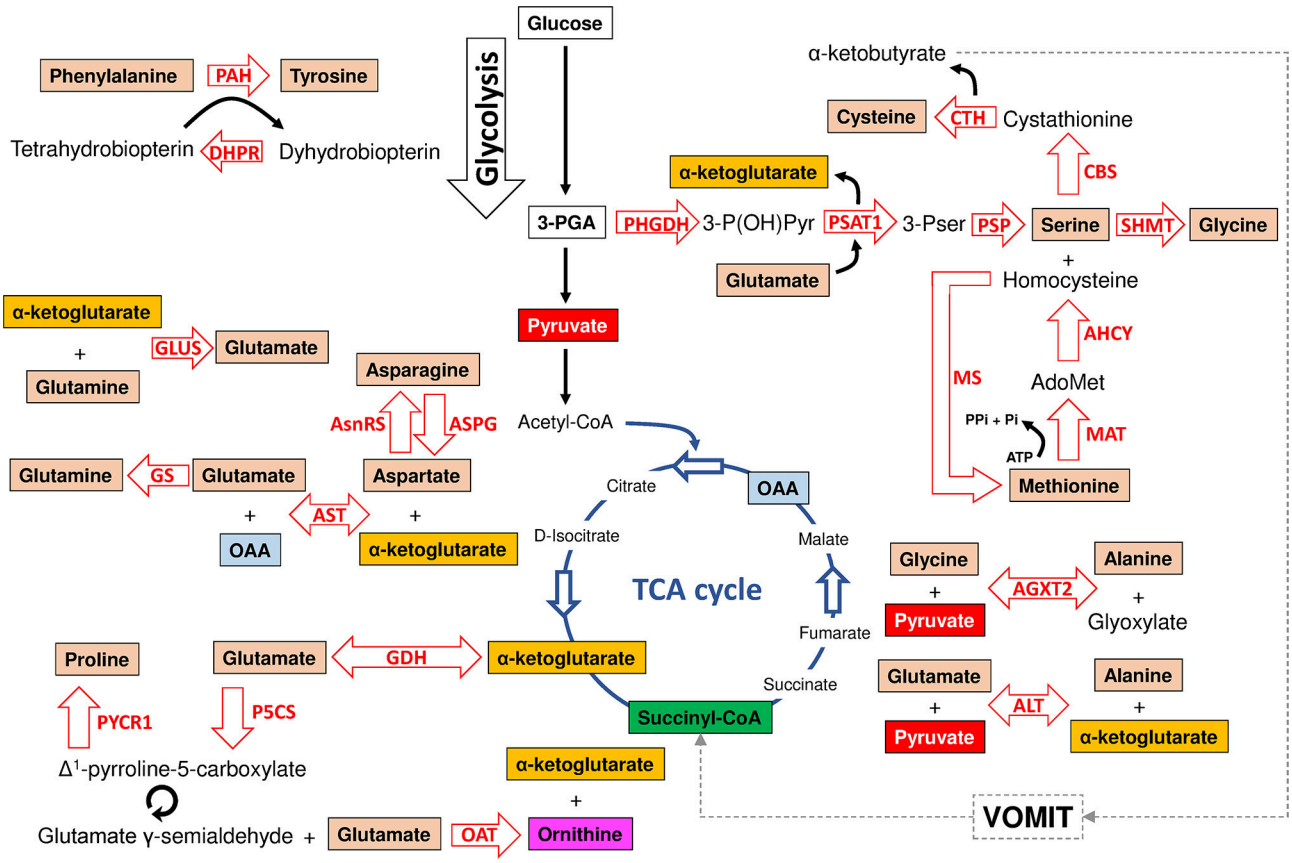

FIGURE 1 | Model of amino acid synthesis in *I. scapularis*. The main enzymes involved in tyrosine, cysteine, serine, glycine, methionine, alanine, glycine, glutamate, glutamine, asparagine, aspartate and proline synthesis that were identified in the genome of *I. scapularis* (**Table 1**) are shown. The interplay between amino acid and glucose metabolism (i.e., glycolysis and TCA cycle) intermediates is also shown. The names of the enzymes were abbreviated as follow: phenylalanine hydroxylase (PAH), dihydropteridine reductase (DHPR), glutamate synthase (GLUS), asparagine synthetase (AsnRS), asparaginase (ASPG), glutamine synthetase (GS), aspartate aminotransferase (AST), glutamate dehydrogenase (GDH), pyrroline-5-carboxylate reductase (PYCR1), delta-1-pyrroline-5-carboxylate synthetase (P5CS), ornithine aminotransferase (OAT), alanine transaminase (ALT), alanine-glyoxylate aminotransferase 2 (AGXT2), methionine adenosyltransferase (MAT), methionine synthase (MS), Adenosylhomocysteinase (AHCY), phosphoserine phosphatase (PSP), serine hydroxymethyltransferase (SHMT), cystathionine beta-synthase (CBS), cystathionase (Cystationine gamma lyase) (CTH), phosphoserine aminotransferase (PSAT1) and 3-phosphoglycerate dehydrogenase (PHGDH). The metabolic intermediates were abbreviated as follow: oxaloacetate (OAA), 3-phosphoglycerate (3-PGA), 3-phosphohydroxypyruvate (3-P(OH)Pyr), 3-phosphoserine (3-Pser), inorganic phosphate (Pi), adenosine triphosphate (ATP) and S-adenosylmethionine (AdoMet). The dashed line represents the VOMIT pathway (where V stands for valine, O for odd-chain fatty acids, M for methionine, I for isoleucine, and T for threonine) that transforms α-ketobutyrate in the TCA cycle intermediate succinyl-CoA. The VOMIT pathway involves three enzymes (propionyl-CoA carboxylase beta chain, methylmalonyl-CoA epimerase and methylmalonyl-CoA mutase) no displayed in the figure (**Table 1**). Glutamate γ-semialdehyde is the open-chain tautomer of Δ^1-pyrroline-5-carboxylate. The circular arrow symbol represents the nonenzymatic and reversible interconversion of Δ^1-pyrroline-5-carboxylate to glutamate γ-semialdehyde.

A. phagocytophilum infection increased the levels of serine, but decreased the levels of alanine (**Figure 5**). Serine is an allosteric activator of pyruvate kinase (PK) isoform M2 (PKM2), which catalyzes the last step of glycolysis to convert PEP to pyruvate and produce one molecule of ATP (Amelio et al., 2014; Yang and Vousden, 2016). The *I. scapularis* PK shares 64% identity with the human PKM2 and is overrepresented in *A. phagocytophilum*-infected ISE6 cells (Cabezas-Cruz et al., 2017a). In serine deprivation conditions, PKM2 activity is lowered, resulting in diversion of the 3-PGA pool into the *de novo* serine synthesis pathway (SSP) (Yang and Vousden, 2016). In contrast, when serine is abundant, PKM2 is fully activated, allowing the consumption of glucose through aerobic glycolysis (Amelio et al., 2014). In contrast, alanine is an allosteric inhibitor of PK (Gaitán et al., 1983; Berg et al., 2002). The high level of serine and low level of alanine suggested that, in addition to PK overrepresentation in *A. phagocytophilum*-infected ISE6 cells, the activity of PK might be enhanced. An activation of PK activity may increase the consumption of its substrate PEP.

To evaluate the effect of infection on the levels of PEP, the intracellular concentration of this metabolite was measured by a colorimetric assay, showing that the levels of PEP increased significantly in *A. phagocytophilum*-infected ISE6 cells (**Figure 6**). Two enzymes are responsible for the production of PEP, the glycolysis enzyme enolase (ENOL) and the

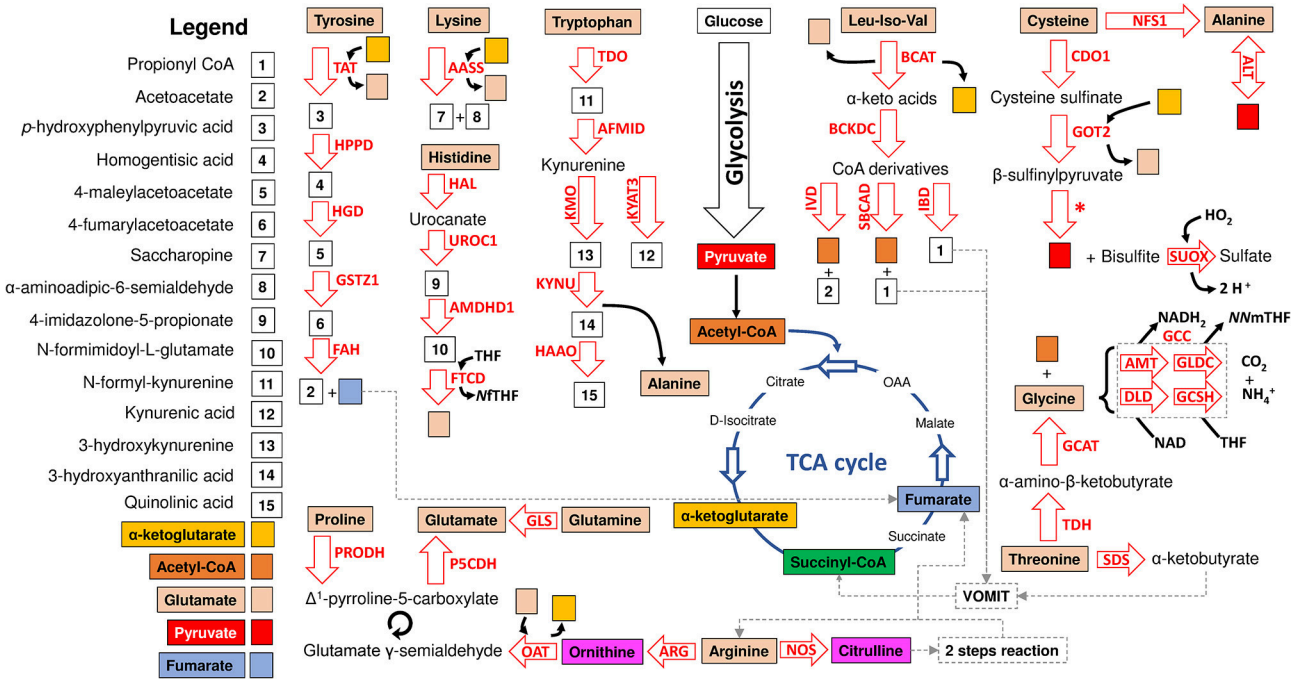

FIGURE 2 | Model of amino acid degradation in *I. scapularis*. The main enzymes involved in tyrosine, cysteine, glycine, alanine, glycine, glutamate, glutamine, arginine, lysine, tryptophan, histidine, threonine, proline, leucine (Leu), isoleucine (Iso) and valine (Val) degradation and identified in the genome of *I. scapularis* (**Table 1**) are shown. The interplay between amino acid and glucose metabolism (i.e., glycolysis and TCA cycle) intermediates is also shown. The names of the enzymes were abbreviated as follow: tyrosine aminotransferase (TAT), hydroxyphenylpyruvate dioxygenase (HPPD), homogentisate 1,2-dioxygenase (HGD), maleylacetoacetate isomerase (GSTZ1), fumarylacetoacetate hydrolase (FAH), α-aminoadipic semialdehyde synthase (AASS), histidine ammonia-lyase (HAL), urocanate hydratase 1 (UROC1), imidazolone propionase (AMDHD1), formiminotransferase cyclodeaminase (FTCD), tryptophan 2,3-dioxygenase (TDO), kynurenine formamidase (AFMID), kynurenine 3-monooxygenase (KMO), kynurenine-oxoglutarate transaminase 3 (KYAT3), kynureninase (KYNU), 3-hydroxyanthranilate 3,4-dioxygenase (HAAO), branched-chain amino acid aminotransferase (BCAT), isovaleryl-CoA dehydrogenase (IVD), short/branched-chain acyl-CoA dehydrogenase (SBCAD), isobutyryl-CoA dehydrogenase (IBD), cysteine dioxygenase 1 (CDO1), GOT2 aspartate aminotransferase (GOT2), cysteine desulfurase (NFS1), alanine transaminase (ALT), sulfite oxidase (SUOX), glycine cleavage complex (GCC) which includes four enzymes: aminomethyltransferase (AMT), glycine dehydrogenase (GLDC), dihydrolipoamide dehydrogenase (DLD) and glycine cleavage system H protein (GCSH), 2-amino-3-ketobutyrate coenzyme A ligase (GCAT), threonine dehydrogenase (TDH), L-serine dehydratase/L-threonine deaminase (SDS), glutaminase (GLS), proline dehydrogenase (PRODH), delta-1-pyrroline-5-carboxylate dehydrogenase (P5CDH), ornithine aminotransferase (OAT), arginase (ARG), nitric oxide synthase (NOS) and branched-chain α-ketoacid dehydrogenase complex (BCKDC) which includes three enzymes (branched-chain α-keto acid decarboxylase, lipoamide acyltransferase and dihydrolipoamide dehydrogenase) no displayed in the figure (**Table 1**). Citrulline is transformed in arginine by a "two-step reaction" including argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) which are not displayed in the figure but are available in **Table 1**. The metabolic intermediates were abbreviated as follow: oxaloacetate (OAA), N^5-N^{10}-methylene tetrahydrofolate (*NN*mTHF), 5-formiminotetrahydrofolate (*Nf*THF) and tetrahydrofolate (THF). The VOMIT pathway (where V stands for valine, O for odd-chain fatty acids, M for methionine, I for isoleucine, and T for threonine) enzymes transform α-ketobutyrate and propionyl CoA into the TCA cycle intermediate succinyl-CoA. The VOMIT pathway enzymes (propionyl-CoA carboxylase beta chain, methylmalonyl-CoA epimerase and methylmalonyl-CoA mutase) are not displayed in the figure but are available in **Table 1**. Glutamate γ-semialdehyde is the open-chain tautomer of Δ^1-pyrroline-5-carboxylate. The circular arrow symbol represents the nonenzymatic and reversible interconversion of Δ^1-pyrroline-5-carboxylate to glutamate γ-semialdehyde. The asterisk shows the position of a desulfurization reaction.

gluconeogenesis enzyme phosphoenolpyruvate carboxykinase (PEPCK). The PEP produced by ENOL may be transformed rapidly to pyruvate by PK to keep the glycolytic flow. There are, however, four major pathways in which the PEP produced by PEPCK plays a key role (Yang et al., 2009). These pathways are gluconeogenesis, glyceroneogenesis, the SSP, and the conversion of the carbon skeletons of amino acids to PEP (via PEPCK) and then to pyruvate (via PK) for subsequent oxidation in the TCA cycle as acetyl-CoA (Yang et al., 2009). A suitable hypothesis to explain the increase in PEP is that all the above pathways that use this metabolite are inhibited in *A. phagocytophilum*-infected ISE6 cells.

Anaplasma phagocytophilum Infection Decreases Gluconeogenesis and Glyceroneogenesis

All eukaryotes have genes for a mitochondrial (PEPCK-M) and a cytosolic (PEPCK-C) PEPCK (Yang et al., 2009). Both enzymes were found in the genome of *I. scapularis* (Cabezas-Cruz et al., 2017a). As previously reported, *A. phagocytophilum* infection inhibits gluconeogenesis and decreases the concentration of glucose by decreasing the protein levels of PEPCK-C (Villar et al., 2015; Cabezas-Cruz et al., 2017a). Gluconeogenesis and glyceroneogenesis are related pathways and both are regulated by PEPCK-C (Berg et al., 2002; Nye et al., 2008).

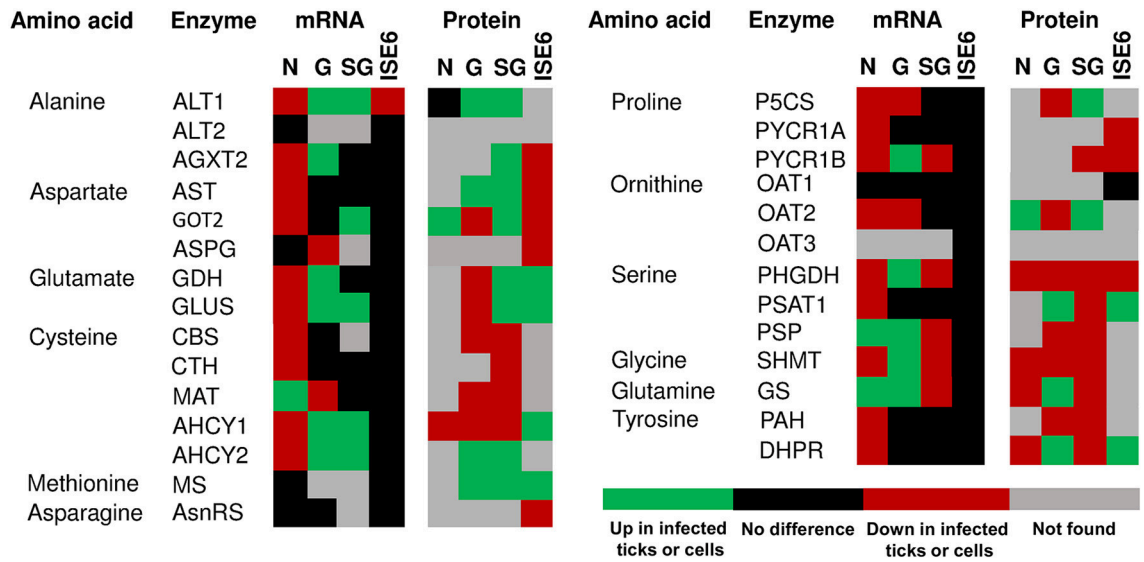

FIGURE 3 | mRNA and protein levels of the *I. scapularis* amino acid synthesis enzymes in response to *A. phagocytophilum* infection. Comparison of amino acid synthesis enzymes mRNA and protein levels in *I. scapularis* nymphs (N), female midguts (G), female salivary glands (SG) and ISE6 cells (ISE6) in response to *A. phagocytophilum* infection. Transcriptomics and proteomics data were obtained from previously published datasets available on the Dryad repository database, NCBI's Gene Expression Omnibus database and ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002181 and doi: 10.6019/PXD002181 (Ayllón et al., 2015; Villar et al., 2015). Name of enzymes were abbreviated as in **Table 1** and **Figure 1**.

Glyceroneogenesis is the *de novo* synthesis of glycerol 3-phosphate (G-3P) from precursors other than glucose and glycerol, including pyruvate, lactate, alanine, and TCA cycle anions (Nye et al., 2008). The decrease in the levels of lactate and alanine in *A. phagocytophilum*-infected ISE6 cells (**Figure 5**) suggested that infection by this pathogen, in addition to gluconeogenesis, also inhibits glyceroneogenesis. To test the effect of *A. phagocytophilum* infection on glyceroneogenesis, the intracellular concentration of G-3P was measured in *A. phagocytophilum*-infected ISE6 cells by a colorimetric assay. The levels of G-3P decreased significantly in infected cells (**Figure 6**).

Dihydroxyacetone phosphate (DHAP) is transformed into G-3P by the enzyme glycerol-3-phosphate dehydrogenase (GPDH) and DHAP can be produced by fructose-bisphosphate aldolase A (ALDA) and triosephosphate isomerase (TPI). The enzyme ALDA was underrepresented in ISE6 cells and salivary glands infected with *A. phagocytophilum* (Cabezas-Cruz et al., 2017a). Interestingly, it was recently shown that PEP competitively inhibits the interconversion of glyceraldehyde-3-phosphate (G3P) and DHAP by TPI and therefore is considered an inhibitor of TPI (Grüning et al., 2014). Therefore, the increase in the intracellular concentration of PEP may act as an additional mechanism to inhibit glyceroneogenesis.

Anaplasma phagocytophilum Infection Decreases *De novo* Serine Synthesis Pathway but Increases the Expression of Serine Transporters in ISE6 Cells

Figure 5 shows that except for valine, serine and proline, *A. phagocytophilum* infection decreased the levels of all glucogenic amino acids found in ISE6 cells (Villar et al., 2015). The levels of valine did not change in response to *A. phagocytophilum* infection, but the levels of serine and proline increased significantly (**Figure 5**). Despite that the levels of proline increased, the levels of glutamate decreased in response to infection (**Figure 5**). Proline is considered to be a glucogenic amino acid because it can be converted into glutamate that enters the TCA cycle through α-ketoglutarate. This finding suggested that during *A. phagocytophilum* infection, proline has little or no contribution to gluconeogenesis.

Furthermore, while the levels of serine increased, the enzyme 3-phosphoglycerate dehydrogenase (PHGDH) that catalyzes the first step in SSP, was underrepresented in ISE6 cells, nymphs, midguts and salivary glands (**Figure 3**). The enzyme PHGDH plays a major role in redirecting the glycolytic intermediate 3-PGA to SSP (Amelio et al., 2014; Samanta and Semenza, 2016). In addition, phosphoserine aminotransferase (PSAT1), the second enzyme of SSP, uses glutamate in a transamination reaction that converts 3-phosphohydroxypyruvate to phosphoserine. As mentioned above, glutamate decreased in infected cells, which suggested that the reaction catalyzed by PSAT1 may be hampered by the low levels of this amino donor. Serine can also be derived from glycine by action of the enzyme serine hydroxymethyltransferase (SHMT), which catalyzes the interconversion of glicine and serine. SHMT was underrepresented in nymphs, midguts and salivary glands and was not found in infected ISE6 cells, but the concentration of glycine was found to decrease in *A. phagocytophilum*-infected ISE6 cells (**Figures 3**, **5**). Therefore, the increase in intracellular serine levels in infected cells cannot be explained by SSP or the activity of SHMT. An alternative explanation is that *A.*

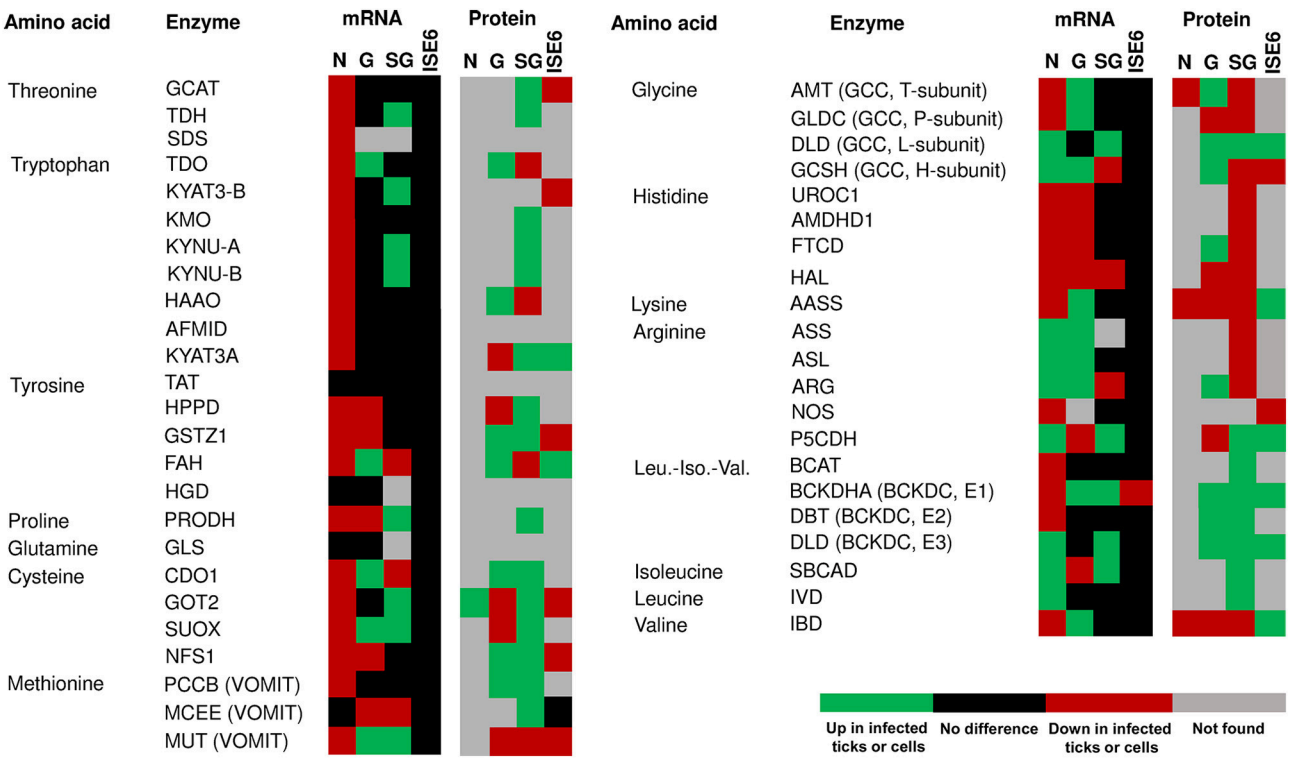

FIGURE 4 | mRNA and protein levels of the *I. scapularis* amino acid degradation enzymes in response to *A. phagocytophilum* infection. Comparison of amino acid degradation enzymes mRNA and protein levels in *I. scapularis* nymphs (N), female midguts (G), female salivary glands (SG) and ISE6 cells (ISE6) in response to *A. phagocytophilum* infection. Transcriptomics and proteomics data were obtained from previously published datasets available on the Dryad repository database, NCBI's Gene Expression Omnibus database and ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002181 and doi: 10.6019/PXD002181 (Ayllón et al., 2015; Villar et al., 2015). Name of enzymes were abbreviated as in **Table 1** and **Figure 2**.

phagocytophilum induces a higher rate of serine uptake through membrane transporters (Yang and Vousden, 2016). Two serine transporters were found in the genome of *I. scapularis* (accession numbers ISCW015439 and ISCW017507). Both transporters were overrepresented in infected salivary glands and ISE6 cells (data not shown), which suggests that serine is taken from the extracellular milieu during *A. phagocytophilum* infection in tick cells.

Contribution of Phenylalanine and Tyrosine to the OAA/PEPCK-M/PEP Node in *A. phagocytophilum*-Infected ISE6 Cells

As mentioned above, *A. phagocytophilum* infection increased the intracellular concentration of PEP and inhibited the pathways that use this metabolite downstream gluconeogenesis (i.e., gluconeogenesis, glyceroneogenesis and SSP). PEP (via PEPCK) is also involved in the cycling of amino acids to PEP and then to pyruvate (via PK) for subsequent oxidation in the TCA cycle as acetyl-CoA (Yang et al., 2009). While PEPCK-C was underrepresented in *A. phagocytophilum*-infected ISE6 cells (Villar et al., 2015; Cabezas-Cruz et al., 2017a), one of the PEPCK-M isoforms found in the *I. scapularis* genome was upregulated and overrepresented in *A. phagocytophilum*-infected ISE6 cells (Cabezas-Cruz et al., 2017a). PEPCK-M cannot replace PEPCK-C in gluconeogenesis (Méndez-Lucas et al., 2013). However, transformation of oxaloacetate (OAA) into PEP from the mitochondria via PEPCK-M can contribute up to 40% of the cytosolic PEP pool (Stark et al., 2009; Yang et al., 2009).

Glucogenic and ketogenic amino acids can enter the TCA cycle at different points and be transformed into OAA, which is the substrate of PEPCK-M to produce PEP. Arginine, histidine, proline, and glutamine are transformed into glutamate, which enters the TCA cycle through α-ketoglutarate (**Figure 5**). Despite that some of the enzymes that transform glutamate into α-ketoglutarate were overrepresented (i.e., GDH and PSAT1) (**Figure 3**), as previously mentioned the concentration of glutamate decreased in *A. phagocytophilum*-infected ISE6 cells (**Figure 5**), suggesting that arginine, histidine, proline and glutamine have a small contribution to the TCA cycle and OAA production.

Another entry point of amino acids (i.e., methionine, valine, threonine, and isoleucine) to the TCA cycle is succinyl-CoA, which results from the VOMIT pathway (**Figure 2**). These four amino acids were not affected (valine and isoleucine), decreased (methionine) or were not found (threonine) in *A. phagocytophilum*-infected ISE6 cells

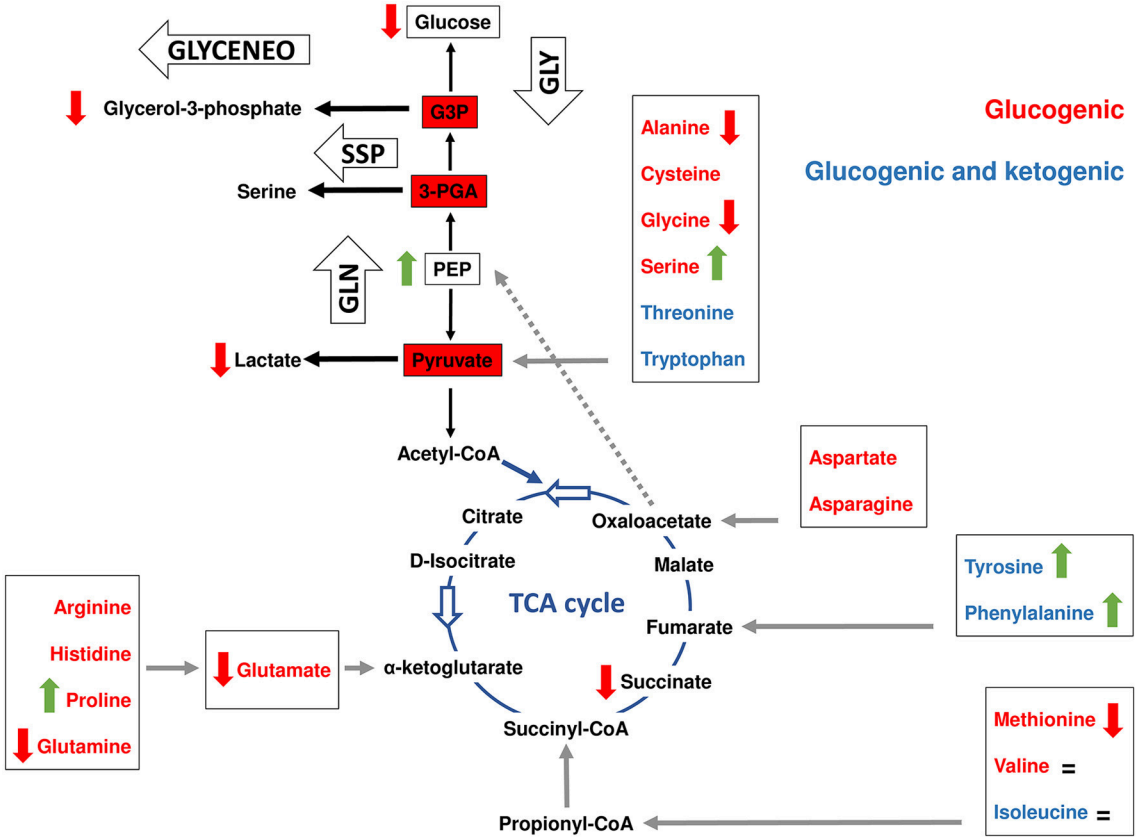

FIGURE 5 | Interplay between amino acid and carbohydrate metabolism in tick ISE6 cells. Simplified gluconeogenesis (GLN), glyceroneogenesis (GLYCENEO), glycolysis (GLY), *de novo* serine synthesis (SSP) and TCA cycle pathways are represented. The amino acids were colored following their classification in glucogenic (red) and those that are both glucogenic and ketogenic (blue). The entry points (gray arrows) of each amino acid group (boxed) to the TCA cycle are shown as well as the amino acids that are transformed to pyruvate. Data on amino acid and metabolic intermediate levels are represented by colored vertical arrows (red decrease, green increase and = no change) was collected from a previous report (Villar et al., 2015), except for phosphoenolpyruvate (PEP) and glycerol-3-phosphate that were measured in the present study. Glyceraldehyde 3-phosphate was abbreviated as G3P.

(**Figure 5**). In addition, the last enzyme of the VOMIT pathway, methylmalonyl-CoA mutase, was underrepresented in *A. phagocytophilum*-infected ISE6 cells (**Figure 4**). These findings suggested that the contribution of amino acids to the succinyl-CoA pool during *A. phagocytophilum* infection is limited. This was supported by the observation that the levels of succinate, the TCA cycle intermediate that results from succinyl-CoA, decreased in infected ISE6 cells (Villar et al., 2015).

As shown in **Figure 1**, the interconversion of aspartate and asparagine is achieved by the enzymatic activity of AsnRS (aspartate to asparagine) and ASPG (asparagine to aspartate). Both enzymes were underrepresented in *A. phagocytophilum*-infected ISE6 cells (**Figure 3**). Aspartate can be transformed into glutamate by AST, an enzymatic reaction that also produces OAA. As shown in **Figure 3**, aspartate aminotransferase (AST) was also underrepresented in *A. phagocytophilum*-infected ISE6 cells. Despite that aspartate and asparagine were not found in uninfected or *A. phagocytophilum*-infected ISE6 cells (Villar et al., 2015), the low protein levels of the enzymes involved in their metabolism suggested that these two amino acids do not contribute to OAA production.

Finally, the degradation of tyrosine and phenylalanine produces fumarate, and the levels of these two amino acids were higher in *A. phagocytophilum*-infected ISE6 cells (**Figures 2**, **5**). The enzyme DHPR, which converts tetrahydrobiopterin into dyhydrobiopterin, a cofactor necessary for the transformation of phenylalanine into tyrosine, was overrepresented in *A. phagocytophilum*-infected ISE6 cells (**Figure 3**). The last enzyme of the tyrosine degradation pathway, FAH, produces fumarate and acetoacetate and was overrepresented in *A. phagocytophilum*-infected ISE6 cells (**Figure 4**). These results suggested that the increase of PEP in *A. phagocytophilum*-infected ISE6 cells was due to the transformation of tyrosine to PEP via TCA cycle and PEPCK-M. To test this hypothesis *A. phagocytophilum*-infected and uninfected ISE6 cells were treated with Nitisinone, an inhibitor of HPPD which is the second enzyme of the tyrosine degradation pathway (**Figure 2**). In agreement with our hypothesis, Nitisinone inhibited the increase of PEP levels induced by *A. phagocytophilum* infection

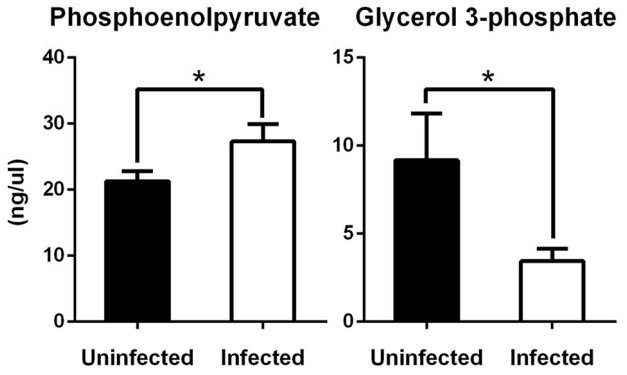

FIGURE 6 | Levels of phosphoenolpyruvate and glycerol 3-phosphate in tick ISE6 cells in response to *A. phagocytophilum* infection. The concentration of phosphoenolpyruvate (PEP) and glycerol 3-phosphate (G-3P) was measured using a colorimetric assay. ISE6 tick cells were inoculated with *A. phagocytophilum* and sampled at 7 days post-infection. G-3P and PEP levels (ng/μl) were compared between untreated and treated cells by Student's *t*-test with unequal variance. Asterisks denote statistical significant differences between groups ($P < 0.05$; $N = 4$).

in ISE6 cells (**Figure 7A**). The effect of Nitisinone on PEP levels was significant during early (24 h) but not late (72 h) *A. phagocytophilum* infection of ISE6 cells (**Figure 7A**). Interestingly, Nitisinone treatment increased the bacterial burden during early and late *A. phagocytophilum* infection (**Figure 7B**). Finally, we measured the percentage of apoptotic cells after Nitisinone treatment in *A. phagocytophilum*-infected ISE6 cells. After 72 h, the Nitisinone treatment increased significantly the apoptosis in *A. phagocytophilum*-infected ISE6 cells (**Figure 7C**).

DISCUSSION

The metabolic crosstalk between hosts and pathogens is more complex than previously realized (Olive and Sassetti, 2016). It was initially thought that the adaptation to a pathogenic lifestyle was exclusively associated to the acquisition of traits to overcome host immunity. However, recent evidences have uncovered that adaptation of pathogens to host metabolism and host metabolism manipulation by pathogens are as important as overcoming host immunity (Olive and Sassetti, 2016). For intracellular pathogens that have to compete for nutrients within the host cells, metabolism is a keystone to the outcome of infection. Several recent studies show that amino acids are central to the host-pathogen metabolic interaction (Belland et al., 2003; Baruch et al., 2014; Olive and Sassetti, 2016; Østergaard et al., 2016). For example, group A *Streptococcus* (GAS) uses asparagine for sensing the host immune status (Baruch et al., 2014). Toxins secreted by GAS trigger endoplasmic reticulum stress response which upregulates asparagine synthetase leading to higher levels of host-derived asparagine. This asparagine then activates the streptococcal invasion locus, which controls genes involved in virulence, growth and metabolism (Baruch et al., 2014). Therefore, it appears that GAS senses the proximity to host cells by monitoring the host response to bacterial toxins (Baruch et al., 2014; Olive and Sassetti, 2016).

An *in vivo* study of *Salmonella enterica* serovar Typhimurium (hereafter *S.* Typhimurium) in mouse revealed that pathogen infection induces a global differential gene expression (Liu et al., 2010). During early infection, *S.* Typhimurium produced a complete shut-off of the oxidative phosphorylation genes, while at a later stage of infection branched chain amino acids (valine, leucine and isoleucine) genes were significantly downregulated by the infection (Liu et al., 2010). Two examples of pathogen dependency on host metabolic status are *Francisella tularensis* and *Mycobacterium tuberculosis*, which are facultative and obligate intracellular pathogens, respectively (Olive and Sassetti, 2016). These pathogens require *de novo* tryptophan synthesis during infection to circumvent the depletion of host tryptophan that occurs during active T cell responses (Chu et al., 2011; Zhang et al., 2013).

One of the best-studied examples of bacterial response to host amino acid modulation is *Chlamydia trachomatis*. This obligate intracellular pathogen has lost the ability to produce several amino acids including tryptophan that it scavenges from the host (Zhang and Rubin, 2013; Olive and Sassetti, 2016). In humans, interferon-γ (IFNγ) induces a specific antimicrobial response through the induction of indoleamine-2,3-dioxegenase (IDO) which in turn depletes local stores of tryptophan (Pfefferkorn, 1984). *Chlamydia trachomatis* senses IDO-mediated tryptophan depletion and responds by differentiating to a viable but non-replicating form which allows the pathogen to cause long-term persistent infections even in the face of ongoing immune responses (Olive and Sassetti, 2016). The presence of an inducible partial tryptophan operon enables *C. trachomatis* to use indole to synthetize tryptophan even in the presence of high levels of the tryptophan-degrading enzyme IDO (Wood et al., 2003). Once the immune response resolves, IFNγ levels decrease, tryptophan accumulates and *C. trachomatis* reverts to active replication (Belland et al., 2003).

Phosphoenolpyruvate (PEP) also plays an important role in host-bacteria interactions. Most studies on the role of PEP in host-bacteria interactions focus in the PEP Phosphotransferase System (PEP-PTS) which is used by many gram-positive and gram-negative bacteria to uptake carbohydrates (Postma et al., 1993; Barabote and Saier, 2005; Khajanchi et al., 2015; Wang et al., 2015; Antunes et al., 2016). Bacterial PEP-PTS uses PEP as the phosphoryl donor for carbohydrate phosphorylation (Postma et al., 1993). Notably, *Borrelia burgdorferi* lacking one of the PEP-PTS components was unable to establish infection in mice by either needle inoculation or tick transmission (Khajanchi et al., 2015). However, the genomes of several Rickettsiales including *Anaplasma marginale* (closely related to *A. phagocytophilum*), *Rickettsia* spp. and *Wolbachia* sp. lack genes encoding identifiable PTS protein homologs (Barabote and Saier, 2005). This suggests that carbohydrate transport and phosphorylation might not be one of the main roles of PEP in Tick-*A. phagocytophilum* interactions.

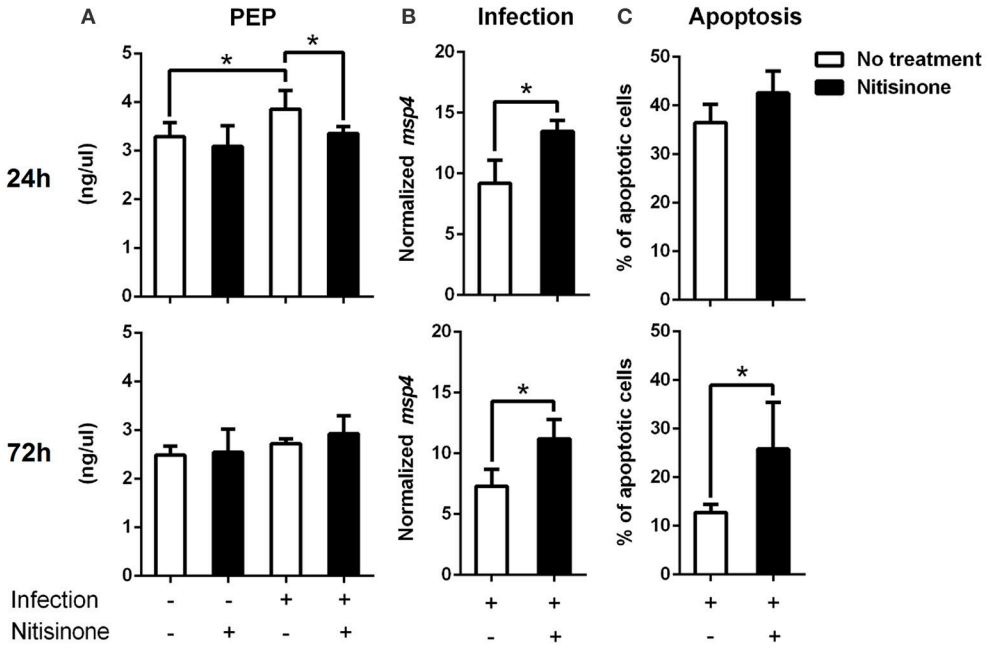

FIGURE 7 | Inhibition of the tyrosine degradation pathway by Nitisinone in *A. phagocytophilum*-infected ISE6 cells. **(A)** ISE6 tick cells were inoculated with *A. phagocytophilum* and sampled at 24 and 72 h post-infection. The concentration of PEP was measured using a colorimetric assay. PEP levels (ng/μl) were compared between untreated and treated cells with Nitisinone to inhibit the activity of HPPD, by Student's *t*-test with unequal variance. **(B)** *A. phagocytophilum* DNA levels were determined in infected ISE6 tick cells untreated or treated with Nitisinone. Bacterial DNA levels were determined by *msp4* real-time PCR normalizing against tick *rps4*. Results are shown as Ave+SD normalized Ct values and were compared between untreated and treated cells by Student's *t*-test with unequal variance. **(C)** The percent (%) of apoptotic cells was determined by flow cytometry in uninfected ISE6 tick cells untreated or treated with Nitisininone. Results were represented as Ave+SD and compared between untreated and treated cells by Student's *t*-test with unequal variance. Asterisks denote statistical significant differences between groups ($P < 0.05$; $N = 4$).

The results of our study showed that *A. phagocytophilum*, an obligate intracellular bacterium, increases the intracellular concentration of PEP which in turn controls bacterial burden. Remarkably, *A. phagocytophilum* increases PEP synthesis by using tyrosine as carbon source. These results suggested a mechanism by which *A. phagocytophilum* infection induces changes at the crosstalk between carbohydrate and amino acid metabolism in the tick vector *I. scapularis* (**Figure 8**). Sequestering host PEP may be critical for this bacterium because its genome lacks the enzymes to actively carry out glycolysis to produce PEP (Dunning et al., 2006), but at the same time high concentration of PEP appears to be deleterious for *A. phagocytophilum*. This was revealed by the fact that high levels of PEP concurred with a lower *A. phagocytophilum* burden, while steady levels of PEP increased *A. phagocytophilum* burden (**Figures 7A,B**). It was previously shown that *A. phagocytophilum* inhibits gluconeogenesis by decreasing the protein levels of cytosolic PEPCK (PEPCK-C), which catalyzes the commitment step of gluconeogenesis (Villar et al., 2015). The inhibition of gluconeogenesis induced by *A. phagocytophilum* infection was confirmed by metabolomics analysis that showed lower levels of glucose in infected ISE6 cells (Villar et al., 2015). In contrast, PEPCK-M was upregulated and overrepresented in infected ISE6 cells (Cabezas-Cruz et al., 2017a). PEPCK-M does not replace the role of PEPCK-C in gluconeogenesis (Méndez-Lucas et al., 2013), but contributes up to 40% of the cytosolic PEP pool (Stark et al., 2009; Yang et al., 2009). Activation of *I. scapularis* PEPCK-C induced apoptosis and reduction of *A. phagocytophilum* levels in ISE6 cells (Villar et al., 2015). Therefore, by downregulation/underrepresentation of PEPCK-C and upregulation/overrepresentation of PEPCK-M, *A. phagocytophilum* infection increases the intracellular levels of PEP without activating gluconeogenesis that is highly detrimental for the pathogen.

That tick cells use tyrosine as a fuel to synthetize PEP during early *A. phagocytophilum* infection (**Figure 7A**) is an appealing hypothesis because it was recently shown that tyrosine accumulation is lethal for blood feeders, such as kissing bugs, mosquitoes, and ticks (Kopáček and Perner, 2016; Sterkel et al., 2016). Thus, the capacity to avoid very high levels of tyrosine is an essential metabolic adaptation to hematophagy (Kopáček and Perner, 2016; Sterkel et al., 2016). Therefore, by activating the tyrosine/OAA/PEPCK-M/PEP node, *A. phagocytophilum* infection may decrease the tyrosine pool which in turn protects the tick host against tyrosine-induced toxicity. In agreement with this hypothesis, here we found that *A. phagocytophilum* infection increases the protein levels of enzymes involved in tyrosine degradation (i.e., HPPD and GSTZ1 in salivary glands, GSTZ1 and FAH in midguts, and FAH in ISE6 cells; **Figure 4**) and decreases the enzymes involved in tyrosine synthesis (i.e., PAH in

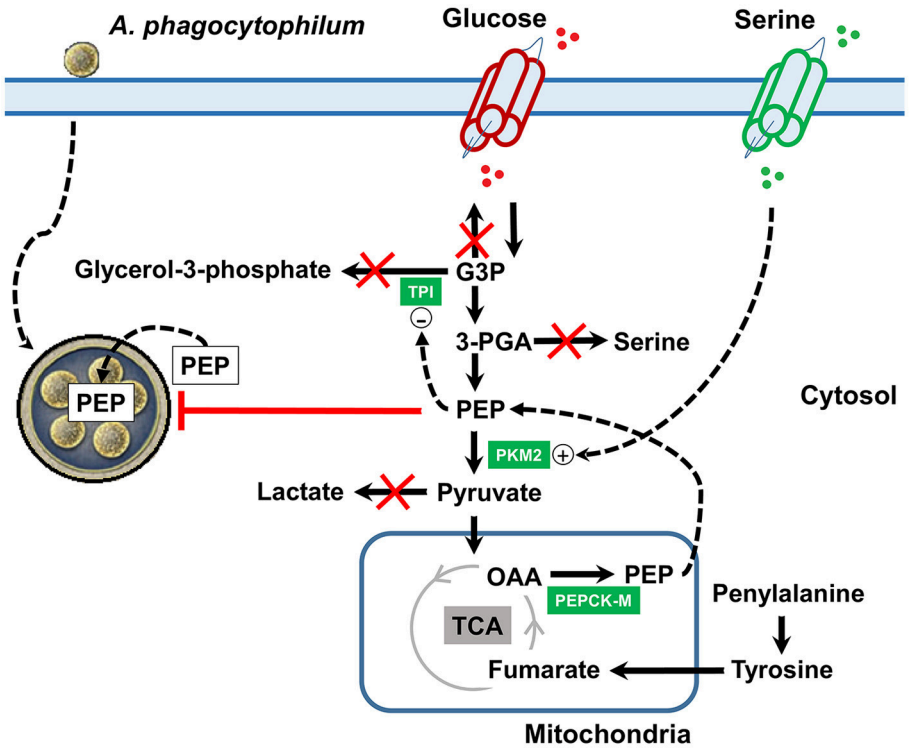

FIGURE 8 | Mechanistic model of metabolic modifications induced by *A. phagocytophilum* infection in tick ISE6 cells. *Anaplasma phagocytophilum* infection increases the protein levels of PKM2 and PEPCK-M in ISE6 cells. TPI is overrepresented in nymphs, midguts and salivary glands, but was not found in ISE6 cells. PEPCK-M transforms mitochondrial OAA into PEP. When the tyrosine degradation pathway (i.e., the enzyme HPPD) was inhibited using Nitisinone, the levels of PEP did not increase in response to *A. phagocytophilum* infection. This strongly suggested that the *A. phagocytophilum*-induced increase in PEP levels is due to activation of the tyrosine/OAA/PEPCK-M/PEP node. Simultaneously, *A. phagocytophilum* infection inhibits glyceroneogenesis (leading to glycerol-3-phosphate), *de novo* serine synthesis pathway (leading to serine) and gluconeogenesis (leading to glucose). PEP is an allosteric inhibitor of TPI and the increase in the levels of PEP may contribute to the inhibition of glyceroneogenesis. Despite that *de novo* serine synthesis pathway is inhibited the levels of serine increased which may be explained by the upregulation of serine transporters located at the cell membrane. Serine is an allosteric activator of PKM2 and the increase in the levels of serine may contribute to the activation of glycolysis. During *A. phagocytophilum* infection, the levels of lactate decrease suggesting that the end product of glycolysis (i.e., pyruvate) is not transformed into lactate, but enters the mitochondria. By this metabolic rearrangement, *A. phagocytophilum* infection increases the cytoplasmic PEP pool which may facilitate the transport of this metabolite inside the parasitophorous vacuole, but at the same time controls the bacterial burden. Name of enzymes were abbreviated as in **Table 1**.

salivary glands, and PAH and DHPR in midguts; **Figure 3**) in a tissue-specific manner.

CONCLUSIONS

In summary, *A. phagocytophilum* infection increased the concentration of PEP by shunting tyrosine into the TCA cycle, which should increase the concentration of OAA that will be transformed into PEP by PEPCK-M (**Figure 8**). Further studies with radio labeled tyrosine should assess whether tyrosine carbons are truly recycled to PEP during *A. phagocytophilum* infection. Sequestering host PEP may be critical for this bacterium because it cannot actively carry out glycolysis to produce PEP. However, as shown here, high concentration of PEP appears to be deleterious for *A. phagocytophilum*. These results provide a more comprehensive view of the major amino acid metabolic pathways involved in the response to pathogen infection in ticks, and provides the basis for further studies to develop novel strategies for the control of human granulocytic anaplasmosis by targeting some of the enzymes involved in the tyrosine/OAA/PEPCK-M/PEP node.

AUTHOR CONTRIBUTIONS

ACC and JF conceived the study. PJE and PA performed the experiments. ACC, PA, DAO, PJE, and JF performed data analyses. ACC and JF wrote the paper, and other co-authors made additional suggestions and approved the manuscript.

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frontiers in Cellular and Infection Microbiology

METHODS
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A Dual Luciferase Reporter System for *B. burgdorferi* Measures Transcriptional Activity during Tick-Pathogen Interactions

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Knowledge of the transcriptional responses of vector-borne pathogens at the vector-pathogen interface is critical for understanding disease transmission. *Borrelia* (*Borreliella*) *burgdorferi*, the causative agent of Lyme disease in the United States, is transmitted by the bite of infected *Ixodes sp.* ticks. It is known that *B. burgdorferi* has altered patterns of gene expression during tick acquisition, persistence and transmission. Recently, we and others have discovered *in vitro* expression of RNAs found internal, overlapping, and antisense to annotated open reading frames in the *B. burgdorferi* genome. However, there is a lack of molecular genetic tools for *B. burgdorferi* for quantitative, strand-specific, comparative analysis of these transcripts in distinct environments such as the arthropod vector. To address this need, we have developed a dual luciferase reporter system to quantify *B. burgdorferi* promoter activities in a strand-specific manner. We demonstrate that constitutive expression of a *B. burgdorferi* codon-optimized *Renilla reniformis* luciferase gene ($rluc_{Bb}$) allows normalization of the activity of a promoter of interest when fused to the *B. burgdorferi* codon-optimized *Photinus pyralis* luciferase gene ($fluc_{Bb}$) on the same plasmid. Using the well characterized, differentially regulated, promoters for flagellin (*flaBp*), outer surface protein A (*ospAp*) and outer surface protein C (*ospCp*), we document the efficacy of the dual luciferase system for quantitation of promoter activities during *in vitro* growth and in infected ticks. Cumulatively, the dual luciferase method outlined herein is the first dual reporter system for *B. burgdorferi*, providing a novel and highly versatile approach for strand-specific molecular genetic analyses.

Keywords: *Borrelia* (*Borreliella*) *burgdorferi*, Lyme disease, tick-pathogen interactions, bioluminescence reporter, *Photinus reniformis* luciferase, *Photinus pyralis* luciferase

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INTRODUCTION

Vector-borne illnesses account for 17% of worldwide infectious diseases, amounting to over one billion cases yearly (World Health Organization, 2016). Ticks are notorious for delivering a diversity of infectious agents to their hosts during the blood meal. Of these pathogens the *Borrelia burgdorferi* sensu lato complex or *Borreliella* genus (Adeolu and Gupta, 2014), the spirochete group

that causes Lyme disease, contributes the highest incidence of arthropod-transmitted bacterial infection worldwide (Schotthoefer and Frost, 2015). Particular to the United States, *Borrelia* (*Borreliella*) *burgdorferi* interaction with and colonization of *Ixodes* species is highly specific (de Silva et al., 2009), with no other natural arthropod vector identified to date.

Newly hatched larval ticks are not colonized with *B. burgdorferi*, as there is currently no evidence to support transovarial transmission of the pathogen (Rollend et al., 2013). Rather, larvae can become infected by feeding on one of the numerous small vertebrates that serve as reservoirs for *B. burgdorferi* in nature, such as the white-footed mouse *Peromyscus leucopus*. Larval ticks then undergo an approximate month-long morphogenesis process and molt into nymphs. All the while, *B. burgdorferi* reside in the tick midgut. Like the larvae, the infected nymphs take a single blood meal from a vertebrate followed by morphogenesis to adults. During nymph feeding, *B. burgdorferi* migrate from the midgut to the tick salivary glands and are transmitted to the vertebrate host, maintaining the spirochete in its enzootic cycle (Radolf et al., 2012). Therefore, it has been proposed that *B. burgdorferi* undergoes three major tick-related events that require complex genetic regulation: acquisition, persistence, and transmission (Iyer et al., 2015; Caimano et al., 2016).

Survival of *B. burgdorferi* in the tick requires that the spirochete overcome a number of environmental stress conditions, such as starvation and assault from tick immune factors (Radolf et al., 2012; Caimano et al., 2016). Recently, open reading frame-based microarray analysis has provided insight into the gene expression changes that occur in the *B. burgdorferi* transcriptome in fed larvae, fed nymphs, and under mammalian host-like conditions in dialysis membrane chambers (Iyer et al., 2015). The unusual structure of *B. burgdorferi*'s genome and its scarcity of characterized transcription factors, further contribute to interest in understanding the mechanisms of stress adaptation and gene regulation that the spirochete employs during its interaction with the tick vector. The *B. burgdorferi* segmented genome, in characterized type strain B31, is composed of an approximate 900 kb linear chromosome and 21 plasmids of size ranges 5–56 kb that include many annotated open reading frames (ORFs) of unknown function (Fraser et al., 1997; Casjens et al., 2012). A recent global examination and 5′ end mapping of the *B. burgdorferi* transcriptome by our laboratory has revealed that the spirochete is rich with "overlapping transcripts" where 63% of total RNA species are transcribed internal and 13% antisense to annotated open reading frames (Adams et al., 2017). Other recent RNA-seq based applications have also described the presence of these transcripts in *B. burgdorferi* (Arnold et al., 2016; Popitsch et al., 2017). These findings are supported by similar analyses in other bacteria, which have revealed complex transcriptomes that include a variety of antisense, intragenic, intergenic, and orphan transcripts, which in some cases represent the majority of transcript types as opposed to mRNAs for annotated open reading frames (Sharma et al., 2010; Kroger et al., 2012; Thomason et al., 2015). These discoveries drive the need for the development of new molecular genetic tools for investigating the expression patterns and functional roles of novel RNA transcripts in a strand-specific manner.

For over three decades, researchers have been isolating, expressing, and adapting bioluminescence genes for biomedical applications (de Wet et al., 1987; Lorenz et al., 1991). These techniques are based on the enzymatic (i.e., luciferase) oxidation of a substrate (i.e., luciferin) to generate light. Transcriptional reporters using bioluminescence read-outs have proven to be robust and sensitive molecular tools for investigating transcript expression (Andreu et al., 2011). Infectious disease-based research has resulted in the development of multiple luciferase systems for a variety of pathogens, and demonstrated that relative luciferase units of constitutively expressed bioluminescence reporters correlate to bacterial numbers (Andreu et al., 2011). Advanced and high-throughput adaptations for transcriptional reporters utilize multiple luciferase enzymes with unique substrates, which are compatible within the same experimental setup. In this manner, one luciferase serves as an experimental readout of promoter activity and the other as the normalization control for cell number (McNabb et al., 2005; Wright et al., 2005). A previously engineered *B. burgdorferi* codon-optimized *Photinus pyralis* (firefly) luciferase gene (Blevins et al., 2007), when fused to a constitutive promoter, has been successful for *in vivo* live imaging of *B. burgdorferi* dissemination during mouse infection (Hyde et al., 2011; Wager et al., 2015). Furthermore, this luciferase reporter has been used to characterize the promoters for a variety of annotated ORFs and novel RNAs during *in vitro* cultivation, *in vivo* mouse infection, and in infected mouse tissues *ex vivo* (Skare et al., 2016; Adams et al., 2017). However, this reporter plasmid is limited in that it does not contain a constitutive control reporter to allow normalization and quantitation of the data. In order to expand the utility of this approach, we engineered a dual luciferase plasmid that carries both a constitutively expressed *B. burgdorferi* codon-optimized *Renilla reniformis* (sea pansy) luciferase gene and the *B. burgdorferi* codon-optimized *Photinus pyralis* (firefly) luciferase gene driven by a promoter of interest. Luciferin, the substrate of *Photinus pyralis* luciferase, emits yellow-green photons (550–570 nm) of light (Marques and Esteves da Silva, 2009), whereas coelenterazine, the substrate of *Renilla reniformis* luciferase, produces light in the blue spectrum (470 nm) (Woo et al., 2008). Functioning on the premise that each luciferase enzyme requires unique substrates for bioluminescence readout, this approach provides a method for quantitative measurement of strand-specific transcription, in an environment of interest. It has been previously demonstrated that coelenterazine-based luciferase reporters are ineffective for *in vivo* live imaging detection of bacterial pathogens during murine infection (Andreu et al., 2010), despite successful *in vivo* applications for mammalian tumor systems (Bhaumik and Gambhir, 2002). Herein, our studies demonstrate the efficacy of the *B. burgdorferi* dual luciferase system for genetic studies during *in vitro* cultivation of spirochetes and analysis of transcriptional activity that occurs at the tick-pathogen interface, which is critical for understanding the interactions of *B. burgdorferi* with the tick vector for the development of novel therapeutic strategies for Lyme disease.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

B. burgdorferi clones used in this study were derived from strain B31. For genetic manipulations infectious low-passage clone A3-68Δ*bbe02* was utilized, which lacks cp9, lp56, and gene *bbe02* on lp25 (Rego et al., 2011), and herein referred to as wild type. Spirochetes were cultivated in liquid Barbour-Stoenner-Kelly (BSK) II medium supplemented with gelatin and 6% rabbit serum (Barbour, 1984) and grown at 35°C with 2.5% CO_2. Luciferase plasmids were engineered in DH5α *E. coli*, grown in LB broth or on LB agar plates containing 300 μg/ml spectinomycin when appropriate, and transformed into *B. burgdorferi* as previously described (Samuels, 1995). Transformants were selected by plating in solid BSKII medium as previously described (Rosa and Hogan, 1992), in the presence of 50 μg/ml streptomycin and/or 200 μg/ml kanamycin, when applicable. All transformants were verified by PCR to contain the plasmid content of the parent clone (Elias et al., 2002; Jewett et al., 2007).

Construction of the Dual Luciferase Plasmids

The *Renilla reniformis* luciferase gene (Lorenz et al., 1991) was codon-optimized for *B. burgdorferi* (*rluc$_{Bb}$*) with the OptimumGene™ algorithm, synthesized, and cloned into the *E. coli* vector pUC18 (Genscript) (Genebank accession number MF043582). All primer sequences are listed in **Table 1**. The *rluc$_{Bb}$* gene was PCR amplified from pUC18-*rluc$_{Bb}$* plasmid DNA using Phusion High-fidelity DNA polymerase (NEB) and primer pair 1732 and 1733. This also resulted in the addition of 27 bp of DNA from the 3′ of the *flaB* promoter to the 5′ of *rluc$_{Bb}$*. Concurrently, a DNA fragment containing the *flaB* promoter sequence with a 24 bp overhang from the 5′ of the *rluc$_{Bb}$* gene was Phusion-PCR amplified using B31 A3 genomic DNA and primer pair 1730 and 1731. The PCR fragments were ligated together by combining Gibson Assembly® Master Mix (NEB) and 0.16 pmol of each PCR fragment and incubating the reaction at 50°C for 1 h. One microliter of assembled product (*flaBp-rluc$_{Bb}$*) was Phusion-PCR amplified using primers 1730 and 1733 and the DNA fragment gel extracted using the QIAquick Gel Extraction kit (Qiagen), and cloned into pCR-Blunt using the Zero Blunt PCR cloning kit (Invitrogen) according to the manufacturer's instructions. The sequence of the *flaBp-rluc$_{Bb}$* cassette was verified by Sanger sequencing.

TABLE 1 | Oligonucleotide primers used in this study.

Number	Name	Sequence (5′-3′)
1730	*flaBp* 5′	TGTCTGTCGCCTCTTGTG
1731	*flaBp* 3′, 24 bp overlay *rlucBb* 5′	AGGATCATAAACTTTACTTGTCATGATTGATAATCAT ATATCATTCCTCCA
1732	*rlucBb* 5′, 27 bp overlay *flaBp* 3′	TGGAGGAATGATATATGATTATCAATCATGACAAGTA AAGTTTATGATCCT
1733	*rlucBb* 3′	TTATTGTTCATTTTTCAATACTCGT
1850	*rlucBb* 3′ KpnI	CTAAGGTACCTTATTGTTCATTTTTCAATACTCGTTC
1910	*flaBp* 5′ BamHI	TGGCCGGATCCTGTCTGTCGCCTCTTGTGGC

The *flaBp-rluc$_{Bb}$* cassette was Phusion-PCR amplified from the pCR-Blunt *flaBp-rluc$_{Bb}$* plasmid using primer pair: 1850 and 1910, introducing BamHI and KpnI restriction sites. *B. burgdorferi* shuttle vectors containing the promoterless *B. burgdorferi* optimized *Photinus pyralis* luciferase gene (*fluc$_{Bb}$*), *flaBp-fluc$_{Bb}$*, *ospAp-fluc$_{Bb}$*, or *ospCp-fluc$_{Bb}$* (Blevins et al., 2007; Adams et al., 2017) were digested with BamHI and KpnI high fidelity enzymes (NEB), gel extracted using the QIAquick Gel Extraction kit (Qiagen), and ligated to the BamHI/KpnI-digested *flaBp-rluc$_{Bb}$* cassette using T4 DNA ligase (NEB), generating plasmids pCFA701, pCFA801, pCFA802, and pCFA803. All plasmid constructs were confirmed by PCR, restriction digest, and Sanger sequencing.

In vitro Dual Luciferase Assay

B. burgdorferi clones were grown to logarithmic phase (3–7 × 10^7 spirochetes/ml) or stationary phase (1–1.2 × 10^8 spirochetes/ml) in 15 ml of BSKII medium and pelleted at 3,210 × g for 10 min. Cells were washed with phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and resuspended in 300 μl of PBS. Eighty microliters of each sample was used to measure the optical density at 600 nm (OD_{600}) using a BioTek Synergy 4. This resulted in an average OD_{600} value of ∼0.25 for logarithmic phase spirochetes and ∼0.36 for stationary phase spirochetes. One hundred microliters of each sample was loaded into a black, solid bottom 96-well plate (Corning) and combined with 700 μM D-luciferin (Regis) in PBS or 3.5 mM water soluble native coelenterazine (NanoLight Technology) in PBS. For samples containing coelenterazine, one well was left empty, in all directions around each sample, to decrease signal overlap between samples. For determining *B burgdorferi Photinus* luciferase ($Fluc_{Bb}$) and *Renilla* luciferase ($Rluc_{Bb}$) sensitivity, spirochetes containing pCFA801 were grown to logarithmic phase in 15 ml of BSKII medium, cell density determined using a Petroff Hauser counting chamber, washed with PBS, and resuspended in PBS to a density of 2 × 10^6 cells/μl. Samples were serial diluted 10-fold and 100 μl of each dilution was loaded into a black, solid bottom 96-well plate (Corning) and combined with 700 μM D-luciferin or 3.5 mM coelenterazine. The relative luciferase units (RLUs) for $Fluc_{Bb}$ and $Rluc_{Bb}$ were determined by measuring photon emission in each well for 1 s, 10 times using the EnVision 2104 Multilabel Reader (PerkinElmer), following the addition of luciferin or coelenterazine substrate, respectively. Background relative $Fluc_{Bb}$ or $Rluc_{Bb}$ units, the average RLUs of the PBS control for either substrate, was subtracted from all experimental measurements, as appropriate. Background-subtracted relative $Fluc_{Bb}$ units were then normalized to the OD_{600} value or 10^8 background-subtracted relative $Rluc_{Bb}$ units of the same sample, when applicable (e.g., 4 × 10^4 $Fluc_{Bb}$ units/0.1 OD_{600} value = 4 × 10^5 relative $Fluc_{Bb}$ units/OD_{600}; 4 × 10^4 $Fluc_{Bb}$ units/0.06 10^8 $Rluc_{Bb}$ units = 6.4 × 10^5 relative $Fluc_{Bb}$ units/10^8 $Rluc_{Bb}$ units). The limit of detection (LoD) and quantification (LoQ) for $Fluc_{Bb}$ and $Rluc_{Bb}$ were established as the average RLUs for PBS alone plus 3 or 10 standard deviations, respectively. All experiments were conducted in biological triplicate.

Ethics Statement

The University of Central Florida is accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care. Protocols for all animal experiments were prepared according to the guidelines of the National Institutes of Health and were reviewed and approved by the University of Central Florida Institutional Animal Care and Use Committee.

B. burgdorferi Infection of Ticks

One week prior to inoculation, and throughout the duration of the study, mice were treated with 5 mg/ml streptomycin and 1 mg/ml Equal® sweetener in their water to maintain selection for the luciferase plasmid in the *B. burgdorferi* clones. Using *B. burgdorferi* carrying pJSB175, pCFA701, pCFA801, pCFA802, or pCFA803, groups of two 6–8 week old female C3H/HeN mice (Envigo) per clone were inoculated with 1×10^5 spirochetes per mouse 80% intraperitoneally and 20% subcutaneously. The inoculum doses were verified by colony forming unit (CFU) counts in solid BSKII medium. All inoculum were PCR verified to contain the endogenous *B. burgdorferi* plasmids of the parent clone as previously described (Elias et al., 2002; Jewett et al., 2007). Three weeks post inoculation mouse infection was confirmed by positive seroreactivity against *B. burgdorferi* protein lysate as previously described (Schwan et al., 1989; Jewett et al., 2007). Groups of approximately 200 naïve *Ixodes scapularis* larvae each (Centers for Disease Control, BEI resources) were fed to repletion on the *B. burgdorferi* infected mice (Jewett et al., 2009). Mice were further confirmed for infection by reisolation of spirochetes from bladder and joint tissues, as described (Showman et al., 2016). Larvae were analyzed for infection (Grimm et al., 2005; Jewett et al., 2009). Briefly, ticks were individually surface sterilized by sequential washes in 100 µl of 3% H_2O_2, 70% ethanol, and sterile H_2O. Subsets of larvae were analyzed for infection by reisolation of spirochetes in BSKII medium containing RPA cocktail (60 µM rifampicin, 110 µM phosphomycin, and 2.7 µM amphotericin B), immediately post feeding to repletion. Approximately 2 weeks following feeding, additional subsets of larvae were crushed and plated in solid BSKII containing RPA cocktail and 50 µg/ml streptomycin to determine CFU counts/larva. The remaining larvae were maintained and allowed to molt into nymphs. Two groups of 10–18 infected nymphs per *B. burgdorferi* clone were fed to repletion on naïve 6–8 week old female C3H/HeN mice (Envigo). These mice were treated with 5 mg/ml streptomycin and 1 mg/ml Equal® sweetener in their water 1 week prior to the feeding, to help sustain the luciferase plasmids in *B. burgdorferi* within the feeding nymphs. Throughout the duration of the study, ticks were stored in glass desiccation jars containing saturated potassium sulfate for to maintain appropriate humidity.

In vivo Tick Dual Luciferase Assay

Approximately 2 weeks post feeding to repletion triplicate groups of 24 fed larvae or 8 fed nymphs per *B. burgdorferi* clone were crushed with a sterile pestle in 250 µl of PBS to generate tick extracts. For tick extracts, which were also plated for CFU counts, the ticks were first surface sterilized as described above, with a final wash in sterile PBS instead of H_2O. Tick debris was allowed to settle and 100 µl of sample was removed and incubated with 700 µM D-luciferin (Regis) in PBS or 3.5 mM water soluble native coelenterazine (NanoLight Technology) in PBS. RLUs were measured as described for *in vitro* grown spirochetes. The limit of quantification (LoQ) for $Fluc_{Bb}$ was established as the average relative $Fluc_{Bb}$ units for PBS alone plus 10 standard deviations. The LoQ for $Rluc_{Bb}$ was established as the average relative $Rluc_{Bb}$ units for infected tick extracts with spirochetes containing pJSB175, which lacks the *rluc*$_{Bb}$ gene, plus 10 standard deviations. Samples with relative $Fluc_{Bb}$ units below the LoQ were given a value of zero; whereas, samples with relative $Rluc_{Bb}$ units below the LoQ were removed from the analysis. Relative $Fluc_{Bb}$ units were normalized to 10^8 relative $Rluc_{Bb}$ units for each sample. One microliter of each fed nymph extract was also plated for CFUs in solid BSKII containing RPA cocktail and 50 µg/ml streptomycin.

Statistical Analysis

GraphPad Prism version 7.02 was used for all statistical analyses. One-way ANOVA was used for analysis of all luciferase assays. For statistical comparison of the relative $Fluc_{Bb}$ units normalized to OD_{600} or 10^8 relative $Rluc_{Bb}$ units, which had an extremely wide distribution ($\sim 10^1$–10^7), all values were first square root transformed prior to statistical analysis. Following ANOVA, all samples were compared to the *B. burgdorferi* clones carrying the promoterless *fluc*$_{Bb}$ control plasmid pJSB161 or pCFA701 using Dunnett's multiple comparison test. To compare two groups (i.e., the same clone in logarithmic versus stationary phase) following ANOVA, Bonferroni's multiple comparison test was applied to determine significance. For association analysis, Pearson correlation coefficient (r) was determined. $p \leq 0.05$ was considered statistically significant for all statistical tests.

RESULTS

Generation of the *B. burgdorferi* Dual Luciferase Plasmid

The *B. burgdorferi* shuttle vector pJSB161 (Blevins et al., 2007) contains a promoterless *B. burgdorferi* codon-optimized *Photinus pyralis* luciferase gene (*fluc*$_{Bb}$) with a BlgII restriction site upstream of a ribosome binding site (RBS) for *fluc*$_{Bb}$ (**Figure 1A**). This reporter plasmid allows a cloned promoter of interest to be analyzed for activity in a strand-specific manner via a bioluminescence detection method (Blevins et al., 2007; Skare et al., 2016; Adams et al., 2017). However, this approach does not allow for quantitative comparative analysis of promoter activity in different environments or between multiple promoters in the same environment due to the lack of an endogenous means to control for spirochete number across samples and conditions. Therefore to improve upon this technique for quantitative applications, we engineered a dual luciferase reporter system to constitutively express *Renilla reniformis* luciferase (*rluc*) (Lorenz et al., 1991), while maintaining *fluc*$_{Bb}$ for quantifying the activity of a promoter of interest. Codon usage in *B. burgdorferi* is biased (Fraser et al., 1997; Nakamura et al., 2000), as the A/T nucleotide frequency is at 71.8% across the genome (Fraser

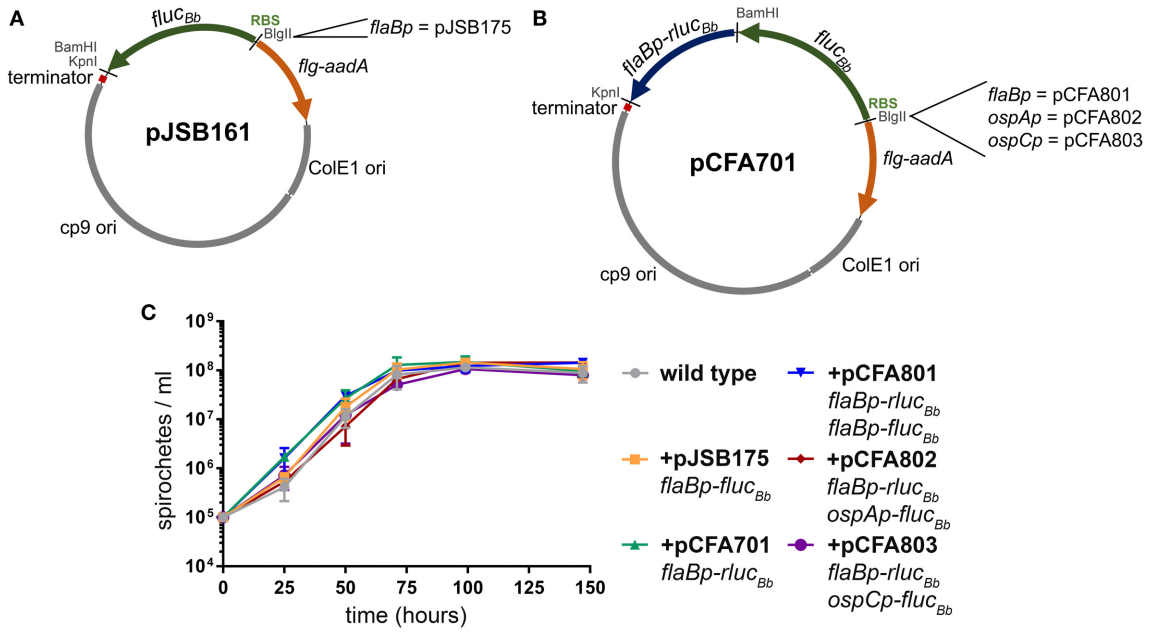

FIGURE 1 | *B. burgdorferi* luciferase plasmids. All of the *B. burgdorferi* luciferase shuttle vectors were derived from pJSB161, which contains a Rho-independent transcription terminator sequence (terminator); ORFs 1, 2, and 3 of the *B. burgdorferi* cp9 replication machinery (cp9 ori); *E. coli* origin of replication (ColE1 ori); and the spectinomycin/streptomycin resistance cassette (*flg-aadA*) (Blevins et al., 2007). **(A)** The *B. burgdorferi* shuttle vector pJSB161 features a promoterless, *B. burgdorferi* codon optimized *Photinus pyralis* luciferase ($fluc_{Bb}$), an upstream ribosome binding site (RBS) and a unique BlgII restriction site (Blevins et al., 2007). The plasmid pJSB175 was generated by addition of the *flaBp* promoter upstream of $fluc_{Bb}$ in pJSB161 (Blevins et al., 2007). **(B)** The *B. burgdorferi* codon optimized *Renilla reniformis* luciferase ($rluc_{Bb}$) gene under the control of the *flaB* promoter (*flaBp-*$rluc_{Bb}$) was added to pJSB161, generating the *B. burgdorferi* dual luciferase shuttle vector, pCFA701. Plasmids, pCFA801, pCFA802, and pCFA803, harbor the *flaB, ospA,* and *ospC* promoters, respectively, upstream of $fluc_{Bb}$. **(C)** The density of *B. burgdorferi* clone A3-68Δ*bbe02* (wild type) alone or harboring various *B. burgdorferi* luciferase plasmids was assessed over a period of 144 h using a Petroff Hauser counting chamber and dark-field microscopy. The data are presented as the mean spirochete density (spirochetes/ml) ± standard deviation over time (hours).

et al., 1997; Adams et al., 2017). Codon optimization has been shown to improve production and activity of non-*B. burgdorferi* proteins expressed in *B. burgdorferi* (Blevins et al., 2007; Hayes et al., 2010). Therefore to prevent rare codons interfering with the *Renilla* luciferase reporter, the OptimumGene™ algorithm (GenScript) was used to refine the codon adaption index (CAI) of *rluc* (Lorenz et al., 1991) for *B. burgdorferi* from 0.64 to 0.85 (where a CAI value of 1.0 indicates the highest proportion of the most abundant codons) and synthesized (GenScript). This codon-optimized *rluc* gene ($rluc_{Bb}$) (Genebank accession number MF043582) was cloned into pJSB161 (Blevins et al., 2007), for use in the dual luciferase reporter system under control of the constitutive promoter *flaBp* and corresponding ribosome binding site, generating pCFA701 (**Figure 1B**).

B. burgdorferi survival in the tick vector is essential for maintenance of the pathogen in its enzootic cycle. The spirochete is known to change its transcriptional profile at different stages of tick colonization including: acquisition, persistence during the molt, and transmission to the mammalian host (Iyer et al., 2015; Caimano et al., 2016). Because of our interest in applying the dual luciferase reporter system to quantitative analysis of *B. burgdorferi* promoter activities in the tick, we selected three well characterized promoters with distinct patterns of expression in the tick environment for proof of principle analysis. The flagellar protein promoter, *flaBp*, is constitutively active (Ge et al., 1997). The promoter for outer surface protein A (*ospAp*) is active during *in vitro* culture and in the tick during acquisition and persistence. In contrast, the promoter for outer surface protein C (*ospCp*) is active in the feeding tick during transmission and the mammalian host during the early stages of infection (Schwan et al., 1995; Schwan and Piesman, 2000; Schwan, 2003; Srivastava and de Silva, 2008). The *flaBp-*$rluc_{Bb}$ cassette was cloned into three previously constructed plasmids, each containing one of these promoters driving the expression of $fluc_{Bb}$ (Blevins et al., 2007; Adams et al., 2017), generating plasmids pCFA801, pCFA802, and pCFA803, respectively (**Figure 1B**). Spirochetes carrying pCFA701, pCFA801, pCFA802, or pCFA803 had no observed *in vitro* growth defect in BSKII medium compared to the wild type or *B. burgdorferi* carrying *flaBp-*$fluc_{Bb}$ alone (pJSB175) (Blevins et al., 2007, **Figure 1C**).

$Rluc_{Bb}$ Selectivity and Limit of Quantification

Photinus pyralis luciferase (Fluc) and *Renilla reniformis* luciferase (Rluc) are compatible for a dual reporter due to the specificity of each enzyme for distinct substrates (Bhaumik and Gambhir, 2002; McNabb et al., 2005). Therefore, we verified the selectivity of the $Fluc_{Bb}$ and $Rluc_{Bb}$ enzymes to recognize luciferin and coelenterazine, respectively. Based on our previous work using the *flaBp-*$fluc_{Bb}$ reporter (Adams et al., 2017), we

performed these analyses with approximately 3×10^8 spirochetes harvested during log phase growth. As expected, the negative control, spirochetes not expressing *fluc*$_{Bb}$ or *rluc*$_{Bb}$ (+pJSB161), demonstrated no significant relative luciferase units for either substrate compared to wild type. Spirochetes expressing *fluc*$_{Bb}$ alone (+pJSB175) demonstrated robust activity when incubated with luciferin, but no significant activity above the background of *B. burgdorferi* carrying pJSB161 when exposed to coelenterazine (**Figure 2A**). Conversely, spirochetes expressing *rluc*$_{Bb}$ alone (+pCFA701) demonstrated strong activity when incubated with coelenterazine, but no significant activity above the negative control background when exposed to luciferin (**Figure 2A**). Spirochetes which express both *fluc*$_{Bb}$ and *rluc*$_{Bb}$ (+pCFA801) demonstrated significant relative luciferase units compared to spirochetes containing pJSB161 for both luciferin and coelenterazine. The background relative luciferase units for wild type and negative control spirochetes exposed to coelenterazine were found to be approximately 10-fold higher than those of the same spirochetes incubated with luciferin. Collectively, these data validated the ability of the codon-optimized Rluc$_{Bb}$ enzyme to effectively oxidize coelenterazine and confirmed the specificity of the Fluc$_{Bb}$ and Rluc$_{Bb}$ enzymes for their respective substrates.

The utility of the dual luciferase reporter system not only depends on the substrate specificity of Fluc$_{Bb}$ and Rluc$_{Bb}$, but also the sensitivity of detecting and quantifying spirochetes expressing *rluc*$_{Bb}$. The limit of detection (LoD) and limit of quantification (LoQ) were established as the number of spirochetes required to achieve relative Rluc$_{Bb}$ units greater than that of phosphate-buffered saline (PBS) alone plus three standard deviations and 10 standard deviations, respectively. Analysis of triplicate samples of 10-fold serially diluted spirochetes, 2×10^8 to 2×10^0, harvested during log phase growth and incubated with coelenterazine, demonstrated 2×10^3 spirochetes to be the lowest detectable number of *flaBp-rluc*$_{Bb}$ expressing spirochetes in the assay (**Figure 2B**). However, the LoQ fell between 2×10^3 and 2×10^4 spirochetes. Saturation of the bioluminescence signal was never reached under the conditions examined, with a linear increase in relative Rluc$_{Bb}$ units from 2×10^3 to 2×10^8 spirochetes ($y = 0.0404x$; $R^2 = 0.9997$). Extrapolating from this linear equation, the LoQ was calculated to be 4.8×10^3 spirochetes. These data indicate that a minimum of $\sim 1 \times 10^4$ *flaBp-rluc*$_{Bb}$ expressing spirochetes are needed to achieve quantifiable relative Rluc$_{Bb}$ units in the assay. Similar to what has been reported previously (Hyde et al., 2011), 2×10^3 spirochetes was also found to be the lowest detectable number of *flaBp-fluc*$_{Bb}$ expressing spirochetes (data not shown).

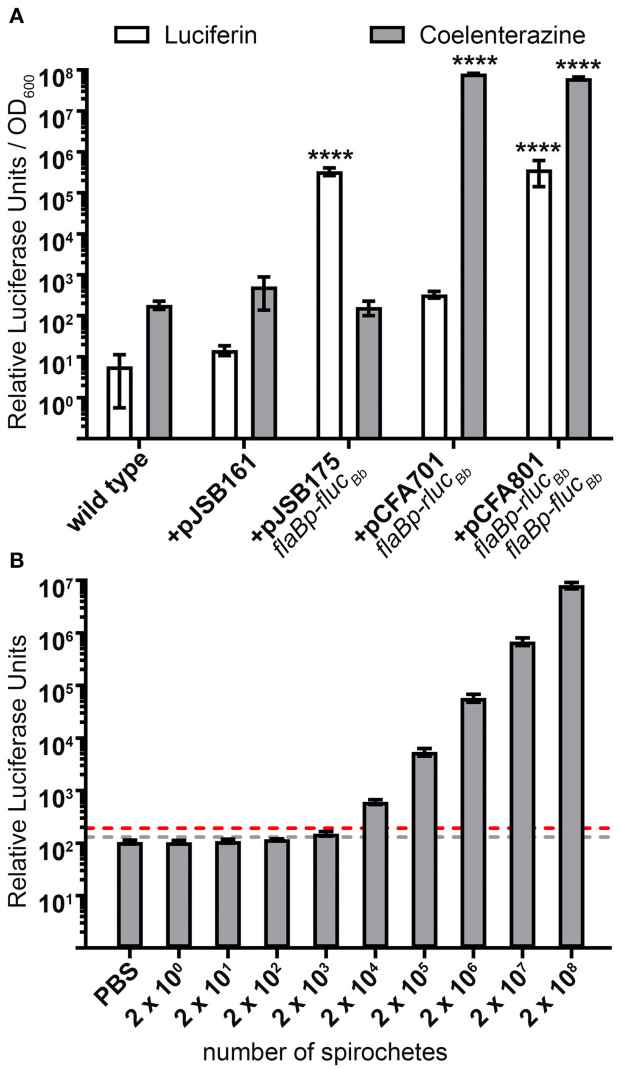

FIGURE 2 | Selectivity and sensitivity of the dual luciferase assay in *B. burgdorferi*. **(A)** *B. burgdorferi* clones were grown to mid-logarithmic phase, and the *in vitro* luciferase assay performed with 700 μM D-luciferin or 3.5 mM coelenterazine. Relative luciferase units were normalized to optical density at 600 nm (OD$_{600}$) and presented as the mean relative luciferase units/OD$_{600}$ ± standard deviation for biological triplicate samples. The data were square root transformed and analyzed with a one-way ANOVA followed by Dunnett's multiple comparison test compared to *B. burgdorferi* containing the promoterless *fluc*$_{Bb}$ (+pJSB161) for each substrate. Unless indicated, means were not significantly different from the control. Significant differences are indicated with asterisks (****$p \leq 0.0001$). **(B)** Mid-logarithmic phase grown *B. burgdorferi* expressing both *flaBp-rluc*$_{Bb}$ and *flaBp-fluc*$_{Bb}$ (+pCFA801) were serial diluted from 2×10^8 to 2×10^0 spirochetes, and incubated with 3.5 mM coelenterazine. The limit of detection (LoD) was established as the mean relative luciferase units for PBS alone plus 3 standard deviations (gray dotted line). The limit of quantitation (LoQ) was established as the mean relative luciferase units for PBS alone plus 10 standard deviations (red dotted line). Data are presented as the mean relative luciferase units ± standard deviation for biological triplicate samples.

The *flaBp-rluc*$_{Bb}$ Reporter is a Robust Constitutive Control for Measuring *B. burgdorferi* Promoter Activities during *In vitro* Growth

Previously, we reported quantification of *in vitro* active *B. burgdorferi* promoters by normalizing relative luciferase units (RLUs) from *fluc*$_{Bb}$ expressing cells to the optical density of the bacterial sample measured at 600 nm (OD$_{600}$) (Adams et al., 2017). In this manner, the OD$_{600}$ measurement reflects the number of spirochetes in the sample allowing normalization of RLUs across samples and assay conditions. To establish the *flaBp-rluc*$_{Bb}$ reporter as an effective alternative for OD$_{600}$ readings in

our assay, first, relative $Rluc_{Bb}$ units were measured for all *rluc*$_{Bb}$-expressing *B. burgdorferi* clones and normalized to the number of spirochetes in the assay by OD_{600} (**Figure 3A**). All clones demonstrated consistent, robust relative $Rluc_{Bb}$ units, ranging from 5×10^7 to 1.68×10^8. There was no significant difference among clones except for *B. burgdorferi* carrying pCFA802, which demonstrated higher relative $Rluc_{Bb}$units/OD_{600} compared to all other clones as well as a difference between logarithmic and stationary phase growth. The same *rluc*$_{Bb}$-expressing clones, were also incubated with luciferin and relative $Fluc_{Bb}$ units were determined by normalizing to OD_{600} (**Figure 3B**). All *fluc*$_{Bb}$ promoter fusions displayed the expected relative $Fluc_{Bb}$ units/OD_{600}, given the known expression patterns of their corresponding mRNA during logarithmic and stationary phase growth (Arnold et al., 2016). Both the *flaB* (+pCFA801) and *ospA* (+pCFA802) promoters demonstrated significant activity above the promoterless *fluc*$_{Bb}$ control (+pCFA701) for both logarithmic and stationary phase growth. The activity of the *ospC* promoter (+pCFA803) during logarithmic phase growth was no different than the promoterless *fluc*$_{Bb}$ control (+pCFA701). Whereas, the *ospC* promoter activity underwent significant induction from logarithmic to stationary phase growth (**Figure 3B**). Normalization of the relative $Fluc_{Bb}$ units to 10^8 relative $Rluc_{Bb}$ units for each clone demonstrated no difference in the trend of the data resulting from this method of analysis compared to the data resulting from $Fluc_{Bb}$ units normalized to OD_{600} (**Figure 3B**). Together these findings establish *flaBp-rluc*$_{Bb}$ as an effective constitutive control reporter, whose quantitation is reflective of spirochete number and is a robust means to normalize data obtained from *fluc*$_{Bb}$ promoter fusions using the dual luciferase reporter system.

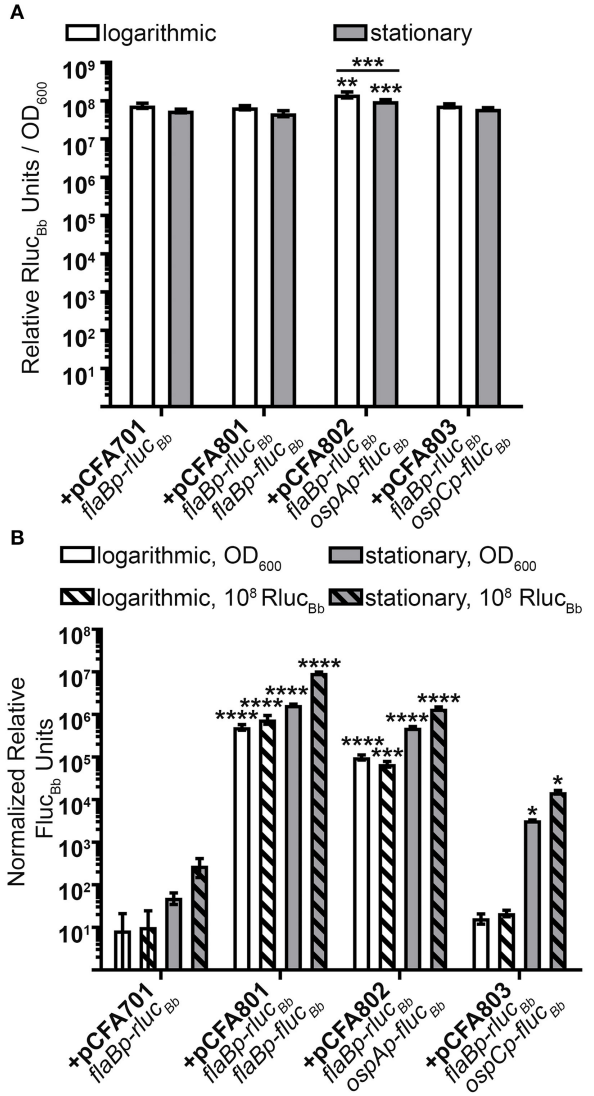

FIGURE 3 | Dual luciferase assay with *in vitro* grown *B. burgdorferi*. Spirochetes were grown to either mid-logarithmic or stationary phase and the *in vitro* luciferase assay performed with 700 μM D-luciferin or 3.5 mM coelenterazine. All data are presented as mean normalized relative luciferase units ± standard deviation for biological triplicate samples. **(A)** Relative $Rluc_{Bb}$ units normalized to OD_{600}. The data set for each growth condition was analyzed with a one-way ANOVA followed by Dunnett's multiple comparison test compared to *B. burgdorferi* expressing *flaBp-rluc*$_{Bb}$ (+pCFA701) and Bonferroni's multiple comparison test to compare the same clone in the two growth phases. **(B)** Relative $Fluc_{Bb}$ units normalized to OD_{600} or 10^8 relative $Rluc_{Bb}$ units of the same sample. Each data set was square root transformed and analyzed with a one-way ANOVA followed by Dunnett's multiple comparison test compared to *B. burgdorferi* expressing *flaBp-rluc*$_{Bb}$ (+pCFA701). Unless indicated, means were not significantly different from the control. Significant differences are indicated with asterisks (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$).

The Dual Luciferase Reporter System Quantifies Promoter Activities during Tick-Spirochete Interactions

Having established the dual luciferase reporter system for use with *in vitro* grown spirochetes, we examined the efficacy of the reporter system for measuring *B. burgdorferi* promoter activities in the tick vector. Naïve *Ixodes scapularis* larval ticks were infected with *B. burgdorferi* carrying the dual luciferase reporter plasmids or *flaBp-fluc*$_{Bb}$, lacking *rluc*$_{Bb}$ (+pJSB175) by feeding on groups of mice infected with the reporter clones via needle inoculation. Immediately following feeding to repletion, the percent of infected larvae per experimental group was determined by spirochete reisolation in BSKII medium. This analysis revealed that 60–90% of each experimental group of larvae successfully acquired *B. burgdorferi* upon feeding on infected mice. As an additional means to determine the percentage of infected larvae and to quantitate the number of spirochetes per tick, individual fed larvae were crushed and plated in solid medium for colony forming units (CFUs). Similar to the spirochete reisolation analysis, the groups of fed larvae were found by CFU analysis to be 66–100% infected. Moreover, although a broad range of spirochetes per tick was detected, there was no statistical difference between the average spirochete load per tick for each of the *B. burgdorferi* clones (**Figure 4**). These data suggest that all of the clones were able to colonize the ticks with the same efficiency.

Based on our quantitation of the average number of spirochetes per tick (**Figure 4**), we estimated that pools of 24

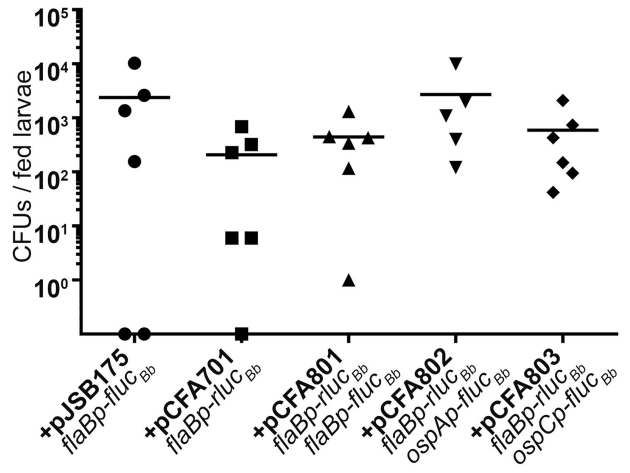

FIGURE 4 | The dual luciferase plasmids do not affect *B. burgdorferi* acquisition efficiency. Groups of ∼200 naïve *Ixodes scapularis* larvae were fed to repletion on mice infected with *B. burgdorferi* containing distinct luciferase plasmids. Subsets of individual fed larvae per *B. burgdorferi* clone were surface sterilized, crushed, and plated in solid BSKII containing RPA cocktail and 50 μg/ml streptomycin. Data points represent the number of colony forming units (CFUs) per individual fed larva. Uninfected larvae (CFU = 0) are represented as data points on the X-axis. No significant differences were detected across the data as determined with a one-way ANOVA.

fed larvae would equate to approximately 10^4 spirochetes per sample, suggesting that the $Rluc_{Bb}$ activity would be quantifiable by our assay (**Figure 2B**). Therefore, 2 weeks following the blood meal, 24 fed larvae per experimental group, in triplicate, were crushed in PBS and relative $Fluc_{Bb}$ and $Rluc_{Bb}$ units measured using the luciferin and coelenterazine substrates, respectively (**Table 2**). The remaining fed larvae were reserved and allowed to molt into nymphs. The unfed, infected nymphs were then fed to repletion on naïve mice. Approximately 2 weeks post feeding, groups of eight fed nymphs were crushed in PBS, in triplicate, and assessed for relative $Fluc_{Bb}$ and $Rluc_{Bb}$ units (**Table 2**). Under the assumption that the spirochete load per fed nymph is increased approximately 10-fold compared to that of fed larvae (Jewett et al., 2007, 2009), we estimated the average spirochete load per fed nymph to be approximately 10^4. Therefore, a pool of eight fed nymphs was estimated to equate to approximately 8×10^4 spirochetes, which is above both the LoD and LoQ of the *in vitro* assay (**Figure 2B**). The actual LoQ for the *in vivo* tick assay was established using the average $Rluc_{Bb}$ units plus 10 standard deviations for tick extracts from fed ticks infected with *B. burgdorferi* lacking $rluc_{Bb}$ but expressing *flaBp-*$fluc_{Bb}$, (+pJSB175), rather than PBS alone. This is due to the observation that this tick extract negative control resulted in lower background relative $Rluc_{Bb}$ units compared to PBS alone (**Table 2**). In contrast, there was no observed difference in the background relative $Fluc_{Bb}$ units between PBS and the tick samples containing *B. burgdorferi* with a promoterless $fluc_{Bb}$ and expressing *flaBp-*$rluc_{Bb}$ (+pCFA701) in the luciferin assay. Therefore, the LoQ for $Fluc_{Bb}$ in the *in vivo* tick assay was determined using the average relative $Fluc_{Bb}$ units for PBS plus 10 standard deviations. Samples that fell below the LoQ threshold for either luciferase enzyme were considered no different than background (**Table 2**). As expected, we detected quantifiable relative $Rluc_{Bb}$ units for all fed larvae samples, and all but two fed nymph samples (**Table 2**), indicating that sufficient spirochetes were present in the samples for the assay. In the pools of fed larvae only samples containing *B. burgdorferi* carrying *flaBp-*$fluc_{Bb}$ (+pCFA801) demonstrated quantifiable relative $Fluc_{Bb}$ units. The activities of *ospAp* and *ospCp* were below the LoQ for $Fluc_{Bb}$ (**Table 2**). In contrast, all three promoters produced quantifiable relative $Fluc_{Bb}$ units in the fed nymphs. Although one of the extracts from the fed nymphs infected with *B. burgdorferi* carrying *ospCp-*$fluc_{Bb}$ (+pCFA803) did not result in quantifiable relative $Fluc_{Bb}$ units, this sample also failed to achieve quantifiable relative $Rluc_{Bb}$ units (**Table 2**), indicating that the number of spirochetes in the sample was insufficient for the assay. The promoter activities of the spirochetes in the fed nymph samples were analyzed by subtracting the average relative $Fluc_{Bb}$ units of PBS from the relative $Fluc_{Bb}$ units of each sample and the average relative $Rluc_{Bb}$units of the infected tick extracts containing the negative control plasmid (+pJSB175) from the relative $Rluc_{Bb}$ units of each sample. Background-subtracted $Fluc_{Bb}$ units were then normalized to the respective background-subtracted relative $Rluc_{Bb}$ units, for all quantifiable values. The $Rluc_{Bb}$-normalized promoter activities reflected the expected corresponding *B. burgdorferi* transcript expression pattern during the fed nymph life stage (**Figure 5A**, Iyer et al., 2015).

As an additional means to validate the method as well as to demonstrate that relative $Rluc_{Bb}$ units are directly reflective of spirochete numbers in the infected tick samples, a portion of each sample from the fed infected nymphs used for $Rluc_{Bb}$ and $Fluc_{Bb}$ quantitation (**Table 2**, **Figure 5A**), was plated in solid BSKII medium for determination of *B. burgdorferi* CFUs. The average CFUs per 100 μl of tick extract, the same volume used for the dual luciferase assay, across all clones, was found to be 3.72×10^5 spirochetes. These data support our rationalization for the use of 8 fed nymphs in the assay. Raw relative $Rluc_{Bb}$ units (**Table 2**) for these samples plotted against their corresponding CFU counts demonstrated a significant positive correlation (**Figure 5B**). Furthermore, this analysis indicated that 1.2×10^3 spirochetes are sufficient to generate relative $Rluc_{Bb}$ units above the LoQ for the *in vivo* tick assay, which is similar to the sensitivity we observed for the *in vitro* assay. Collectively, we have described a valuable new method to determine the activity of *B. burgdorferi* promoters of interest under *in vitro* growth conditions and in infected ticks. This is the first application of a dual reporter system for *B. burgdorferi* and, to the best of our knowledge, the first quantification of spirochete promoter activities in the tick vector.

DISCUSSION

Promoter fusion reporter systems are elegant, simple, and powerful tools to quantitate bacterial promoter activities in environments of interest. Herein we have established a new

TABLE 2 | *In vivo* tick dual luciferase assay.

Tick life stage	Plasmid	Luciferase cassette(s)	Relative $Rluc_{Bb}$ units/biological replicate[a]			Relative $Fluc_{Bb}$ units/biological replicate[b]		
			1	2	3	1	2	3
Fed Larvae[e]	PBS	n/a[c]	85.2	89.6	92.8	20.0	24.8	24.4
	pJSB175	*flaBp-fluc*$_{Bb}$	37.6	36.4	34.4	137.6	245.6	138
				LoQ[d] = 52.3			LoQ = 49.7	
	pCFA701	*flaBp-rluc*$_{Bb}$	185.6	108.8	208.0	22.4*	24.0*	24.0*
	pCFA801	*flaBp-rluc*$_{Bb}$*; flaBp-fluc*$_{Bb}$	603.6	1,502.4	1,187.6	56.0	136.0	100.8
	pCFA802	*flaBp-rluc*$_{Bb}$*; ospAp-fluc*$_{Bb}$	144.4	153.2	176.0	26.0*	25.2*	24.0*
	pCFA803	*flaBp-rluc*$_{Bb}$*; ospCp-fluc*$_{Bb}$	346.8	341.2	234.8	22.4*	23.6*	19.6*
Fed Nymph[f]	PBS	n/a	82.4	93.2	83.2	19.6	20.4	18.8
	pJSB175	*flaBp-fluc*$_{Bb}$	31.6	31.6	30.0	504.8	1,700.8	881.6
				LoQ = 40.3			LoQ = 27.6	
	pCFA701	*flaBp-rluc*$_{Bb}$	41.6	29.6*	296.0	18.0*	19.6*	18.0*
	pCFA801	*flaBp-rluc*$_{Bb}$*; flaBp-fluc*$_{Bb}$	2,416.8	2,560.0	780.4	956	552.8	388
	pCFA802	*flaBp-rluc*$_{Bb}$*; ospAp-fluc*$_{Bb}$	1,001.2	2,257.6	338.4	107.6	262	36
	pCFA803	*flaBp-rluc*$_{Bb}$*; ospCp-fluc*$_{Bb}$	1,500.8	1,239.6	34.8*	21.2*	32.8	16.4*

[a] *Relative $Rluc_{Bb}$ units from three independent tick extracts incubated with 3.5 mM coelenterazine.*
[b] *Relative $Fluc_{Bb}$ units from three independent tick extracts incubated with 700 μM luciferin.*
[c] *Not applicable.*
[d] *Limit of Quantification (LoQ) defined as the average background signal for each assay plus 10 standard deviations.*
[e] *Extract from groups of 24 fed larvae crushed in PBS.*
[f] *Extract from groups of 8 fed nymphs crushed in PBS.*
**Samples that fell below their respective LoQ.*

dual luciferase reporter method using the *Renilla* (sea pansy) and *Photinus* (firefly) luciferase enzymes for measurement of *B. burgdorferi* promoter activities *in vitro* and in the feeding tick during spirochete acquisition from an infected vertebrate host and transmission to a naïve vertebrate host. We demonstrate that constitutive expression of the *B. burgdorferi* codon-optimized *Renilla* luciferase gene (*rluc$_{Bb}$*) is a specific and sensitive measurement of spirochete numbers for normalization of *Photinus* luciferase gene (*fluc$_{Bb}$*) expression under the control of a promoter of interest.

Several reporter genes have been applied to *B. burgdorferi* including chloramphenicol acetyl transferase (*cat*) (Sohaskey et al., 1997), genes encoding a variety of fluorescent proteins (Eggers et al., 2002; Carroll et al., 2003; Schulze and Zuckert, 2006), the *Photinus pyralis* luciferase gene (*fluc$_{Bb}$*) (Blevins et al., 2007), and *lacZ* encoding β-galactosidase (*lacZ$_{Bb}$*) (Hayes et al., 2010). Here we describe the first use of a dual reporter system for *B. burgdorferi*. The combined application of the *Renilla* and *Photinus* luciferase genes has several advantages compared to other *B. burgdorferi* reporter systems as well as other methods of gene expression quantitation such as RT-qPCR. No sample extraction or purification is required to achieve detectable bioluminescence signals, allowing for rapid assay read out with little sample manipulation. Our data indicate that the *rluc$_{Bb}$* gene under the control of the strong, constitutive *flaB* promoter results in relative $Rluc_{Bb}$ units reflective of the number of live spirochetes. This allows relative $Rluc_{Bb}$ units to serve as the endogenous control against which the relative luciferase units of promoter fusions to *fluc$_{Bb}$* on the same plasmid, in the same sample, can be normalized. It is even possible to measure $Fluc_{Bb}$ and $Rluc_{Bb}$ signals back-to-back in the same assay well using firefly luciferase quenching reagents, such as Stop & Glo by Promega (McNabb et al., 2005) and therefore little sample material is required. Use of optical density at 600 nm (OD_{600}) to quantitate sample turbidity as a measure of cell number does not distinguish between live and dead cells in the sample and therefore may not accurately reflect the number of live cells that contribute to the bioluminescence signal. Furthermore, OD_{600} cannot be used for complex biological samples such as extracts from fed ticks. We demonstrate a significant positive correlation between relative $Rluc_{Bb}$ units and numbers of live spirochetes both *in vitro* and in ticks. The *B. burgdorferi* clone containing pCFA802 exhibited statistically different relative $Rluc_{Bb}$ units *in vitro* when normalized to OD_{600} compared to the other clones. However, the relative $Fluc_{Bb}$ units/10^8 relative $Rluc_{Bb}$ units for this clone followed the expected pattern of *ospA* expression *in vitro* and in nymphs. Furthermore, the relative $Rluc_{Bb}$ units for spirochetes carrying pCFA802 correlated to the number of live spirochetes in fed nymph extracts from this clone, suggesting that the observed difference may not result in a biologically significant effect. Utilizing *flaBp-rluc$_{Bb}$* as an endogenous constitutive control provides new opportunities for the development of novel high-throughput genetic screening approaches. DNA libraries engineered to drive expression of *fluc$_{Bb}$* could be effectively screened for active promoters in various growth conditions of interest and relative $Fluc_{Bb}$ units normalized to relative $Rluc_{Bb}$ units. Further, the dual luciferase reporter plasmid can be manipulated to engineer $Fluc_{Bb}$ translational fusions to quantitate protein production and stability in growth conditions of interest. An additional important benefit of the dual luciferase

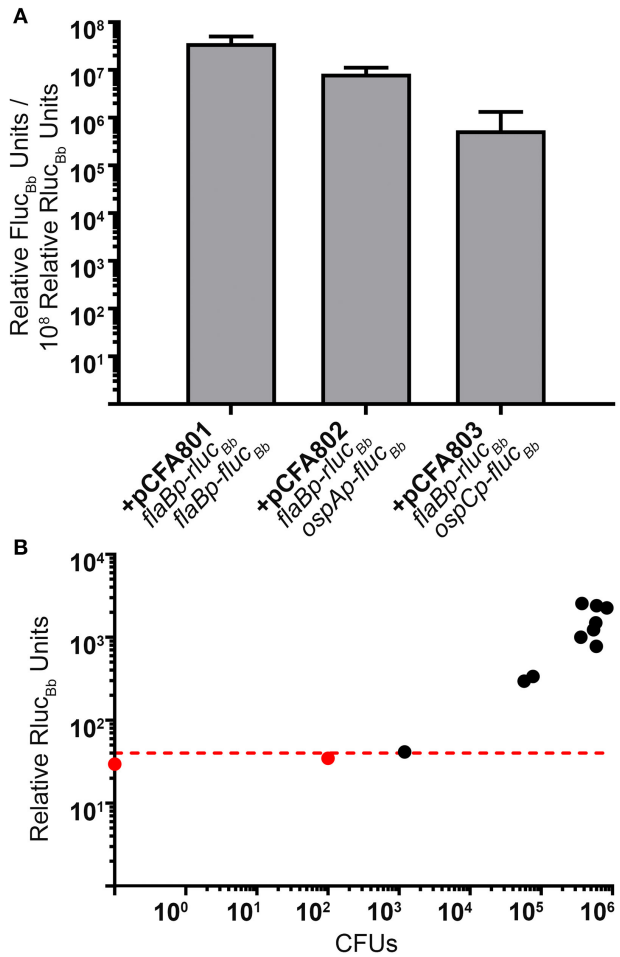

FIGURE 5 | The dual luciferase assay quantitates known patterns of *B. burgdorferi* transcript expression in nymphs. **(A)** Groups of eight fed nymphs per *B. burgdorferi* clone were surface sterilized and crushed in 250 μl of PBS and the *in vivo* tick luciferase assay performed with 100 μl of tick extract and 700 μM D-luciferin or 3.5 mM coelenterazine. The data are presented as the mean relative $Fluc_{Bb}$ units per 10^8 relative $Rluc_{Bb}$ units ± standard deviation ($n = 3$; *B. burgdorferi* carrying pCFA803 $n = 2$). **(B)** Relative $Rluc_{Bb}$ luciferase activity is reflective of spirochete numbers in extracts from fed infected nymphs. 1 μl of fed nymph extract was plated for CFUs in solid BSKII containing RPA cocktail and 50 μg/ml streptomycin. The number of CFUs/100 μl of tick extract (CFUs) was plotted against the relative $Rluc_{Bb}$ units for the same extract. The red dotted line indicates the established LoQ for relative $Rluc_{Bb}$ units. Black symbols represent extracts with quantifiable relative $Rluc_{Bb}$ units and red symbols represent extracts with non-quantifiable $Rluc_{Bb}$ units. A nymph extract with no detectable CFU (CFU = 0) is represented as the data point on the Y-axis. A significant positive correlation was detected between CFU and relative $Rluc_{Bb}$ units (Pearson coefficient, $r = 0.8022$, $p = 0.0017$).

reporter assay is the ability to quantitate the promoter activity of a transcript in a strand-specific manner. We and others have recently reported recognition of novel RNA transcripts in the *B. burgdorferi* genome (Arnold et al., 2016; Adams et al., 2017; Popitsch et al., 2017). Through global 5′ end mapping of the *B. burgdorferi* transcriptome, we have predicted promoter sequences for previously unannotated RNAs, including antisense and intragenic transcripts, and validated their activities in a variety of environments (Adams et al., 2017). Application of the dual luciferase reporter system now provides a robust means for quantitative comparative analysis of strand-specific *B. burgdorferi* transcription in complex regions of the genome at the tick-pathogen interface.

For the correct interpretation of molecular techniques it is important to define the lowest level of a measurement, in this case relative luciferase units, which can be reliably analyzed. The limit of detection (LoD) is the lowest amount of measurable signal above background and the limit of quantification (LoQ) signifies the lowest interpretable signal above background. Effective use of LoD and LoQ are based off the standard deviation (SD) of background readings and assume at least 95% of analyzed values are true measurements in the biological assay (Armbruster and Pry, 2008). We have stringently defined LoD as the $mean_{background\ RLUs}$+ 3SD and LoQ as the $mean_{background\ RLUs}$ + 10SD. Thereby LoQ should be calculated for each luciferase substrate and each independent application of the *B. burgdorferi* dual luciferase assay to best distinguish low but quantifiable bioluminescence signals from background. It is also important to define the appropriate background controls in the context of the assay. Indeed, our studies have demonstrated that background relative $Rluc_{Bb}$ units were ∼60% decreased in fed tick extracts compared to PBS alone. Therefore, extracts from fed ticks infected with *B. burgdorferi* lacking *rluc*$_{Bb}$ expression (+pJSB175) served as the background control to calculate the LoQ for $Rluc_{Bb}$ in ticks. Conversely, this was not observed for the background relative $Fluc_{Bb}$ units for fed tick extracts and PBS alone served as the negative control for these measurements. We hypothesize that the biological matrix of the fed tick extracts contributes, in part, to alteration of the $Rluc_{Bb}$ signal by inhibiting non-specific activation of the coelenterazine substrate.

We found that not all samples with quantifiable relative $Rluc_{Bb}$ units, also had quantifiable relative $Fluc_{Bb}$ units. In some cases, the finding that a promoter fusion has non-quantifiable relative $Fluc_{Bb}$ units may accurately reflect the weak to no biological activity of that promoter in a particular environment and/or non-quantifiable relative $Fluc_{Bb}$ units may result from low numbers of spirochetes, albeit quantifiable relative $Rluc_{Bb}$ units. These challenges may be overcome by increasing the number of spirochetes used in the assay. This is evident in the data we present for the *in vivo* tick assay, in which the fed larvae samples for all *B. burgdorferi* clones achieved quantifiable relative $Rluc_{Bb}$ units; however, the clone containing *flaBp-fluc*$_{Bb}$ (+pCFA801), but not the clones containing *ospAp-fluc*$_{Bb}$ (+pCFA802) or the *ospCp-fluc*$_{Bb}$ (+pCFA803), produced quantifiable relative $Fluc_{Bb}$ units. This finding was not surprising for the *ospC* promoter, given that the *ospC* transcript is known to have weak to no activity in fed larvae following *B. burgdorferi* acquisition from infected mice. This finding was, however, unexpected for the *ospA* promoter, whose transcript is known to have strong activity in this environment (Caimano et al., 2015). Yet, the average number of spirochetes in the *ospAp-fluc*$_{Bb}$ (+pCFA802) and *ospCp-fluc*$_{Bb}$ (+pCFA803) containing clone extracts, as reflected by the average relative $Rluc_{Bb}$ units (1.6×10^2 and 3.1×10^2, respectively), were approximately 10-fold and 4-fold

less than that of the *flaBp-fluc*$_{Bb}$ (+pCFA801) containing clone (1.1×10^3), suggesting that spirochete number may contribute, in part, to the non-quantifiable relative $Fluc_{Bb}$ units for these spirochetes. In contrast, the fed nymph extracts contained comparable average numbers of spirochetes regardless of the clone, as reflected by both the average relative $Rluc_{Bb}$ units ($1.5 \times 10^3 \pm 380$) and CFU counts ($4.4 \times 10^5 \pm 2.2 \times 10^5$) and all *fluc*$_{Bb}$ promoter fusions achieved quantifiable relative $Fluc_{Bb}$ units. Furthermore, while it was one of our goals to measure promoter activities for spirochetes in unfed-flat nymphs post-molt, we found the luciferase signals for these samples to be below the limit of quantification of our assay. We again hypothesize that the spirochete loads in the ticks at this point in the infectious cycle may be below the number of spirochetes necessary for the assay. To examine this possibility we crushed and plated for CFU a subset of individual unfed nymphs infected with spirochetes carrying both *flaBp-rluc*$_{Bb}$ and *flaBp-fluc*$_{Bb}$ (pCFA801). The average spirochete load was determined to be ∼27 spirochetes/unfed nymph. This was approximately 10-fold lower than the average spirochete load in the fed larval ticks for the same clone ($\sim 4.4 \times 10^2$ spirochetes/fed larvae) and approximately 10,000-fold lower than that of fed nymphs ($\sim 1.6 \times 10^5$ spirochetes/fed nymph). Considering that pools of 24 fed larvae and 8 fed nymphs, and therefore $\sim 10^4$ and $\sim 10^6$ spirochetes carrying pCFA801, respectively, were used for the luciferase assays, nearly 400 up to 40,000 unfed nymphs would be required to achieve equivalent relative luciferase units. The difficulties of studying *B. burgdorferi* transcription in unfed nymphs was also shown by a recent microarray study, where even with an amplification step, transcript analysis in this tick life-stage was precluded (Iyer et al., 2015). RT-qPCR does remain an alternative approach for gene expression analysis in unfed nymphs, having several documented successes in determining *B. burgdorferi* transcript levels (Wang et al., 2002; Bykowski et al., 2007; Showman et al., 2016), albeit lacking strand specificity. It should be noted that the endogenous copies of the *flaB, ospA*, and *ospC* genes and their promoters are present in the genetic background of all of the *B. burgdorferi* clones that were analyzed. This raises the possibility that a reduction in $Fluc_{Bb}$ or $Rluc_{Bb}$ signals could have occurred due to titration of transcription factors away from the promoter fusions by the endogenous promoters. However, expression of *flaB, ospA*, and *ospC* are essential for survival of *B. burgdorferi* throughout its infectious cycle (Samuels, 2011; Sultan et al., 2013) and thus these experiments could not be conducted in the absence of these genes.

While dual *fluc* and *rluc* reporter systems have been used successfully for live imaging and quantitation of eukaryotic tumor cells in mice (Bhaumik and Gambhir, 2002), the use of *Renilla* luciferase and the coelenterazine substrate for live imaging of microbial infections in mice has proven challenging (Andreu et al., 2011) and few publications report exploration of the use of dual *Renilla* and *Photinus* luciferase reporters in the context of infectious disease applications. There is great interest in applying a luciferase dual reporter system to quantification of *B. burgdorferi* promoter activities during an active mammalian infection. We and others have demonstrated the power of the *fluc*$_{Bb}$ reporter for tracking *B. burgdorferi* dissemination and qualitative detection of promoter activities over time in live mice (Hyde et al., 2011; Chan et al., 2015; Adams et al., 2017). By extension we investigated the efficacy of the dual luciferase reporter system for live imaging applications with *B. burgdorferi* in infected mice. Exhaustive examination of available coelenterazine substrates including: h-Coelenterazine-SOL *in vivo* (NanoLight), Inject-A-Lume h-Coelenterazine (NanoLight), ViviRen™ *in vivo* Renilla Luciferase Substrate (Promega), and XenoLight RediJect Coelenterazine h (PerkinElmer) as well as various substrate concentrations, substrate injection methods and imaging times, resulted in no significant $Rluc_{Bb}$ signals above background (data not shown). Unlike for applications for solid cancers, use of luciferase substrates for *in vivo* detection of microbial pathogens relies on the substrates to be available in excess, systemically throughout the animal. Luciferin has been documented to rapidly distribute throughout the mouse (Contag et al., 1997), but the bioavailability of coelenterazine may be more limited (Luker et al., 2002). In addition, we found coelenterazine to have an extraordinary high background signal. Indeed, $Rluc_{Bb}$ signals following coelenterazine delivery were observed for mice infected with spirochetes lacking *rluc*$_{Bb}$ entirely, which were not able to be overcome in mice infected with spirochetes expressing *flaBp-rluc*$_{Bb}$ (data not shown). These findings are consistent with what has been reported for attempted *in vivo* imaging applications using coelenterazine and *Mycobacterium smegmatis* expressing *Gaussia* luciferase (Andreu et al., 2010). Rather, alternative methods of normalization may be used, such as determining spirochete loads of infected tissues immediately following $Fluc_{Bb}$ imaging (Skare et al., 2016), in instances where quantification of promoter activity during murine infection is warranted.

B. burgdorferi has been shown to colonize *Ixodes scapularis* via a biphasic mode of dissemination which is believed to involve complex interactions between the pathogen and the arthropod vector (Dunham-Ems et al., 2009). We are still discovering many of the mechanisms *B. burgdorferi* employs to survive throughout its enzootic cycle. Additionally, the recently sequenced *Ixodes scapularis* genome opens new areas of study for host-pathogen interactions (Gulia-Nuss et al., 2016). Successful and reliable techniques for analysis of spirochete biology in the tick are critical to drive understanding of these interactions. The dual luciferase system presented here is a simple and powerful approach for measuring transcript expression, which can be easily modified to meet the needs of the researcher and adds to the ever growing molecular genetic toolbox for investigation of *B. burgdorferi* transcription and gene regulation.

AUTHOR CONTRIBUTIONS

PA and MJ conceived the study and designed experiments; PA and CF performed experiments; PA, CF, and MJ interpreted results; PA and MJ wrote the manuscript; all authors critiqued and edited the final manuscript.

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REVIEW
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Relapsing Fevers: Neglected Tick-Borne Diseases

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Relapsing fever still remains a neglected disease and little is known on its reservoir, tick vector and physiopathology in the vertebrate host. The disease occurs in temperate as well as tropical countries. Relapsing fever borreliae are spirochaetes, members of the *Borreliaceae* family which also contain Lyme disease spirochaetes. They are mainly transmitted by *Ornithodoros* soft ticks, but some species are vectored by ixodid ticks. Traditionally a *Borrelia* species is associated with a specific vector in a particular geographical area. However, new species are regularly described, and taxonomical uncertainties deserve further investigations to better understand *Borrelia* vector/host adaptation. The medical importance of *Borrelia miyamotoi*, transmitted by *Ixodes* spp., has recently spawned new interest in this bacterial group. In this review, recent data on tick-host-pathogen interactions for tick-borne relapsing fevers is presented, with special focus on *B. miyamotoi*.

Keywords: relapsing fever, *Borrelia*, *Ornithodoros*, hard ticks, *Borrelia miyamotoi*, neurotropism, antigenic variations

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INTRODUCTION

Tick-borne borreliosis are vector-borne diseases including Lyme disease, the most important tick-borne disease from the northern hemisphere, but also relapsing fevers (RF) especially prevalent in temperate and tropical areas (Ogden et al., 2014). Within tick-borne relapsing fevers (TBRF), the vectors are mainly argasid vectors also known as "soft ticks" of the genus *Ornithodoros.* Interestingly, some species are transmitted by ixodid vectors or "hard ticks." The bacteria are all maintained in enzootic cycles with the human as accidental host except *B. duttonii* in Africa which seems strictly human and has no identified animal reservoir (Lopez et al., 2016). Whereas, Lyme disease continues to be extensively studied, TBRF, although known for ages, remain neglected diseases with only few studies elucidating the interactions between host, tick and pathogens.

In this review, we will present the most recent data on host/vector/pathogen interactions in soft tick-borne RF (STBRF) and in hard tick-borne RF (HTBRF), with a special focus on *Borrelia miyamotoi*, discovered recently as a human pathogen (Platonov et al., 2011). Concentrating on TBRF agents, the louse borne infection caused by *B. recurrentis* will not be further discussed. First, we will present the different pathogenic species of these spirochaetes and their characteristic adaptive strategies in the reservoir, the vector, and the host, as well as the phylogenetic evolution compared with their ecological features. Then, the

vertebrate host/TBRF borreliae interactions of this zoonotic disease transmission system will be reviewed, with special interest in the human disease and mechanisms of immune evasion. We will describe the tick/TBRF borreliae interactions involved in the transmission of spirochaetes to vertebrates. Finally, we will provide an overview of the state of the art in HTBRF.

MULTIPLE PATHOGENIC SPECIES OF TBRF AND ADAPTATION TO SPECIFIC ECOLOGICAL NICHES

Systematics and Phylogeny of TBRF Borreliae

TBRF are spirochaetes of the *Borrelia* genus within the family of *Borreliaceae* (Gupta et al., 2013). Recently, the *Borrelia* and *Borreliella* genera, which respectively contain the TBRF-associated species and the Lyme disease pathogens (lately denominated "TB-RF borreliae" and "LD borreliellae") have been divided into two taxonomical groups. This distinction is based upon their nucleotide and protein signatures, their phylogeny of the 16S rRNA gene/conserved proteins and their phylogenomic metrics (Adeolu and Gupta, 2014; Oren and Garrity, 2015). Their arthropod vectors as well distinguish these two genera because schematically hard ticks transmit Lyme disease pathogens whereas soft ticks transmit TBRF pathogens. But this rule has some exceptions given that several RF agents are transmitted only by hard ticks (e.g., *B. miyamotoi*) and one species, *B. recurrentis*, is louse transmitted.

The taxonomy of TBRF borreliae species was historically based on a concept of co-speciation bacteria/tick (Wang and Schwartz, 2011), but the latest species descriptions rely on molecular methods (Fingerle et al., 2016). Currently, there are 22 validly published species names in the genus (Wang and Schwartz, 2011; Adeolu and Gupta, 2014; **Table 1**). Six other taxa were also proposed ("*B. merionesi,*" "*B. lonestari,*" "*B. microti,* "*Canditatus* B. texasensis," "*Candidatus* B. algerica," and "*Candidatus* B. kalaharica") but none could be validated according to the current taxonomic rules, because the deposit of different strains in collections was not possible (Lin et al., 2005); indeed cultivation remains difficult for some species.

Although they harbor differences in their rates of evolution and robustness, several molecular chronometers of RF borreliae housekeeping genes (e.g., 16S rRNA, flagellin, glycerophosphodiester phosphodiesterase GlpQ) and non-coding sequences from the linear chromosome are quite congruent to delineate TBRF phylogenesis (Fukunaga et al., 1996; Ras et al., 1996; Scott et al., 2005; Oshaghi et al., 2011). Single gene phylogenetic analyses are supported by studies of multiple loci (2 to 7 among *rrs*, *flaB*, *glpQ*, *groEL*, *p66*, *recG*, and 16S−23S rRNA intergenic spacer IGS) (Toledo et al., 2010; Trape et al., 2013; Naddaf et al., 2017), extended multilocus phylogenetic analysis (MLPA) panel of 25 conserved coding DNA sequences (Adeolu and Gupta, 2014) and phylogenetic analysis based on 266 sets of single-copy orthologues present in all genomes (Di et al., 2014). According to the highest resolutive methods, TBRF borreliae embrace four lineages also harboring common ecological features, including a vector of *Ixodidae* ("Hard-ticks") or *Argasidae* ("Soft-ticks") family and/or geographic distribution: (1) Old-World TBRF borreliae, (2) New-World TBRF borreliae, (3) the worldwide avian TBRF borreliae (i.e., *B. anserina*) and (4) the HTBRF group (**Table 1**; Adeolu and Gupta, 2014; Di et al., 2014). Other borreliae species may be virtually attached to these phylogenetic groups by analysis of SLPA/MLPA-based studies mostly preserving the ecological specificities of each clade (**Figure 1**). Phylogenetic positions of some recent strains retain ambiguities after the sequencing of too few genes due to incongruities between genes ("*Ca.* B. kalaharica" and a new clinical *Borrelia* sp. in Iran) (Fingerle et al., 2016; Naddaf et al., 2017) and would require additional phylogenetic analysis to clarify relationships between lineages. In addition, several TBRF borreliae species could not be included in any phylogeny comparison because no DNA sequences have been available so far (*B. venezuelensis*, *B. caucasica*, *B. harveyi*, *B. dugesii*, *B. braziliensis*, *B. graingeri*, *B. mazzottii*, *B. tillae*, and *B. baltazardii*).

Concerning the species level, African RF borreliae (*B. crocidurae*, *B. duttonii*, and *B. recurrentis*) are closely related species and may correspond to the ecotypes of a unique genomospecies regarding their average nucleotide identity (ANI $>$ 96%) as suggested by Elbir et al. (2014), corroborating the genomic deep-analysis concluding that the genome of *B. recurrentis* was a degraded subset of *B. duttonii* (Lescot et al., 2008). Similarly, the North American *B. parkeri* and *B. hermsii* are also very closely related species regarding their ANI values (Adeolu and Gupta, 2014). Up to now, the classification of RF borreliae as presented herein remains official (**Table 1**) but may probably be further revised due to the taxonomically admitted bacterial concept of "genomic species" or genospecies as suggested by several authors (Ras et al., 1996; Wang and Schwartz, 2011; Elbir et al., 2014). The harmonization of RF borreliae taxonomy within current rules and the recognition of ecotypes could be achieved by re-classifications of the species and subspecies levels. Nevertheless, the current systematics of RF borreliae reflects very well the characteristics of vector-bacteria-host association of this genus that can be considered as a remarkable case-study of adaptation in correlation with the bacterial concept of "ecotype species" whatever the name of the taxonomic level. The availability of new completed genome sequences as well as genetic population studies of strains from different origins may in the coming years, increase the understanding of the phylogeny of RF borreliae and also clarify taxonomic issues.

Bacterial Features and Vector/Host Associated Lifestyles

RF borreliae are motile, chemo-organotrophic, microaerophilic and host-associated bacteria (Kelly, 1971; Barbour and Hayes, 1986; Adeolu and Gupta, 2014). These spirochaetes dwell extracellularly in ticks as well as in the blood and organs of their vertebrate hosts. They usually grow at temperatures between 33 and 35°C corresponding to mammalian host temperature, but they are also able to multiply at 22°C (tick temperature)

TABLE 1 | Valid and proposed (in bold) species in the genus *Borrelia*.

***Borrelia* species (year of description)**	**Vectors**	**Geographical distribution**	**Hosts**	**Human diseases**	**References**
OLD-WORLD RF BORRELIAE					
'*Candidatus* B. algerica' (2015)	Unknown	Algeria	Human	TBRF	Fotso Fotso et al., 2015
B. baltazardii (1979)	Unknown (*O. tholozani* in lab experiment only)	Iran	Human	TBRF, Thrombocytopenic purpura	Karimi et al., 1979; Naddaf et al., 2017
B. caucasica (1945)	*O. verrucosus*	Azerbadjan, Georgia, Armenia	Rodents, Human	TBRF	Maruashvili, 1945; Felsenfeld, 1965; Assous and Wilamowski, 2009
B. crocidurae (1917)	*O. sonrai*	Western and Northern Africa	Insectivores, Rodents, Human	TBRF, Mild symptomatology	Leger, 1917; Felsenfeld, 1965; Vial et al., 2006a; Trape et al., 2013
B. duttonii (1906)	*O. moubata* s.l. complex	East, Central and Southern Africa, Madagascar	Human	TBRF, Neurological signs, Ocular complications, Neonatal infections,	Novy and Knapp, 1906; Felsenfeld, 1965; Cadavid and Barbour, 1998; Larsson et al., 2006; Rebaudet and Parola, 2006
B. graingeri (1953)	*O. graingeri*	Kenya	Rodents, Human	Flu-like syndrome	Heisch, 1953; Felsenfeld, 1965; Wang and Schwartz, 2011
B. harveyi (1947)	Unknown	Kenya	Monkey	Unknown	Garnham, 1947, 1950
B. hispanica (1926)	*O. erraticus, O. marocanus*	Maghreb, Spain, Portugal, Greece, Cyprus	Rodents, Insectivores, Weasels, Foxes, Bats, Jackals, Dogs, Human	TBRF, Ocular complications, Neurological signs (rare)	Buen, 1926; Felsenfeld, 1965; Cadavid and Barbour, 1998; Trape et al., 2013
'*Candidatus* B. kalaharica' (2016)	Unknow 'likely a soft tick'	Southern Africa	Human	TBRF	Fingerle et al., 2016
B. latyschewii (1941)	*O. tartakovskyi*	Central Asia, Middle East	Rodents, Human	Flu-like syndrome	Sofiev, 1941; Baltazard, 1952; Goubau, 1984; Assous and Wilamowski, 2009
'*B. merionesi*' (1948)	*O. merionesi*, *O. costalis*	Morocco	Rodent, Monkeys	No	Blanc and Maurice, 1948; Felsenfeld, 1965; Diatta et al., 2012; Trape et al., 2013
'*B. microti*' (1947)	*O. erraticus*	Iran	Human	TBRF	Rafyi, 1947; Felsenfeld, 1965; Naddaf et al., 2012
B. persica (1913)	*O. tholozani*	Middle East, Egypt, Central Asia, India	Rodents, Dogs, Cats, Human	TBRF, Neurological signs (rare), Respiratory distress syndrome (rare)	Dschunkowsky, 1913; Cadavid and Barbour, 1998; Yossepowitch et al., 2012; Baneth et al., 2016
B. recurrentis (1874)	*Pediculus humanus*	Virtually worldwide, Currently Ethiopia, Sudan	Human	Louse-borne RF, Neurological signs	Lebert, 1874; Cadavid and Barbour, 1998; Hoch et al., 2015
B. tillae (1961)	*O. zumpti*	Southern Africa	Rodents	No	Zumpt and Organ, 1961; Felsenfeld, 1965

(Continued)

TABLE 1 | Continued

***Borrelia* species (year of description)**	**Vectors**	**Geographical distribution**	**Hosts**	**Human diseases**	**References**
New-World RF borreliae					
B. brasiliensis (1952)	*O. brasiliensis*	Brazil	Human	TBRF	Davis, 1952; Lopez et al., 2016
B. coriaceae (1987)	*O. coriaceus*	USA	Rodents, Deer	Likely not pathogenic	Johnson et al., 1987; Nieto et al., 2012; Nieto and Teglas, 2014; Lopez et al., 2016
B. dugesii (1949)	*O. dugesi*	Mexico	Unknown	Unknown	Mazzotti, 1949; Wang and Schwartz, 2011
B. hermsii (1942)	*O. hermsi*	British Columbia (Canada), Western USA	Rodents, Deer, Dog, Human	TBRF, Neurological signs (rare), Neonatal infections (rare)	Davis, 1942; Cadavid and Barbour, 1998; Schwan et al., 2007; Centers for Disease Control Prevention, 2012; Nieto et al., 2012; Kelly et al., 2014; Nieto and Teglas, 2014
B. mazzottii (1956)	*O. talaje*	Mexico and Guatemala	Unknown	Likely TBRF	Davis, 1956; Lopez et al., 2016
B. parkeri (1942)	*O. parkeri*	Western USA	Rodents, Horses, Human	TBRF	Davis, 1939, 1942; Walker et al., 2002
B. turicatae (1933)	*O. turicata*	British Columbia (Canada), US, Mexico	Rodents, Dog, Human,	TBRF, Ocular complications, Neurological signs	Brumpt, 1933; Cadavid and Barbour, 1998; Schwan et al., 2005; Lopez et al., 2016
B. venezuelensis (1921)	*O. rudis*	Panama, Columbia, Venezuela, Ecuador, Paraguay	Unknown	TBRF	Brumpt, 1921; Goubau, 1984
WORLDWIDE AVIAN RF BORRELIAE					
B. anserina (1891)	*Argas* spp.	Worlwide	Birds	No	Sakharoff, 1891; Marchoux and Salimbeni, 1903; Fabbi et al., 1995; Hovind-Hougen, 1995
HARD TICK-BORNE RF BORRELIAE					
'*B. lonestari*' (1996)	*Amblyomma americanum* (lone star tick)	USA	Birds, Deers	No	Barbour et al., 1996; Jordan et al., 2009; Castellaw et al., 2011
B. miyamotoi (1995)	*Ixodes persulcatus*, *I. ricinus*, *I. scapularis*	Asia, Europe, USA	Rodents, Birds, Human	Flu-like syndrome, TBRF, Neurological signs	Fukunaga et al., 1995; Platonov et al., 2011; Gugliotta et al., 2013; Wagemakers et al., 2017
'*Candidatus* B. texasensis' (2005)	*Dermacentor variabilis* (American dog tick)	USA (Texas)	Unknown	Unknown	Lin et al., 2005
B. theileri (1903)	*Rhipicephalus* spp.	Africa, Australia, North, and South America	Cattle, Sheep, Goats	No	Laveran, 1903; Theiler, 1905; McCoy et al., 2014

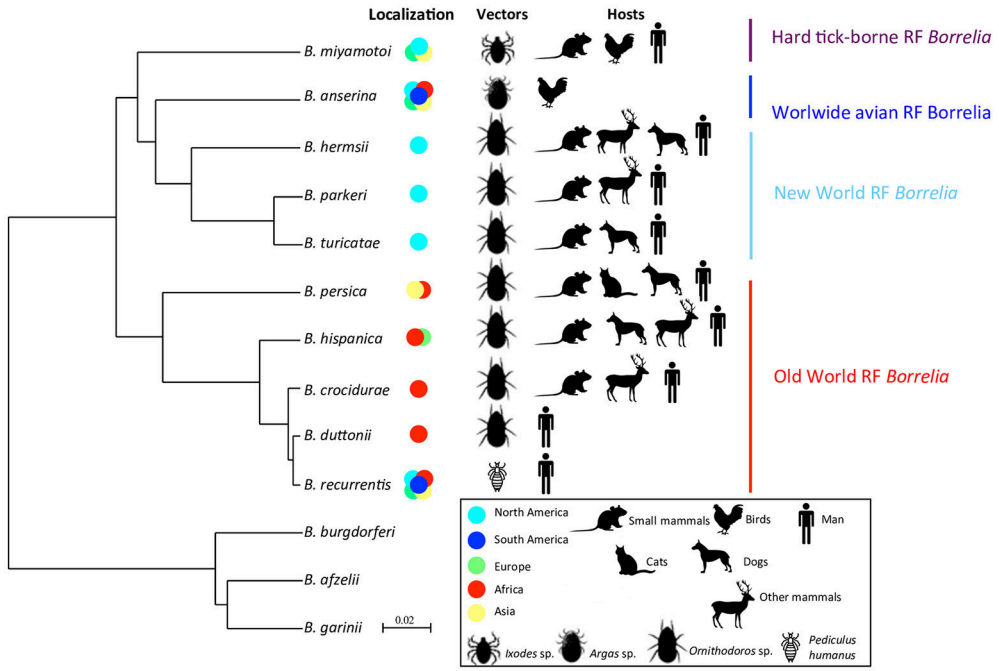

FIGURE 1 | Phylodendrogram of Average nucleotide identity values among RF borreliae genomes. LD *Borreliella* genomes have been taken as outgroup. The phylodendrogram has been obtained by pairwise comparison of average nucleotide identity (ANI) between two genome sequences of each RF borreliae species sourced from Adeolu and Gupta (2014) and Elbir et al. (2014). Topology created on http://genomes.urv.cat/UPGMA/. The horizontal and vertical lines represent genetic distance, with the scale bar indicating 2% of difference from ANI = 100%. Other related species in SLPA or MLPA based on literature review are: (1) "*B. microti,*" "*B. merionesi*" and *Ca.* B. algerica in the Old world RF group (Naddaf et al., 2012; Trape et al., 2013; Fotso Fotso et al., 2015), (2) *B. coriaceae* and *Ca.* B. texasensis in the New world RF group (Fukunaga et al., 1996; Lin et al., 2005; Naddaf et al., 2012), and (3) "*B. lonestari*" and *B. theileri* in the Hard-tick-borne RF group (Lee et al., 2014; Hagen et al., 2018).

as proven *in vitro* for *B. turicatae* (Wilder et al., 2016). Genes encoding enzymes for the synthesis of most amino acids, fatty acids, enzyme cofactors, and nucleotides are absent in the RF borreliae genomes as shown in LD borreliellae (Fraser et al., 1997; Adeolu and Gupta, 2014). The *Argasidae* and *Ixodidae* cuticle, which contains chitin derived from the polymerization of N-Acetyl Glucosamine (NAG), might be an important nutrient source for *Borreliaceae* during the arthropod-associated phase (Hackman and Goldberg, 1985; Tilly et al., 2001).

The genome size of RF borreliae (1–1.5 Mb) is smaller than the one in other pathogenic bacteria which have a more versatile lifestyle (e.g., *P. aeruginosa*, 6.3 Mb). Evolution by genome reduction is well correlated to the degenerated lifestyle of several well-adapted pathogens (Dobrindt and Hacker, 2001), that are likely to include RF borreliae which have a restricted niche in the vector and in the vertebrate host. Correlating to their size, RF borrelia genomes have a limited repertoire which very well reflects their adapted lifestyle, including only few virulence-associated genes (Fraser et al., 1997; Adeolu and Gupta, 2014). These genomes harbor a linear megaplasmid of 160 kb having a fairly conserved synteny among *B. duttonii* (lp165), *B. hermsii* (lp174), and *B. turicatae* (lp150) strains which is not found in LD borreliellae (Miller et al., 2013). The *B. turicatae* megaplasmid is subject to a shift in its transcriptional profile between *in-vitro* tick-like growth conditions and murine infected blood, identifying a cluster encoding for putative surface lipoproteins likely involved in vector colonization and host-vector interactions (Wilder et al., 2016).

Geographic Distribution

The four main species responsible for STBRF in Europe are: *B. hispanica*, *B. persica*, *B. caucasica* and *B. crocidurae* (Rebaudet and Parola, 2006). In Africa, the main circulating bacteria are *B. crocidurae* in Western and Northern Africa, *B. duttoni* in Eastern, Central and Southern Africa and *B. hispanica* in the Maghreb (Felsenfeld, 1965; Rebaudet and Parola, 2006; Vial et al., 2006a; Trape et al., 2013). In the United States, three predominant species have been identified and were particularly studied in the western part: *B. hermsii*, *B. parkeri*, and *B. turicatae* (Barbour, 2005; Sonenshine and Roe, 2013). The STBRF borreliae species of African (*B. hispanica*, *B. crocidurae*, and *B. duttonii*) and American clades (*B. hermsii*, *B. parkeri*, and *B. turicatae*) have evolved in narrow geographic areas, probably due to the presence of specific reservoirs and arthropod vectors. In Iran, several species are associated with human STBRF cases, including "*B. microti*," *B. persica* and another genotype close to the complex *B. duttonii*/*B. recurrentis* by 16S−23S rRNA intergenic spacer sequence (Naddaf et al., 2015).

In HTBRF, the distribution of the particular species is not limited to a single continent, since besides Asian

Borrelia sp. ("*lonestari*-like") and American borreliae ("*B. lonestari,*" "*Candidatus* B. texasensis"), worldwide spread taxa (*B. miyamotoi*, *B. theileri*) are also found (**Table 1**).

VERTEBRATE HOST-RF BORRELIAE INTERACTION

Host Specificity

Vertebrate host specificity is variable between RF *Borrelia* spp., since most of the species can infect small mammals and human, and some species can infect birds, domestic or wild mammals without a clear lineage specificity (**Table 1**, **Figure 1**). Inversely, strong association has been described between RF borreliae and the vector species, to such an extent that some authors proposed soft tick vectors as the original natural reservoir for RF borreliae (Barbour, 2005). Regarding the nidicolous or endophilous lifestyle of soft ticks, which colonize sheltered underground habitats like nests, burrows, caves or cracks inside buildings (Sonenshine, 1993; Vial, 2009), it seems obvious that their vertebrate hosts are likely to be rodents or other small vertebrates directly present inside or around the habitats. Such preference for peculiar habitats may drive the host specificity of ticks and their associated RF borreliae. RF borreliae-tick-vertebrate host interrelationships were summarized as follows by Hoogstraal (1979): "*Borrelia* develops as symbiont of soft ticks but act as parasites in mammals and birds, which serve as borrelial reservoirs and amplifiers following bites by infected ticks."

However, two remarkable host associations are reported for RF borreliae. The spirochaete *B. anserina* has a host restriction in birds. It was experimentally proven to be infectious to chicken and various birds but not to rodents and non-human primates (McNeil et al., 1949; Lisbôa et al., 2009). In parallel, the RF borreliae of the *B. duttonii*/*B. recurrentis* complex has only been associated with human cases up to now, although *B. duttonii* can infect chicken and rodents under laboratory conditions (Kervan, 1947; Yokota et al., 1997; Larsson et al., 2009). The DNA of *B. duttonii* has also been detected in the blood of domestic chickens and pigs in Tanzania (McCall et al., 2007).

Host Sensitivity

The characterization of RF borreliae has been conducted for a long time through experimental infection of laboratory animals such as the mouse, the rat, the rabbit, the guinea pig or the monkey, and through the description of resulting pathogenicity (Goubau, 1984). However, not all the infections do automatically result in the death of the animal, and symptoms can vary depending on the inoculated bacterial load as well as the age and the metabolism of the animal (Hindle, 1935). Likewise, some mammals have been found naturally infected by RF borreliae, without any clinical signs and during a long time, and are supposed to be vertebrate reservoirs of RF borreliae (Rhodain, 1976). Several rodent species, squirrels, foxes, shrews, porcupines, opossums, armadillos, and also some domestic animals, have been mentioned as possible reservoirs worldwide (Hindle, 1935; Barbour, 2005). Their bacteraemia is very low and cannot be detected by classical thick blood smear, to such a point that Burgdorfer and Baltazard (1954) classified these infections as "occult." Only the inoculation of blood or organ homogenate, such as brain or spleen, to sensitive animals can lead to sufficient and detectable amplification of RF borreliae. Due to the proximity of rodents to human habitations, especially in rural areas, such organisms may be the most important carriers for RF borreliae (Hindle, 1935). In the United States, pine squirrels and chipmunks are considered major reservoirs for RF borreliae (Trevejo et al., 1998; Paul et al., 2002; Fritz et al., 2004). However, in West Africa, Mathis et al. (1934) noticed that the insectivorous formerly called *Crocidura stampfii* showed high spirochetemia in blood and suggested that it could be a better reservoir than small rodents for *B. crocidurae*, since soft ticks were more likely to become infected through biting such animals.

Reservoir Host Dynamics and RF Borreliae Transmission

The specificities of small mammal reservoirs, as well as those of soft tick vectors, supposedly the original hosts for RF borreliae, can influence the transmission dynamics of such pathogens to humans, who remain an occasional host. Host-vector-pathogen interactions here will focus only on RF borreliae transmitted by *Ornithodoros* soft ticks since they are the most investigated worldwide.

After their contamination through biting on infectious vertebrate reservoirs, *Ornithodoros* ticks can remain infected for several weeks, months, or years (Hindle, 1935; Gaber et al., 1984; Goubau, 1984). Because of multiple blood feeding along their life cycle and a very long lifespan of adult development stages (Morel, 2003; Gray et al., 2014), soft ticks have many occasions to transmit borreliae. Their blood feeding is short, from a few minutes to a few hours for most stages, except for larval stages of the former genus *Alectorobius* (e.g., *O. talaje*) that feed for several days (Loomis, 1961; Morel, 2003), which results in very short parasitic phases on their vertebrate host. Furthermore, these ticks are typically nidicolous or endophilous and photophobic, thus predestined to live in the underground and sheltered microhabitats (Sonenshine, 1993; Vial, 2009). Both features may lead to very low tick dispersal, as suggested by previous genetic or biological studies (Chabaud, 1954; Vial et al., 2006b). Small mammals being reservoirs of TBRF and hosts for ticks are also considered very sedentary because of their territorial behaviors (Powell, 2000). In view of these specificities, RF borreliae transmission is expected to occur locally. This was actually reported in the United States (Trevejo et al., 1998; Dworkin et al., 2002; Paul et al., 2002; Schwan et al., 2003; Fritz et al., 2004), Israel (Sidi et al., 2005), and in Jordan (Babudieri, 1957), where patients are mainly exposed to RF borreliosis during their visit and sleeping in rustic cabins or caves, which are colonized by infected ticks and rodent reservoirs. In Africa, local transmission of RF borreliae is also observed, and patients are generally contaminated in their own houses especially in rural areas where rodents commonly burrow undisturbed inside buildings (Cutler, 2006; Vial et al., 2006a).

Interestingly, a longitudinal monitoring study of human borreliosis over 14 years in Dielmo village, Senegal, highlighted

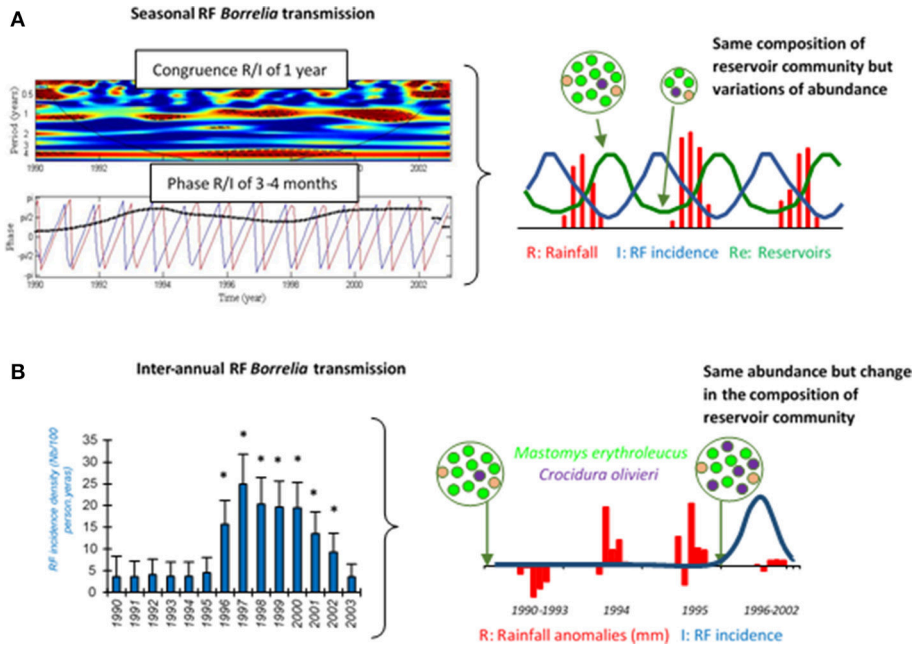

FIGURE 2 | Seasonal **(A)** and inter-annual **(B)** RF borreliae transmission to human in Dielmo village, Senegal, from 1990 to 2003. In **(A)** left panel, the medium red shape in the first graph indicates the congruence between RF borreliae transmission and seasonal rainfall, and the black dotted line in the second graph indicates the 3–4 month phase between both time-series (RF borreliae transmission in blue and rainfall in red). In **(B)** left panel, the annual incidence density of RF borreliosis is expressed as the number of new infections per 100 persons per year, 95% confidence intervals are indicated and significant differences of incidence are reported by stars (*). In right panels, the schemes expose the likely interpretations for seasonal and inter-annual RF incidence variations, respectively, in relation to the dynamics of the small mammal reservoirs. The reservoir community is represented by a circle, including several dots of different colors as the different small mammal species, and the relative number of dots reflects the relative abundance of each species.

a spatial clustering of cases in and close to compounds where *O. sonrai* ticks were systematically collected, 10 years apart (Vial et al., 2006a). During this period, ticks seem to have lightly dispersed to a third site, which resulted in a new temporary focus of TBRF cases (Vial et al., 2006a). The compound-specific effect was significant on RF borreliosis incidence ($p < 0.01$ using random-effect Poisson regression). TBRF transmission to humans should show a seasonal pattern, corresponding to seasonal dynamics of rodent and tick populations. In Dielmo, Senegal, infections with TBRF rose throughout the year, but were actually most common in March and least common in October (Vial et al., 2006a). A wavelet analysis, which can be used to perform a timescale decomposition of ecological or epidemiological time series (Cazelles et al., 2014), showed a strong congruence between rainfall annual cycles and TBRF human cases, with a phase of 3–4 months (**Figure 2A**, left panel). This indicates that maximum transmission of TBRF to humans occurred 3–4 months before the rainy season, meaning at the end of the dry season when rodent reservoir populations decrease because of annual mortality related to the scarcity of food resources (Sicard and Fuminier, 1996; **Figure 2A**, right panel). *Ornithodoros* tick ecology could explain this non-intuitive result. Because of their endophilous lifestyle, tick populations are only slightly influenced by external climatic variations especially in subtropical regions, and it is accepted that the probability of tick biting inside one habitat should be more or less even along the year. In addition, *Ornithodoros* ticks do not show any host preferences, as a possible adaptation to host scarcity in their habitats (Morel, 2003; McCall et al., 2007; Palma et al., 2013). Consequently, ticks may mainly engorge on rodents when they are available in abundance directly in tick microhabitats, leading to a lower transmission of RF borreliae to humans due to lower tick biting pressure; inversely, when rodent populations decline, ticks may preferentially engorge on humans and RF borreliae transmission conjointly increases (**Figure 2A**, right panel). Apart from endemic TBRF in the rural African environment, seasonal RF borreliae transmission can also occur but it may then be rather due to human habits, with higher transmission during summer when people settle in tick-infested locations for holidays or increase their outdoor activities (Fihn and Larson, 1980; Dworkin et al., 2002; Masoumi Asl et al., 2009; Moemenbellah-Fard et al., 2009).

In temperate regions, *Ornithodoros* ticks can suffer from low temperatures during winter although they remain protected by their sheltered habitat, and can consequently stop questing and feeding (Skruinnik, 1939; Kadatskaia and Shashnikova, 1963; Oleaga-Pérez et al., 1990; Morel, 2003), which may result in a decrease of RF borreliae transmission.

Some authors indeed reported human RF outbreaks, which might not be a simple consequence of local transmission or improved surveillance, but highlighting a real increase in RF borreliae transmission. Factors sparking such outbreaks are

always a matter of speculation because it is not possible to monitor them before first evidence of the impact. At the North Rim of the Grand Canyon, United States, populations of rodent hosts for *O. hermsi* were drastically reduced in 1973, possibly due to epizootic plague, and might have resulted in higher tick biting on humans and higher RF borreliae transmission; the same pattern was also observed in 1990 although there was no documented epizooty of plague (Paul et al., 2002). As already demonstrated for Lyme disease, biodiversity loss in small mammal reservoirs can contribute to promoting RF borreliae transmission based on the general concept of a dilution effect (Ostfeld and Keesing, 2000; LoGiudice and Gosain, 2003). In Dielmo village, Senegal, a RF outbreak was reported from 1996 to 2002, with two to six times more cases than previously (Vial et al., 2006a; **Figure 2B**, left panel). Small mammal captures conducted inside houses before and after these outbreaks in 1990 and 2002, revealed changes in the composition of the rodent community with the partial replacement of the common rodent species *Mastomys erythroleucus* by commensal shrews *Crocidura olivieri* (**Figure 2B**, right panel). RF borreliae detection in captured animals revealed higher prevalence of infection in *C. olivieri* than in any other rodent species, suggesting that this shrew species is a very competent reservoir for RF borreliae (Vial et al., 2006a). Other authors (Mathis et al., 1934; Burgdorfer and Mavros, 1970; Nieto and Teglas, 2014) also proposed this assumption of differential reservoir ability for maintaining RF borreliae between the different small mammals. Since *Ornithodoros* ticks have no host preference, they may have engorged proportionally much more on *C. olivieri* than on others and this might have resulted in higher tick infection and increased RF borreliae transmission to humans (**Figure 2B**, right panel). The relatively high longevity of *C. olivieri* (2–3 years) and its competitiveness against other commensal rodents (Churchfield, 1990) are both features that could favor persistent outbreaks for several years. Inversely, *M. erythroleucus* like many rodents is an annual species that depends on climatic conditions, with possible pullulating events due to abundant rainfall and vegetation growth (Sicard and Papillon, 1996). This might be the case for 1993–1994 (**Figure 2B**, right panel). Such abnormal population increase is usually followed by density-dependent crash (Leirs et al., 1997), leading to empty suitable niches for the development of *C. olivieri*.

Human-*Borrelia* Interactions

Clinical Manifestations

One of the major difficulties in RF diagnosis and investigation of medical history is the variable disease presentation, i.e., variable clinical manifestations. The outcome may also be very different with a more or less asymptomatic presentation or a lethal progression, but it is usually severe among small children (Southern and Sanford, 1969). The feeding behavior of Argasid (soft) ticks, i.e., short period of feeding and the fact that the patients do not recognize the tick bite makes it hard to perform a correct clinical diagnosis. Although variable, the incubation time is about 1 week, i.e., between tick bite and the first manifest symptoms (Southern and Sanford, 1969), which result in high fever in the temperature range between 38.7 and 40°C (or even 41°C). The first fever period is usually the longest and lasts for about 4–7 days (Bryceson et al., 1970; Felsenfeld, 1971). This initial febrile episode is followed by a series of relapses (9 to 13 in STBRF), corresponding to peaks of spirochaetemia interspaced by a few days of remission. This is usually described during the course of non-fatal infections in absence of antibiotic treatment (Cutler, 2015).

The typical symptoms are flu-like with malaise and general ache, often involving myalgia and headache. Very often patients experience nausea with vomiting or diarrhea. Patients may also display different types of skin rashes that might have petechial or haemorrhagic manifestations. In mouse model, many of the Old-World RF species can bind to erythrocytes and generate cell aggregates that disrupt the microcirculation. These aggregates then create micro-thrombosis in arterioles anywhere in the body that affects the blood flow during spirochetemia (Shamaei-Tousi et al., 1999). During the spirochetemic peak, more or less severe bleeding can be observed. In cases with more severe haemorrhagic phenomena, bleeding from different organs may be seen, including nose bleed, haemoptysis, bloody diarrhea, haematuria. During severe RF borreliosis, hemorrhages may also be seen of the retina and cerebrum as well as bleeding into the sub-arachnoid space. Internal organs, such as the liver may also be affected, which can cause enlargement and tenderness that can be followed by jaundice. Relapsing fever may also affect the spleen causing micro-abscesses, enlargement and rupture. This systemic disease often causes respiratory symptoms with a cough as well as myocarditis further proving the difficulties to establish a differential diagnosis from influenza virus infection. The disease can resolve itself in the absence of treatment but mortality (around 5%) is observed in epidemics (Rebaudet and Parola, 2006; Ogden et al., 2014).

Severe Presentations

Neurological symptoms are common during RF, but the presentation and severity of symptoms are variable, with infections caused by *B. duttonii* and *B. turicatae* being the most neurotropic. The most common symptoms are meningitis and facial palsy. Many RF cases with neurological involvement, including fatal ones, encompass sequelae with oedema and subarachnoid and parenchymal brain hemorrhages (Judge et al., 1974; Salih et al., 1977; Ahmed et al., 1980). Ocular complications have been reported as well (Cadavid and Barbour, 1998). In addition, depending on the infecting strain and changing with the phases of the disease, several hematological effects can be seen. Among those, a striking and pronounced thrombocytopenia as well as low hemoglobin and erythrocyte counts can be seen. If RF borreliosis remains untreated, a progressive waning of the general condition can occur at later stages, which is often accompanied with severe weakness and weight loss.

Penetration of Tissues and Barriers

Borrelia spirochaetes are known to readily penetrate biological barriers, which can be partly attributed to their helical shape and movement. Thus, flagellar mutants affect their helical shape and movement resulting in a deficient penetration through endothelial layers (Sadziene et al., 1991). However, the use

of the host protease system to penetrate biological barriers during infection has been demonstrated in several studies. No endogenous protease has so far been attributed to *Borrelia* spirochaetes (Klempner et al., 1996), but they have the capacity to bind factors of the plasminogen activation system (PAS) such as the plasminogen (plg) and plg-activator. Klempner and coworkers revealed that the binding of human serine proteases can initiate a proteolytic activity that will be helpful for the efficient invasion during RF borreliosis (Klempner et al., 1996). Several *in vitro* and *in vivo* studies show the importance of the PAS during infections caused by *Borrelia* spirochaetes. These studies revealed that it was involved in the degradation of several substrates (Coleman and Benach, 2000) and also enhanced spirochaetes penetration through the endothelial cell layers (Coleman et al., 1995). In addition, the importance of the PAS was further shown in a series of animal experiments using plasminogen knockout mice ($plg^{-/-}$). These *in vivo* studies revealed that there was a delay of RF borreliae spreading to tissues when the PAS is absent. This effect on penetration and invasion also reduced the bacterial amount in both brain and heart of infected animals (Gebbia et al., 1999; Nordstrand et al., 2001). However, the PAS activity is not the only mechanism in place as there are also indications that there is an additional activation of matrix metalloproteinases (Gebbia et al., 2001). Thus, several host proteases enable the dissemination through biological barriers, but the activity is not a critical factor for initially reaching the circulation, since the $plg^{-/-}$ knockout mice develop, although delayed, spirochaetemia similar to wild-type mice (Gebbia et al., 1999).

Relapsing Fever in Pregnancy

Reports from international organizations claim that approximately 10–15% of neonatal deaths in the World are caused by serious infections (Oza et al., 2015; United Nations Inter-agency Group for Child Mortality, 2017). A part of those infections is possibly caused by RF *Borrelia* in endemic regions. The consequences of RF borreliosis on pregnancy and subsequent pregnancy complications can either be mild with a slight decrease in birth weight and preterm delivery or severe resulting in miscarriage or neonatal death (Goubau and Munyangeyo, 1983; Brasseur, 1985; Melkert and Stel, 1991; Dupont et al., 1997; Jongen et al., 1997; van Holten et al., 1997). Interestingly, Dupont and coworkers reported that in Congo, more than 6% of pregnant women seeking healthcare were diagnosed with RF borreliae (Dupont et al., 1997). Several studies indicated that RF infection might be more severe during pregnancy, which is true for many infections (Goubau and Munyangeyo, 1983; Melkert, 1988). Still, no clear evidence has been presented supporting this statement. In contrast, Fuchs and Oyama published a study in which the mother had an uncomplicated mild RF borreliosis infection with low-grade fever, 3 weeks before labor but with a child fatal outcome only 36 h after delivery (Fuchs and Oyama, 1969). In a pregnancy animal model, it was revealed that RF borreliae can infect the fetus *in utero*. In this model, the *B. duttonii* infection can result in intrauterine growth retardation as well as placental damage with inflammation. Spirochaetes efficiently cross the maternal-fetal barrier, causing an infection of the fetus. It was shown that over 70% of fetuses became infected in the uterus of *B. duttonii* infected mice (Larsson et al., 2006). These infected mice exhibited noticeable intrauterine growth retardation, possibly caused by the histologically observed placental damage and inflammation. In addition, the impaired fetal circulation caused by spirochaete and erythrocyte interactions (see below) as well as lowered maternal hemoglobin was causing the described pregnancy complications in addition to the actual bacterial invasion of the placenta (Larsson et al., 2006).

Antigenic Variations

In response to an exogenous change such as vertebrate immune response, a microorganism can modify its immunodominant antigens by a switch in gene expression of multiphasic surface proteins. RF borreliae represent a well-studied model for these antigenic variations. Once spirochetemia is high, RF borreliae are neutralized by IgM antibody immune response. A small part of bacteria harboring less prevalent antigens can escape the immune system and rises to cause a spirochetaemic relapse (Barbour and Restrepo, 2000; Alugupalli et al., 2003a).

Asymptomatic incubation in human RF borreliosis is estimated at 3–10 days, probably depending on the 4–5 h generation time of spirochaetes in the blood (mouse model) (Crowder et al., 2016). Then, an initial febrile episode followed by a series of relapses (3–5 in LBRF and 9–13 in TBRF) interspaced by a few days remissions are usually described during the course of non-fatal infections in absence of antibiotic treatment (Cutler, 2015). Schematically, each relapse corresponds to the rise of a new immunogenic variant of RF borreliae harboring a changed "Variable major protein" (Vmp) on its surface (**Figure 3**). There are two different families of Vmps, the Variable large proteins (Vlps) of ≈40 kDa and the Variable small proteins (Vsps) of ≈20 kDa. Both Vlps and Vsps families are encoded on linear plasmids (Hinnebusch et al., 1998). Each genome contains a collection of silent 600 bp *vsp* and 100 pb *vlp* copies on linear plasmids, while only one duplicate copy of these archived genes is transcriptionnaly active in a unique telomeric expression site on the same or another linear plasmid. Several mechanisms of gene conversion, DNA rearrangements, hypermutations and change in transcription locus appear to be involved in the replacement of the active *vsp* or *vlp* gene (Meier et al., 1985; Plasterk et al., 1985; Penningon et al., 1999; Dai et al., 2006; Raffel et al., 2014). The repertoire of Vmp encoding genes is highly diverse with 27 (17 *vlp*/10 *vsp*), 59 (38/21) and 82 (68/14) silent cassettes detected in the genomes of *B. recurrentis*, *B. hermsii*, and *B. duttonii*, respectively (Dai et al., 2006; Lescot et al., 2008).

The mechanism of antigenic variation is likely to be a common feature in all the different RF borreliae, since several orthologs of silent cassettes have been found. Up to now, these multiphasic changes have been demonstrated in the following species: *B. hermsii* (Plasterk et al., 1985), *B. turicatae* (Ras et al., 2000), and more recently in *B. miyamotoi* (Wagemakers et al., 2016).

Vsps and Vlps are phylogenetically related to the LD borreliellae surface proteins OspC (Outer Surface protein C) and VlsE (vmp-like sequence E), respectively (Zhang et al., 1997;

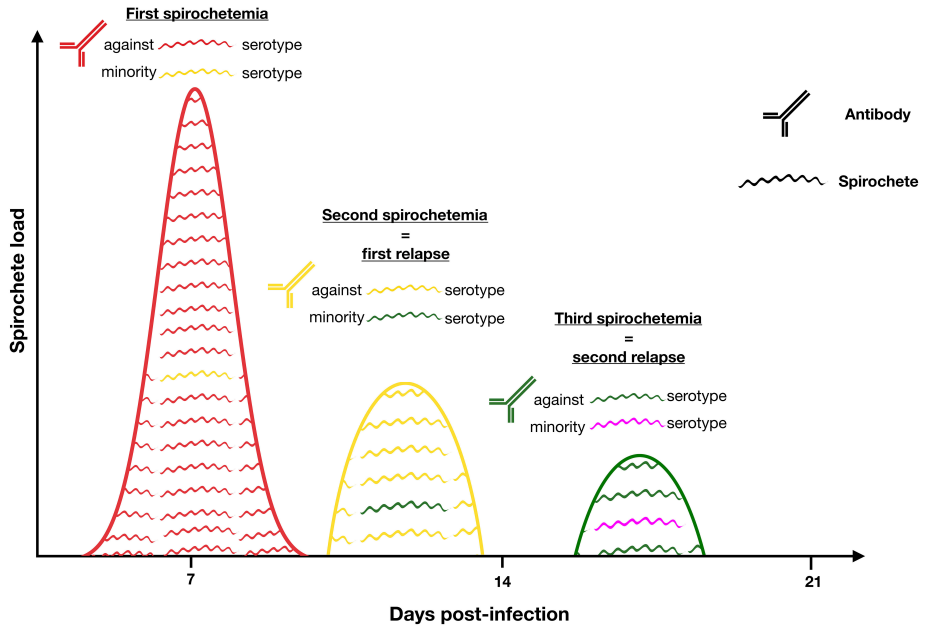

FIGURE 3 | RF increases its persistence in blood by shifting the surface protein Vmp. When antibodies are mounted against the initial serotype (red) all bacteria expressing it are killed by Vmp-specific antibodies. Only those that have shifted to another serotype (yellow) survive and multiply to cause the first relapse. This battle continues until the host dies or the bacteria are eradicated from the blood. Antigenic variation is the mechanism causing the recurring fever which gave the disease its name. Remember that relapses rarely consist of one, single serotype.

Zuckert et al., 2001). In mammals, OspC is involved in the early phase of infection and in replacement VlsE is expressed later for the host immune evasion (Steere et al., 2016).

Beyond the host immune evasion, *in vivo* antigenic variations of *B. turicatae* are correlated to the emergence of different populations of serotypes well adapted to particular organs (e.g., NCS or joints) only depending on the expression of their surface Vmp (Cadavid et al., 1994). These distinctions in tissue tropism for 2 isogenic but antigenically distinct serotypes could be explained at least in part by differences in the ability of Vsps to bind extracellular matrix molecules of the host in link with their respective electrostatic surface properties (Magoun et al., 2000; Lawson et al., 2006). Similar results arguing for the existence of Vmp-related pathotypes are also reported among *B. hermsii* isogenic serotypes and by Vmp mutant experiments (Mehra et al., 2009; Raffel et al., 2014). In addition, Vmps of *B. recurrentis* expressed in the host can act as major TNF-inducing factors likely involved in extremely serious Jarisch-Herxheimer reaction following antibiotic treatment of louse-borne relapsing fever (Vidal et al., 1998).

Persistence

Borreliae spirochaetes are well adapted to persist as long as possible in a susceptible host, thus increasing the possibility of transmission to a naïve host by an arthropod vector. RF borreliae are blood-borne pathogens and when entering a blood vessel they will momentarily be transported to any blood perfused tissue. At the distal ends of the circulatory system, the blood flow is reduced and the speed of transportation of the spirochaetes will be decreased, resulting in a transmigration and invasion of spirochaetes through the endothelium into neighboring tissues and organs. *In vivo* studies on neurotropic RF *Borrelia* species revealed that these species could persist for a long time in the brain without causing any harm in the infected host. Neither any relapsing fever symptoms nor any spirochaetes in the blood were detected indicating a silent infection (Cadavid and Barbour, 1998; Larsson et al., 2006). This phenomenon of silent and persistent infections has not been proven in humans yet. But Cutler (2006) reported a low *B. duttonii* spirochaetemia in the blood of apparently healthy people in a village in Tanzania, demonstrating the importance and ability of RF borreliae to hide in immune privileged sites and cause silent infections.

Interactions in the Circulation

RF borreliae multiply in the circulation, where they can lead to very high spirochaetemia (**Figure 4A**). Besides, some of the Old-World RF *Borrelia* species, eg. *B. duttonii*, *B. crocidurae*, *B. persica* and *B. hispanica* (but not *B. recurrentis*), frequently interact with erythrocytes, causing them to aggregate, a phenomenon called erythrocyte rosetting. Rosetting was early observed by Mooser (1958), and *in vitro* models later suggested that this interaction is a way for the spirochaete to cover itself to escape the immune response. *B. crocidurae* which easily makes rosetting with erythrocytes, has a longer duration of the initial spirochetemia and also a delayed antibody response when compared to the non-rosetting strain *B. hermsii*, indicating that this may be one of the advantages of this strategy (Burman et al., 1998). Another hypothesis concerning the erythrocyte interaction is that the spirochaete picks up nutrients from the cells. In the murine model of *B. duttonii* infection, Larsson

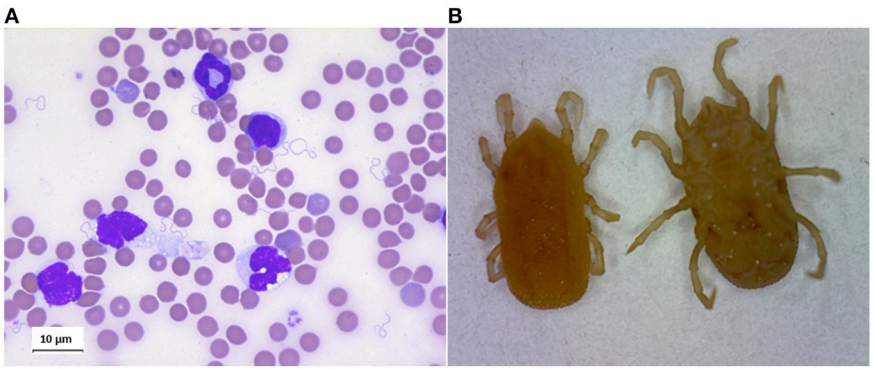

FIGURE 4 | (A) Giemsa staing of blood smear of mouse infected by *Borrelia crocidurae*. **(B)** *Ornithodoros erraticus* adult, dorsal, and ventral views.

et al. have calculated as many as one billion bacteria in every milliliter of blood (Larsson et al., 2006), and obviously a lot of nutrients are needed to maintain the growth of such a population. This grazing theory is supported by the finding that the RF borreliae, in contrast to Lyme disease borreliellae, contain genes for utilization of purines as substrates for RNA and DNA synthesis (Pettersson et al., 2007). Since purine hypoxanthine is produced by red blood cells and abundant in human plasma, the purpose of the interaction with these cells is likely to supply *Borrelia* growth with this and other metabolites (Pettersson et al., 2007). These genes are missing in Lyme disease borreliellae which exceptionally reach such high density in the blood. As anemia and low concentration of erythrocytes are typical features of RF, spirochaete rosetting is likely to affect the red blood cells, as well as other cells in the circulation. Then this might cause a premature removal of affected cells. However, this theory has not been proven yet. Then again, thrombocytopenia, which also a typical consequence of RF borreliosis, is probably caused by spirochaete-platelet interactions such as *B. hermsii* attachment to platelets, resulting in increased platelet loss and prolonged bleeding. The depth of the thrombocytopenia was also linked to the degree of spirochetemia (Alugupalli et al., 2003b).

Finally, RF borreliae interactions with cells in the circulation might be a strategy to increase and prolong the time the pathogen can be maintained within the host. In addition, erythrocyte rosettes can protect the spirochaetes from the host immune defense and the subsequent loss of platelets will facilitate the penetration process into distant tissues.

TICK-*BORRELIA* INTERACTIONS

Vector Specificity

The classification of RF borreliae was historically based on the concept of the specific relationship of arthropod-spirochete, meaning that a given bacterial species was carried by a particular vector and assuming a co-speciation (Wang and Schwartz, 2011). Numerous studies using gene sequencing have confirmed the genotype association both for RF borreliae strains and vectors [e.g., *B. crocidurae-O. sonrai*, (Trape et al., 2013); *B. turicatae-O. turicata* (Schwan et al., 2005)]. Several epidemiological studies have reported other tick-spirochaete associations which are in contradiction to the above vector specificity paradigm. As examples from soft ticks collected in North Africa, *B. crocidurae* DNA was detected in *O. erraticus* (Bouattour et al., 2010), DNA from *B. hispanica* in *O. marocanus*, *O. occidentalis* and *O. kairouanensis,* and *B. merionesi* DNA in *O. costalis* (Trape et al., 2013). Furthermore, some of the genomic species of RF borreliae share their vector with other *Borrelia* species (e.g., *B. microti* and *O. erraticus*; Naddaf et al., 2012). In some cases, the vector is even still unknown (e.g., *Candidatus B. algerica*; Fotso Fotso et al., 2015). However, studies reporting RF borreliae DNA in tick should be interpreted with caution given the fact that DNA traces found in a tick are not necessarily synonymous with vector competence. The proof of concept for vector specificity lies in the vector competence assays (Kahl et al., 2002).

For genomically distant species like *B. duttonii* and *B. anserina*, the transmission was not efficient by exchange of their respective natural *O. moubata* and *Argas* sp. ticks (Nicolle et al., 1928; Felsenfeld, 1971). Regarding closely-related RF borreliae, the cross transmissions of the North American *B. hermsii*, *B. turicatae* and *B. parkeri* have not been experimentally proven into *Ornithodoros* specimens other than their natural vectors *O. hermsi*, *O. turicata* and *O. parkeri*, respectively (Barbour and Hayes, 1986). However, *B. crocidurae* could be efficiently transmitted via a *O. erraticus* blood meal on rodents (Gaber et al., 1984), which is a natural vector of the other African species *B. hispanica* and "*B. microti.*"

Tick Midgut and Salivary Gland Environments

During the multi-host development, soft ticks pass the usual three tick stages: larva, nymphs and male and female adults. While hard ticks have a unique nymphal stage, several nymph stages are present in soft ticks; they may pass through six or more nymphal stages, and females feed several times. After each blood meal they proceed for oviposition (Mehlhorn, 2001). The complete life cycle may last 20 years with prolonged periods of starvation. *Ornithodoros* ticks are also characterized by a quick engorgement, completing the blood meal within five to 60 min after host attachment, generally at night (Sonenshine and Roe, 2013).

As described previously, the STBRF borreliae comprise two main clusters: (1) The New-World TBRF borreliae, and (2) the Old-World TBRF borreliae (**Table 1**). At least 21 different species

of STBRF Borreliae have been identified, associated with their own specific tick vector (Schwan and Piesman, 2002). Specific mechanisms of vector competence have evolved between species of RF borreliae and *Ornithodoros* ticks (**Figure 4B**).

The RF borreliae transmission by soft ticks is characterized by inoculation of saliva during the infective blood meal but also by the secretion of a pathogen-containing liquid from the coxal glands. However, only little work has been done so far to well understand the potential role of soft tick saliva in this process, while many more studies have been done in the hard *Ixodes* ticks, the vector responsible for the transmission of Lyme disease spirochaetes (Hovius et al., 2008; Kazimírová and Štibrániová, 2013; Liu and Bonnet, 2014).

Soft Tick Saliva

The first study performed on *Ornithodoros* saliva demonstrated the presence of antihemostatic activity and of apyrase (Ribeiro et al., 1991). Later on, thanks to the progress in proteomics and transcriptomic techniques, a few more investigations on soft tick saliva were published (Mans et al., 2002; Oleaga et al., 2007; Francischetti et al., 2008, 2009).

As described for hard ticks (*Ixodidae*), argasid saliva supports the feeding process by providing a cocktail of anti-hemostatic, anti-inflammatory and immunomodulatory molecules. The salivary transcriptome of the soft tick *Ornithodoros parkeri* refers to the presence of genes of the lipocalin family, as well as of several genes containing Kunitz domains indicative of serine protease inhibitors. Novel protein families with sequence homology to the insulin growth factor-binding protein (prostacyclin-stimulating factor), adrenomedulin, serum amyloid A protein precursor were characterized in soft ticks. The sialotranscriptome of *O. parkeri* confirms that gene duplication process is a common event in blood-feeding arthropods. Numerous homologies were found with the transcriptome of ixodid ticks (Francischetti et al., 2008). Using proteomic techniques (ED-gels and mass spectrometry analysis), molecules isolated from tick saliva of O. *moubata* and *O. erraticus* were characterized although not as efficiently as with the transcriptomic technique (Oleaga et al., 2007).

Soft Tick Midgut

The midgut of the tick is the first interface encountered by the bacteria during an infective blood meal. Therefore, proteomics studies have also been made on midgut tissue, allowing the identification of concealed antigens in the midgut which might serve as potential candidates for an anti-tick vaccine (Manzano-Román et al., 2006).

In a recently conducted thorough proteome study on *O. moubata*, the main vector of *B. duttonii* in Eastern and Southern Africa, a comparison between fed and unfed midguts was made. Interestingly, it revealed similarities between the blood digestion in hard ticks and in soft ticks (Oleaga et al., 2017). This study completed an earlier investigation on the transcriptome of *O. erraticus*, the vector of relapsing fever in South-Europe and also in Africa (Oleaga et al., 2015).

Bacteria Inside the Tick

Soft Ticks-Borrelia Interactions

Involvement of soft ticks in RF transmission was first described in Africa by Livingstone during his explorations in West-Africa as early as 1857 for *B. duttonii* transmitted by *O. moubata*. Then, Dutton and Todd published in 1905 the detection of a systemic infection of soft ticks with spirochaetes affecting the midgut, synganglion, malpighian tubules, salivary glands, ovary and coxal organs. Interestingly, a Burgdorfer's study in 1951, showed that the transmission occurred not only via tick bite but also by contamination with infected coxal fluid (Dutton and Todd, 1905; Burgdorfer, 1951). In addition, the mode of spirochaete transmission was different in the various tick stages: while the nymph transmits the bacteria with the saliva, the adults mainly transmit via the coxal fluid (Schwan and Piesman, 2002). These works also showed the initial midgut colonization, followed by the migration and the colonization of the salivary glands a few weeks later. Thus, in contrast to infected *Ixodes* where Lyme borreliellae persist in the gut only, RF bacteria infect the tick midgut in unfed ticks, and disseminate to other sites including salivary glands (Schwan and Piesman, 2002).

In the United States, three main species of *Borrelia* are found: *B. turicatae*, *B. hermsii* and *B. parkeri* transmitted respectively by *O. turicata*, *O. hermsii* and *O. parkeri* (Lopez et al., 2016). In Eurasia, the highest infection risk has been identified on the Iberian Peninsula and in Minor Asia (Rebaudet and Parola, 2006). The three main borreliae species there are: *B. hispanica*, *B. crocidurae*, and *B. duttonii* transmitted by *O. erraticus, O sonrai* and *O. moubata* respectively (**Table 1**). However, most of the studies conducted on the interaction tick-*Borrelia* have been accomplished in American models of TBRF.

B. hermsii

It is the primary cause of tick-borne relapsing fever in North America. *B. hermsii* is mainly contracted in remote areas of the Western United States and of British Columbia (Canada). The typical tick habitats are forested mountains at altitudes above 900 m. The main reservoir is rodents (Ogden et al., 2014). To investigate the interaction between *B. hermsii* and *O. hermsii*, a number of significant studies have been conducted by Schwan and collaborators. Looking first into the natural reservoirs, chipmunks and tree squirrels inhabiting coniferous forests, they demonstrated a rapid blood meal of 15–90 min occurring at night. The infected ticks were shown to keep the pathogens for years and to constitute real reservoirs in nature (Schwan and Piesman, 2002). All tick stages, the different nymph stages and the adults, transmitted the pathogen, although transovarial transmission was rare. Coxal transmission does not exist for this *Borrelia* species: *B. hermsii* is only transmitted by tick bite. However, systemic tick infection was demonstrated with these borreliae. An antigenic variation occurring during the process of transmission from the vertebrate host to the tick, was clearly described. The synthesis of a variable tick protein (Vtp formerly Vsp33 or Vmp33) occurs, likely under the influence of environment changes within the tick (pH, temperature and bacteria density) (Schwan and Hinnebusch, 1998). Like OspC for Lyme borreliosis spirochaetes (Grimm et al., 2004; Tilly et al.,

2007), Vtp seems to be essential for the transmission to the vertebrate host. However, Vtp is expressed on all the bacteria in the tick, since in RF the transmission takes place within minutes. While the spirochaetes inside the tick vector express one unique protein, Vtp, the latter spirochaetes found in the blood of infected animals are able to express a multitude of variable major proteins (Vmps).

More precisely, in *B. hermsii*, Vtp production is higher in the spirochaetal population of the salivary glands than in midgut spirochaetes which express mainly Vmp. The proportion of Vtp+ spirochaetes from the salivary glands reaches 50% 35 days post infection and 90% 116 days post infection. A Δ*vtp* mutant of *B. hermsii* remains able to colonize *O. hermsi* but Vtp is not produced at the surface of spirochaetes during the vector phase and the tick-borne transmission is lost. Thereby, Vtp has an essential role in the *B. hermsii* tick-borne transmission (Raffel et al., 2014).

In *B. hermsii*, the interaction of spirochaetes, depleted in variable major proteins (Vmp) with specific antibodies, led to the identification of another tick protein, Alp (BHA128) (Marcsisin et al., 2012). This protein, identified by mass spectrometry, seems to be specifically expressed in the tick since it was produced at a higher level at 23°C than at 34°C. It is strongly expressed in tick salivary glands and expressed at a very low level in the blood of infected animals. Unlike Alp, *vtp* genes of *B. hermsii* share a high diversity among field spirochaetal population and are likely involved in horizontal genomic transfer and recombination events between strains (Porcella et al., 2005). This may be in part explained by a pathogen strategy influencing Vtp variability to avoid the vertebrate immune memory against previous transmitted Vtp+ spirochaetes (Marcsisin et al., 2016).

In this same model, *B. hermsii–O. hermsi*, a vaccine was tested using the Vtp antigen (Krajacich et al., 2015). The *vtp* gene from two isolates of *B. hermsii* was cloned and expressed as recombinant Vtps to vaccinate mice. Mice were protected only if they were challenged by *O. hermsi* ticks that were infected with the homologous strain of *B. hermsii* from which the *vtp* gene originated. Such a vaccine points out the difficulty to set up a protective vaccine against bacteria with such a high-protein diversity.

B. turicatae

This *Borrelia* is transmitted by *O. turicata* and present in the southern United States and in Latin America. Risks for exposure to *O. turicata* and *O. parkeri* occur primarily in semi-arid plains. *O. turicata* are parasites of ground dwelling and burrowing animals including tortoises (Adeyeye and Butler, 1989; Donaldson et al., 2016). In a mouse model used to investigate the process of transmission, the authors analyzed the dissemination in the blood by qPCR, dark field microscopy and serological responses. The transmission was found to occur within a minute, and dissemination into the blood was also very rapid. Inside the tick, *B. turicatae* entered the midgut and invaded the salivary glands during the following weeks (Boyle et al., 2014). This is clearly distinct from the LD borreliellae that remain in the midgut only. The blood meal on the vertebrate host triggers the migration of Lyme spirochaetes toward the salivary glands due to physico-chemical changes (pH, temperature, nutrients) (Schwan et al., 1995; Piesman et al., 2001; Schwan and Piesman, 2002). A more recent study by the same group completed these data, using the recent technology of the green fluorescent protein (gfp). It confirmed the systemic infection of ticks and also a persistent infection of the tick midgut and salivary glands for at least 18 months (Krishnavajhala et al., 2017). The spirochaetes were shown to be maintained transstadially, i.e., during six or more nymphal stages before molting to adults. After blood feeding on the vertebrate host, the salivary gland lumen of infected ticks remained positive. This indicates that the *Borrelia* inoculum is probably low, as described for Lyme borreliosis spirochaetes (Kern et al., 2011; Bockenstedt et al., 2014). The midgut remains positive after the blood meal as well. This midgut population likely replenishes the salivary glands after the infective blood meal. It also explains the rapid transmission of bacteria to the vertebrate host during the timely short blood meal of soft ticks (Krishnavajhala et al., 2017).

Using microarrays on *B. turicatae* grown *in vitro* at 22°C, thus mimicking the tick environment, a protein of 40 kilodaltons was identified and designated *Borrelia* repeat protein A (BrpA) due to the repetition of a particular amino acid motif. Deletion of the respective *brpA* gene did neither avoid the infection of mice when inoculated by needle, nor inhibit further colonization of the *O. turicata* salivary glands and the subsequent transmission (Lopez et al., 2013).

Finally, it has been postulated that the tick salivary glands might constitute a selective environment for a particular *Borrelia* species. Indeed, in different tick species (*O. hermsi*, *O. parkeri*, and *O. turicata*) engorged on mice infected with *Borrelia hermsii*, only the association *O. hermsi-B. hermsii* was able to further transmit *Borrelia* to naïve mice (Schwan, 1996). *Borrelia*-gfp should help to understand the mechanisms responsible for the specificity of the interactions vector-pathogen (Krishnavajhala et al., 2017).

HARD TICK-TRANSMITTED RELAPSING FEVERS

Although the majority of RF spirochaetes infections occur through soft ticks, few spirochaetes species are transmitted by hard ticks. This includes *B. theileri*, *B. miyamotoi* and *B. lonestari* which are transmitted by *Rhipicephalus* spp., *Ixodes* spp. and *Amblyomma americanum* ticks, respectively (**Table 1**). Very few studies have been conducted on these systems, likely because their pathogenicity to humans is not clearly established except for *B. miyamotoi* (Platonov et al., 2011). Considering all RF borreliae, a relative specificity can be postulated since the phylogenetically close species *B. miyamotoi*, *B. theileri*, and "*B. lonestari*" are associated with hard tick as vectors, while others are transmitted by soft ticks (*Argas* spp. for *B. anserina* or *Ornithodoros* spp.). Interestingly, newly described genotypes close to "*B. lonestari*/*B. theileri*" have been detected in hard ticks in Japan (*Haemaphysalis* sp. or *Amblyomma* sp.) and in Ethiopia (*Rhipicephalus* sp.) (Takano et al., 2012; Kumsa et al., 2015; Furuno et al., 2017), but "*Ca.* B. texasensis" close to the

North American *B. parkeri*/*B. turicatae* according to *flaB* and *rrs* sequences is associated with the dog hard tick *Dermacentor variabilis* rather than an *Ornithodoros* soft tick (Lin et al., 2005).

Borrelia miyamotoi

Although considered as worldwide species, the evolution of *B. miyamotoi* may also have been under the influence of its global distribution because this species is represented by the Siberian, European and American genotypes (*glpQ*, 16S rDNA, and/or *flaB*. *SLPA*) (Mun et al., 2006; Crowder et al., 2014; Takano et al., 2014). Geller and collaborators (Geller et al., 2012) have shown that the Asian genotype of *B. miyamotoi* could be associated as well with *I. ricinus* as with *I. persulcatus* ticks in a sympatric region of Estonia. In addition, *B. miyamotoi* genotypes are characterized by a very low diversity within an area, and even considered as genetically clonal isolates after MLSA whatever the sources *I. persulcatus*, *I. pavlovskyi* ticks or vertebrates in Hokkaido, Japan (Takano et al., 2014).

Reservoir

There is little information concerning *B. miyamotoi* reservoirs, as this bacterium shares common characteristics with the bacteria belonging to the *B. burgdorferi* sl complex, it can be easily hypothesized that they share the same reservoir hosts. Small rodents such as the white-footed mouse (*Peromyscus leucopus*) in North America (Bunikis and Barbour, 2005; Barbour et al., 2009; Hamer et al., 2012); *Apodemus argenteus* (Fukunaga et al., 1995), *Apodemus speciosus*, *Myodes rufocanus*, *Myodes rutilus* (Taylor et al., 2013) in Japan; *Apodemus flavicollis* (Szekeres et al., 2015; Hamšíková et al., 2017), *Myodes glareolus* (Hamšíková et al., 2017; Wagemakers et al., 2017), *Apodemus sylvaticus*, *Microtus arvalis* (Wagemakers et al., 2017) have been found to harbor *B. miyamotoi* DNA. Identically *B. miyamotoi* DNA has been found in birds like *Meleagris gallopavo* (Scott et al., 2010), *Carduelis chloris* and *Parus major* (Wagemakers et al., 2017). Only *Apodemus* spp. mice, *M. glareolus* (Burri et al., 2014) and *P. leucopus* (Scoles et al., 2001) are experimentally proven reservoir hosts, indeed *B. miyamotoi* horizontal transmission to naïve ticks has been observed. However, there is a lower rate of transmission than the one noticed for the *B. burgdorferi* sl complex bacteria. It has also been observed that *B. miyamotoi* infection rate of wild rodents is age-independent whereas *B. burgdorferi* sl infection rate is age-dependent, these data suggest that *B. miyamotoi* is shortly maintained by the small rodents (Taylor et al., 2013). Conversely to what is observed for the *B. burgdorferi* sl complex bacteria, domestic ruminants do not seem to eliminate *B. miyamotoi* (Richter and Matuschka, 2010) and may play a role in its dissemination.

Interaction Vertebrate Host Bacteria

Until 2011, pathogenicity of *B. miyamotoi* was unknown. Interest in this bacterium has grown up since 2011 when a series of 46 Russian cases were published (Platonov et al., 2011). Its pathogenicity was then precise with the report of two meningoencephalitis cases in highly immunocompromised patients (Gugliotta et al., 2013; Hovius et al., 2013), an additional case was later reported in Germany (Boden et al., 2016). The three cases of *B. miyamotoi* meningoencephalitis seem to display common characteristics, indeed they were treated for non-Hodgkin lymphoma with a CHOP protocol (cyclophosphamide, doxorubicin, vincristine and prednisolone), they also received an anti-CD20 monoclonal antibody (rituximab) which is known to deplete B-cell lymphocytes. All 3 cases of *B. miyamotoi* presented a cerebrospinal fluid pleocytosis with an elevation of proteins as observed for the Lyme neuroborreliosis. Clinically there is a disparity between the German case who had acute symptoms (Boden et al., 2016) and the other two (Gugliotta et al., 2013; Hovius et al., 2013) who evolved on a more chronic mode. These reports highlight that *B. miyamotoi* has a neurotropism whose physiopathology remains unknown.

Data describing the typical *B. miyamotoi* disease (BMD) in apparently immunocompetent patients come from 2 series of cases from Russia (Platonov et al., 2011) and from the United States (Molloy et al., 2015). The most reported clinical form is an acute febrile viral-like illness occurring around 2 weeks after the tick bite which can evolve toward one or more relapses (Platonov et al., 2011) for 11% of patients in the Russian cohort. In addition to fever, the symptoms include chills, headache, myalgia, arthralgia, malaise and fatigue. BMD and human anaplasmosis share common clinical characteristics and must be the subject of a differential diagnosis (Chowdri et al., 2013; Molloy et al., 2015). *B. miyamotoi* broadens the circle of the aetiological agents responsible for post-tick bite febrile syndromes.

The study of the physiopathology of diseases relies on experimental models and *in vivo* studies allow a global approach of the host/bacterial interactions. There are few data on the animal model for *B. miyamotoi* infection. It has been shown that SCID mice develop a prolonged spirochaetemia (up to 20 days) following an intra-peritoneal injection of *B. miyamotoi* culture (Krause et al., 2015; Wagemakers et al., 2016). C3H/HeN mice develop a peak in spirochaetemia 2 days after an intraperitoneal injection, followed by spirochaetemic relapse of a lower intensity in three out of the eight C3H/HeN mice used for the experiment (Wagemakers et al., 2016).

Concerning the interaction of *B. miyamotoi* with the host immune system, like other pathogens responsible for STBRF (*B. parkeri*, *B. duttonii*, and *B. hermsii*), *B. miyamotoi* is resistant *in vitro* to the human complement whereas *B. anserina*, a non-pathogenic STBRF for humans, is sensitive to the human complement (Teegler et al., 2014; Wagemakers et al., 2014). It has been observed that the activated complement components (i.e. C3, C5, C7, C8, C9, and the membrane attack complex) poorly or do not bind to *B. miyamotoi* under *in vitro* conditions, suggesting that the inhibition of the complement cascade occurs at the C3 activation level (Teegler et al., 2014). This mechanism was initially poorly understood since a protein assimilated to OspE, identified in *B. miyamotoi*, is unable to bind to factor H (McDowell et al., 2003). Recently, a protein factor CbiA (complement binding and inhibitory protein A) (Röttgerding et al., 2017) has been identified as binding and inhibiting the human complement at different levels. CbiA is a *B. miyamotoi* outer surface protein which binds to the complement regulators factor H and C4b-binding protein. Moreover, factor H bound

to CbiA can interact with factor I to inactive C3b. Additionally, CbiA binds to C3, C3b, C4b, and C5 in a dose dependent manner. Finally, CbiA inhibits activation of the classical and terminal complement pathway.

Borrelia lonestari

Reservoir

Borrelia lonestari reservoir host is not clearly identified. As the white-tailed deer (*Odocoileus virginiatus*) is a reservoir host for several *A. americanum*-associated pathogens (Allan et al., 2010) it can be easily hypothesized that the white-tailed deer could be a reservoir for *B. lonestari* in nature. Moreover, it has been shown to develop a bacteraemia following a *B. lonestari* injection (Moyer et al., 2006) and *B. lonestari* was detected in its blood compartment (Moore et al., 2003). However, serological studies supporting contact between *O. virginiatus* and *B. lonestari* are contradictory (Krishnavajhala et al., 2017). Interestingly, the DNA of a *Borrelia* species very close to *B. lonestari* was detected in *C. nippon yesoensis* (Lee et al., 2014) suggesting that cervids could be, indeed, reservoir hosts for *B. lonestari* and closely related species. Other observations mentioned that *B. lonestari* DNA was found in several birds particularly in turkeys (Jordan et al., 2009). Further investigations must be led to better understand the ecology of *B. lonestari*.

Interaction Vertebrate Host Bacteria

Borrelia lonestari is found in *Amblyoma americanum* also known as the Lone Star tick and gave its name to this relapsing fever *Borrelia*. Initially researchers hypothesized that the southern tick-associated rash illness (STARI) was caused by *B. lonestari*. Indeed, patients frequently bitten by *A. americanum* developed symptoms similar to erythema migrans observed in Lyme disease (Armstrong et al., 1996; Kirkland et al., 1997). The parallel between erythema migrans/*B. burgdorferi* sl bacteria, and, STARI/*B. lonestari* can easily be done and *B. lonestari* DNA was found in the skin biopsy of a patient with a STARI (James et al., 2001). However, since its discovery (Lin et al., 2005) *B. lonestari* implication in the STARI has been discussed. More recent observations (Wormser et al., 2005; Feder et al., 2011) demonstrate that STARI occurs without proof of *B. lonestari* implication. So far, there is no proof of *B. lonestari* implication in human or animal pathology.

Borrelia theileri

Reservoir

Borrelia theileri has been observed in the blood compartment of cattle (Laveran, 1903), sheep (Theiler, 1905), and in a horse (Callow, 1967). However, horses had previously been reported as non-susceptible to this infection (Theiler, 1905). The cattle can be considered as the main reservoir host for *B. theileri* since it has been demonstrated that the cattle can infect naïve ticks (Theiler, 1909; Brumpt, 1919; Trees, 1978) and since *B. theileri* is vectored by ticks belonging to the genus *Rhipicephalus* (*R. annulatus*, *R. microplus*, *R. decoloratus*, *R. evertsi*) which preferentially parasites the cattle (Guglielmone et al., 2014).

Interaction Vertebrate Host Bacteria

To our knowledge there is no report of human infection by *B. theileri*. Its pathogenicity is expressed in cattle but remains low (Callow, 1967; Smith et al., 1978). Work by L. L. Callow reports a transitory rise of the rectal temperature and occasionally a slight depression with anorexia and hemoglobin level decrease has been observed in a splenectomized calf (Callow, 1967).

CONCLUSION

Compared to Lyme borreliosis, RF diseases remain poorly investigated. The phylogeny of RF bacteria still deserves further investigation to better understand the complex interactions *Borrelia*-tick-vertebrate host and establish adapted models. It is particularly true for bacteria transmitted by soft ticks which represent most of the bacteria in RF. For example, the soft tick saliva is not so well studied. While numerous saliva tick proteins have been characterized in hard ticks and demonstrated essential in pathogen transmission, very few ones have been identified in *Ornithodoros* ticks. Similarly, the process of transmission and persistence of RF bacteria in vertebrate host is not clarified, although antigenic variations and erythrocytes rosetting have been described as potential virulence factors. The emergence of *B. miyamotoi* transmitted by hard ticks these last years might draw more attention to these diseases which are present in tropical as well as temperate countries.

AUTHOR CONTRIBUTIONS

ET-R, PB, SB, LV, and NB conducted the literature research, wrote the paper and prepared the figures and tables. All authors provided critical reviews and revisions.

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The Distinct Transcriptional Response of the Midgut of *Amblyomma sculptum* and *Amblyomma aureolatum* Ticks to *Rickettsia rickettsii* Correlates to Their Differences in Susceptibility to Infection

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Rickettsia rickettsii is a tick-borne obligate intracellular bacterium that causes Rocky Mountain Spotted Fever (RMSF). In Brazil, two species of ticks in the genus *Amblyomma*, *A. sculptum* and *A. aureolatum*, are incriminated as vectors of this bacterium. Importantly, these two species present remarkable differences in susceptibility to *R. rickettsii* infection, where *A. aureolatum* is more susceptible than *A. sculptum*. In the current study, *A. aureolatum* and *A. sculptum* ticks were fed on suitable hosts previously inoculated with *R. rickettsii*, mimicking a natural infection. As control, ticks were fed on non-infected animals. Both midgut and salivary glands of all positively infected ticks were colonized by *R. rickettsii*. We did not observe ticks with infection restricted to midgut, suggesting that important factors for controlling rickettsial colonization were produced in this organ. In order to identify such factors, the total RNA extracted from the midgut (MG) was submitted to next generation RNA sequencing (RNA-seq). The majority of the coding sequences (CDSs) of *A. sculptum* differentially expressed by infection were upregulated, whereas most of modulated CDSs of *A. aureolatum* were downregulated. The functional categories that comprise upregulated CDSs of *A. sculptum*, for instance, metabolism, signal transduction, protein modification, extracellular matrix, and immunity also include CDSs of *A. aureolatum* that were downregulated by infection. This is the first study that reports the effects of an experimental infection with the highly virulent *R. rickettsii* on the gene expression of two natural tick vectors. The distinct transcriptional profiles of MG of *A. sculptum* and *A. aureolatum* upon infection stimulus strongly suggest that molecular factors in this organ are responsible for delineating the susceptibility to *R. rickettsii*.

Functional studies to determine the role played by proteins encoded by differentially expressed CDSs in the acquisition of *R. rickettsii* are warranted and may be considered as targets for the development of strategies to control the tick-borne pathogens as well as to control the tick vectors.

Keywords: spotted fever, tick, rickettsiae, *Rickettsia rickettsii*, *Amblyomma*, midgut, RNA-seq, transcriptome

INTRODUCTION

Rocky Mountain Spotted Fever (RMSF), also known as Brazilian Spotted Fever (BSF), is a severe tick-borne illness caused by *Rickettsia rickettsii*. In Brazil, *R. rickettsii* is transmitted to humans by *Amblyomma sculptum*, formerly named *Amblyomma cajennense* (Nava et al., 2014), and *Amblyomma aureolatum* (Labruna, 2009). The prevalence rates of *R. rickettsii*-infected ticks in endemic areas are low, oscillating around 1%. These low prevalence rates seems to be associated with lower reproductive and survival rates of infected ticks, suggesting that *R. rickettsii* is also pathogenic to its vectors (Burgdorfer, 1988; Niebylski et al., 1999; Labruna et al., 2008). Previous experimental infections with *R. rickettsii* have demonstrated that 80–100% of *A. aureolatum* ticks from a laboratory colony acquire this bacterium, whereas only 10 to 60% of *A. sculptum* ticks become infected (Labruna et al., 2008). These results showed that *A. aureolatum* is more susceptible to rickettsial infection than *A. sculptum*.

The midgut (MG) is the first tick organ that blood meal-acquired microbes (including tick-borne pathogens) interact with. The neutral pH and the nutrients of the host blood favor microbial proliferation in the lumen of the tick MG. In addition, microbes do not directly contact digestive enzymes, as digestion in ticks is intracellular (Sojka et al., 2008). Therefore, to efficiently prevent microbial growth, ticks must present a robust immune response in MG (Hajdusek et al., 2013). Indeed, different antimicrobial peptides (AMPs) and/or their transcripts have been detected in this organ, such as hemocidins (host hemoglobin-derived antimicrobial peptides) (Fogaca et al., 1999; Nakajima et al., 2003; Sonenshine et al., 2005; Belmonte et al., 2012), defensins (Nakajima et al., 2001, 2002; Hynes et al., 2005; Rudenko et al., 2005; Ceraul et al., 2007; Zhou et al., 2007), and lysozymes (Kopacek et al., 1999; Grunclova et al., 2003; Simser et al., 2004; Ceraul et al., 2007). Moreover, transcriptomics data suggest that tick MG contains proteins involved in the production of reactive oxygen species (ROS), which may also help to control microorganism growth (Anderson et al., 2008). To be successfully transmitted to another host, tick-borne pathogens must then be able to resist to the immune reactions of tick MG, transpose this barrier, and reach the salivary glands through hemolymph.

In the current work, we present the first global transcription profile of MG of *A. sculptum* and *A. aureolatum* in response to infection with *R. rickettsii*. Importantly, ticks were infected by feeding on hosts previously inoculated with *R. rickettsii*, mimicking a natural infection. The analyses were performed using next generation RNA sequencing (RNA-seq) and data were validated by quantitative polymerase chain reaction preceded by reverse transcription (RT-qPCR). The proteins encoded by differentially expressed CDSs in the colonization of tick MG by *R. rickettsii* should be functionally characterized and may be considered as targets for the development of strategies to control the tick borne pathogens as well as to control the tick vectors.

Abbreviations: MG, Midgut; SG, salivary glands; AsC, *Amblyomma sculptum* control; AaC, *Amblyomma aureolatum* control; AsI, *Amblyomma sculptum* infected; AaI, *Amblyomma aureolatum* infected; CDSs, coding sequences.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were performed in strict accordance with the Institutional Animal Care and Use Committees from of the Faculty of Veterinary Medicine and Animal Husbandry (protocol number 1423/2008) and the Institute of Biomedical Sciences (protocol number 128/11) of the University of São Paulo, São Paulo, Brazil. Animal purchases and euthanize procedures were performed as described in Galletti et al. (2013).

R. rickettsii-Infected and Non-infected Ticks

Ticks were obtained from laboratory colonies of *A. sculptum* (Pedreira strain; state of São Paulo, Brazil) and *A. aureolatum* (Atibaia strain; state of São Paulo, Brazil). *A. sculptum* larvae, nymphs, and adults were fed on rabbits (*Oryctolagus cuniculus*), as previously described (Pinter et al., 2002). Feeding of *A. aureolatum* developmental stages was accomplished following the methodology detailed in Galletti et al. (2013). To obtain infected ticks, larvae were fed on hosts previously infected with the highly virulent Taiaçu strain of *R. rickettsii*, using the procedure described by Pinter and Labruna (2006) and Galletti et al. (2013). Off-host phases were held in an incubator at 25°C and 95% relative humidity.

Adult ticks from the *R. rickettsii*-infected and non-infected groups were manually removed from the vertebrate hosts after 3 days of feeding. The ticks were washed in 70% ethanol and sterile phosphate-buffered saline (PBS) (10 mM NaH_2PO_4, 1.8 mM KH_2PO_4, 140 mM NaCl, and 2.7 mM KCl, pH 7.4) for 10 min each. Midgut (MG) and salivary glands (SG) of each tick were dissected and separately transferred to 100 μL of RNA*later*® Solution (ThermoFisher Scientific).

Nucleic Acid Extraction

The SG and MG of each adult tick were homogenized and submitted to a simultaneous isolation of genomic DNA (gDNA) and total RNA using the InviTrap® Spin Cell RNA Mini Kit (Stratatec), according to the manufacturer's specifications.

R. rickettsii Quantification

gDNA was used to quantify the total number of rickettsiae in tick MG by real-time quantitative PCR (qPCR) using a hydrolysis probe for the citrate synthase gene (*glt*A) of *R. rickettsii*, as previously described by Galletti et al. (2013). All samples were analyzed in three technical replicates. Samples of gDNA extracted from control ticks were also tested to confirm the absence of infection.

RNA-Seq, Assembly and Annotation

To perform mRNA sequencing (RNA-seq) analysis, total RNA extracted from the MG of adult females were pooled, according to the following description: eight *A. sculptum* harboring $2.2 \times 10^7 \pm 2.4 \times 10^7$ rickettsiae and ten *A. aureolatum* presenting $4.8 \times 10^7 \pm 2.7 \times 10^7$ rickettsiae composed the infected samples (AsI and AaI, respectively); and eight non-infected adult females of *A. sculptum* and 10 non-infected *A. aureolatum* composed the control samples (AsC and AaC, respectively). Each tick contributed equally for the composition of the RNA pool samples. Samples were tagged and multiplex sequenced in four lanes using a HiSeq™ sequencing system (Illumina) at the North Carolina State University facility (North Carolina, USA).

Near 567 million reads of 101 base pairs were obtained using the single read mode (these reads also include the transcriptomes of SG of *A. aureolatum* and *A. sculptum*; data not shown). Reads for each species were assembled together using Abyss and Soapdenovo Trans programs with *K*-values varying from 21 to 91 (in 10 interval increments). Resulting assemblies were concatenated and clustered using the blastn tool (performed locally from executables obtained at the NCBI FTP site ftp://ftp.ncbi.nih.gov/blast/executables/) (Altschul et al., 1990) and CAP3 assembler (Huang and Madan, 1999) by a decreasing word size inclusion strategy, as described by Karim et al. (2011), starting at 300 and ending in 60. Coding sequences (CDSs) were extracted as detailed by Karim et al. (2011), based on matches to public databases or longer open reading frames with a signal peptide as an indicative of secretion. Data were organized in a hyperlinked spreadsheet as described by Ribeiro et al. (2004). The blastx (Altschul et al., 1997) tool was used to compare the predict amino acid sequences translated from the nucleotide sequences to the NR protein database of the NCBI and to the Gene Ontology (GO) database (Ashburner et al., 2000). The tool reverse position-specific BLAST (rpsblast) (Altschul et al., 1997) was used to search for conserved protein domains in the Pfam (Bateman et al., 2000), SMART (Schultz et al., 2000), KOG (Tatusov et al., 2003), and conserved domains databases (CDD) (Marchler-Bauer et al., 2002). To identify putative secreted proteins, predicted proteins starting with a methionine residue were submitted to the SignalP server (Nielsen et al., 1997). In addition, the program NetOGlyc (Julenius et al., 2005) was used to predict glycosylation sites on the proteins. The functional annotation of the CDSs was carried out according to the their *e*-values and their order of appearance on the comparisons described above, as detailed in Karim et al. (2011).

To compare the gene expression between samples [*A. sculptum* infected (AsI) vs. non-infected (AsC) and *A. aureolatum* infected (AaI) vs. non-infected (AaC)], paired comparisons of the number of reads hitting each contig were calculated by X^2 tests to detect significant differences between samples, where the minimum considered value was larger than 5 and $p < 0.05$. Normalized fold-ratios of the sample reads were computed by adjusting the numerator by a factor based on the ratio of the total number of reads in each sample, and adding one to the denominator to avoid division by zero.

The complete dataset of *A. sculptum* and *A. aureolatum* can be downloaded from the National Center for Biotechnology Information (NCBI). The raw data were deposited to the Sequence Read Archives (SRA) of the NCBI under bioprojects numbers PRJNA343654 (*A. sculptum*, raw reads runs SRR4277085, SRR4277086, SRR4277087, SRR4277088, and SRR4277089), and PRJNA344771 (*A. aureolatum*, raw reads runs SRR4301100, SRR4301108, SRR4301110, and SRR4301120). This Transcriptome Shotgun Assembly (TSA) project has been deposited at DDBJ/EMBL/GenBank under the accession GFAA00000000 (for *A. sculptum*) and GFAC00000000 (for *A. aureolatum*). Only CDS representing 90% of known proteins or larger than 250 amino acids were deposited.

When required, phylogenetics and topology analyses of predicted proteins were performed using the program Clustal omega (European Bioinformatics Institute; EMBL-EBI) with default parameters.

Real-Time Quantitative PCR Preceded by Reverse Transcription (RT-qPCR)

Five hundred nanogram of the total RNA extracted from the MG of infected (AsI and AaI) or control (AsC and AaC) ticks were treated with RQ1 RNase-free DNase (Promega) and reverse transcribed (RT) in cDNA using M-MLV Reverse Transcriptase (ThermoFisher Scientific), as detailed by manufacturer. Resulting cDNA was used as template in qPCR with the Maxima SYBR Green/ROX qPCR MasterMix (ThermoFisher Scientific) and specific primers for selected CDSs (Supplementary Table 1). Primers were designed using Primer3 (Rozen and Skaletsky, 2000) and synthesized by ThermoFisher Scientific. qPCR was performed on a StepOnePlus™ Real-Time PCR System (ThermoFisher Scientific), using the following program: 95°C for 10 min followed by 40 cycles at 95°C for 15 s, 60°C for 60 s, and 72°C for 20 s. In addition, a melting curve analysis was carried out to check the specificity of the primers. To determine the efficiency of each pair of primers, standard curves were generated using different concentrations of cDNA (400–3.12 ng; 2-fold dilution).

The $2^{-\Delta\Delta Ct}$ equation was utilized to calculate the relative expression of select CDSs in infected vs. non-infected ticks (Livak and Schmittgen, 2001). The CDS of the ribosomal protein S3A was used as reference (Supplementary Table 1). Eight biological replicates from *A. sculptum* and nine from *A. aureolatum* were analyzed. Each biological replicate corresponds to one tick MG. Student's *t*-test was used to statistically validate the differentially expressed CDSs. To verify the reproducibility of both gene expression measurement techniques, we calculated the Pearson's correlation coefficient between RNA-seq and RT-qPCR.

RESULTS

Initially, adult females of *A. sculptum* and *A. aureolatum* infected with *R. rickettsii* were obtained by a laboratory-controlled experimental infection. The presence of rickettsiae in midgut (MG) and salivary glands (SG) of ticks was assessed by qPCR. Only fifteen among 100 *A. sculptum* adult females (15%) were positively infected, while 27 from 29 *A. aureolatum* adult females (93%) acquired *R. rickettsii*. Importantly, both MG and SG of all infected ticks were colonized by *R. rickettsii*.

The RNA extracted from the MG of control and infected *A. sculptum* and *A. aureolatum* [*A. sculptum* control (AsC), *A. aureolatum* control (AaC), *A. sculptum* infected (AsI), and *A. aureolatum* infected (AaI)] were multiplex sequenced using an Illumina HiSeq platform. The sequencing of *A. scultpum* RNA samples resulted in 324 million reads of 101 base pairs, which were assembled in 9,560 CDSs. For *A. aureolatum*, near 242 million reads were obtained and assembled in 11,906 CDSs (**Table 1**).

To identify coding sequences (CDSs) differentially expressed by infection with *R. rickettsii*, we compared the number of reads mapped to each contig in infected vs. non-infected *A. sculptum* (**Table 2** and Supplementary Tables 2, 3) and *A. aureolatum* (**Table 3** and Supplementary Tables 4, 5). Infection modulated 479 CDSs in the MG of *A. sculptum*, among which 416 were upregulated (Supplementary Table 3) and only 63 were downregulated (Supplementary Table 2). Differently, from 310 CDSs of *A. aureolatum* modulated by infection, 237 were downregulated (Supplementary Table 4) and only 73 were upregulated (Supplementary Table 5). In order to validate the transcription pattern obtained by RNA-seq, selected CDSs were analyzed by RT-qPCR (**Tables 2**, **3** for *A. sculptum* and *A. aureolatum*, respectively). The correlation between transcriptional data obtained by these two methods was 0.69 ($p = 0.037$) for *A. sculptum* and 0.73 ($p = 0.039$) for *A. aureolatum*, strengthening the transcriptional data obtained by RNA-seq.

Most of the CDSs upregulated by *R. rickettsii* infection in *A. sculptum* belongs to metabolism, protein modification, secreted, signal transduction, transporter and channels, and transposon elements functional categories (**Figure 1**). Differently, CDSs in these same functional categories were majorly downregulated in *A. aureolatum*. Moreover, CDSs of *A. sculptum* in protein synthesis and storage categories were majorly downregulated by infection, while CDSs in immunity, proteasome, transcription factor, and virus categories were mostly upregulated. In *A. aureolatum*, CDSs in cytoskeletal category were remarkably downregulated by infection, whereas CDSs in transcription machinery and detoxification categories were upregulated (**Figure 1**).

In metabolism category, 11 CDSs were upregulated by infection in *A. sculptum* (Supplementary Table 3), while none was downregulated (Supplementary Table 2). On the other hand, 10 CDSs inside this same category were downregulated in *A. aureolatum* (Supplementary Table 4), while three were upregulated (Supplementary Table 5). Among them, only one share the same annotation (pancreatic lipase-like) in both *A. sculptum* (Acaj-69206) and *A. aureolatum* (Ambaur-63833). In addition, two sequences coding glucose dehydrogenases were downregulated in *A. aureolatum* (AmbarSigP-52021 and AmbarSigP-66386; **Table 3** and Supplementary Table 4). In *A. sculptum*, two glycolate oxidase CDSs (AcajSigP-74567 and AcajSigP-79349) were upregulated (**Table 2** and Supplementary Table 3).

In protein modification category, three CDSs of serine carboxipeptidases (Acaj-72629, Acaj-72628, and Acaj-72627) were upregulated by infection in *A. sculptum* MG (**Table 2** and Supplementary Table 3), while none was downregulated. Differently, in *A. aureolatum*, two serine carboxipeptidases CDSs (Ambaur-58705 and AmbarSigP-15638, this last one included in secreted category) were downregulated (**Table 3** and Supplementary Table 4) and one (AmbarSigP-13149) was upregulated (**Table 3** and Supplementary Table 5). Moreover, 15 metalloprotease CDSs (predicted to be secreted or not) were upregulated in *A. sculptum* (Supplementary Table 3), while eight were downregulated in *A. aureolatum* (Supplementary Table 4). None metalloprotease CDS was detected to be downregulated in *A. sculptum* or upregulated in *A. aureolatum*. Phylogenetics and amino acid alignment analysis showed that, among the metalloproteinases of *A. aureolatum*, six are reprolysins and two are astacins (Supplementary Figure 1). In relation to the metalloproteases of *A. sculptum*, 14 are reprolysins and only one is astacin (Supplementary Figure 1).

Among the differentially expressed sequences that code secreted proteins with annotated function, we highlight lipocalins, serine proteinase inhibitors, principally members of Kunitz family, and mucins. Twenty six lipocalin CDSs were upregulated in *A. sculptum* MG (Supplementary Table 3) and only two in *A. aureolatum* (Supplementary Table 5). On the other hand, 30 lipocalin CDSs were downregulated in *A. aureolatum* (Supplementary Table 4), while none was downregulated in *A. sculptum*. In addition, five sequences encoding Kunitz inhibitors were downregulated by infection in *A. aureolatum* (**Table 3** and Supplementary Table 4) and only one was upregulated (**Table 3** and Supplementary Table 5). An opposite panorama was observed in *A. sculptum*, with eight CDSs of Kunitz inhibitors upregulated by *R. rickettsii* infection (**Table 2** and Supplementary Table 3) and none downregulated. In relation to sequences encoding mucins, one CDS (Acaj-39037) was detected to be downregulated in *A. sculptum* by RNA-seq analysis (**Table 2** and Supplementary Table 2). In *A. aureolatum*, four secreted mucin CDSs (Ambaur-60941, Ambaur-63342, Ambaur-63341, and AmbarSigP-64116) were downregulated (**Table 3** and Supplementary Table 4).

In signal transduction category, *R. rickettsii* upregulated the transcription of 11 CDSs in *A. sculptum* (Supplementary Table 3) and downregulated 13 CDSs in *A. aureolatum* (Supplementary Table 4). Two sequences inside this category, coding K^+-dependent Ca^{2+}/Na^+ exchanger NCKX1, are common to both *A. sculptum* (AcajSigP-12807) and *A. aureolatum* (Ambaur-41603). Transporter and channels category also contains CDSs of *A. sculptum* that were upregulated by infection as well as CDSs of *A. aureolatum* that were downregulated (Supplementary Tables 3, 4, respectively). Nonetheless, none of those CDSs shares the same annotation in both tick species.

TABLE 1 | Functional classification of CDSs identified in the MG of *A. sculptum* and *A. aureolatum* by RNA-seq.

Functional categories	*A. sculptum*				*A. aureolatum*			
	Number of CDSs	Number of reads in infected ticks	Number of reads in control ticks	Sum of mapped reads	Number of CDSs	Number of reads in infected ticks	Number of reads in control ticks	Sum of mapped reads
cytoskeletal	303	6.41E + 05	9.79E + 05	1.62E + 06	326	7.62E + 05	1.49E + 06	2.25E + 06
detoxification	96	1.48E + 05	2.28E + 05	3.76E + 05	228	4.59E + 05	7.41E + 05	1.20E + 06
extracellular matrix	317	9.15E + 05	1.39E + 06	2.30E + 06	341	6.97E + 05	1.48E + 06	2.17E + 06
immunity	138	3.06E + 05	4.72E + 05	7.78E + 05	145	2.52E + 05	4.69E + 05	7.21E + 05
metabolism	551	1.44E + 06	2.56E + 06	4.00E + 06	1,070	2.14E + 06	4.62E + 06	6.76E + 06
nuclear export	32	6.56E + 04	1.05E + 05	1.71E + 05	47	9.11E + 04	1.46E + 05	2.38E + 05
nuclear regulation	208	2.17E + 05	3.55E + 05	5.72E + 05	355	5.28E + 05	7.56E + 05	1.28E + 06
protein export machinery	305	4.35E + 05	6.65E + 05	1.10E + 06	403	6.31E + 05	1.08E + 06	1.71E + 06
protein modification	198	5.13E + 05	9.67E + 05	1.48E + 06	344	6.96E + 05	1.41E + 06	2.11E + 06
proteasome machinery	178	2.76E + 05	4.61E + 05	7.38E + 05	269	5.68E + 05	8.90E + 05	1.46E + 06
protein synthesis	288	2.11E + 06	3.90E + 06	6.01E + 06	438	2.44E + 06	4.36E + 06	6.80E + 06
secreted	2,535	4.13E + 06	6.11E + 06	1.02E + 07	2,523	4.83E + 06	7.43E + 06	1.23E + 07
signal transduction	825	1.02E + 06	1.38E + 06	2.39E + 06	1,051	1.23E + 06	2.19E + 06	3.42E + 06
storage	20	4.74E + 05	6.72E + 05	1.15E + 06	13	1.89E + 05	2.86E + 05	4.75E + 05
transposon element	576	1.34E + 05	1.52E + 05	2.86E + 05	518	2.02E + 05	3.86E + 05	5.87E + 05
transcription factor	133	1.20E + 05	1.60E + 05	2.81E + 05	172	1.55E + 05	2.79E + 05	4.34E + 05
transcription machinery	430	8.73E + 05	1.37E + 06	2.25E + 06	680	2.00E + 06	2.00E + 06	4.00E + 06
transporter and channels	420	4.49E + 05	5.81E + 05	1.03E + 06	473	5.22E + 05	1.01E + 06	1.53E + 06
unknown conserved	1,035	1.31E + 06	1.89E + 06	3.20E + 06	1,568	2.18E + 06	2.69E + 06	4.87E + 06
unknown	933	9.05E + 05	1.37E + 06	2.28E + 06	907	1.10E + 06	9.77E + 05	2.08E + 06
virus	39	4.56E + 04	4.59E + 04	9.15E + 04	35	3.59E + 04	7.19E + 04	1.08E + 05
TOTAL	9,560	1.65E + 07	2.58E + 07	4.23E + 07	11,906	2.17E + 07	3.48E + 07	5.65E + 07

TABLE 2 | Selected CDSs of *A. sculptum* midgut differentially expressed by infection with *R. rickettsii*.

CDS	Annotation	RNA-seq	RT-qPCR
		Fold-change	Fold-change
Acaj-52926	superoxide dismutase	0.17	NA
Acaj-76542	structural constituent of cuticle	0.03	0.19
Acaj-75348	cuticle protein	0.03	NA
Acaj-80620	cuticle protein	0.02	NA
Acaj-81446	chitin binding peritrophin	0.13	NA
AcajSigP-85359	peritrophin	0.17	215.94**
Acaj-36823	tumor necrosis factor receptor-associated factor	0.14	NA
Acaj-43466	tumor necrosis factor receptor-associated factor	0.05	NA
Acaj-70988	tumor necrosis factor receptor-associated factor	0.16	NA
Acaj-51987	tumor necrosis factor receptor-associated factor	6.06	NA
Acaj-33728	lysozyme	163.20	NA
Acaj-48379	tick defensin	5.31	4.90*
Acaj-74395	antimicrobial peptide	11.24	47.50**
Acaj-60304	peptidoglycan recognition protein	8.85	18.31*
AcajSigP-81204	peptidoglycan recognition protein	5.83	NA
AcajSigP-6765	ML domain protein	11.68	85.90**
AcajSigP-19478	vitellogenin receptor	0.13	NA
AcajSigP-26714	vitellogenin receptor	0.13	0.11*
AcajSigP-74567	glycolate oxidase	9.13	NA
AcajSigP-79349	glycolate oxidase	8.05	38.05*
Acaj-72627	serine carboxypeptidase	31.63	NA
Acaj-72628	serine carboxypeptidase	33.88	NA
Acaj-72629	serine carboxypeptidase	47.20	NA
Acaj-25905	BPTI/Kunitz domain-containing protein	9.15	NA
Acaj-32963	tick_Kunitz_108	13.74	NA
Acaj-62359	tick_Kunitz_108	6.96	NA
Acaj-67782	Kunitz-type peptidase inhibitor	271.74	NA
Acaj-84823	tick_Kunitz_101	6.04	NA
AcajSigP-26186	tick_Kunitz_115	8.85	NA
AcajSigP-45672	tick_Kunitz_101	8.27	NA
AcajSigP-57222	Kunitz domain protein	7.99	NA
Acaj-39037	secreted mucin MUC17	0.15	NA
AcajTE-85265	hypothetical protein PGCG_00421	0.03	0.10
AcajSigP-78265	hypothetical secreted protein	13.22	3.42

Only CDSs with statistically significant differences in expression in the midgut of infected vs. control (non-infected) ticks, identified by RNA-seq analysis, are presented. Some CDSs were additionally analyzed by RT-qPCR ($p < 0.05$ and **$p < 0.01$; Student's t-test). NA: relative expression not analyzed by RT-qPCR.*

TABLE 3 | Selected CDSs of *A. aureolatum* midgut differentially expressed by infection with *R. rickettsii*.

CDS	Annotation	RNA-seq	RT-qPCR
		Fold-change	Fold-change
Ambaur-58882	cuticular protein	0.08	NA
Ambaur-64626	cuticle protein	0.18	NA
Ambaur-18924	peptidoglycan recognition protein	5.08	NA
Ambaur-45342	lysozyme	5.15	4.06**
AmbarSigP-48279	ixoderin precursor	5.29	NA
Ambaur-58673	tumor necrosis factor receptor-associated factor	0.19	NA
AmbarSigP-52021	glucose dehydrogenase	0.04	NA
AmbarSigP-66386	glucose dehydrogenase	0.11	NA
Ambaur-5106	glutamine synthetase	0.15	0.33
Ambaur-61820	homocysteine S-methyltransferase	25.55	1.40
Ambaur-53938	chymotrypsin-elastase inhibitor ixodidin	0.16	NA
Ambaur-58705	serine carboxypeptidase	0.08	NA
Ambaur-60941	secreted mucin MUC17	0.19	NA
Ambaur-63341	secreted mucin MUC17	0.08	NA
Ambaur-63342	secreted mucin MUC17	0.12	NA
AmbarSigP-64116	secreted mucin	0.18	NA
Ambaur-19862	5.3 kda antibacterial peptide	0.09	NA
AmbarSigP-15638	serine carboxypeptidase	0.05	NA
AmbarSigP-13149	serine carboxypeptidase	33.46	NA
AmbarSigP-19966	Kunitz/Bovine pancreatic trypsin inhibitor domain	0.03	NA
AmbarSigP-54335	BPTI/Kunitz domain-containing protein 5	0.03	NA
Ambaur-28274	Kunitz-type protease inhibitor	0.01	NA
Ambaur-43435	BPTI/Kunitz family of serine protease inhibitors	0.02	NA
Ambaur-59675	BPTI/Kunitz domain-containing protein 5	5.09	NA
Ambaur-54534	secreted metalloprotease	0.17	1.00
Ambaur-25926	evasin	11.21	1.39
AmbarSigP-64894	tick transposon	0.18	0.34*
AmbarSigP-70666	ceramidase	0.12	0.33*
Ambaur-62064	hypothetical protein PGCG_00421	56.03	1.52
Ambaur-4532	ABC transporter	14.06	1.76

Only CDSs with statistically significant differences in expression in the midgut of infected vs. control (non-infected) ticks, identified by RNA-seq analysis, are presented. Some CDSs were additionally analyzed by RT-qPCR ($p < 0.05$ and **$p < 0.01$; Student's t-test). NA: relative expression not analyzed by RT-qPCR.*

Cuticle proteins CDSs, which belong to external matrix category, were downregulated by infection in both *A. sculptum* (Acaj-76542, Acaj-80620, and Acaj-75348; **Table 2** and Supplementary Table 2) and *A. aureolatum* (Ambaur-64626 and Ambaur-58882; **Table 3** and Supplementary Table 4). In this same category, two peritrophin CDSs (Acaj-81446 and Acaj-SigP85359) were downregulated exclusively in infected *A. sculptum* (**Table 2** and Supplementary Table 2).

In *A. sculptum*, infection downregulated two vitellogenin receptor CDSs (AcajSigP-26714 and AcajSigP-19478), which are comprised in storage category (**Table 2** and Supplementary Table 2). Moreover, two CDSs in transcription factors

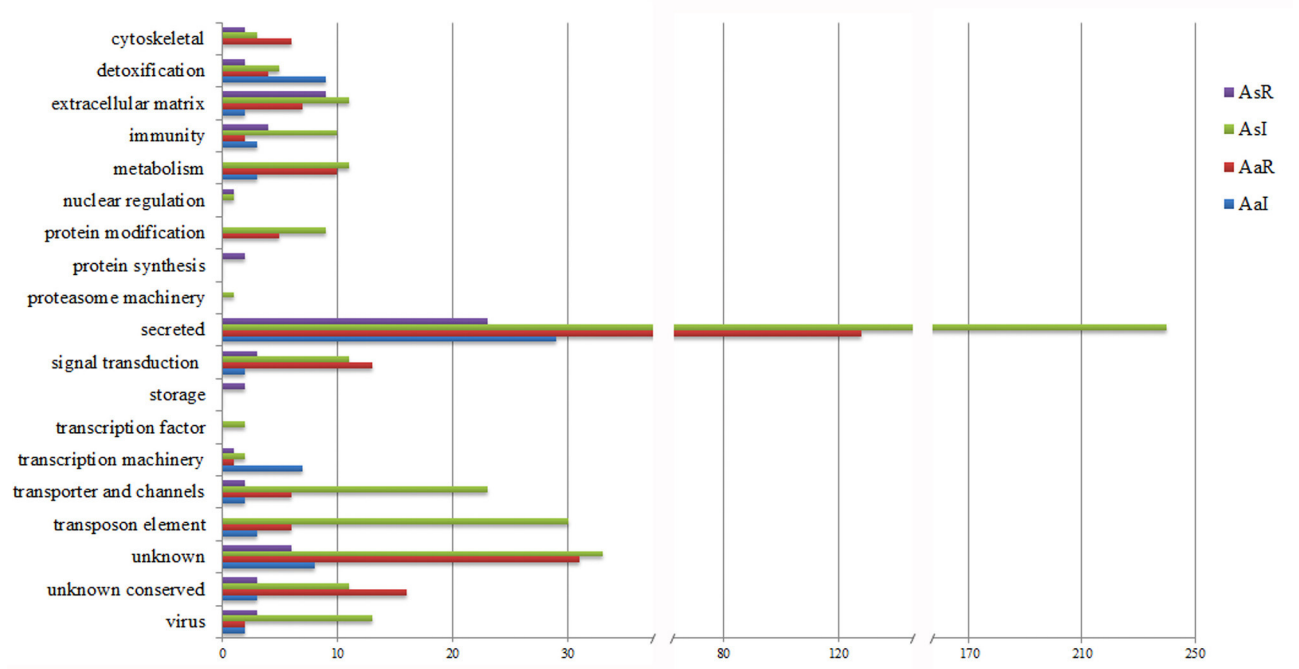

FIGURE 1 | Functional classification of CDSs differentially expressed in the midgut of *R. rickettsii*-infected ticks. AsR, downregulated CDSs of *A. sculptum*; AsI, upregulated CDSs of *A. sculptum*; AaR, downregulated CDSs of *A. aureolatum*; AaI, upregulated CDSs of *A. aureolatum*.

(Acaj-72151: alcohol dehydrogenase transcription factor Myb/SANT-like; and Acaj-62642: transcription factor NERF) and one in proteasome machinery (AcajTE-85582: E3 ubiquitin -protein ligase RBBP6-like isoform X3) were exclusively upregulated in *A. sculptum* (Supplementary Table 3). In *A. aureolatum*, nine CDSs in detoxification category were upregulated by infection, among which seven code cytochrome P450 (Supplementary Table 5). In *A. sculptum*, two cytochrome P450 CDSs (AcajSigP-19690 and AcajSigP-26888) were also upregulated by infection (Supplementary Table 3), while one superoxide dismutase (SOD) CDS (Acaj-52926) was downregulated (Supplementary Table 2). Seven CDSs in transcription machinery category, among which six code the regulator of rDNA transcription protein 15, were also upregulated by *R. rickettsii* in *A. aureolatum* MG (Supplementary Table 5).

Infection upregulated a higher number of CDS of immune-related proteins in *A. sculptum* than in *A. aureolatum* (**Figure 1**). Lysozyme, ixoderin, and peptidoglycan recognition protein (PGRP) CDSs were upregulated by infection in both tick species (**Tables 2, 3**, and Supplementary Tables 3, 5). In *A. sculptum*, *R. rickettsii* also upregulated the expression of two CDSs (Acaj-48379 and Acaj-74395) that code antimicrobial peptides (AMPs) similar to the defensin of *Amblyomma americanum*, named amercin (**Table 2** and Supplementary Table 3). In addition, one CDS of a protein containing ML domain (MD-2-related lipid-recognition) (AcajSigP-6765) was upregulated in *A. sculptum* by infection (**Table 2** and Supplementary Table S3). In *A. aureolatum*, sequences coding one 5.3 kDa AMP (Ambaur-19862) and one AMP similar to the ixodidin of *Rhipicephalus microplus* (Ambaur-53938), which exhibit chymotrypsin-elastase inhibitory activity, were downregulated (**Table 3** and Supplementary Table 4). In *A. sculptum*, three tumor necrosis factor receptor-associated factor (TRAF) CDSs (Acaj-43466, Acaj-36823, and Acaj-70988; **Table 2** and Supplementary Table 2) were downregulated, while one was upregulated (Acaj-51987; **Table 2** and Supplementary Table 3). In *A. aureolatum*, only one CDS encoding TRAF (Ambaur-58673) was detected as downregulated (**Table 3** and Supplementary Table 4).

As expected, several differentially expressed sequences encode hypothetical proteins or unknown products in both tick species (**Figure 1** and Supplementary Tables 2–5). Remarkably, the majority of those sequences encode proteins that are predicted to be secreted in *A. sculptum* (140 among 204 sequences) and in *A. aureolatum* (61 among 117 sequences).

DISCUSSION

In the current study, laboratory colonies of two different species of ticks that are vectors of *R. rickettsii* in Brazil were fed on infected hosts, mimicking a natural infection. While 93% of *A. aureolatum* females acquired *R. rickettsii*, only 15% of *A. sculptum* females were positively infected. This result reinforces the differences in susceptibility of these two tick species to infection with *R. rickettsii*, as previously reported by Labruna et al. (2008).

It is known that pathogens acquired within the blood meal have to overcome several barriers in ticks to be successfully transmitted to another host (Kopacek et al., 2010; Hajdusek et al.,

2013). Firstly, the pathogen must colonize MG and later the salivary glands (SG) through hemolymph. Our data showed that both MG and SG of all positively infected *A. aureolatum* and *A. sculptum* ticks were colonized by *R. rickettsii*. We did not observed ticks with infection restricted to MG, suggesting that if this barrier is broken, this pathogen is able to reach the SG. Therefore, the tick MG probably plays a key role in controlling rickettsial infection. To get better insights on the factors that control infection in tick MG, the global gene expression profile of infected vs. non-infected (control) ticks was determined by RNA sequencing (RNA-seq) and validated using RT-qPCR. The correlation between RNA-seq and RT-qPCR (around 70%) strengthened the transcriptional findings of the current study.

Most CDSs of *A. aureolatum* that were differentially expressed by infection with *R. rickettsii* were downregulated (87%). On the contrary, the majority of CDSs of infected *A. sculptum* were induced (76%). Interestingly, many upregulated CDSs of *A. sculptum* belong to the same functional categories of most downregulated CDSs of *A. aureolatum*, for instance, metabolism, protein modification, and secreted proteins. Further studies are required to determine the role played by CDSs within those functional categories in delineating the differences in susceptibility of these two tick species to infection.

Among CDSs associated to metabolism that were induced by infection in *A. sculptum*, we highlight two glycolate oxidases. In plants, this enzyme converts glycolate to oxalate producing hydrogen peroxide (H_2O_2) (Rojas and Mysore, 2012). The upregulation of such oxidases upon infection might represent an attempt of ticks to control *R. rickettsii* through production of H_2O_2. Indeed, production of ROS, including superoxide (O_2^-) and H_2O_2, is an ancient immune response against invader pathogens (Zug and Hammerstein, 2015). It was previously reported that *R. rickettsii* infection induces a pro-oxidant response in human endothelial cells (Santucci et al., 1992; Devamanoharan et al., 1994), causing oxidative cell injury (Eremeeva et al., 2001). The stimulation of hemocytes of the tick *R. microplus* with the Gram-positive bacterium *Micrococcus luteus* also leaded to production of ROS. This activity was abolished by addition of superoxide dismutase (SOD) and catalase, suggesting that both superoxide and H_2O_2 are produced upon microbial challenge (Pereira et al., 2001). Besides upregulation of glycolate oxidase, one SOD CDS was downregulated by infection in *A. sculptum*, strengthening that the MG is under oxidative stress upon infection. In *A. aureolatum*, two glucose dehydrogenase encoding genes were downregulated. This enzyme reduces NADP to NADPH. Electrons from NADPH can be used by glutathione reductase to regenerate the intracellular pool of reduced glutathione (Mishra and Imlay, 2012), which, in turn, is important to reduce H_2O_2 into H_2O by action of glutathione peroxidase (Wang et al., 2013). Alternatively, intracellular NADPH can act as the electron donor to the NADPH oxidase complex to catalyze the reduction of molecular oxygen to superoxide in phagocyte membranes (Wang et al., 2013). Therefore, glucose dehydrogenase play an important role in maintaining the cellular redox homeostasis. In a previous study, our research group showed that antioxidant enzymes of *R. rickettsii* (thioredoxin peroxidase 1, glutaredoxin 3, ferredoxin, and also one hypothetical protein A1G_00185 with a thioredoxin domain) are upregulated in MG of fed *A. aureolatum*, which may protect *R. rickettsii* against the deleterious effects of free radicals (Galletti et al., 2013). Then, it is plausible to suppose that the MG of fed infected *A. aureolatum* is under oxidative stress.

It is known that the main proteolytic activities in the MG of ticks are acidic aspartic and cysteine proteinases, whereas exopeptidases (the cysteine amino- and carboxy-dipeptidases cathepsins C and B, respectively) may participate in a later stage of digestion (Horn et al., 2009; Cruz et al., 2010). In addition, two monopeptidases, namely serine carboxypeptidase (Motobu et al., 2007) and leucine aminopeptidase (Hatta et al., 2006), would be possibly involved in the final stage of digestion, liberating free amino acids (Horn et al., 2009). Digestion of hemoglobin, as well as other proteins within the blood meal, is important not only to provide nutrients and energy for molting and vitellogenesis processes, but also to generate fragments with antimicrobial properties (Fogaca et al., 1999; Nakajima et al., 2003; Sonenshine et al., 2005; Belmonte et al., 2012). These antimicrobial fragments, named hemocidins, are probably involved in protection of tick MG against proliferation of pathogens (Kopacek et al., 2010; Hajdusek et al., 2013). We did not detect the transcriptional regulation of aspartic and cysteine proteinases by infection. However, three serine carboxipeptidase CDSs were upregulated in *A. sculptum*, while two were downregulated in *A. aureolatum*. As serine carboxypeptidases are involved in the final stage of hemoglobin digestion (Motobu et al., 2007), studies to determine the contents of hemocidins in MG of *Amblyomma* ticks are warranted to determine the role of these peptides in controlling infection.

Infection also modulated the expression of metalloproteases in tick MG, upregulating 15 CDSs in *A. sculptum* and downregulating eight CDSs in *A. aureolatum*. No metalloprotease CDS was detected to be upregulated in *A. aureolatum* or downregulated in *A. sculptum*. It is known that metalloproteinases of tick saliva, which belong to metzincin family, are essential for both the initial and late feeding stages (Francischetti et al., 2003, 2005a). During the initial feeding stage, these enzymes inhibit blood clotting and degrade extracellular matrix proteins, which is essential for the preparation of the feeding site. As metalloproteases also present anti-angiogenic activity, they are also important in the late feeding stage, inhibiting host tissue repair. Metalloproteases from metzincin family present a zinc-binding consensus motif (HEXXHXXG/NXXH/D, where X corresponds to any amino acid residue) in the active site and a conserved methionine-containing turn (Met-turn) that underlies the active site. According to their structure, metzincins are subdivided in other families, among which are reprolysin and astacin family (Gomis-Ruth, 2009), where tick metalloproteases are included. It was previously reported that reprolysins are majorly expressed in the SG of the tick *R. microplus*, while astacin transcripts are more abundant in the MG and ovaries (Barnard et al., 2012). Importantly, the knockdown of both reprolysins and astacins leaded to a reduction of both oviposition and egg weight, demonstrating that those proteins correspond to promising targets for development of strategies to protect the cattle

against ticks (Barnard et al., 2012). Indeed, the immunization of bovines with the reprolysin BrRm-MP4 diminished the feeding and reproductive rates of *R. microplus* females (Ali et al., 2015), strengthening the potential of metalloproteinases as vaccine candidates. Among the metalloproteinases from *A. aureolatum*, six are reprolysins and two are astacins, while 14 of the metalloproteases of *A. sculptum* are reprolysins and only one is astacin.

Many CDSs of both annotated and hypothetical/unknown function proteins that contain signal peptide for secretion were modulated by infection. Importantly, most of the proteins predicted to be secreted were downregulated in *A. aureolatum* and induced in *A. sculptum*. Among them, lipocalins, proteins containing Kunitz domain, and mucins are prominent. *R. rickettsii* infection upregulated 26 lipocalin CDSs in *A. sculptum* and downregulated 30 CDSs in *A. aureolatum*. It is known that lipocalins bind histamine and serotonin (Paesen et al., 1999; Sangamnatdej et al., 2002; Francischetti et al., 2009), which in high concentrations on the feeding pool can affect attachment, feeding efficiency, and reproductive success (Kemp and Bourne, 1980; Paine et al., 1983). Therefore, these anti-inflammatory proteins are extensively detected to be present in tick saliva (Francischetti et al., 2005b; Ribeiro et al., 2006; Alarcon-Chaidez et al., 2007; Karim et al., 2011; Karim and Ribeiro, 2015). The unique non-salivary gland lipocalin described in ticks is savicalin from hemocytes of *Ornithodoros savignyi*. Importantly, savicalin transcripts were also detected to be present in MG and ovaries (Cheng et al., 2010). Therefore, additional studies should be addressed to determine the site of production and the role played by lipocalins in protection of *Amblyomma* ticks against infection.

In addition, CDSs of secreted proteins encoding serine protease inhibitors, predominantly Kunitz inhibitors, were upregulated in *A. sculptum* and downregulated in *A. aureolatum*. Serine proteinase inhibitors were already enrolled in immune reactions of arthropods, mediating both coagulation and melanization processes of the hemolymph and also the production of AMPs (Gulley et al., 2013). These inhibitors may also control pathogen proliferation by inhibiting proteinases they use to colonize the host tissues and evade immune system (Armstrong, 2001). In addition, Kunitz inhibitors are widely described to be produced by ticks, inhibiting blood coagulation and facilitating tick feeding (Corral-Rodriguez et al., 2009). The differential expression of tick Kunitz inhibitors by *Dermacentor variabilis* upon an infection with the avirulent *Rickettsia montanensis* was previously reported (Ceraul et al., 2008). Moreover, it was shown that this Kunitz inhibitor exhibits a bacteriostatic effect against *R. montanensis* (Ceraul et al., 2008) and that its transcription knockdown leads to an increase in the susceptibility of *D. variabilis* to infection (Ceraul et al., 2011).

Some mucins predicted to be secreted were also detected to be differentially expressed in infected ticks. These proteins are lipid binding proteins that constitute an acellular barrier that promote protection of the MG epithelium. Mucins were already reported to be present in MG of ticks, such as *D. variabilis* (Anderson et al., 2008), as well as in SG (Karim et al., 2011; Ribeiro et al., 2011). Interestingly, *D. variabilis* mucins exhibit similarity to insect peritrophins (Anderson et al., 2008). Two peritrophin CDSs and one secreted mucin CDS were detected to be downregulated in *A. sculptum* by RNA-seq analysis. Peritrophin encoding sequences were not detected to be modulated by infection in *A. aureolatum*. However, four secreted mucin CDSs were downregulated in this tick species. It was previously reported that the knockdown of the peritrophin 1 of *Ixodes scapularis* reduces the thickness of the peritrophic matrix, impairing the colonization of the tick MG by *Borrelia burgdorferi*, causative agent of Lyme disease (Narasimhan et al., 2014). Similar effects of peritrophin 1 knockdown was observed when ticks were treated with antibiotics, altering the MG microbiota (Narasimhan et al., 2014). Indeed, ticks harbor a microbiota that can be modified, for instance, upon the treatment with antibiotics (Narasimhan et al., 2014; Clayton et al., 2015; Abraham et al., 2017). Interestingly, a recent study showed that infection with *Anaplasma phagocytophilum*, causative agent of human granulocytic anaplasmosis, also alter the composition of the microbiota of *I. scapularis* MG through the upregulation of the tick anti-freeze glycoprotein (iafgp) (Abraham et al., 2017). Iafgp binds bacterial peptidoglycan, negatively affecting biofilm formation and, consequently, altering microbiota. Differentially from the effect previously observed for *B. burgdorferi* (Narasimhan et al., 2014), the remodeling of the microbiota of *I. scapularis* MG, which diminishes the thickness peritrophic matrix, increased the load of *A. phagocytophilum* (Abraham et al., 2017). It is possible that the MG of *A. sculptum* and *A. aureolatum* harbor a distinct microbiota, which might play a role in delineating susceptibility to infection. In addition, the distinct transcriptional profile triggered by *R. rickettsii* infection may also exert a different impact on the MG microbiota of these two ticks, resulting in differences in susceptibility. Then, studies to determine the composition of the microbiota of the MG of *A. sculptum* and *A. aureolatum* infected or not with *R. rickettsii* are warranted.

It is known that *R. rickettsii* exerts a detrimental effect on ticks, reducing both survival and reproductive rates (Burgdorfer and Brinton, 1975; Niebylski et al., 1999; Labruna et al., 2008; Soares et al., 2012). Our data showed that CDSs of cuticle proteins were downregulated by infection in both *A. sculptum* and in *A. aureolatum*. The expansion of the cuticle is crucial for the engorgement of the tick female (Flynn and Kaufman, 2011), assuring that an enough amount of blood be acquired for egg production. In addition to cuticle proteins, two vitellogenin receptor CDSs were detected to be downregulated by infection in *A. sculptum*. Therefore, it is possible that the downregulation of cuticle proteins and vitellogenin receptors might be involved in the reduction of both oviposition and survival rates in infected ticks.

Infection modulated CDSs of immune-related proteins in both species of ticks. Ixoderins, which are recognition proteins that possesses a C-terminal domain with a high homology to fibrinogen and fibrinogen-related proteins (FREPs), were upregulated by infection in both *A. sculptum* and *A. aureolatum*. Two isoforms of ixoderins, A and B, were identified in the hard tick *Ixodes ricinus* (Rego et al., 2005) and possess similarity to the lectin Dorin M of the soft tick *Ornithodorus moubata*

(Kovar et al., 2000). Infection with *R. rickettsii* also induced expression of PGRP CDSs in MG of both *A. sculptum* and *A. aureolatum*. Depending on the presence of an amidase catalytic site, those proteins are classified into non-catalytic or catalytic. Non-catalytic PGRPs function as pathogen pattern recognition receptors and activate the Toll and Imd pathways in *Drosophila* upon infection, while catalytic PGRPs cleaves peptidoglycan, acting, therefore, as negative regulators of the immune response (by removing peptidoglycan) or as effectors (by killing bacteria) (Palmer and Jiggins, 2015). The PGRPs of both *A. sculptum* and *A. aureolatum* exhibit amidase catalytic site, suggesting they might play a role as effectors or negative regulators of tick immune signaling pathways. One protein containing ML domain was also induced in *A. sculptum* by infection. Genes encoding ML-domain containing proteins were detected to be expressed in MG of *I. ricinus* (Rudenko et al., 2005; Horackova et al., 2010). The function of such proteins in tick gut is not known, but is likely that they might be involved in response against invader pathogens and/or lipid metabolism (Inohara and Nunez, 2002).

The production of AMPs in tick MG is important, as the slow intracellular digestion of nutrients and neutral pH may favor the proliferation of microorganisms (Kopacek et al., 2010; Hajdusek et al., 2013). One lysozyme CDS was induced by infection in both *A. sculptum* and *A. aureolatum*. In *A. sculptum*, infection also induced the expression of two CDSs that code AMPs similar to the defensin of *A. americanum*, named amercin (Todd et al., 2007). Conversely, sequences encoding one 5.3-kDa AMP and one AMP with chymotrypsin-elastase inhibitory activity, similar to the ixodidin of *R. microplus* (Fogaca et al., 2006), were downregulated in the MG of infected *A. aureolatum*. These results suggest that the MG of *A. sculptum* might be more hostile to *R. rickettsii* than the MG of *A. aureolatum*. Then, functional studies are required to determine the importance of AMPs in protection of *Amblyomma* ticks against rickettsiae.

In insects, it is well-known that transcription of AMPs, as well as other immune effectors, is mainly regulated by the intracellular signaling pathways Toll, Immune deficiency (Imd), JNK (Jun-N-terminal kinase), and Jak/Stat (Ferrandon et al., 2007; Souza-Neto et al., 2009; Kleino and Silverman, 2014). Much less information is available on the immune signaling pathways in ticks. Immune signaling pathway components were identified in the genome of *I. scapularis* (Smith and Pal, 2014; Kotsyfakis et al., 2015; Gulia-Nuss et al., 2016). It was recently reported that Toll and Jak-Stat insect immune signaling pathway components are well conserved in ticks. Conversely, ticks lack some IMD signaling pathway components, such as the adaptor protein IMD, its associated molecule FAAD (Fas associated protein with death domain), the caspase DREDD (death related ced-3/Nedd2-like), and the negative regulators Pirk (poor IMD response upon knock-in) and Dnr1 (defense repressor 1) (Rosa et al., 2016). This same study has also shown that *A. marginale* downregulates the expression of immune signaling pathway components in an embryonic cell line (BME26) of *R. microplus*, its biological vector, while other microbial stimuli, including *R. rickettsii*, induce their expression. In addition, it was previously reported that the Jak/Stat pathway regulates the expression of 5.3-kDa AMP, which is essential to prevent *A. phagocytophilum* infection in *I. scapularis* (Liu et al., 2012). Jak/Stat pathway was also enrolled in controlling the production of peritrofin-1, which is essential for the successful colonization of *I. scapularis* MG by *B. burgdorferi* (Narasimhan et al., 2014). Interestingly, TRAF, a downstream member of the Toll pathway, was differentially expressed in both *A. sculptum* and *A. aureolatum*. Therefore, functional studies are warranted to determine the role played by immune pathways in the control of rickettsial infection in *Amblyomma* ticks.

In conclusion, the current study shows that infection with *R. rickettsii* elicits a distinct transcriptional profile in the MG of *Amblyomma sculptum* and *A. aureolatum*, which might be responsible for their differences in susceptibility to infection. The proteins encoded by differentially expressed CDSs should be functionally characterized and might be targets for development of blocking vaccines. As the genome of *A. sculptum* and *A. aureolatum* is unavailable, this study provides a rich source of sequences in the genus *Amblyomma*.

AUTHOR CONTRIBUTIONS

Designed the experiments: ACF. Generated biological samples: LM, MG, and FC. Performed the experiments: LM and MG. Analyzed RT-qPCR data: LM, MG, and AF. Performed bioinformatics data analysis: JR. Performed statistic data analysis: JR, AF. Contributed reagents/materials/analysis tools: JR, AF, ML, SD, and ACF. Wrote the paper: LM, MG, and ACF. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Emerging Tick-Borne Viruses in the Twenty-First Century

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Ticks, as a group, are second only to mosquitoes as vectors of pathogens to humans and are the primary vector for pathogens of livestock, companion animals, and wildlife. The role of ticks in the transmission of viruses has been known for over 100 years and yet new pathogenic viruses are still being detected and known viruses are continually spreading to new geographic locations. Partly as a result of their novelty, tick-virus interactions are at an early stage in understanding. For some viruses, even the principal tick-vector is not known. It is likely that tick-borne viruses will continue to emerge and challenge public and veterinary health long into the twenty-first century. However, studies focusing on tick saliva, a critical component of tick feeding, virus transmission, and a target for control of ticks and tick-borne diseases, point toward solutions to emerging viruses. The aim of this review is to describe some currently emerging tick-borne diseases, their causative viruses, and to discuss research on virus-tick interactions. Through focus on this area, future protein targets for intervention and vaccine development may be identified.

Keywords: tick, virus, emerging, transmission

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INTRODUCTION

Many arthropods, including ticks, transmit diseases that cause morbidity, and mortality amongst humans, livestock, companion animals, and/or wildlife. This in turn can cause major economic costs particularly to the owners of livestock affected by disease. The relationship between the tick, its host and pathogens has been shown to be complex and each may benefit or suffer detrimental effects due to the combination of physiological and immune mediated processes each elicits during infestation and infection (de la Fuente et al., 2016). Viruses form a major constituency of the pathogens transmitted by ticks (for review see Labuda and Nuttall, 2004). Although, the presence of microorganisms in ticks appears to have little impact on the tick, presumably there is an energetic cost to harboring them and studies have demonstrated that ticks do respond in a coordinated fashion to infection with bacterial (Mercado-Curiel et al., 2011; Ayllón et al., 2015) and protozoal (Antunes et al., 2012) pathogens. Recent studies using *in vitro* models are beginning to identify transcriptional responses in ticks cell during infection with viruses (Mansfield et al., 2017).

Viruses that are transmitted by ticks belong to a range of virus families with different characteristics and tick vectors. One of the first tick-borne viruses identified was the flavivirus, louping ill virus, the causative agent of encephalitis in sheep and grouse, a disease recognized in livestock for hundreds of years. The demonstration that ticks were the source of infection for the virus was described almost 100 years ago (Stockman, 1918). Since this time, many more pathogenic viruses have been identified around the world. However, new examples of emerging tick-borne viral diseases that affect man are constantly being reported. Another striking feature

that favors pathogen transmission by ticks is the multi-stage lifecycle of ticks. Most species of *Ixodid* ticks have three states, larva, nymph and adult that each requires a blood-meal, sometimes requiring days to complete, in order to develop to the next stage. *Argasid* ticks, such as *Ornithodoros* sp. by contrast only require minutes to feed but each stage may feed multiple times. This ensures that the tick can become infected at any stage of its lifecycle and that infection persists through each developmental stage via transstadial transmission (Karbowiak et al., 2016) and then has the opportunity to transmit that virus back to a mammalian host (**Figure 1**). When blood feeding requires attachment to the host for a number of days, this provides ample opportunity for transfer of virus from tick to host within infected saliva. Horizontal transmission occurs following consumption of blood from an infected mammalian host, whilst co-feeding is a form of transmission that occurs when multiple ticks feed in close proximity enabling virus to transfer between ticks without infection in the host (Jones et al., 1987). Vertical transmission of pathogens between generations of ticks has been observed (transovarial transmission) for viruses such as tick-borne encephalitis virus (Reháček, 1962) and African swine fever virus (Rennie et al., 2001).

Currently, there are no effective therapeutic agents or vaccines for many tick-borne viruses with the exception of vaccines for louping ill virus, tick-borne encephalitis virus, and Kyasanur Forest disease virus, thus avoidance or control of the vector are the principal means of preventing disease. Emerging tick-borne diseases that affect humans and livestock will continue to challenge public and veterinary health. Most current research is limited to a small number of *Ixodid* tick species, mainly *Ixodes scapularis* and *Ixodes ricinus*, vectors of pathogens such as *Borrelia burgdorferi (sensu stricto* and *sensu lato)*, *Anaplasma phagocytophilum,* and tick-borne encephalitis virus, a virus that is currently increasing in range across Europe (Mansfield et al., 2009). However, with over 900 species of ticks in the world (Horak et al., 2002), many capable of transmitting viruses, future research on the interactions between the pathogen, its vector and the mammalian host will need to consider a greater number of virus-tick associations. This article provides an overview of some of the emerging tick-borne viruses (summarized in **Table 1** and located in **Figure 2**) that have arisen in the twenty-first century.

EMERGING TICK—BORNE BUNYAVIRUSES

Emergence of Severe Fever with Thrombocytopenia Syndrome Virus in China

Severe fever with thrombocytopenia syndrome (SFTS) was first documented in humans in 2007 when three patients were admitted to a hospital in Henan Province, China, with acute fever and severe leucopenia (Liu et al., 2014). Further cases were reported in Henan and Hubei provinces between 2008 and 2010

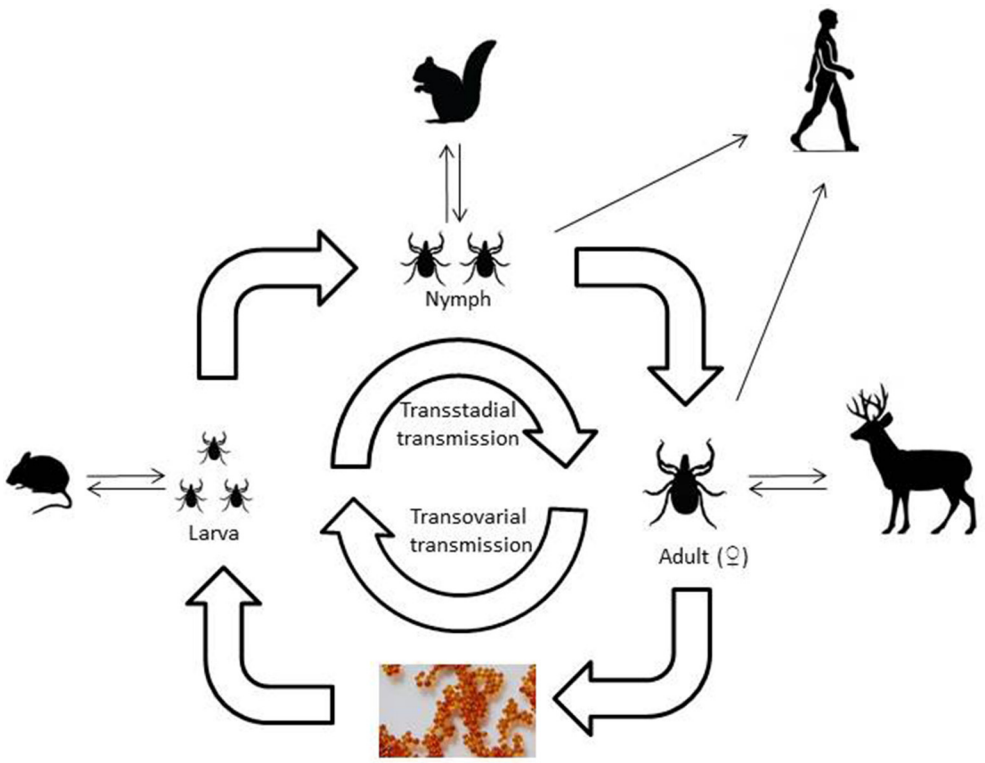

FIGURE 1 | A schematic of the three host *Ixodid* tick lifecycle illustrating the type of vertebrate hosts involved in each life stage of the tick.

TABLE 1 | Summary of emerging viruses discussed in this review.

Virus	Abbreviation	Classification (family, genus)	Description of genome	Distribution	Primary tick vector
Severe Fever with Thrombocytopenia syndrome virus	SFTSV	Bunyaviridae, *Phlebovirus*	Tri-segmented negative strand RNA	East Asia, North America	*Haemaphysalis longicornis*[a]
Heartland virus	HRTV	Bunyaviridae, *Phlebovirus*	Tri-segmented negative strand RNA	North America	*Amblyomma americanum*
Crimean congo haemorrhagic fever virus	CCHFV	Bunyaviridae, *Nairovirus*	Tri-segmented negative strand RNA	Africa, Central Asia, Mediterranean Basin	*Hyalomma marginatum*
Powassan virus	POWV	Flaviviridae, *Flavivirus*	Positive strand RNA	North America, Russian Federation	*Ixodes scapularis, Ixodes cookei*[b]
Deer tick virus	DTV	Flaviviridae, *Flavivirus*	Positive strand RNA	North America	*Ixodes scapularis*
Kyasunar forest disease virus	KFDV	Flaviviridae, *Flavivirus*	Positive strand RNA	India	*Haemaphysalis spinigera*
Alkhurma haemorrhagic fever virus	AHFV	Flaviviridae, *Flavivirus*	Positive strand RNA	Saudi Arabia	*Ornithidoros savigny*
African swine fever virus	ASFV	Asfarviridae, *Asfivirus*	Double-stranded DNA	Africa, Central Europe, Sardinia	*Ornithodoros moubata*[c]

[a] *In China.*
[b] *In North America.*
[c] *In Africa.*

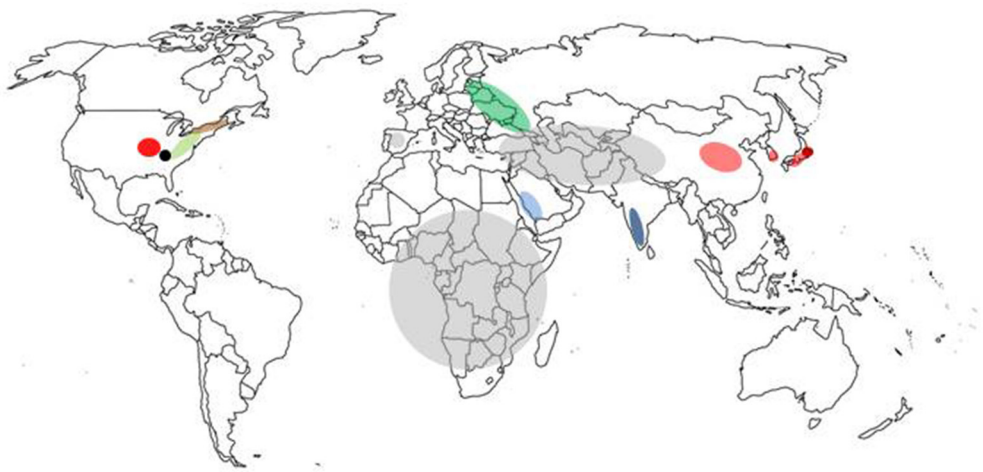

FIGURE 2 | Map of the world showing location of tick-borne disease outbreaks caused by emerging viruses. The regions marked are severe fever and thrombocytopenia syndrome virus (pale red), Heartlands virus (red), Crimean-Congo haemorrhagic fever virus (gray), Kysanur Forest disease virus (blue), Alkurma haemorrhagic fever virus (light blue), Powassan virus (brown), deer tick virus (pale green), Bourbon virus (black), Tofla virus (brown), and African swine fever virus (green).

with a similar clinical presentation and a case-fatality rate of over 10%. In 2009, the etiological agent of these infections was isolated from a patient's blood during the outbreaks of SFTS in Xinyang City in Henan province and identified as a bunyavirus related to viruses in the genus *Phlebovirus* (Zhang et al., 2012a,b; Lam et al., 2013). The virus was subsequently named severe fever with thrombocytopenia syndrome virus (SFTSV). Phleboviruses belong to the family Bunyaviridae, characterized by viruses with a segmented single-stranded negative sense genome divided into three segments, a small (S), medium (M), and large (L) segment coding for the nucleoprotein (N), surface glycoproteins (Gn/Gc), and RNA-dependent RNA polymerase (L), respectively. Related viruses include the mosquito-borne Rift Valley fever virus (Mansfield et al., 2015), the sandfly-borne Toscana virus (Charrel et al., 2012), and the tick-borne Uukuniemi virus (Matsuno et al., 2015). At present, infections with SFTSV have been reported in at least 13 provinces in China, including Henan, Hubei, Anhui, Shandong, Jiangsu, and Zhejiang (Li, 2013). Preliminary investigation of the first outbreak revealed that patients with SFTS were predominantly individuals who worked outdoors such as farmers indicating a rural source for the infection. Numerous patients presented with a history of tick bites (Zhang et al., 2012a). Subsequently, cases of SFTS were reported in North Korea, South Korea, and Japan (Liu et al., 2014).

In order to identify the reservoir of SFTSV, ticks were collected from livestock around the localities where patients originated and SFTSV was detected in *Haemaphysalis longicornis* (Zhang and Xu, 2016). Further surveys of *H. longicornis* provided strong

evidence that this tick species was the definitive reservoir and vector responsible for SFTSV transmission to humans working in rural locations (Zhang et al., 2012b). Experimental studies also demonstrated transstadial transmission of the virus between the different life stages of *H. longicornis* and transmission to mice during feeding (Luo et al., 2015). With *H. longicornis* being the source of infection and associated with feeding on livestock, it was highly likely that domestic animals would be exposed to infection and seroprevalence studies in China have now confirmed this (Zhang and Xu, 2016). However, disease in livestock has not been reported suggesting that humans are particularly susceptible to infection with SFTSV.

Emergence of Heartland Virus in the USA

Heartland virus (HRTV) was first reported in 2009 from North America when two farmers from Missouri with a clinical presentation similar to SFTS were admitted to hospital (McMullan et al., 2012). Symptoms included fever, fatigue, headache, myalgia, arthralgia, anorexia and diarrhea. The source of the infection was identified as likely resulting from tick bites, and the isolated virus shared 73% sequence identity with SFTSV by alignment of the polymerase gene. Diagnosis of HRTV infection may be challenging but can be achieved through detection of viral RNA in blood or tissue or by demonstrating a four-fold or greater increase in virus-specific plaque reduction neutralization antibody titre between acute and convalescent serum specimens (McMullan et al., 2012). Seroprevalence studies of livestock in Minnesota State reported seropositivity rates ranging from 10 to 18%, for example 15.5% in cattle, suggesting widespread exposure to infected ticks (Xing et al., 2013).

Surveillance for HRTV in Missouri has identified virus in the lone star tick *Amblyomma americanum* (Savage et al., 2016), an abundant tick species in many regions of the United States. Transmission of virus to a vertebrate host, transstadial and transovarial transmission between lifecycle stages has been demonstrated in *A. americanum* (Godsey et al., 2016). Both SFTSV and HRTV are related phleboviruses and have emerged in the past 10 years. However, the viruses exist in different tick species and have emerged in different continents. Human encroachment, probably combined with environmental conditions that influence both tick abundance and behavior may have led to the simultaneous emergence of these viruses.

The Spread of Crimean Congo Haemorrhagic Fever Virus around the Mediterranean Basin

The virus now known as Crimean Congo Haemorrhagic fever virus (CCHFV) was first described during an outbreak of haemorrhagic fever in Red Army soldiers in the Crimea during the final years of the Second World War (Ergönül, 2006). This virus was shown to be antigenically similar to a second virus isolated from a human in what is now the Democratic Republic of the Congo (Simpson et al., 1967). Worldwide, many tick species have been found infected with CCHFV and could be implicated in transmission of this virus (Mertens et al., 2013). The virus is primarily transmitted by ticks of the genus *Hyalomma* (Morikawa et al., 2007). By the late twentieth century, CCHFV had one of the most extensive distributions of any tick-borne virus with infections being reported from the Middle East, Asia, and sub-Saharan Africa. However, during the late twentieth and early twenty-first century, cases of CCHFV have been reported in new geographical locations around the Mediterranean Basin. CCHFV belongs to the family Bunyaviridae, genus *Nairovirus*. Other members of this genus include Hazara virus and Nairobi sheep disease virus. The virus genome is a tri-segmented negative sense RNA genome that encodes four structural proteins, the nucleoprotein (N), glycoproteins (GN and GC), and the RNA-dependent RNA polymerase (L). The virus is particularly virulent for humans but has little or no pathogenic effect on livestock. Infection following a tick bite is followed by a short incubation period of between 3 and 7 days leading to a brief febrile illness characterized by fever, headache and myalgia. Haemorrhagic manifestations of disease develop 4–5 days after the development of fever and include bleeding from the nose, gastrointestinal system and urinary tract. The fatality rate ranges from 3 to 30%. Treatment is supportive, including replacement of fluids and blood constituents. There is no licensed antiviral treatment or licensed vaccine for CCHF.

Reports of infection around the Mediterranean Basin began around 2002 with cases reported in Turkey (Karti et al., 2004) and the Balkans (Papa et al., 2005). In Europe, the main tick vector is *Hy. marginatum*, a species found throughout the Iberian Peninsula, southern France, Italy, the Balkans, and Turkey. In 2010, the virus was detected in *Hy. lusitanicum* ticks in Spain (Estrada-Peña et al., 2012), followed 6 years later with two human cases of autochthonous transmission (Garcia Rada, 2016). Phylogenetic analysis of virus detected in ticks indicated that the virus present in Spain shared greater identity with CCHFV in West Africa than that spreading in the Balkans. This suggested that it was a separate introduction into Europe with the most likely mechanism of entry being by tick-infested migrating birds (Estrada-Peña et al., 2012; Palomar et al., 2016). *Hy. marginatum* have been detected on birds migrating into Europe on numerous occasions (Capek et al., 2014) including as far north as the United Kingdom (Jameson et al., 2012). It is likely that the severity of the winters prevents establishment of this tick species in countries of northern Europe. However, the existence of populations of *Hyalomma* spp. in Spain, Portugal, France, and Italy puts these countries at greater risk of CCHFV persisting if introduced.

EMERGING TICK-BORNE FLAVIVIRUSES

Interstate Spread of Kyasanur Forest Virus in India

Kyasanur Forest disease virus (KFDV) was isolated following an outbreak of haemorrhagic fever amongst villagers living in the Kyasanur Forest area in 1957 (Work and Trapido, 1957). The Forest is found in the Shimoga distict of Karnataka State in the south-west of India and has been the epicenter of the disease ever since with between 400 and 500 human cases reported annually (Holbrook, 2012). Initial studies demonstrated that the virus shared similar properties to Russian spring

summer encephalitis virus (RRSEV), a variant of tick-borne encephalitis virus. However, the main clinical manifestation of disease is haemorrhagic fever unlike the encephalitis associated with RSSEV. KFDV is a member of the genus *Flavivirus*, family Flaviviridae. The flaviruses consist of a positive-sense single-stranded RNA genome that codes for three structural and seven non-structural proteins. Virion particles are enveloped with a single envelope protein projecting from the virus. KFDV is closely related to Alkhurma virus (AHFV) (Charrel et al., 2007) and a virus isolated in Yunnan Province, China (Wang et al., 2009). Phylogenetic studies on KFDV and AHFV suggest that they diverged over 700 years ago (Dodd et al., 2011) and that the virus has spread slowly within Karnataka Forest, reflecting dissemination by the tick vector that primarily feeds on small to medium sized mammals. Long distance movement that has separated the progenitors of both viruses could have been mediated by infected ticks attaching to migrating birds (Mehla et al., 2009).

The association with tick-borne viruses suggested a tick vector as the source of infection. Another early observation was mortality of non-human primates such as macaques and langurs in the area where human cases were reported. *Haemaphysalis* spp. ticks were collected from primate carcases and KFDV was isolated from *Haemaphysalis spinigera* (Trapido et al., 1959). Following the detection of KFDV, a second virus, Kaisodi virus, was isolated from *H. spinigera* sampled in India (Bhatt et al., 1966; Pavri and Casals, 1966). *Haemaphysalis* spp. are three-host ticks with a larval, nymphal and adult stage, each taking a blood meal prior to metamorphosis into the next stage, or egg development in the case of adult females. Although transovarial transmission of KFDV has been demonstrated in *H. spinigera* (Singh et al., 1963), it is the nymph and adult stages that are critical for the transmission of virus to small mammals and humans as these are the hosts favored by these life stages of the tick.

In recent years there have been reports of KFDV infections in both Karnataka province (Mourya et al., 2013; Yadav et al., 2014) and in the neighboring provinces of Kerala (Tandale et al., 2015; Sadanandane et al., 2017), Tamil Nadu and Maharashtra (Mourya and Yadav, 2016). As in previous reports, outbreaks are often preceded by disease in monkeys, and contact with carcases can lead to infection (Mourya et al., 2013).The appearance of dead monkeys, particularly target species such as the red-face bonnet monkey (*Macaca radiata*) and the black-faced langur (*Semnopithecus entellus*) are considered sentinels for the presence of KFDV at a site (Murhekar et al., 2015). The spread of KFDV in recent years could reflect further gradual spread of the virus by the tick vector, improvements in diagnosis (Mourya et al., 2012) and surveillance leading to increased frequency of reporting. Increased human exploitation of the environment leading to greater contact between humans and ticks could also contribute to this apparent spread. All of the affected regions form part of the Western Ghats, a mountain range running north-south parallel to the west coast of India. This in turn suggests that the areas affected share climatic features that are favorable to the tick vector, currently poorly studied, and that its presence could indicate a risk of KFDV. Further surveillance assessing the distribution and abundance of *H. spinigera* could reveal the true extent of KFDV in India.

Emergence of Alkhurma Haemorrhagic Fever in Saudi Arabia

Alkhurma haemorrhagic fever virus (AHFV) is a recently described tick-borne virus within the genus *Flavivirus* and family Flaviviridae (Horton et al., 2016). AHFV was first isolated in 1995 from a patient with haemorrhagic manifestations and fever in the city of Alkhurma in Saudi Arabia (Zaki, 1997). The whole genome of AHFV has been derived, and shares 89% sequence identity with KFDV (Charrel et al., 2001). This suggests that AHFV is a variant genotype of KFDV but with a distinct geographical distribution. AHFV is classified as biosafety level 3 agent (Charrel et al., 2007).

To investigate the tick-borne nature of AHFV, ticks were collected in both western and southern Saudi Arabia and assessed by reverse transcriptase PCR (Eraksoy, 2015). At present, AHFV has been identified in both the *Argasid* tick *Ornithodoros savignyi* and the *Ixodid* tick *Hyalomma dromedarii* (Charrel et al., 2007). As a recently emerged virus, the geographic range of AHFV is poorly understood. The wide distribution of *Ornithodoros* and *Hyalomma* spp. ticks suggests that the geographic limits of AHFV may be larger than presently assumed. The clinical case from Najran and report of AHFV in ticks from the Horn of Africa supports this view (Memish et al., 2005; Horton et al., 2016).

Increased Human Incidence of Powassan Virus in New England

Powassan virus (POWV) causes fatal encephalitis in a proportion of humans that become infected with it. Between 2013 and 2015, 8 cases of POWV encephalitis were reported from hospitals in Massachusetts and New Hampshire (Piantadosi et al., 2016). This represented the most recent evidence of an increasing trend for human cases of POWV in the United States where 9 cases were reported between 1999 and 2005 (Hinten et al., 2008). Similar trends were observed for other tick-borne diseases such as Lyme borreliosis (Bacon et al., 2008) and infection with *Babesia microti* (Vannier et al., 2015). POWV is a flavivirus, causing a febrile disease that can develop into severe meningoencephalitis. The virus was first reported from a fatal case in a 5 year old child in Powassan, Ontario in 1958 (McLean and Donohue, 1959).

Preliminary virological analysis suggested an association with tick-borne viruses such as (RSSEV) pointed researchers in the direction of ticks as the vector. Subsequent field surveillance detected POWV in pools of *Ixodes cookei* ticks and provided evidence that small mammals such as groundhogs (*Marmota monax*) contribute to the maintenance of virus (McLean et al., 1967). Surveillance by groups in the United States detected POWV in Colorado (Thomas et al., 1960) and New York State (Whitney and Jamnback, 1965). Numerous examples of POWV have now been isolated from the black-legged tick *I. scapularis* (Anderson and Armstrong, 2012) indicating that this tick species may be the most abundant vector of the virus across the eastern states of the US and southern states of Canada. In a further twist, POWV has been reported from the eastern province of Russia, Primorsky Krai, and is suspected of being established across a larger geographical area (Leonova et al., 2009; Deardorff et al., 2013). Here, the tick vector includes *Ixodes* spp. such as

I. persulcatus. Phylogenetic analysis of North American POWV strains indicates that POWV diverged from a common ancestor present ∼500 years ago (Pesko et al., 2010). The viruses in Russia could be an introduction due to their similarity with American isolates. One possible means of introduction of the virus could be the importation of North American species such as mink (*Neovison vison*) with infected ticks during the expansion of the Russian fur trade.

The number of human cases of Powassan virus infection across its range has increased in recent years. The underlying cause is unclear but could be due to an increased awareness amongst clinicians and diagnosticians. Alternatively, the increase could be related to ecological factors that lead to the increase in abundance of the tick population driven in turn by increases in the number and range of mammalian hosts such as deer. This is believed to have been behind the increase in Lyme disease, caused by *B. burgdorferi*, across many States in the USA (Barbour and Fish, 1993). However, the nymphal form of the tick is suspected of being responsible for most cases of transmission and abundance of this life stage is more dependent on the availability of small mammals such as rodents, for example the white-footed mouse (*Peromyscus leucopus*). Therefore, factors that influence rodent abundance such as predator decline, mediated by the red fox (*Vulpes vulpes*) for example, may have greater impact on disease transmission by ticks (Levi et al., 2012).

The Spread of the Deer Tick Virus in North America

Deer tick virus (DTV), a flavivirus in the tick-borne encephalitis group, is a genetically distinct lineage (subtype) of POWV that can cause neuroinvasive infection in humans in parts of North America. DTV was originally isolated from the Rocky Mountain wood tick, *Dermacentor andersoni,* but is mainly found in *I. scapularis* collected from states in the north-east of the US (Telford et al., 1997; Aliota et al., 2014) and has since been responsible for a human case of encephalitis (Tavakoli et al., 2009). Although the first recognized human case of DTV encephalitis occurred in 1997, evidence of the causative virus, based on sequence data, was not available until 2001 (Gholam et al., 1999; Kuno et al., 2001). The DTV genome shares 84% sequence identity with POWV and 94% amino acid identity between the virus polyproteins. However, DTV and POWV are regarded as antigenically indistinguishable and the infecting virus cannot be determined by serological testing, and genotypic analysis is needed to make a definitive diagnosis. DTV is maintained in an enzootic cycle between *I. scapularis* and the white-footed mouse (*Peromycus leucopus*) (Ebel et al., 2000). Until now there have been a small number of published cases of proven DTV induced encephalitis (Kuno et al., 2001), one from Canada and three from USA. Based on detection of virus genome, prevalence with DTV ranged up to 5% in *I. scapularis* from several geographic areas including Hudson Valley, Nantucket Island and Prudence Island (Aliota et al., 2014). The increase in the number of human cases of Powassan virus encephalitis that have been reported since 2010 is remarkable. Most of these cases were diagnosed by serological assays (Khoury et al., 2013) and it is possible that DTV could be responsible for some of these cases.

THE EMERGENCE OF ASFARVIRUS IN EUROPE

Establishment of African Swine Fever Virus in Eastern Europe

African swine fever virus (ASFV) is the causative agent of African swine fever (ASF), an acute haemorrhagic fever that causes severe morbidity and high mortality in domestic pigs. ASFV is classified within the genus *Asfivirus* and family Asfarviridae, and consists of a double-stranded DNA genome coding for ∼150 proteins. The genome is surrounded by a protein capsid and a host-derived envelope. Virus particles are very robust and can survive for days in the environment or months within pork meat. ASF was first described in Kenya and is found across sub-Saharan Africa (Thomson, 1985). The epidemiology of ASF in Africa is driven by two cycles. The first is sylvatic with bush pigs (*Potamochoerus larvatus*) and warthogs (*Phacochoerus africanus*) being infected following infestation with *Argasid* ticks of the genus *Ornithodorus*, particularly *O. moubata*. Infections in wild species do not result in clinical signs of disease. The second cycle involves infection of domestic pigs (*Sus scrofa*) that are highly susceptible to infection and shed virus in excreta. Initial infection is by tick bite but is then amplified by pig-to-pig transmission, either through contact or consumption of contaminated food. ASF is a notifiable disease in Europe (http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2016/) and significant effort is directed at preventing its introduction through import control of livestock and food stuffs. Outbreaks resulting from importation have occurred in domestic pigs on a number of occasions across Europe. Significantly in the case of Sardinia, following introduction in the late 1970s. Repeated attempt to eliminate the disease have failed and the disease is endemic (Mur et al., 2016). More recently, ASFV was introduced into the Caucasus region in 2007 (Rowlands et al., 2008) and led to extensive spread into neighboring countries including Armenia, Azerbaijan, and Russia. By 2016, ASFV has been detected throughout Eastern Europe and the Baltic region. Pig-to-pig transmission appears to be the main driver for the spread of the epidemic but wild boar, a species which is also susceptible to infection, could also contribute to spread (Guinat et al., 2016). A further concern for Europe is the infection of indigenous *Argasid* ticks such as *O. erraticus* that are present throughout the Mediterranean Basin and Balkans, and feed preferentially on domestic pigs, although less so on wild boar (Pietschmann et al., 2016). ASFV infects and replicates in *O. erraticus* (Basto et al., 2006; Ribeiro et al., 2015) and has been shown to experimentally transmit ASFV to pigs (Boinas et al., 2011). Widely distributed *Ixodid* ticks in Europe such as *I. ricinus* and *Dermacentor reticulatus* are unable to support ASFV replication and presumably do not contribute to disease spread (de Carvalho Ferreira et al., 2014). The spread of ASFV has been primarily caused by human activities including long distance transport of

livestock. The presence of a susceptible wildlife host, wild boar, has further complicated efforts to control the disease and it is likely that it will continue to spread across the continent.

TICK SALIVA-ASSISTED TRANSMISSION AND POTENTIAL ANTIGENIC TARGETS FOR CONTROL

The components of saliva are critical to the successful completion of a blood meal by all life stages of ticks. As a result, tick saliva is a highly complex mixture of proteins, peptides, and other bioactive compounds. Transcriptomic analysis suggests that tick feeding leads to the upregulation of thousands of protein transcripts (Karim and Ribiero, 2015; Ribeiro et al., 2017) that when secreted promote attachment to the host, inhibit host responses such as blood clotting and inhibit microbial growth (Hovius et al., 2008). Tick saliva has also been shown to promote transmission of tick-borne viruses to the mammalian host. Early studies demonstrated that salivary gland extracts (SGE) from a range of tick species enhanced transmission of Thogoto virus and TBEV to guinea-pigs (Jones et al., 1989, 1992; Labuda et al., 1993). Recent studies have shown a similar enhancement by *O. porcinus* SGE of ASFV infection in pigs (Bernard et al., 2015) and *I. scapularis* SGE of POWV infection in mice (Hermance and Thangamani, 2015). One mechanism suggested for this enhancement is the increased attraction of macrophages and other antigen presenting cells to the site of tick attachment. These appear to rapidly disseminate the virus throughout the mammalian host. It is also possible that rather than being passively transmitted by the tick, viruses actively modulate tick salivary gland transcripts. In a study investigating feeding by *I. scapularis* nymphs, infection with Langat virus modulated tick salivary gland transcriptional responses during 3 days of feeding (McNally et al., 2012). Further analysis of virus-induced transcripts could lead to the identification of those proteins that promote virus transmission and in turn could be molecular targets for tick control and prevention of virus transmission. The concept of a tick vaccine has been discussed for decades and has led to a number of approaches based on specific tick salivary proteins that have been used as vaccine formulations that inhibit tick feeding (Labuda et al., 2006; Garcia-Varas et al., 2010). The advent of "omics" technologies using both the sialotranscriptome (Maruyama et al., 2017) and the proteome (Villar et al., 2017) have dramatically expanded the number of targets available for vaccine development. The application of such vaccines to livestock or even key wildlife species could provide an opportunity to suppress tick abundance and reduce the frequency of pathogen transmission in order to suppress incidence of disease.

CONCLUSIONS

The emergence of tick-borne viruses is driven by a range of factors, often inter-related, that lead to the appearance and/or increase in human or veterinary cases of disease. *Ixodid* tick species have multiple life stages (**Figure 1**) with each feeding off a different host, and often a different host species. Factors that influence each life stage will affect their ability to transmit pathogens. Key amongst these factors are those associated with the tick vector, including its presence in an area, feeding behavior, abundance, and contact with humans or livestock. Studies on TBEV suggest that co-feeding favors virus transmission between immature tick stages (larva/nymphs) and environmental factors that promote co-feeding will drive infection rates up via horizontal transmission and increase the risk of transmission to humans (Randolph et al., 1999). This leads to the paradoxical situation whereby decreases in numbers of mammalian hosts can lead to an increase in disease transmission. Seasonal variation in temperatures can also dramatically affect transmission to humans. Low temperatures in winter and warm summer temperatures between 2009 and 2012 have been identified as the reason for the increased incidence of TBEV in Sweden enhanced by co-feeding by *I. ricinus* on small mammals (Jaenson et al., 2012). Warm summer temperatures also encourage humans to spend more time outdoors and wear less clothing, both factors that could increase the risk of encountering ticks. The presence of large ruminants such as deer can increase the abundance of ticks and indirectly indicate risk of virus transmission (Carpi et al., 2008). Conversely, activities that reduce tick abundance will reduce virus transmission.

The distribution of a tick-borne disease is usually dictated by that of the tick vector. This appears to be the case for viruses such as CCHFV. However, when driven by anthropomorphic factors, such viruses can emerge in new locations, enabled by an alternative mechanism of transmission. This appears to have led to the emergence of ASFV in Central Europe where tick-borne transmission has been replaced by contact transmission between pigs, driven in part by the presence of wild boar (Guinat et al., 2016).

Human factors are a major influence on disease emergence. The paradigmatic example of this is the emergence of Omsk haemorrhagic fever virus in central Russia due to the importation of muskrats (*Ondatra zibethicus*) from North America in the nineteenth century (Růžek et al., 2010). The virus existed in the area within the *D. reticulatus* tick population but was rarely, if ever, encountered by humans. By introducing an exotic mammalian host that was highly susceptible to infection and regularly handled by humans, clusters of haemorrhagic disease occurred. A similar scenario appears to have resulted in the introduction of POWV in Eastern Russia and the activities of the farming industry in Eastern Europe has propelled the spread of ASFV. A further human factor that can increase detection rates of emerging tick-borne diseases is awareness by clinicians. Once a cluster of cases of disease have been reported, further cases come to light. However, a lack of disease awareness among clinicians and veterinarians can hamper the control of a disease epidemic, which could potentially be the case for ASFV where clinical signs can be similar to those observed with other diseases of swine (Guinat et al., 2016). Therefore, an increase in awareness and data sharing of new and emerging tick-borne diseases among medical and veterinary professionals is essential. Trans-national bodies such as the World Health Organization and the World Organization for Animal Health (OIE) play a key role in

information sharing and setting standards for disease detection and reporting.

Tick-borne diseases continue to cause a burden to both animal and human health. As the twenty-first century progresses there will certainly be more examples of tick-borne virus spread and novel virus emergence. New viruses associated with ticks are being discovered as a result of human infection, such as Bourbon virus (Kosoy et al., 2015) or before there is any association with disease such as Tofla virus (Shimada et al., 2016; de Figueiredo et al., 2017). Early recognition of those that are pathogens will be critical to their control in addition to measures to control potential tick vectors. Understanding the interactions between these emerging viruses and the tick species that transmit them to vertebrate hosts represents both a colossal challenge and a great opportunity to identify targets for future control of tick-borne diseases. A greater understanding of tick feeding has identified both the complexity of the feeding process and its role in promoting pathogen transmission (Randolph, 2009). This understanding has also resulted in the identification of protein components within tick saliva that can act as antigens for vaccines that suppress tick infestation and protect against tick-borne virus transmission (Labuda et al., 2006). Such generic approaches may assist in protecting humans and livestock from emerging tick-borne viruses.

AUTHOR CONTRIBUTIONS

NJ conceived the review. KM, LJ, LP, and NJ wrote the paper. All authors reviewed the final version of the manuscript.

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PERSPECTIVE
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Tick-Virus Interactions: Toll Sensing

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Ticks are important vectors of viruses that infect and cause disease in man, livestock, and companion animals. The major focus of investigation of tick-borne viruses has been the interaction with the mammalian host, particularly the mechanisms underlying disease and the development of vaccines to prevent infection. Only recently has research begun to investigate the interaction of the virus with the tick host. This is striking when considering that the virus spends far more time infecting the tick vector relative to the vertebrate host. The assumption has been that the tick host and virus have evolved to reach an equilibrium whereby virus infection does not impede the tick life cycle and conversely, the tick does not restrict virus replication and through blood-feeding on vertebrates, disseminates the virus. The development and application of new technologies to tick-pathogen interactions has been fuelled by a number of developments in recent years. This includes the release of the first draft of a tick genome, that of *Ixodes scapularis*, and the availability of tick-cell lines as convenient models to investigate interactions. One of the by-products of these investigations has been the observation of familiar proteins in new situations. One such protein family is Toll and Toll-like receptors that in vertebrates play a key role in detection of microorganisms, including viruses. But does Toll signaling play a similar role in detection of virus infection in ticks, and if it does, how does this affect the maintenance of viruses within the tick?

Keywords: ticks, Toll, Toll-like receptors, virus, immunity

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TICK-MICROORGANISM COEXISTENCE

The phylum Arthopoda emerged during the "Cambrian explosion" (540–485 million years ago) creating numerous groups, many that have survived to the present day. One of these, the Chelicerata, contains the order Acari, which in turn contains species that obtain nutrition through blood feeding on vertebrates, collectively termed ticks. Fossil records indicate that ticks have been present from at least the Cretaceous period (146–65 million years ago) where they could feed on mammals (de la Fuente, 2003), and likely evolved earlier to take blood meals from reptiles and then birds (Nava et al., 2009). Irrespective of the precise date that hematophagous behavior evolved, it is clearly measured in millions of years and implies a long period over which ticks were in turn parasitized by microorganisms (viruses, bacteria, and protozoa) that are found in abundance in ticks extant today (Vayssier-Taussat et al., 2015). The presence of microorganisms in ticks appears to have little impact on the tick, although presumably there is an energetic cost to harboring such microorganisms. Some authors have characterized this as a combination of conflict and cooperation (de la Fuente et al., 2016). However, recent studies demonstrated that ticks do respond in a coordinated fashion to infection with pathogens of mammals (Alberdi et al., 2016), at least in order to control infection if not eliminate it.

One of the major groups of microorganisms associated with transmission by ticks is the viruses (Labuda and Nuttall, 2004). The interaction of most tick-borne viruses with vertebrate hosts leads to a transient infection that causes morbidity and mortality. Occasionally, viruses are found that appear avirulent in humans, such as the flavivirus Langat virus (LGTV), although these are the exception and provide a useful model for more virulent viruses (Tsetsarkin et al., 2016). Infection with a virulent virus in a vertebrate host is usually short-lived and, if the host survives, eliminated by the rapid induction of antibodies and subsequent development of cell-mediated responses. By contrast, the interaction with the tick appears more benign and long-lived (Nuttall, 2009). Indeed, for ticks to act as the reservoir for viruses, the virus must persist in the tick for long periods, potentially years, without harming the tick or preventing completion of its various life stages. In addition, viruses can be transmitted transovarially to the next generation of ticks.

Such a harmonious arrangement contrasts completely with the virus-vertebrate interaction and recent investigations suggest that the virus-tick relationship is more dynamic. Preliminary findings have demonstrated both transcriptomic and proteomic responses to infection with flaviviruses such as tick-borne encephalitis virus (TBEV) and LGTV (Weisheit et al., 2015). A subsequent proteomics study of *Ixodes scapularis* cells infected with LGTV demonstrated increased expression of proteins associated with metabolic pathways (Grabowski et al., 2016). This may represent a cellular response to stress or manipulation, by the virus, of the host cells metabolic machinery. What other potential responses does the tick have in response to virus infection?

ANTIVIRAL RESPONSES IN TICKS

Arthropods have an array of antiviral mechanisms to prevent and control infection (reviewed by Kopáček et al., 2010) . These include RNA interference (Schnettler et al., 2014), antiviral peptides such as defensins (Talactac et al., 2017) and detection through Toll receptors (Rükert et al., 2014). This last group have been extensively studied in vertebrates. Toll-like receptors (TLRs) are a recognized family of pattern-recognition receptors that form part of the innate immune system of vertebrates (Akira and Takeda, 2004). In addition to binding to a diverse range of pathogen motifs, they also provide a signaling function that activates immune responses to infection. A distinctive feature of the TLRs is their conserved structure composed of an N-terminal leucine-rich repeat (LRR) ectodomain, a transmembrane domain, and toll-interleukin receptor (TIR) signaling domain (Bell et al., 2003). Multiple LRRs, ranging from 19 to 25 in human TLRs, create a long stretch of beta-sheet that forms a horseshoe-shaped structure that enables pattern-recognition (Botos et al., 2011). The importance of TLRs to the control of infection is highlighted by the widespread presence of these proteins in both invertebrates and vertebrates (Buchmann, 2014).

Toll-like proteins evolved early in the evolution of life and the proteins present in extant species can be found in most multicellular organisms, including many ancient invertebrates (Buchmann, 2014). This is not the case for all innate immune proteins. RIG-like receptors (RLRs), including proteins such as RIG-I, LGP2, and MDA5, have not been found in arthropod genomes although they are present in other invertebrate animals, suggesting the early loss of RLR precursors in the phylum's evolution (Mukherjee et al., 2013). However, TLR genes are often present in numerous copies within the genome of many species and have evolved to fulfill a number of roles including structural development (Anderson et al., 1985) and immunity against pathogens, including viruses (Ferreira et al., 2014). However, the mechanism of action of arthropod Toll differs from the pattern recognition receptor function of mammalian TLRs. Insect Toll is activated as a result of cleavage of an endogenous ligand protein, Spätzle, following engagement with carbohydrates of microbial origin (Arnot et al., 2010). Cleavage causes conformation change in Spätzle enabling it to engage with the Toll receptor. It is likely that Toll functions through a similar mechanism in ticks but what is the evidence for this?

A ROLE FOR TOLL IN TICK ANTIVIRAL RESPONSES

Firstly, is there a gene encoding tick Toll in the tick genome? The completion of a detailed draft of the *I. scapularis* genome

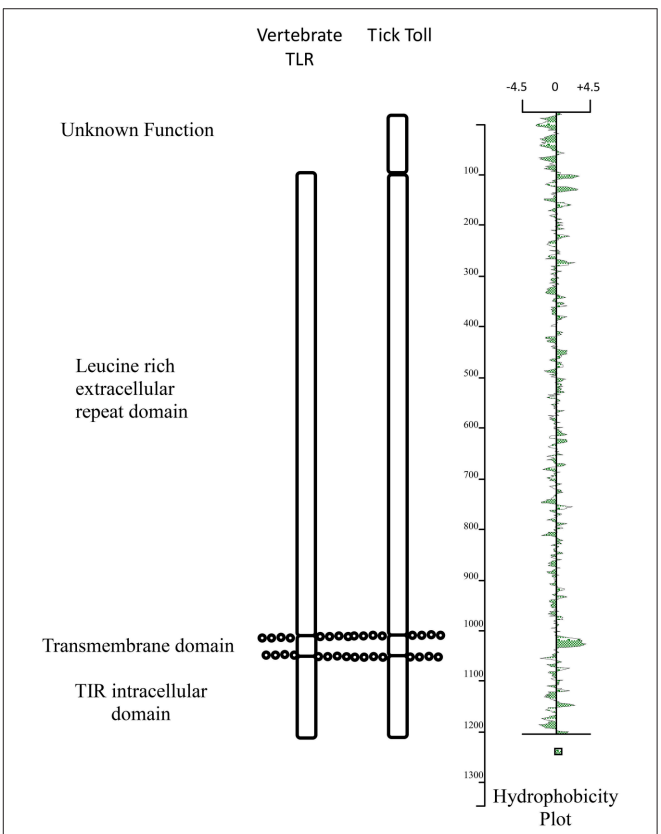

FIGURE 1 | Schematic comparison of the main structural domains present in *Ixodes ricinus* toll (ISWC02240) and human TLR3.

(Gulia-Nuss et al., 2016) suggests that multiple isoforms of tick Toll exist. A recent review on the subject of immunity genes in *I. scapularis* reported 13 copies of *Toll* genes and 2 copies of the *Spätzle* gene (Smith and Pal, 2014). This compares favorably with the nine Toll receptors that exist in *Drosophila melanogaster* (Arnot et al., 2010). However, it is too early to assume that all of the genes identified in ticks produce a functional protein and have a role in immunity. Some may be pseudogenes or produce proteins with a developmental function.

An alternative approach is to measure Toll activity in ticks in response to microbial infection. Mansfield et al. (2017) have recently compared the transcriptional response to infection of *Ixodes ricinus* cells with a bacterium, *Anaplasma phagocytophilum*, to infection with two flaviviruses, louping ill virus (LIV) and TBEV. One striking observation from this study was the up-regulation of a single Toll-like protein transcript (ISCW022740) following infection with the viruses but not the bacterium. Three other Toll transcripts showed very little change to infection with the pathogens. This suggests that transcript ISCW022740 may play a role in the antiviral response. This strongly mirrors the mammalian response to infection where Toll-like receptors such as TLR3 are upregulated in response to infection (McKimmie et al., 2005). Structurally, the protein encoded by transcript ISCW022740 shares many of the characteristics of TLRs including a large leucine rich domain composed of numerous LRR motifs, a transmembrane domain and a putative TIR domain (**Figure 1**). In addition, there appears to be a one hundred amino acid domain at the amino terminus of the protein (extracellular) that increases the size of tick toll in comparison with mammalian TLRs. The role of this extra domain is unknown although appears to be shared with other arachnid toll proteins and may play a role in engaging with the homolog of the Spätzle protein.

CONCLUSIONS

The association between ticks and viruses is a fascinating one and a growing field of investigation. Ticks harbor a vast array of endogenous viruses (Bell-Sakyi and Attoui, 2013; Li et al., 2015). However, it is not clear what impact this infection has on the tick and there is little evidence that this impact is deleterious in the way that certain viruses are to insects (Carlson et al., 2006; Chen and Siede, 2007; Xu and Cherry, 2014). Ticks encode Toll proteins and there is early evidence that at least one of these proteins could play some role in the tick response to virus infection. This may take the role of actively controlling virus and *in vitro* infection of tick cells with tick-borne viruses shows no apparent cellular changes in stark contrast to the lytic cytopathic effect observed in many mammalian cells infected with the same virus. However, an alternative interpretation could be that infection stresses the cell. Cell-lines used in such studies are often derived from embryonic tissue and stress could lead to induction of transcripts associated with a developmental response. Ticks appear to tolerate virus infection but further investigation is required to understand what mechanisms tick cells use to control virus infection and why this does not lead to elimination of the virus analogous to the response in vertebrate hosts.

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The author confirms being the sole contributor of this work and approved it for publication.

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Crimean-Congo Hemorrhagic Fever: Tick-Host-Virus Interactions

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Crimean-Congo hemorrhagic fever virus (CCHFV) is transmitted to humans by bite of infected ticks or by direct contact with blood or tissues of viremic patients or animals. It causes to humans a severe disease with fatality up to 30%. The current knowledge about the vector-host-CCHFV interactions is very limited due to the high-level containment required for CCHFV studies. Among ticks, *Hyalomma* spp. are considered the most competent virus vectors. CCHFV evades the tick immune response, and following its replication in the lining of the tick's midgut, it is disseminated by the hemolymph in the salivary glands and reproductive organs. The introduction of salivary gland secretions into the host cells is the major route via which CCHFV enters the host. Following an initial amplification at the site of inoculation, the virus is spread to the target organs. Apoptosis is induced via both intrinsic and extrinsic pathways. Genetic factors and immune status of the host may affect the release of cytokines which play a major role in disease progression and outcome. It is expected that the use of new technology of metabolomics, transcriptomics and proteomics will lead to improved understanding of CCHFV-host interactions and identify potential targets for blocking the CCHFV transmission.

Keywords: Crimean-Congo hemorrhagic fever virus, tick, humans, interactions, immune response

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INTRODUCTION

Crimean-Congo hemorrhagic fever virus (CCHFV, genus Nairovirus, family *Bunyaviridae*) circulates in nature in an enzootic cycle between ticks and non-human vertebrates and poses a significant public health threat due to its high pathogenicity to humans. Humans are infected by bite of infected Ixodid ticks (mainly *Hyalomma* spp.), or by contact with blood or tissues of viremic patients or animals. The disease (CCHF) is characterized by abrupt onset of fever, headache, fatigue, and myalgia, as well as gastrointestinal symptoms, such as nausea, vomiting, and diarrhea. Severe cases present hemorrhagic manifestations ranging from petechiae, epistaxis, ecchymosis, and gingival hemorrhage to severe hemorrhages from various systems. The fatality rate is up to 30%. Wild and domestic animals present a short viremia (2–15 days) and they do not develop clinical illness.

CCHFV is a negative sense, single-stranded RNA virus with a tri-segmented genome consisting of the small (S), medium (M), and large (L) segments which encode the nucleocapsid (N) protein, the glycoprotein precursor (which gives rise to the envelope glycoproteins Gn and Gc) and the L protein, respectively. The CCHFV genome is encapsidated by multiple copies of N protein to form a ribonucleocapsid complex which is critical for the virus replication cycle (Carter et al., 2012). The L protein contains a viral RNA-dependent RNA polymerase domain and an ovarian tumor (OTU) domain with deubiquitinating and deISGylating activities, which is thought to suppress immune signaling (Frias-Staheli et al., 2007).

CCHF endemic foci are present in Africa, Asia, and Europe. Its geographic distribution is associated with that of *Hyalomma* spp. ticks (mainly *H. marginatum, H. rufipes, H. anatolicum,* and *H. asiaticum*) which are the main competent vectors of the virus. The term "vector competence" is used to describe the ability of a vector to acquire, maintain and transmit a pathogen. *H. marginatum* is present in southern Europe and some parts of Asia and Africa. It is characterized by its aggressiveness in seeking human hosts. In CCHF endemic areas, where the climatic and environmental factors are suitable for *H. marginatum* ticks (and their animal hosts), their population is increased in spring and summer, accounting for >30% of tick species in the area. CCHFV has been detected or isolated from additional tick species, but studies are needed to show whether they are competent virus vectors, or merely coincidental unmaintained tick infection from recent feeding on an infected animal or co-feeding (feeding on an uninfected vertebrate host in close proximity with an infected tick) or the result of a recent blood meal on an infected animal. For an arthropod to be incriminated as an actual vector, several criteria must be met; such as vector competence in laboratory studies, and evidence that the arthropod species feeds in nature on a host that develops an appropriate viremia and that it is active at the time of the year that viral transmission is occurring (Reeves, 1957; Turell, 2007). The virus persists in ticks for the duration of the tick lifespan, while the overwintering of the infected ticks plays a critical role in the maintenance of epidemic foci.

As in most arboviral infections, the main players in CCHF are the vector, the pathogen and the host, resulting in the infection (or not) of the host. The co-evolution of the ticks, hosts and pathogens results in conflict or cooperation between them, benefiting ticks and pathogens and, to a lesser extent, hosts (de la Fuente et al., 2016). CCHFV-infected humans may present asymptomatic, mild, severe, or even fatal disease. The course and the outcome of the disease depend on the individual characteristics of the vector, the virus strain and the host, but also on the vector -pathogen-host interactions. The laboratory studies about these interactions are limited due to the high-level containment required for CCHFV and the lack of an animal model, until recently. In this review we will examine the recent findings on CCHFV and discuss the potential contribution of the new technologies to future research in order to better understand the molecular and cellular basis of these interactions.

TICK-PATHOGEN INTERACTIONS

Ticks serve as vectors and reservoirs of CCHFV which can be maintained by transovarial and transstadial (from larva to nymph and adult), and, less efficiently, by venereal transmission (Gonzalez et al., 1992). Ixodid ticks, particularly members of the *Hyalomma* genus, are considered main competent vectors, while additional tick species may maintain the enzootic foci of CCHFV circulation between ticks and wild and domestic mammals (Hoogstraal, 1979). The virus has to overcome the midgut and the salivary gland barriers in the tick body (**Figure 1**). Tick vector competence is influenced by the ability of transmitted pathogens to evade tick innate immune response (Hajdusek et al., 2013).

The molecular events at the tick-pathogen interface are not known. Most likely, the first step is the interaction of CCHFV envelope glycoproteins and the epithelial cells of the ticks. The glycoprotein Gc was shown to be a class II viral fusion protein (Garry and Garry, 2004). Like other invertebrates, ticks do not present adaptive immunity, and they rely on innate immune response consisting of phagocytosis, encapsulation, nodulation, and secretion of humoral factors in the hemolymph (McNally and Bloom, 2014). An additional important mechanism of innate antiviral defense of arthropods (including ticks) against arboviruses, RNA interference (RNAi), was investigated on Hazara nairovirus, which is considered as a surrogate CCHFV model. It was shown that small interfering RNAs (siRNAs), targeting Hazara nairovirus N protein mRNA, inhibited virus replication, and the antiviral effect was stronger when siRNAs were combined with ribavirin (Flusin et al., 2011). The exact role of RNAi in tick-CCHFV interactions remains to be elucidated.

Following a blood meal, CCHFV evades the tick humoral and cellular immune responses and replicates in the lining of the tick's midgut; then it is disseminated to the hemolymph and infects various tissues, with highest viral titers being observed in the proliferating tissues (e.g., salivary glands and reproductive tissues) (Dickson and Turell, 1992). The minimum virus titer necessary to infect the ticks varies among tick species (Shepherd et al., 1991). Following intracoelomic inoculation of CCHFV, virus titer is not affected by tick's sex and feeding status (unfed or engorged), but it is positively related with blood feeding (Dickson and Turell, 1992). CCHFV replication in tissues of an infected tick may be stimulated by tick attachment and feeding on a susceptible host, probably by reducing the stress on a tick induced by viral replication while the tick is waiting to find a vertebrate host, but increase the potential for viral transmission once a host had been acquired (Turell, 2007).

Using a transmission model for CCHFV and next generation sequencing it was shown that many mutations in CCHFV were recovered from ticks after only a single transstadial transmission, whereas no mutations were detected in CCHFV recovered from the mammalian host, with greater viral intra-host diversity in the tick rather than the vertebrate host (Xia et al., 2016).

CCHFV is generally not the sole microbe in ticks; endosymbionts and several pathogens may be present at the same time (Papa et al., 2017). Metagenomic studies showed that the microbiome has an effect on tick fitness and pathogen infection and transmission. As an example, *Francisella*-like endosymbionts have been detected in *Hyalomma* spp. ticks (Ivanov et al., 2011; Szigeti et al., 2014). Although the presence of additional pathogens or endosymbionts may affect the physiology and immune response of the ticks, there are no related studies.

Viral infections in ticks are not entirely silent and may affect the tick survival, behavior and gene expression (McNally and Bloom, 2014). Next generation sequencing of infected and uninfected ticks microbiome may give more insights into the interactions between pathogens and ticks.

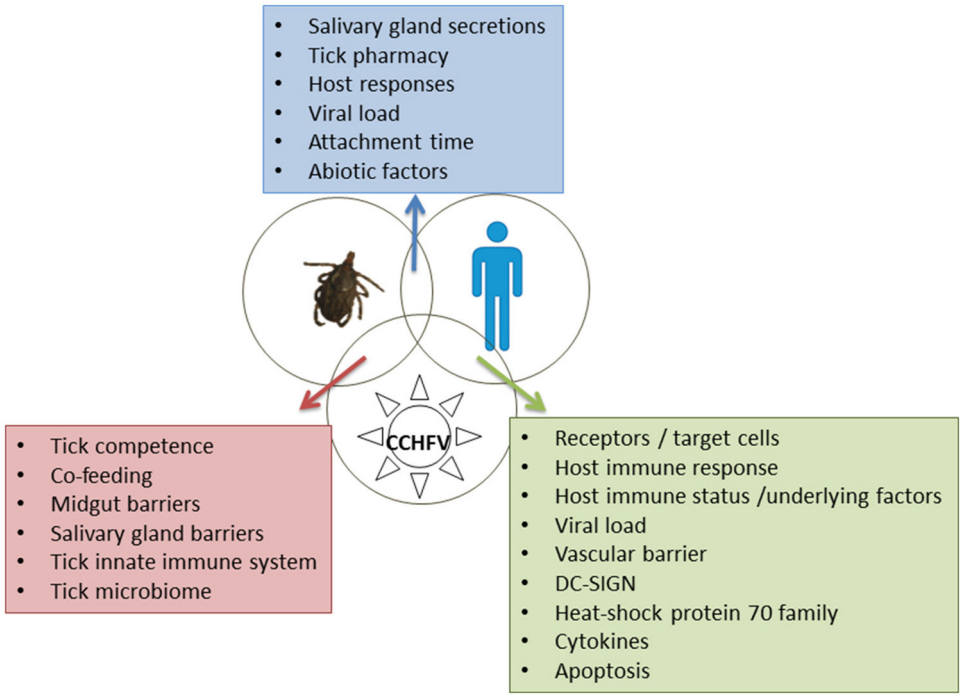

FIGURE 1 | Tick-host-CCHFV interactions. Factors playing a major role are shown in the boxes.

TICK-HOST INTERACTIONS

The general issues concerning tick-host interactions likely apply to CCHFV. The first contact between the tick and the host occurs during the tick bite and the prolonged complex process of the tick feeding on the host. The introduction of salivary gland secretions into the feeding lesion is the major, if not exclusive, route via which pathogens and toxins access the vertebrate host and mediate the host reactions (Kaufman, 1989). Despite the host's hemostatic, inflammatory and immune responses, the tick manages to remain attached for blood-feeding via the pharmacy located in its salivary glands and secreted in saliva. Anticoagulants, cytolytic substances, vasoactive mediators (such as prostaglandins) and cement, which anchors the mouthparts to the skin, are among the secreted agents. Saliva activated transmission, subsequently renamed saliva-assisted transmission (SAT), affects the host in ways that are exploited by many pathogens to facilitate infection (Nuttall, 1999); SAT is thought to play an additional critical role facilitating the infection of uninfected ticks feeding at the same time on the same host in the absence of an overt host viremia (co-feeding or mechanical transmission) (Gordon et al., 1993). There are no reports on the role of SAT on CCHFV. Because the salivary glands are the most important route for pathogen transmission by arthropod vectors, it is expected that the volume of saliva secreted into the host would be a major factor determining the efficacy of transmission (Kaufman, 2010). Time of attachment may also affect the level of tick-host interaction. Abiotic (environmental and climatic) factors are involved indirectly in the tick-host interactions by playing a role in the abundance and aggressiveness of ticks, thus affecting the chance of a host to be bitten by ticks (**Figure 1**).

HOST-PATHOGEN INTERACTIONS

CCHFV must overcome the epithelium and preferentially escape at the basolateral membrane of epithelial cells to establish infection (Connolly-Andersen et al., 2007). CCHFV replicates to high titers at the site of inoculation, in epithelial cells, dendritic cells, and tissue resident macrophages. The productive infection of these cells facilitates spread of the virus and results in early infection of local lymph nodes and peripheral blood-borne monocytes supporting systematic spread of the virus (Burt et al., 1997; Connolly-Andersen et al., 2009; Akinci et al., 2013).

To date the receptor of CCHFV in target cells is not known. The viral glycoproteins Gn and/or Gc are involved in the initial attachment of CCHFV to the cell plasma membrane. It was suggested that Gc is responsible for binding to the cellular receptors, and mediates fusion later, during the early step of replication cycle. An interaction between CCHFV glycoproteins and cell surface nucleolin, a protein found predominantly within nucleoli, has been suggested as putative entry factor; however, more investigations are needed to support the involvement of nucleolin in CCHFV internalization (Xiao et al., 2011). CCHFV enters the cells using clathrin- and the clathrin pit adaptor protein-2 complex, but not caveolin-1 (Simon et al., 2009a; Garrison et al., 2013). Internalization is cholesterol- and pH-dependent (Simon et al., 2009b). Then, CCHFV particles are

transported to early endosomes and to multivesicular bodies where the fusion of the virus envelope with cellular membranes takes place. These processes use components of the endosomal sorting complex required for transport regulators (Shtanko et al., 2014).

Cytoskeleton components, including microtubulin and actin filaments, are essential for CCHFV internalization, replication and progeny virus production (Andersson et al., 2004; Simon et al., 2009a). The predicted actin-interacting domain is localized within the central stalk region of the CCHFV N protein adjacent to the coiled-coil motif. The key residue responsible for N protein-actin interaction, D219, and is also crucial for self-association of the N protein (Levingston Macleod et al., 2015). Furthermore, it has been recently shown that the CCHFV N protein interacts with cellular chaperones of the heat shock protein 70 family (including actin), which, in association with DnaJ cofactor adapter proteins, play roles that relate to correct folding and transport of newly synthesized and misfolded proteins and to the assembly of multicomponent complexes (Surtees et al., 2016). One other protein which has been recently demonstrated to be involved in CCHFV replication is aquaporin 6, a water channel that facilitates fluxes of water and small solutes across membranes (Molinas et al., 2016).

The infection of endothelial cells and peripheral blood-borne monocytes results in extravasation into parenchymal tissue enabling the virus to interact with basolateral cells receptors in target organs (Connolly-Andersen et al., 2007). Secondary replication in these organs facilitates the systemic spread of the virus in humans (Akinci et al., 2013). This theory is supported by studies in animal models, which showed that on the first day of infection, the viral replication occurs in the blood, on the second day in spleen and liver, and then spreads systemically to the lungs, kidneys, and brain (Bente et al., 2010).

The virus enters the blood stream overcoming the vascular endothelial surface barrier and the endothelial junctions (Becker et al., 2010). Endothelial cells are targeted either directly by the virus, or indirectly, by virus-induced host-derived soluble mediators that cause endothelial activation (Connolly-Andersen et al., 2011). This has been previously demonstrated for other viral hemorrhagic fevers (Schnittler and Feldmann, 2003). To date, it is not known how CCHFV causes microvascular instability. It is more likely that it is mediated indirectly by increased levels of proinflammatory cytokines, or by a combination of virus infection and the cytokine storm (Connolly-Andersen et al., 2007; Papa et al., 2016).

The endothelial damage is responsible for hemostatic failure by stimulating aggregation and degranulation of the platelets, and activation of the intrinsic coagulation cascade (Weber and Mirazimi, 2008; Bodur et al., 2010). During CCHFV infection, apart from the activated macrophages, an increase in the numbers of natural killer cells and CD3+ CD8+ T cells is observed (Yilmaz et al., 2008; Akinci et al., 2009). But as the disease progresses, the uncontrolled apoptosis of lymphocytes contributes to a depletion in lymphocyte counts, which is presented as lymphopenia (Bente et al., 2010). It has been demonstrated that CCHFV infection can induce apoptosis indirectly, through the release of cytokines from infected cells (Karlberg et al., 2015). This finding fits nicely with the hypothesis described above. Recently it has been shown that CCHFV codes for a non-structural protein, NSs, which may induce apoptosis via both intrinsic and extrinsic pathways (Barnwal et al., 2016).

Soon after the presentation of CCHFV antigen to host cells, innate and adaptive immune responses are activated (**Figure 1**). DC-SIGN (a calcium-dependent [C-type] lectin cell-surface molecule), which is expressed in the antigen-presenting dendritic cells, was suggested as probable entry factor for CCHFV (Suda et al., 2016). *In vitro* studies showed that RIG-I acts as a pattern recognition receptor for CCHFV and mediates a type I interferon (IFN) antiviral response via the cellular adaptor MAVS (Spengler et al., 2015). As in other viral hemorrhagic fevers, replicating CCHFV delays substantially the IFN response, possibly by interfering with the activation pathway of IRF-3, allowing the rapid viral spread in the host (Andersson et al., 2008). Downregulation of IFN-I signaling pathways relies on the cleavage of ubiquitin and ISG15 from various host proteins (Frias-Staheli et al., 2007). It is of interest that the related CCHFV OTU proteases show clear preferences for ISG15s from certain mammalian species (Deaton et al., 2016).

Several cytokines and chemokines are released during the course of CCHF, especially in severe cases (Ergonul et al., 2006; Papa et al., 2006, 2015, 2016; Saksida et al., 2010). Preliminary analysis showed that the expression of microRNAs related to regulation of cytokine expression is altered in CCHF patients (Demir et al., 2017). Genetic factors and immune status of the host, as well as genetic differences in CCHFV strains, may play a significant role in the virus-host interface, however, there are no studies available.

TOOLS FOR RESEARCH ON TICK-HOST-CCHFV INTERACTIONS

Tick cell lines are now available to enable the CCHFV studies *in vitro*, offering an alternative approach to understand the way that tick cells respond to virus infection (Bell-Sakyi et al., 2012). The recent development of CCHF virus-like particle (VLP) systems can be used to study cell entry and viral transcription and replication (Devignot et al., 2015; Zivcec et al., 2015). The fact that VLPs are non-infectious will greatly facilitate the tick-pathogen interaction studies under non-BSL-4 conditions. The widespread adaptation of RNA interference (RNAi) will aid in studying tick gene functions (de la Fuente et al., 2005). Interferon response knockout mice have been recently described as animal models for CCHF (Bente et al., 2010; Bereczky et al., 2010; Zivcec et al., 2013). An *in vivo* transmission model for CCHFV in a BSL4 biocontainment was established recently using interferon knockout mice, which is an additional tool to study the transmission and interaction of CCHFV with its tick vector (Gargili et al., 2013). Advances in the study of molecular events at the tick-host-pathogen interface are expected by the increasing number of available genomic resources, including metabolomics, transcriptomics and proteomics. Mathematical

and relational models are being constructed for the challenging integration of multi-source datasets from biological systems and cellular networks that would improve our understanding of CCHF pathogenesis (Vidal et al., 2011; Gomez-Cabrero et al., 2014).

FUTURE PERSPECTIVES

Over the last decades considerable progress has been made in the identification of the cellular components involved in tick-host-pathogen interactions. However, there is limited knowledge so far in the case of CCHFV due to the high-level containment required for studies with the virus. The identification of the molecular drivers that promote CCHFV survival in the tick, persistence and pathogen transmission provides the opportunity to disrupt these processes and lead to a reduction in tick burden and prevalence of tick-borne diseases (De la Fuente et al., 2017), while the identification of the molecular signaling pathways taking place during the CCHFV-host interactions provides the opportunity to design novel control and vaccine strategies for CCHF. Potential targets could be the cell fusion step during virus entry to the host cells (Garry and Garry, 2004), the pattern recognition receptors for CCHFV (Spengler et al., 2015), the chaperones of the HSP70 family (Surtees et al., 2016), the OTU domain (Frias-Staheli et al., 2007), and the immunogenic factors (Papa et al., 2016). Scientists now have tremendous opportunities to utilize new technologies and *in vitro* models to increase our understanding of CCHFV pathogenesis for the good of Public Health.

AUTHOR CONTRIBUTIONS

AP wrote the first draft of the article. KTse, KTsi, and AM contributed to the writing of the article. All authors worked for the final version of the article.

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Deciphering *Babesia*-Vector Interactions

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Understanding host-pathogen-tick interactions remains a vitally important issue that might be better understood by basic research focused on each of the dyad interplays. Pathogens gain access to either the vector or host during tick feeding when ticks are confronted with strong hemostatic, inflammatory and immune responses. A prominent example of this is the *Babesia* spp.—tick—vertebrate host relationship. *Babesia* spp. are intraerythrocytic apicomplexan organisms spread worldwide, with a complex life cycle. The presence of transovarial transmission in almost all the *Babesia* species is the main difference between their life cycle and that of other piroplasmida. With more than 100 species described so far, *Babesia* are the second most commonly found blood parasite of mammals after trypanosomes. The prevalence of *Babesia* spp. infection is increasing worldwide and is currently classified as an emerging zoonosis. *Babesia microti* and *Babesia divergens* are the most frequent etiological agents associated with human babesiosis in North America and Europe, respectively. Although the *Babesia*-tick system has been extensively researched, the currently available prophylactic and control methods are not efficient, and chemotherapeutic treatment is limited. Studying the molecular changes induced by the presence of *Babesia* in the vector will not only elucidate the strategies used by the protozoa to overcome mechanical and immune barriers, but will also contribute toward the discovery of important tick molecules that have a role in vector capacity. This review provides an overview of the identified molecules involved in *Babesia*-tick interactions, with an emphasis on the fundamentally important ones for pathogen acquisition and transmission.

Keywords: tick-pathogen interaction, *Babesia* spp., vector, babesiosis, tick-borne diseases

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INTRODUCTION

Parasites from the genus *Babesia* are responsible for causing an emerging zoonotic disease called babesiosis. Transmission occurs mainly through the bite of a *Babesia*-infected tick and, less commonly, by blood transfusion (Leiby, 2006; Ord and Lobo, 2015).

At least four Ixodidae genus are recognized as *Babesia* vectors: *Rhipicephalus*, *Ixodes*, *Haemaphysalis*, and *Hyalomma* (Sonenshine and Michael Roe, 2014). This disease has a considerable impact on the health and economy of the livestock industry, mainly in tropical and subtropical climates, with *Rhipicephalus microplus* and *Rhipicephalus annulatus* the main vectors of *Babesia bovis* and *Babesia bigemina,* the etiological agents of bovine babesiosis (Bock et al., 2004). In small ruminants, infections can be caused by several *Babesia* species, such as *B. ovis*, transmitted to sheep usually by the tick *R. bursa*

(Shayan et al., 2007; Ranjbar-Bahadori et al., 2012; Ferrolho et al., 2016). Dogs are susceptible of infection by *B. canis vogeli* and *B. gibsoni*, primarily transmitted by *R. sanguineus* (Solano-Gallego et al., 2016; Chao et al., 2017). Human babesiosis, caused largely by *Babesia microti* and *Babesia divergens*, is not acknowledged as a tropical neglected disease, but there is a growing concern globally regarding this emerging zoonosis (Ord and Lobo, 2015).

Despite the fact that *Babesia* infections tend to impair tick development, an adaptive tolerance to *Babesia* has been described in *R. microplus* suggesting a balance between tick defense mechanisms and tick-pathogen mutual interaction(s) (Cen-Aguilar et al., 1998; Chauvin et al., 2009; Florin-Christensen and Schnittger, 2009; Lack et al., 2012; Gou et al., 2013; de la Fuente et al., 2016).

The development of improved tick and tick-borne disease control measures are essential to overcome the lack of data regarding which tick molecules are important and how they may be suitable as study targets. Based on this, herein we will discuss the functional roles of several molecules involved during the infection of tick tissues by *Babesia* spp.

TICK MIDGUT MOLECULES WITH A ROLE IN *BABESIA* ACQUISITION

Once ingested *Babesia*-infected red-blood cells reach the tick midgut many parasites will be destroyed or degenerate, but a small number will evolve to gametocytes, essential for zygote fusion and penetration of the midgut peritrophic membrane (Sonenshine and Hynes, 2008; Chauvin et al., 2009; Maeda et al., 2017). Recently, it was proposed that during the *Babesia* spp. sexual phase, some specific proteins with known functional roles in recognition and adhesion are expressed, including glycosylphosphatidylinositol (GPI) anchored proteins that interact with specific targets in the epithelial cells (Bastos et al., 2013; Alzan et al., 2016).

In the *R. microplus* midgut, proteomic analysis has identified a mitochondrial voltage-dependent anion-selective channel (**BmVDAC**) polypeptide, also known as mitochondria porin that binds to *B. bigemina* sexual stage proteins (Mosqueda et al., 2004; Rodríguez-Hernández et al., 2012). VDAC was first described as located in the external mitochondrial membrane that regulates the flux of small molecules into the mitochondrial space membrane having a role in cell metabolism and apoptosis (Young et al., 2007). In mosquitoes, VDAC plays a role during *Plasmodium* sp. invasion of the midgut; likewise, the dissemination of *B. burgdorferi* through the tick midgut might be associated with the ability of VDAC to bind a tissue-type plasminogen activator (Coleman et al., 1997; Ghosh et al., 2011). Under *Babesia* invasion this protein was found over-represented in the *R. microplus* midgut (Rodríguez-Hernández et al., 2012).

The tick receptor of the outer surface protein A (**TROSPA**) was firstly identified in the *I. scapularis* midgut epithelium as a receptor for *B. burgdorferi*, suggesting it has the potential to control bacterial infections in ticks (Pal et al., 2004; Konnai et al., 2012; Urbanowicz et al., 2016). In *R. annulatus,* an orthologue of *trospa* gene was over-expressed during *B. bigemina* infection and gene knockdown significantly reduced *B. bigemina* infection levels by 70 and 83% in *R. microplus* and *R. annulatus,* respectively (Antunes et al., 2012). In addition, *B. bigemina*-infected cattle vaccinated with TROSPA revealed close to an 80% decrease in pathogen transmission to ticks (Merino et al., 2013). In *R. annulatus*, this receptor was found not only in the midgut, but also in the salivary glands (SGs) and ovaries (Antunes et al., 2014).

During protozoal invasion, the tick innate immune response leads to the rapid, synthesis of **defensins** and tick antimicrobial peptides (**AMPs**). These constitute an important humoral defense mechanism, which is also active against intracellular bacteria and fungi (Antunes et al., 2012; Hajdusek et al., 2013; Tonk et al., 2015). The midgut defensin-like protein, **longicin**, was first identified in the tick *Haemaphysalis longicornis* and has a role in *Theileria equi* proliferation (Tsuji et al., 2007). Merozoite *in vitro* cultures were inhibited in the presence of recombinant longicin while the inoculation of this protein led to a reduction of *B. microti* parasitaemia in infected mice. Also, *longicin* silencing led to an increase in *B. gibsoni* parasitaemia in several tick tissues, including midgut, ovaries and eggs. Accumulated data on the function of this protein indicate that longicin has a babesiacidal effect. **Microplusin** was the first fully characterized member of a family of cysteine-rich AMPs in *R. microplus* (Fogaça et al., 2004); in *R. annulatus,* was found over represented in response to *B. bigemina* infection (Antunes et al., 2012).

Other molecules present in the midgut that also protect the tick from pathogen invasion are the **MD-2-related lipid-recognition** (ML)-domain containing proteins related with lipid recognition (Rudenko et al., 2005), proteases and protease inhibitors (Sonenshine and Hynes, 2008; Kopacek et al., 2010; Antunes et al., 2012; Hajdusek et al., 2013). **Longipain,** a *H. longicornis* midgut cysteine protease, has shown similar effects to longicin. Recombinant longipain was also able to inhibit the proliferation of *T. equi* merozoites, and gene silencing resulted in an increase of protozoa in the midgut lumen, ovaries and hatched larvae (Tsuji et al., 2008). Also in *H. longicornis*, a **leucine-rich repeat domain-containing protein (LRR)** has been identified as over represented in all tick tissues, with the exception of the ovary, where it is constitutively expressed. *In vitro*, a specific recombinant LRR has demonstrated a growth inhibitory effect on *B. gibsoni* with similar or better results than traditional anti-babesial drugs (Maeda et al., 2015).

Tick **Kunitz-type protease inhibitors** may restrict pathogen infection, presumably via the inhibition of microbial proteinases (Sasaki and Tanaka, 2008; Antunes et al., 2012). This group of genes was upregulated in response to infection (Antunes et al., 2012; Heekin et al., 2013), but its influence in *Babesia* acquisition was only related to ovary infection (Rachinsky et al., 2007; Bastos et al., 2009).

Bm86 is a glycoprotein, recognized for the first time in *R. microplus*, and present in midgut cells, that is likely to be involved in the endocytosis of the blood ingested by ticks (Gough and Kemp, 1993; Bastos et al., 2010; Rodríguez-Mallon, 2016). Regardless of the efficiency of Bm86 against tick infestation, some studies aimed to evaluate the role of Bm86 in *Babesia*

infection (Bastos et al., 2010; Rodríguez-Mallon et al., 2013). RNA interference (RNAi) studies carried out in *R. microplus* females showed that *Bm86* silencing significantly reduced the number of ticks; by contrast, silencing did not affect the efficiency of transovarial transmission of *B. bovis* (Bastos et al., 2010). In a different study using Gavac®, a vaccine based on the Bm86 antigen, naïve nymphs that co-fed on immunized dogs presented lower levels of *B. canis,* (Rodríguez-Mallon et al., 2013). It is conceivable that the lysis of midgut cells inhibited the entry of zygotes and/or their posterior differentiation into motile ookinetes, compromising *B. canis* acquisition by the nymphs.

Subolesin, firstly identified in *I. scapularis* ticks as an orthologue of akirin in insects and vertebrates (Almazán et al., 2003; Galindo et al., 2009), is a highly conserved protein in eukaryotes, including many tick species (Moreno-Cid et al., 2013; Antunes et al., 2014), suggesting its potential as a candidate antigen for an anti-tick and tick-borne pathogen (TTBP) vaccine. Subolesin family proteins are transcriptional factors, regulating protein expression in cellular pathways involved in the response to pathogen infection (de la Fuente et al., 2013; Sultana et al., 2015). *Subolesin* silencing mediated by RNAi led to a lower *B. bigemina* infection in *R. microplus* (Merino et al., 2011) but, in contrast, in *R. annulatus*, silencing did not lead to a significant decrease in *B. bigemina* levels (Antunes et al., 2012). Vaccination using subolesin and a chimera containing subolesin protective epitopes (Q38) revealed an effect on *B. bigemina* transmission to feeding ticks (Merino et al., 2013). *Subolesin* expression and subolesin-mediated innate immunity varies according to the pathogen and tissue (Zivkovic et al., 2010), which explains the variation in the results. However, it seems that targeting subolesin by vaccination or its gene by RNAi would result in lower *Babesia* infection levels.

The tick midgut is one of the few major organs that defines vector competence since it is the first obstacle that several pathogens, including *Babesia*, have to cross. Still, our understanding of the interplay between an infective pathogen and the tick midgut continues to be poor and requires further studies to better define this important interaction.

TICK HAEMOLYMPH AND OVARY MOLECULES ACTING IN *BABESIA* DISSEMINATION

After the successful invasion of the midgut epithelium, *Babesia* zygotes go through meiosis and differentiate into motile ookinetes that go across the haemocoel, with the help of haemolymph; in the haemocoel, the parasite undergoes asexual reproduction, resulting in several sporokinetes spread for all tick organs throughout all tick life stages (transstadial transmission) (Schnittger et al., 2012).

When a tick experiences microbial invasion, for example from a protozoa like *Babesia* spp., the hemocytes increase their circulating number to destroy and control the invader, phagocytizing small particles and microbes (Inoue et al., 2001; Villar et al., 2015). Besides phagocytosis, other processes including nodulation and encapsulation, and molecules like AMPs, lysozymes, proteases, protease inhibitors, and lectins, that exist in the haemolymph act directly on the pathogen (Esteves et al., 2008; Kotsyfakis et al., 2015). *B. bigemina* exhibits motility when reaching the haemolymph and adheres to *R. microplus* haemocyte membranes (de Rezende et al., 2015), however there is no information about how *Babesia* spp. invasion is controlled at the haemolymph level.

In female ticks, effective infection of ovaries and the eggs allow transovarial transmission of almost all *Babesia* species, a distinctive characteristic of this genus (Homer et al., 2000; Chauvin et al., 2009) that can be interpreted as an adaptation to efficiently persist in the ecosystem (Chauvin et al., 2009). The first ovarian proteomic profile of *R. microplus* infected with *B. bovis* identified a small number of differentially represented proteins. Among these proteins were calreticulin, glutamine synthetase and a family of Kunitz-type serine protease inhibitors; whereas between the less represented proteins were a tick lysozyme and a group of small proteins that may belong to a family of AMPs (Rachinsky et al., 2007). Ovarian genes involved in the stress response, detoxification and immune responses were found potentially regulated by *B. bovis* infection (Heekin et al., 2013); many of these genes translate into proteases and protease inhibitors that participate in the ovarian immune response. A putative *immunophilin* (*Imnp*) and a putative *Kunitz-type serine protease inhibitor* (*Spi*) genes were found to be up regulated when tick ovaries were infected (Rachinsky et al., 2007) and the *Imnp* knockdown revealed a significant increase of larval infection, suggesting that this molecule might control the protozoan invasion of tick ovaries, and subsequent larval progeny. **Immunophilin** proteins, also known as cyclophilins, are associated with multiple cellular processes, like protein folding, trafficking and defense mechanisms (Wang and Heitman, 2005), however their role(s) during *Babesia* infection is still unknown.

The *H. longicornis* **vitellogenin receptor** (VgR) has been associated with the transovarial transmission of *B. gibsoni*. *VgR* silencing results in the absence of *B. gibsoni* infection and development of abnormal eggs (Boldbaatar et al., 2008) confirming its influence on oogenesis acting on heme detoxification and egg maturation (Boldbaatar et al., 2010; Perner et al., 2016). These results may suggest that *Babesia* molecules have ligand-binding activity for tick VgR, consequently invading the developing oocyte (Boldbaatar et al., 2008).

Ovarian proteins can affect tick biology by decreasing oogenesis and embryogenesis, which reduce tick reproduction rates and TBP transmission by blocking transovarial transmission, making these molecules promising targets for vaccine development.

TICK SALIVARY GLAND MOLECULES THAT INTERVENE IN *BABESIA* TRANSMISSION

When *Babesia* kinetes reach the SGs they undergo a final step of multiplication to produce sporozoites, the vertebrate host-infective stage. SGs can be considered as the last barrier that

parasites must overcome to complete their life cycle in the vector, facing similar obstacles to those of the midgut (Chauvin et al., 2009).

Different SGs transcriptomes, commonly referred to as sialomes, from soft and hard ticks have been published (Francischetti et al., 2008, 2011; Anatriello et al., 2010; Karim et al., 2011; Ribeiro et al., 2011; Garcia et al., 2014; Yu et al., 2015; de Castro et al., 2016), showing genes encoding AMPs, such as defensins, microplusin/hebraein, Kunitz domain-containing proteins, lipocalins, proteases and other molecules related to tick defense mechanisms. Despite their importance for transmission, reports describing the influence of SG molecules on *Babesia* infection are absent.

The sialome of the soft tick *Ornithodoros parkeri* contains a putative **serum amyloid A** protein, whose orthologue was also found in the *I. scapularis* genome. In vertebrates, this protein is involved in the acute phase of an inflammatory response (Francischetti et al., 2008; Antunes et al., 2012). Vertebrate serum amyloid A protein was found increased in cattle with more resistance to tick infections, suggesting its involvement in the stress response induced by tick infestations (Ferreira et al., 2004). The expression of a putative *serum amyloid A* gene was increased in response to *B. bigemina* infection in *R. annulatus* and gene knockdown resulted in a reduction of 66 and 86% of the infection levels, in *R. microplus* and *R. annulatus*, respectively (Antunes et al., 2012).

Calreticulin, has been identified in tick ovaries, midgut and SGs (Antunes et al., 2012, 2015). The role of this molecule in ticks is still not clear but some studies support its presence in the SGs and saliva is presumably related to a mechanism to avoid vertebrate host defense responses (Jaworski et al., 1995; Ferreira et al., 2004; Antunes et al., 2015) and may lack the anti-thrombotic and complement-inhibiting characteristics that suppress host defense actions (Kim et al., 2015). The gene encoding this protein was found to be over expressed in *R. annulatus* infected with *B. bigemina*. *Calreticulin* knockdown had a significant effect on pathogen infection in *R. microplus,* but not in *R. annulatus* ticks, affecting the body weight in both tick species (Antunes et al., 2012). According to this and to other reports, it is thought that calreticulin acts during blood feeding (Ferreira et al., 2002; Antunes et al., 2012) and may alter calcium metabolism during *Babesia* infection. *Babesia* sp. may need calcium ions to invade tick cells as shown for *T. equi* (previously classified as *B. equi*). A pilot immunization trial in cattle using recombinant calreticulin failed to reduce tick infestation, probably due to the low immunogenicity of the protein (Ferreira et al., 2002). More recently, serum with anti-calreticulin antibodies also failed to promote a significant decrease in *B. bigemina* infection in *R. microplus* (Antunes et al., 2015). In this study, calreticulin immunolocalization assays have shown that this molecule can be found in the tick midgut, ovaries and SGs, suggesting that it might have a role in *Babesia* infection in all these tissues.

Other molecules, such as TROSPA, already discussed, have been also identified in tick SGs, where it may function as a receptor for *Babesia* parasites. Tick SG proteins are of extreme

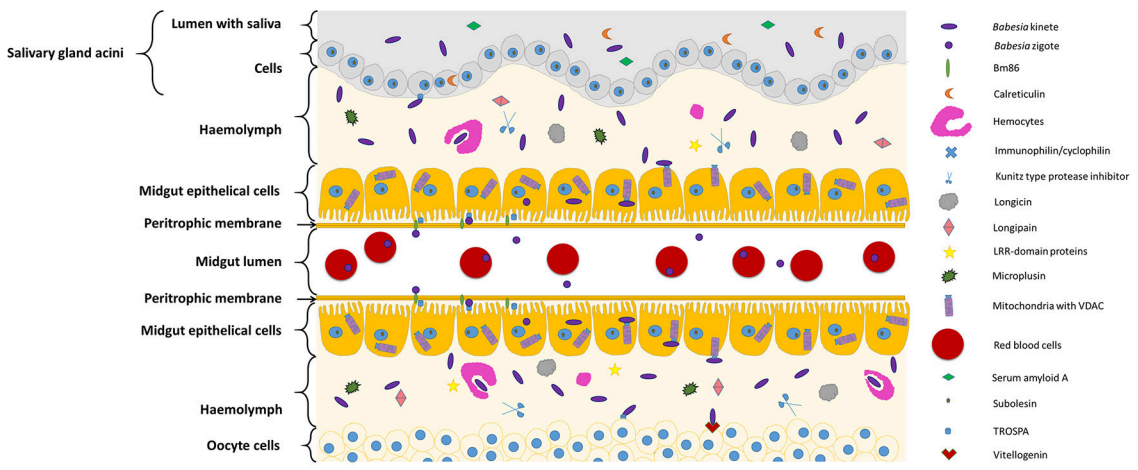

FIGURE 1 | Diagram representing tick molecules implicated in *Babesia* spp. acquisition and transmission by the vector. When ticks feed on *Babesia*-infected animals, parasites within red blood cellsreach and penetrate the tick midgut peritrophic membrane to invade the epithelial cells (in the figure center). Once these cells are infected, transcriptional factors, such as subolesin, can regulate protein expression in several cellular pathways, facilitating *Babesia* infection. In the microvilli of the midgut cells, parasite zygotes will probably interact with a tick glycoprotein (Bm86) and a tick receptor of the outer surface protein A (TROSPA). Inside the epithelial cells, mitochondria porins (VDAC) can bind to *Babesia* kinete proteins promoting plasminogen activation in the cell surface, allowing their passage to the haemolymph. Once here, the haemocytes can phagocyte circulating parasites and the tick antimicrobial molecules such as, longicin, micropulsin, longipain, LRR-domain and Kunitz-type protease inhibitors are activated potentially reducing the infection in the vector. If the infectious parasites surpass these barriers of defense, they will be capable to spread across the tissues and invade ovaries (represented in the bottom of the figure) and SGs (represented in the top of the figure). In the ovary, the interaction of *Babesia* molecules with tick vitellogenin and TROSPA receptors may contribute for the occurrence of transovarial transmission; while in the SGs, *Babesia* interacts with TROSPA and calreticulin.

TABLE 1 | Tick molecules interfering with *Babesia* spp. infection.

Protein name	Assigned function	Proteins localization				Described effect in *Babesia* spp. infection	References
		MD	HL	OV	SG		
Mitochondrial voltage-dependent anion-selective channel (BmVDAC)	Cell metabolism and apoptosis regulation	X				Enhance acquisition	Rodríguez-Hernández et al., 2012, 2015
Tick receptor of the outer surface protein A (TROSPA)	Factor for tick gut colonization	X		X	X	Enhance acquisition	Antunes et al., 2012; Merino et al., 2013; Urbanowicz et al., 2016
Longicin	Defensin	X				Control acquisition and transovarial transmission	Tsuji et al., 2007
Microplusin	Antimicrobial peptide	X					Antunes et al., 2012
Longipain	Cysteine protease	X					Tsuji et al., 2008
Leucine-rich repeat domain-containing proteins	Component of the innate immune system	X	X	X	X	Control infection	Maeda et al., 2015
Kunitz-type protease inhibitors	Blood coagulation	X			X	Enhance transovarial transmission	Rachinsky et al., 2007; Bastos et al., 2009; Antunes et al., 2012
Bm86	Blood coagulation and cell growth	X		X		Enhance acquisition	Bastos et al., 2010
Subolesin	Transcriptional factor involved in the immune signaling pathways	X				Enhance acquisition	Merino et al., 2011; de la Fuente et al., 2013
Immunophilin	Protein folding, trafficking and defense		X			Control transovarial transmission	Rachinsky et al., 2007; Wang and Heitman, 2005
Vitellogenin receptor (VgR)	Vitellogenin uptake			X		Enhance transovarial transmission	Boldbaatar et al., 2008, 2010
Serum amyloid A	Response to inflammation	X				Enhance acquisition	Antunes et al., 2012
Calreticulin	Protein folding and signaling	X		X	X	Enhance acquisition.	Antunes et al., 2012

MD, Midgut; HL, Haemolymph; OV, Ovaries; SG, Salivary Glands.

importance during *Babesia*-vector-host interactions and it seems likely that more molecules will emerge as key players in these vector-parasite networks in the near future.

Figure 1, **Table 1** summarizes the so far identified tick molecules networking with *Babesia* spp. showing their localization and suggested interaction.

CONCLUSIONS

The major critical point for the development of vaccines is the identification of new targets. In this review, our objective was to gather relevant information about the tick molecules involved with *Babesia* parasite infections. During the last decade, several studies using "omics" and systems biology approaches have greatly improved our knowledge of the interactions taking place at the tick-pathogen interface. The *Babesia*-tick interactome is still neglected with scattered information, and only a few tick proteins have been shown to influence the acquisition, dissemination and transmission of the parasite. From this short list, subolesin, having a role in the tick innate immune response, stands out as a potential candidate antigen for a universal anti-vector vaccine. During *Babesia* infection, this molecule produced positive results, making it a candidate antigen for a transmission-blocking vaccine. Other proteins involved in *Babesia* acquisition, including the TROSPA receptor, are also promising candidates for a multi-antigenic vaccine. Some of these datasets were obtained through use of transcriptomic, proteomic, and systems biology approaches. These and future technologies will be fundamental to the improvement and development of new control strategies and more effective vaccines.

AUTHOR CONTRIBUTIONS

SA, JF, CR, and AD conducted the literature research and wrote the paper. SA and CR prepared the table and JC prepared the figure. All authors critically read and revised the manuscript.

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Rhipicephalus bursa Sialotranscriptomic Response to Blood Feeding and *Babesia ovis* Infection: Identification of Candidate Protective Antigens

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Ticks are among the most prevalent blood-feeding arthropods, and they act as vectors and reservoirs for numerous pathogens. Sialotranscriptomic characterizations of tick responses to blood feeding and pathogen infections can offer new insights into the molecular interplay occurring at the tick-host-pathogen interface. In the present study, we aimed to identify and characterize *Rhipicephalus bursa* salivary gland (SG) genes that were differentially expressed in response to blood feeding and *Babesia ovis* infection. Our experimental approach consisted of RNA sequencing of SG from three different tick samples, fed-infected, fed-uninfected, and unfed-uninfected, for characterization and inter-comparison. Overall, 7,272 expressed sequence tags (ESTs) were constructed from unfed-uninfected, 13,819 ESTs from fed-uninfected, and 15,292 ESTs from fed-infected ticks. Two catalogs of transcripts that were differentially expressed in response to blood feeding and *B. ovis* infection were produced. Four genes coding for a putative vitellogenin-3, lachesin, a glycine rich protein, and a secreted cement protein were selected for RNA interference functional studies. A reduction of 92, 65, and 51% was observed in *vitellogenin-3, secreted cement*, and *lachesin* mRNA levels in SG, respectively. The *vitellogenin-3* knockdown led to increased tick mortality, with 77% of ticks dying post-infestation. The reduction of the secreted cement protein-mRNA levels resulted in 46% of ticks being incapable of correctly attaching to the host and significantly lower female weights post-feeding in comparison to the control group. The *lachesin* knockdown resulted in a 70% reduction of the levels associated with *B. ovis* infection in *R. bursa* SG and 70% mortality. These results improved our understanding of the role of tick SG genes in *Babesia* infection/proliferation and tick feeding. Moreover, lachesin, vitellogenin-3, and secreted cement proteins were validated as candidate protective antigens for the development of novel tick and tick-borne disease control measures.

Keywords: sialotranscriptomics, *Rhipicephalus bursa*, *Babesia* spp., RNA interference, vaccine, vector-pathogen interactions

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INTRODUCTION

Ticks are widely distributed obligate hematophagous ectoparasites, which have recognized effects on host species. During blood feeding, ticks secrete varying substances into the host bloodstream acting as remarkable vectors of numerous pathogens, some of which can cause severe diseases in vertebrate hosts, including humans (Jongejan and Uilenberg, 2004; Domingos et al., 2013; Sonenshine and Michael Roe, 2014). Reflecting the progress of feeding, salivary glands (SG) increase ∼25-fold in mass and protein content, as the glands are responsible for the production of complex saliva that is capable of quelling host innate and adaptive immune responses (Sauer et al., 2000; Kazimírová and Stibraniova, 2013; Kotál et al., 2015; Šimo et al., 2017). SG play an essential role in tick survival and success as parasites by modulating host haemostasis and complement systems (Sauer et al., 2000; Francischetti et al., 2009; Kazimírová and Stibraniova, 2013). In addition to being involved with osmoregulation (Kaufman, 2010), this tissue is also responsible for the production of cement, which is an adhesive substance that surrounds the mouthparts and the host skin that ensures tick attachment (Sauer et al., 2000; Francischetti et al., 2009; Kazimírová and Stibraniova, 2013; Šimo et al., 2017). SG are also pivotal in tick pathogen interactions, because pathogens need to cross the physical barrier of SG epithelium and endure the salivary biochemical environment to gain access to the next host. Remarkably, to increase their proliferation and transmission, pathogens adapted to SG in a way that exploits tick salivary molecules (Ramamoorthi et al., 2005; Kaufman, 2010). Therefore, these features make SG an exceptional target for the identification of new candidate protective antigens that are relevant to biological functions associated with tick development, fertility, feeding, and pathogen infection and transmission (Merino et al., 2013; Shahein et al., 2013).

Research that examined tick SG made the characterization of a large number of tick salivary compounds possible, but the function of several of these molecules remains unknown (Francischetti et al., 2009). The sialomes of some tick species have been described (Francischetti et al., 2008, 2011; Anatriello et al., 2010; Karim et al., 2011; Tan et al., 2015; de Castro et al., 2016; Moreira et al., 2017), and this information represents an important data source for functional studies and analyses of gene expression dynamics during tick feeding. Moreover, high-throughput technologies have also enabled researchers to study the effects of sex, physiological stages, and different tick statuses such as the presence of pathogens in tick tissues (Chmelar et al., 2016).

Rhipicephalus bursa is a multi-host tick that is mainly associated with ruminants, but it can occasionally parasitize other animals such as wild ungulates and small mammals (Walker et al., 2000; de la Fuente et al., 2004; Santos-Silva et al., 2011; Mihalca et al., 2012). *R. bursa* is recognized as the primary vector of *Babesia ovis* (Moltmann et al., 1982a), but it transmits other pathogens such as *Rickettsia* spp. and *Anaplasma* spp. (Raele et al., 2015; Dahmani et al., 2016; Ferrolho et al., 2016b), thus demonstrating its importance in animal health, particularly in livestock. *B. ovis*, an intraerythrocytic apicomplexan parasite, is the main etiological agent of ovine babesiosis, which is a tick-borne disease of small ruminants, and its geographical distribution overlaps with that of *R. bursa* (Walker et al., 2000; Ranjbar-Bahadori et al., 2012; Erster et al., 2015; Ferrolho et al., 2016a). This highly pathogenic organism is characterized by low parasitaemia, and it causes severe infections (Habela et al., 1990; Sevinc et al., 2013; Hurtado et al., 2015). *B. ovis* is extremely well adapted to the vector, and it survives in the tick during several successive generations (Yeruham et al., 2001) using horizontal and vertical transmission (Friedhoff, 1988). Microscopy studies in the 1980's discovered that the *B. ovis* cycle within the tick is similar to other *Babesia* spp. (Moltmann et al., 1982a,b). Briefly, *Babesia* penetrates the tick midgut, undergoes meiosis, and differentiates into motile ookinetes that propagate via haemolymph to reach all tick organs. *B. ovis* kinetes reach SG within 48 h post-infestation, and they undergo a final step of multiplication to produce sporozoites (Moltmann et al., 1982a; Antunes et al., 2017). Adult ticks are the main vector, and both females and males are implicated in the transmission of the hemoparasite. However, females present a higher threat due to transovarial transmission and extended feeding periods (Friedhoff, 1988).

The importance of the *R. bursa-B. ovis* system was emphasized in a disease outbreak that resulted in animal morbidity and mortality (Hurtado et al., 2015). Pathogen and vector control methods are limited to the common usage of imidocarb dipropionate (to manage animal disease) and acaricides (McHardy et al., 1986; Belloli et al., 2006; Domingos et al., 2013). Safer and effective alternatives are urgently needed, including the development of vaccines that may reduce tick infestations and block pathogen transmission (Merino et al., 2013; Liu and Bonnet, 2014; Neelakanta and Sultana, 2015). Studies of the molecular interactions associated with the tick-pathogen interface represent a bridge for the identification of antigenic targets to implement vaccination strategy. Information about the *R. bursa* and *B. ovis* interactome is scarce. Thus, in the present study, SG of *R. bursa* adult females were used to assess the transcriptomic response to blood feeding and *B. ovis* infection. Fed-infected, fed-uninfected, and unfed-uninfected female ticks were produced, SG were isolated and used for RNA extraction. RNA-seq and *de novo* transcriptome assembly approaches were used to construct the sialotranscriptome of fed-infected, fed-uninfected, and unfed-uninfected *R. bursa* specimens. These catalogs were analyzed, and four genes were selected for further functional studies, thus allowing the evaluation of encoded proteins for inclusion in anti-tick and tick-borne pathogen vaccines. These data are essential for vaccinomics pipelines, which could enhance our knowledge of the dynamic processes that occur at the tick-pathogen-host interface.

MATERIALS AND METHODS

Ethics Statement

Animal experiments were conducted with the approval of the Divisão Geral de Alimentação e Veterinária (DGAV), Portugal, under Art° 49, Portaria n°1005/92 from 23rd

October (permit number 0421/2013) and the Council of Ethics of the Instituto de Higiene e Medicina Tropical (IHMT). Animals were maintained and manipulated following protocols compliant with the national and European Animal Welfare legislation, in frame with DL 113/2013 and Directive 2010/63/EUbased on the principle of the Three R's, to replace, reduce, and refine the use of animals for scientific purposes.

Rhipicephalus bursa Colony

R. bursa colony was established under laboratory conditions and further maintained. For colony initiation, adult ticks were collected either in naturally infested domestic animals or by dragging/flagging the vegetation and kept in a chamber regulated at 25 ± 1°C, 70 ± 10% relative humidity and a photoperiod of 16:8 (light: dark). During oviposition, the dark period was increased to improve female egg laying. After oviposition, each female and a sample of eggs were tested by conventional PCR for pathogens detection (*Babesia* spp., *Anaplasma* spp., *Ehrlichia* spp.) during two generations using the protocols and primers described elsewhere (Inokuma et al., 2000; de la Fuente et al., 2003; Aktaş et al., 2005; Harrus et al., 2011). Ticks were fed on Hyla breed rabbits at Centro de Estudos de Vetores e Doenças Infeciosas, Instituto Nacional de Saúde Doutor Ricardo Jorge (CEVDI/INSA) in appropriate conditions. Ten lineages of *R. bursa* were selected in order to reduce interbreeding.

In Vitro Babesia ovis Cultures

In vitro B. ovis cultures were established at IHMT in biosafety level 2 facilities, following a protocol adapted from Vega et al. (1985). Briefly, cryopreserved *B. ovis* (Israeli strain) infected red blood cells (RBC) were used to initiate the culture. *B. ovis* merozoites were cultured in lamb erythrocytes maintained in 20% lamb serum-containing medium, in an atmosphere of 5% CO_2/2% O_2/93% N_2 at 37°C, as described elsewhere (Horta et al., 2014). Half of the medium was replaced daily and cultures monitored for parasitaemia by preparing thin blood smears stained with Hemacolor® Rapid staining of blood smear (EMD Millipore, Darmstadt, Germany). Intraerythrocytic parasites were observed under a 400x original magnification of a Nikon eclipse 80i fluorescence microscope.

Salivary Glands and RNA Samples for RNA-Seq

Fed and Unfed *R. bursa*

Thirty adult female ticks were carefully removed from the rabbits ear 10–12 days post attachment. Equally, thirty unfed adult female ticks were also obtained. Ticks were individually rinsed in distilled water, after in 75% (v/v) ethanol, once more in water and dissected under a stereoscopic microscope at 4x magnification (Motic SMZ-171B, China) using sterile conditions in ice-cold phosphate-buffered saline (PBS). The SG were stored in RNAlater (Ambion, Austin, TX, USA) and afterwards pooled, resulting in two samples for the fed condition and other two for the unfed. Total RNA was extracted from each sample using Tri-reagent (Sigma–Aldrich, St. Louis, MO, USA). RNA quantity was estimated using the ND-1000 Spectrophotometer (NanoDrop ND1000, Thermo Fisher Scientific, Whaltman, MA, USA).

Fed-*B. ovis* Infected *R. bursa*

A batch of 60 female ticks were inoculated with *B. ovis* in the trochanter—coxae articulation and allowed to feed on rabbits. After drop off, SG were carefully isolated and DNA/RNA extracted has mentioned previously. Genomic DNA was used to amplify a 549 bp fragment of *B. ovis 18S* ribosomal DNA (18S rRNA) using primers and conditions described elsewhere (Aktaş et al., 2005). RNA from positive samples (Supplementary Figure 1) were used for the production of two RNA pools with fifteen samples each. All samples were promptly shipped in dry ice to Parque Cientifico de Madrid for sequencing. The tick infection model and vector competence was evaluated. *B. ovis* inoculated *R. bursa* were allowed to feed in a naïve lamb. The lamb was monitored every two days for babesiosis clinical symptoms and blood collected for *B. ovis* detection by PCR (Supplementary Figure 1) using the above mentioned conditions. After 8 days, the ticks were recovered for analysis.

RNA-Seq

RNA quality was assessed using an Agilent RNA 6000 bioanalyzer (Agilent Technologies, CA, USA). Libraries preparation was performed with "NEBNext Ultra Directional RNA Library Prep" kit (New England Biolabs, Ipswich, MA, USA) following manufacturer instructions. Briefly, prior to cDNA library construction magnetic beads with oligo (dT) were used to enrich poly (A) mRNA from 1 μg of total-RNA. Next, the purified mRNAs were disrupted into short fragments, and double-stranded cDNAs were immediately synthesized. The cDNAs were subjected to end-repair and adenilation, then connected with sequencing adapters. Suitable fragments, purified by size selection protocol with AMPure XP beads (Beckman Coulter), were selected as templates for PCR amplification. The final library sizes and qualities were evaluated electrophoretically using an Agilent High Sensitivity DNA kit (Agilent Technologies, CA, USA); the mean fragment size was 510 bp. Subsequently, the library was sequenced using a HiSeq 2500 sequencer (Illumina, CA, USA) in rapid run mode. Cluster generation was performed, followed by 2 × 100 cycle sequencing reads separated by a paired-end turnaround. Image analysis was performed using the HiSeq control software version 1.8.4. The raw fastq files were deposited in the Sequence Read Archives (SRA) of the National center for Biotechnology information (NCBI) under the accession numbers SRR4428986, SRR4428987 and SRR4428988, Biosamples SAMN05916213, SAMN05916214, and SAMN05916215, regarding the unfed-uninfected, fed-uninfected and fed-infected populations, respectively, of Bioproject PRJNA348674. The Transcriptome Shotgun Assembly (TSA) projects have been deposited at DDBJ/EMBL/GenBank under the accessions GFZD00000000, GFZJ00000000, and GFZK00000000. The versions described in

this paper are the first versions, GFZD01000000, GFZJ01000000, and GFZK01000000.

Transcriptomic Data of Female *R. bursa* Sialome

Assembly and Analysis of Transcripts

This project comprised *de novo* assembly of six transcriptomes. Three conditions and two replicas per condition: F, SG from fed ticks; NFni, SG from unfed-uninfected ticks; and Fi, SG from fed-*B. ovis* infected ticks. Subsequently, two comparisons were performed: F *vs.* NFni (response to blood feeding) and F *vs.* Fi (response to *B. ovis* infection). Quality analysis of the raw reads was done with Prinseq tool (Schmieder and Edwards, 2011). Pre-processing of reads included: (a) right trimming where quality < Q30; (b) left trimming of the first base; (c) filtering out reads with Ns; (d) quality analysis of the processed data. For each of the four transcriptomes three *de novo* assemblies were made with three different *k*-values using the *de novo* transcriptome assembler Oases (Velvet, version: 1.2.10) (Schulz et al., 2012). The annotation of each transcript was done based on the Basic Local Alignment Search Tool (BLAST) results comparing the transcript to a database of reference proteins. The set of reference proteins was selected from UniProt database from all the organisms belonging to the taxon "Ixodidae". In total 76, 475 proteins were used as reference proteins. A set of unigenes for each sample was obtained. The assignment of each transcript to a protein was based on BLAST similarity. Rich functional annotation for each unigene extracted from the UniProt protein in which the read clustering process has been centered for this unigene is provided. Afterwards a unigene expression quantification was performed using eXpress. To compare the transcripts from the samples, the transcripts were clustered by protein. The protein driven transcript clusters that were done using UniProt proteins, were furtherly clustered by UniRef90 proteins. The mapping from the UniProt proteins to UniRef90 was done using UniProt retrieval tool. The quantification per UniRef90 cluster was calculated adding the quantification per protein included in each UniRef90 cluster. *P*-value calculation of the *Z*-test was based on the raw counts (total exon reads per gene). Genes were considered significantly differentially expressed if the *P*-value was below 0.05. Functional annotation of these genes was manually done by compiling information from UniProt, RefSeq, GO, Panther, KEGG, Pfam, and NCBI databases.

Gene Ontology Assignments

Functional data for each identified protein was obtained using Blast2GO platform version 4.0.7 available at https://www.blast2go.com (Conesa et al., 2005; Götz et al., 2008). Homology to the protein sequences was searched by BLAST against Arthropoda (nr subset) [arthropoda, taxa:6656] from 30.01.2017 as well as against to InterPro protein signature databases, using InterProScan. To retrieve gene ontology (GO) terms, a mapping step was performed gathering GO annotations and evidence codes (EC). Annotation to assign functional terms was performed next. At this step, the most specific and reliable annotation was considered. Finally, to map a set of annotations to high level GO terms, GO slim option was used. GO frequency charts were constructed using the Microsoft Office 2016 Excel tool. The most up and down-regulated genes in response to feeding and infection ($P < 0.1$) were analyzed using the same approach.

Validation of RNA-Seq Data

A total of 18 transcripts with differential regulation and belonging to different functional classes with a potential interference in response to blood feeding and *B. ovis* infection, were chosen for RNA-Seq validation through qPCR using the minimum information for publication of qPCR experiments (Bustin et al., 2009). Ten individual *R. bursa* SG, from each condition studied, were used to extract total RNA using the GRS FullSample Purification kit, Grisp™ (Porto, Portugal), which included DNAse treatment and 60 ng/μL of each sample were used to synthesize cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, CA, USA). qPCR reactions of 10 μL were performed in triplicate using IQ™ SYBR® Green Supermix kit (Bio-Rad, CA, USA) in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The cycling conditions were as follows: an initial cycle of denaturation at 95°C for 10 min; followed by 45 cycles of 95°C for 15s and temperature of each primer set for 45s. Fluorescence readings were taken at 62°C after each cycle and a dissociation curve (60–95°C) was performed. Negative controls were prepared with water. To determine the reaction efficiency standard curves were constructed with five-fold serial dilutions of cDNA from *R. bursa*. Reactions specificity was assured by the absence of PCR product in control reactions and by the dissociation curves (60–95°C) run at the end the cycling protocol. The average expression stability (*M*-value) of the reference genes, *β tubulin*, *β actin*, *elongation factor,* and *16S*, was assessed based in geNorm algorithm (Vandesompele et al., 2002) included in the CFX Manager™ Software (Bio-Rad, CA, USA) and gene relative quantification was evaluated using the CFX Manager™ Software including the *Pfaff* method (Pfaffl, 2001) using the above-mentioned reference genes for normalization. Normalized Cq-values were compared between conditions by Student's t test ($P < 0.05$). Primers were design using Primer3 platform (http://bioinfo.ut.ee/primer3-0.4.0/) and their conditions are summarized in Supplementary Table 1. Pearson's correlation was used to compare the expression values between RNA-Seq and qPCR methods for the 18 selected genes.

RNA Interference Assays

Lamb Infection With *B. ovis*

A six-month old lamb bred and maintained at the Instituto Nacional de Investigação Agrária e Veterinária (INIAV) animal facility was splenectomized and, 45 days after, intravenously inoculated with 1 mL of cryopreserved *B. ovis* culture with 9% parasitemia. The *B. ovis* infection was monitored daily by blood screening. Genomic DNA was extracted from lamb blood using the NZY Blood gDNA Isolation Kit (NZYTech, Lisboa, Portugal) as per manufacturer instructions. As previously mentioned, *B. ovis* infection was screened using conventional PCR with primers and conditions described elsewhere (Aktaş et al., 2005).

PCRs were performed in 25 µl reactions with Supreme NZYTaq 2× Green Master Mix (NZYTech), 1 µM primers and 5 µl of template DNA. A negative control with water and a positive *B. ovis* (Israeli strain) control were added. The PCR was carried out with a thermal cycling profile of 95°C for 2 min, and 35 cycles of 95°C for 30 s, 62°C for 45 s and 72°C for 45 s, followed by a 72°C extension for 5 min, in a T-100® Thermal Cycler (Bio-Rad, CA, USA). Resulting amplicons were checked on a 0.5X TBE, 1.2% (w/v) agarose gel.

Synthesis of dsRNA

Specific primers containing T7 promoter sequences (5′-TAATACGACTCACTATAGGGTACT-3′) at the 5′- end were manually designed using as template available sequences, in particular, GACK01008016 from *Rhipicephalus pulchellus*, GBBO01000019 from *Rhipicephalus microplus*, GBBR01000108 from *R. microplus*, and GACK01007634 from *R. pulchellus* and synthesized by StabVida (Lisbon, Portugal) (Supplementary Table 2). *R. bursa* cDNA was synthetized using the iScript cDNA synthesis (Bio-Rad) following the manufacturer instructions and further used as template to amplify fragments of interest by PCR. Amplifications of target DNA fragments were achieved using the iProof High Fidelity PCR kit (Bio-Rad) in a 50 µl of final volume reaction, including 200 mM of each primer. Cycling conditions were for 40 cycles: 30 s at 94°C, 30 s at specific annealing temperature and 30 s at 72°C with a final extension step of 7 min at 72°C (Supplementary Table 2). All PCR assays were performed in a T100 thermal cycler (Bio-Rad). Amplification results were analyzed on a 0.5x TBE, 1.2 % (w/v) agarose gel. Amplicons were purified using the NZYGelpure kit (NZYtech) and sent for Sanger sequencing at StabVida (Lisbon, Portugal). The obtained sequences were aligned and compared to reference sequences. After validation of the amplified sequences the MEGAscript RNAi Kit (Ambion, Austin, TX, USA) was used to synthesize dsRNA according to manufacturer's instructions. The resulting dsRNA was purified and analyzed by spectrometry and agarose gel.

Inoculation of dsRNA and Tick Infestation

R. bursa adult female ticks from the established colony at CEVDI/INSA were cleaned and placed ventral side up on double sticky tape, affixed to a plane wood table. Thirty female ticks per group were injected in the trochanter-coxae articulation with 69 nL of gene specific dsRNA (1×10^{11} to 1×10^{12} molecules) or unrelated dsRNA as control, using the nano-injector (Nanoject, Drummond Scientific, PA, USA). The mouse *β-2-microglobulin* dsRNA (ds*β2M*) (GenBank: NM_009735) was used as control (Couto et al., 2017). After dsRNA injection, female ticks were held in a humidity chamber for 4 h after which they were allowed to feed on the splenectomized lamb infected with *B. ovis* together with 30 male ticks per feeding cell. Tick-feeding cells (450 × 400 mm) (cotton fabric) were glued to shaved skin using Pattex® contact glue (Henkel Nederland, Nieuwegein, Netherlands) on the day before infestation. Ticks were monitored daily and allowed to feed in the infected lamb for 8 days. After this period, attached ticks were manually removed.

Analysis of Tick Biological Parameters After Gene Knockdown

Tick mortality was evaluated as the ratio of dead ticks to the total number of initial ticks. To analyze tick mortality, the Chi-square test ($P > 0.05$) was used with the null hypothesis that tick mortality was independent of gene knockdown. The ability to attach to the vertebrate host was also evaluated as the ratio of attached ticks and the total number of live ticks. The Chi-square test ($P > 0.05$) was also used in this analysis. Tick weight was determined in individual female ticks collected after feeding and further compared between ticks injected with test genes dsRNA and control dsRNA by Student's *t*-test with unequal variance ($P > 0.05$).

Gene Knockdown Assessment and Determination of *B. ovis* Infection by qPCR

To assess gene knockdown efficiency in tick SG ten ticks per group were randomly selected and tissues dissected and further used to extract total RNA and DNA and synthetize cDNA, as described previously. Quantity and quality of the RNA samples was estimated using the QIAxcel Advanced system (Qiagen™, Hilden, Germany). qPCR assays were performed under the conditions aforementioned. Gene expression was analyzed by the CFX Manager™ Software (Bio-Rad) as previously referred. Infection levels in tick SG were estimated using qPCR by evaluation of the levels of *B. ovis 18S* ribosomal DNA (*18S* rRNA) normalized against tick *16S* rDNA, as described previously for other *Babesia* spp. (Antunes et al., 2012). The primers used for detection of *B. ovis* were the same used previously for conventional PCR. The cycling conditions are described in the Supplementary Table 1. Normalized Cq-values were compared between ticks injected with dsRNA and control ticks by Student's *t*-test with unequal variance ($P > 0.05$).

Antigenicity Prediction

Antigenicity of the selected molecules was estimated *in silico* using VaxiJen Server (Doytchinova and Flower, 2007) to allow antigen classification based on the physicochemical properties of proteins without resorting to sequence alignment. Complete sequences of the proteins were retrieved from UniProt in FASTA format and antigenicity estimated using the settings of parasite as target organism and threshold level 0.4.

RESULTS

Assembly and Annotation of Female *R. bursa* Sialomes

R. bursa female ticks representing the three conditions were produced and used for SG dissections, which were followed by DNA and RNA extractions. RNA qualitative and quantitative analysis are summarized in Supplementary Table 3. Infection of protozoan-exposed group (Fi) was confirmed prior to experimentation, and total RNA was used in RNA-Seq analyses. Data were collected as two sets of matched 100-bp

reads and quality analysis and raw read pre-processing were performed. The *de novo* assembly statistics are presented in **Table 1**.

A substantial increase in the number of contigs was observed in fed-uninfected samples compared to unfed-uninfected samples. The fed-*B. ovis* infected samples exhibited the highest number of contigs (**Table 1**). Each transcript was annotated based on BLAST results that compared the transcript to a database of reference proteins. The complete list of results can be accessed in Supplementary Datasheets 1, 2.

The obtained transcriptomes were analyzed using the Blast2GO tool and a public Arthropoda database (nr subset) (arthropoda, taxa: 6656; from 30.01.2017). Molecular functions (**Figure 1A**) and biological processes (**Figure 1B**) of the three transcriptomes were analyzed.

The molecular functions represented in the three sialotranscriptomes included DNA, RNA, protein, and ion binding properties as well as kinase, oxidoreductase, peptidase, and transmembrane transporter activities (**Figure 1A**). The remaining functions represented molecular functions that were present in both fed-uninfected and fed-infected catalogs, with the exception of nucleoside-triphosphatase and structural molecule activities that were exclusive to the unfed-uninfected sialotranscriptome. Ion binding was the most represented molecular function in all three datasets (**Figure 1A**). Biological processes such as catabolic, cellular protein modification, single-organism cellular, small molecule metabolic processes, translation, and signal transduction were also overrepresented in all sialotranscriptomes (**Figure 1B**). Anatomical structure development, chromosome organization, macromolecular complex assembly, response to stress, ribonucleoprotein complex biogenesis, vesicle-mediated transport, and DNA, RNA, and lipid metabolic processes were represented in the two sialotranscriptomes associated with fed-uninfected and fed-infected conditions (**Figure 1B**). Single-organism development is a feeding-exclusive process, while cellular component assembly, organelle organization, transport, and nucleic acid metabolic processes were exclusive to the unfed-uninfected samples.

Profile of SG Transcriptomic Dynamics in Response to Tick Feeding and *B. ovis* Infection

To clarify the response of *R. bursa* sialotranscriptomes to *Babesia* infection and blood feeding, an analysis that focused on the most ($P < 0.1$) up-regulated and down-regulated transcripts (Supplementary Figure 2) and as well as significantly differentially expressed ($P < 0.05$) genes (**Figures 2**, **3**) was conducted.

In total, 7,272 and 13,819 different expressed sequence tags (ESTs) were obtained from the SG of unfed and fed ticks, respectively. From these, 5,188 were found in both conditions, 2,884 were exclusive to the unfed population, and 8,631 were only present in the SG of fed *R. bursa* females. The sialotranscriptome associated with the fed-uninfected condition was compared to the fed-infected one. The results of RNA-Seq analyses indicated that 13,819 ESTs were obtained from the sialotranscriptome of the fed sample, and 15,292 ESTs were obtained from the fed-infected sample. Of these, 9,722 ESTs were present in both samples. A total of 4,097 ESTs were exclusive to the fed-uninfected ticks, and 5,570 ESTs were only present in the SG of the fed *R. bursa* females.

Analysis of the most up-regulated and down-regulated transcripts ($P < 0.1$) (Supplementary Figure 2) indicated that 500 and 216 ESTs were differentially regulated upon feeding and infection, respectively. The diversity of molecular functions and biological processes was higher in response to blood feeding compared to infection conditions. Regarding molecular functions, hydrolase activity was the only *Babesia* infection-exclusive function, and it was completely down-regulated. The blood-feeding exclusive functions were anion, metal ion, heterocyclic, and organic cyclic compound, and protein binding activities, and these functions were only associated with up-regulated transcripts. Regarding biological processes, *B. ovis* infection resulted in the induction of biosynthetic processes, cellular protein metabolic processes, gene expression, macromolecular complex assembly, organelle organization, and symbiosis (encompassing mutualism through parasitism). However, infection was also associated with the down-regulation

TABLE 1 | Assembly statistics of the six examined *Rhipicephalus bursa* sialotranscriptomes.

Assembly	NFni (1)	NFni (2)	F (1)	F (2)	Fi (1)	Fi (2)
# contigs (≥0 bp)	9,832	3,433	16,931	14,051	18,801	17,032
# contigs (≥1,000 bp)	1,317	345	3,200	2,143	3,224	2,655
# contigs (≥200 bp)	9,824	3,429	16,911	14,043	18,785	17,003
Total length (≥0 bp)	5,924,670	1,825,230	12,261,455	9,122,113	13,243,635	11,318,332
Total length (≥1,000 bp)	2,478,605	589,891	6,455,940	4,306,138	6,743,456	5,408,930
Total length (≥200 bp)	5,923,273	1,824,505	12,258,149	9,120,798	13,240,893	11,313,392
Largest contig	12,501	9,307	10,194	11,098	14,540	12,991
GC (%)	51.20	55.34	57.58	51.10	50.23	51.15
# N's per 100 kbp	0.02	0.00	0.00	0.00	0.02	0.25

NFni (1) and (2) correspond to the replicates of the pools of SG mRNA collected from unfed-uninfected females; F (1) and (2) correspond to the replicates of the pools of SG mRNA from fed-uninfected females; and Fi (1) and (2) correspond to the replicates of the pools of SG mRNA from fed-Babesia ovis infected female ticks.

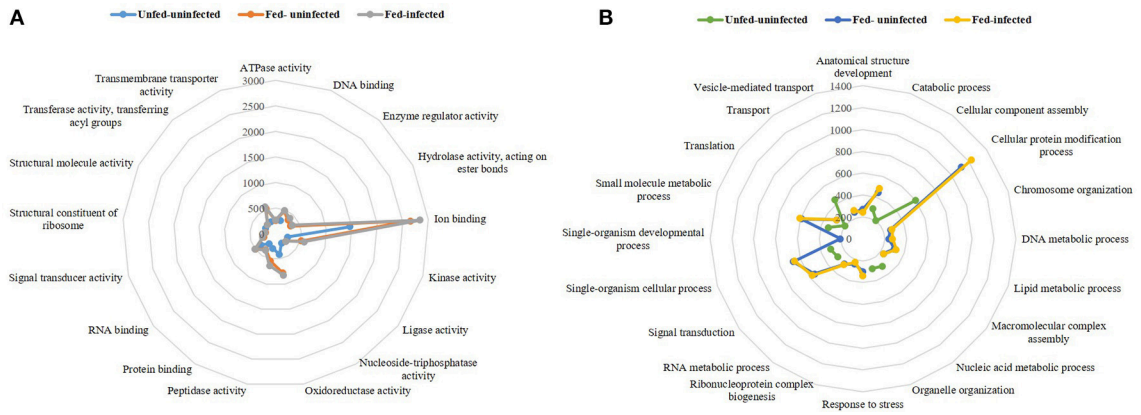

FIGURE 1 | Radar plots of the three transcriptomes per represented molecular functions **(A)** and biological processes **(B)**. The lines represent a pattern of the three transcriptomes unfed-uninfected, fed-uninfected and fed-*Babesia ovis* infected, allowing a visual comparison between conditions.

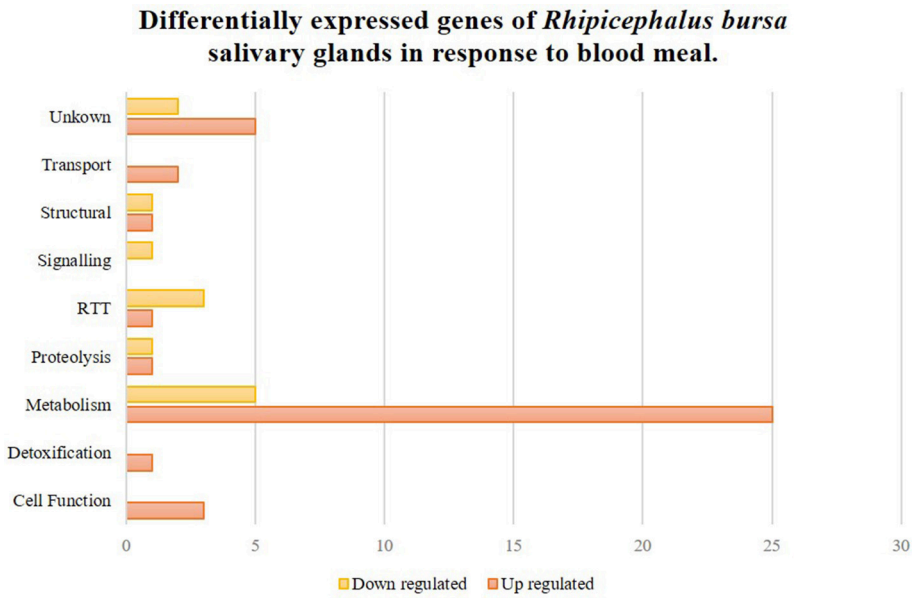

FIGURE 2 | *Rhipicephalus bursa* SG transcriptional response to blood meal based on Gene Ontology functional classes assignments of encoded proteins. Yellow bars represent down regulated genes, orange bars represent up regulated genes with statistical significance ($P < 0.05$).

of catabolic processes, cellular component organization, lipid metabolic and single-organism cellular process, and transmembrane transport. *R. bursa* blood meals predominantly induced biological processes such as oxidation-reduction, organic substance biosynthetic, and cellular biosynthetic processes, and cellular amino acid metabolic process and signal transduction were down-regulated.

SG Gene Differential Expression in Response to Blood Feeding

Fifty-two genes were considered significantly differentially expressed ($P < 0.05$), and these were classified based on GO for biological process and molecular functions (**Figure 2**). Seventy-five percent of these genes were up-regulated, and metabolism was the most up-regulated functional class in response to blood feeding. Functional classes such as transport, detoxification, and cell functions were only up-regulated, while signaling was down-regulated. Transcripts from structural, RTT (replication-transcription-translation), proteolysis, and metabolism functional classes were also differentially regulated during blood meals.

SG Gene Differential Expression in Response to *B. ovis* Infection

Thirty-six genes were considered differentially expressed ($P < 0.05$) and classified by functional classes as previously

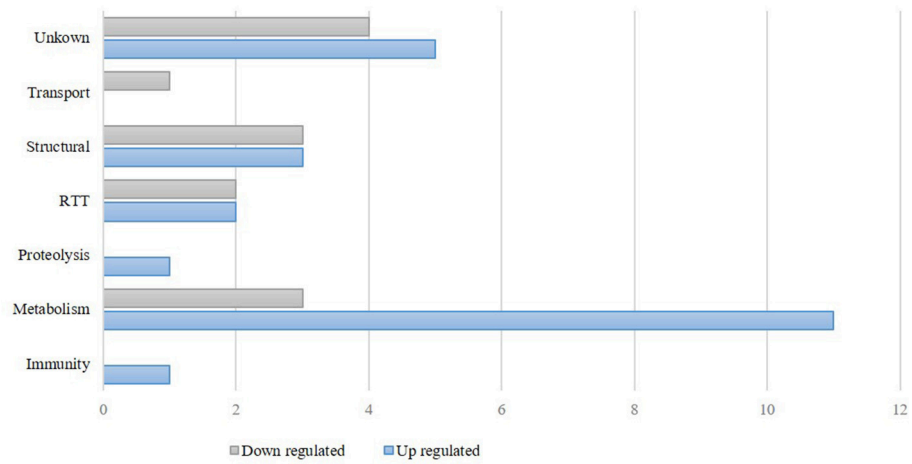

FIGURE 3 | *Rhipicephalus bursa* SG transcriptional response to *Babesia ovis* infection based on Gene Ontology functional classes assignments of encoded proteins. Gray bars represent down regulated genes and blue bars represent up regulated genes with statistical significance ($P < 0.05$).

described (**Figure 3**). Further analyses revealed that 64 and 36% of the differentially expressed genes were up-regulated and down-regulated, respectively. Metabolism was a highly represented functional class that was associated with both up- and down-regulated genes. Structural and RTT functional classes were also affected in the *R. bursa* sialome by *Babesia* infection. Proteolysis and immunity were exclusively up-regulated, while transport was down-regulated.

Validation of RNA-Seq Results

Sixteen genes identified as differentially expressed in response to infection and blood feeding in RNA-Seq were selected for data validation by qPCR analysis. From the RNA-Seq catalog derived from the comparison of fed vs. unfed populations, nine transcripts that encoded the following proteins were selected: annexin (UniProt ID: A0A023FX57), aspartic protease (UniProt ID: Q2WFX6), yolk cathepsin (UniProt ID: Q56CZ1), a putative hydroxysteroid 17-beta dehydrogenase (UniProt ID L7M196), hirudin-like (UniProt ID: F0JA28), lachesin (UniProt ID: L7M018), lipocalin 9 (UniProt ID: A0A034WWJ8), a putative scinderin-like (UniProt ID: L7MCZ6), and vitellogenin-3 (UniProt ID: A0A034WWF8) (**Figure 4**). Regarding the RNA-Seq data obtained from the comparison of infected and uninfected SG, eight genes encoding the following proteins were selected: a putative chondroitin sulfate synthase 1-like (UniProt ID: V5H7Q8), lachesin (UniProt ID: L7M018), laminin receptor (UniProt ID: E2J6W6), a putative glycine rich protein (UniProt ID:L7M1K6), a mucin-like protein (UniProt ID: C9W1L9), a putative ornithine decarboxylase antizyme (UniProt ID: A0A023FCB3), a secreted cement protein (UniProt ID: A0A034WWS7), and a putative yurt (UniProt ID: V5HE08) (**Figure 5**). A moderate positive correlation between the mRNA levels by both RNA-Seq and qPCR methods was obtained (Pearson's correlation coefficient 0.5394, $P = 0.025$).

Selection of Genes for RNA Interference Studies

Genes for RNAi functional studies were selected based on their potential role in the condition studied and fold change of expression. The gene encoding a putative *vitellogenin*-3 (*Vg-3*) was identified herein as up-regulated in response to feeding in both RNA-Seq (fold-change 17.51, $P = 0.025$) and qPCR (fold-change 98.05, $P < 0.001$) evaluations. The GO analysis assigned the encoded protein to a lipid transporter activity function (molecular function), belonging to the lipid transport biological process. *Lachesin,* which was also selected for functional analysis, was found to be up-regulated in the RNA-Seq analysis (fold-change = 15.14, $P = 0.045$) in response to blood feeding, while it was down-regulated based on the qPCR analysis (fold-change = −3.83, $P < 0.001$). This gene was also identified in the transcriptomic response to infection (fold-change = −0.80, $P = 0.857$), so its expression during *B. ovis* infection was also verified by qPCR (fold-change = −2.6427, $P = 0.955$). Lachesin belongs to the UniRef90_A0A1E1X7K6 cluster that is related to neural cell adhesion molecules. The gene designated as *secreted cement* encodes a component that is potentially involved in cement cone formation and tick attachment, and it was up-regulated in response to infection based on both RNA-Seq (fold-change = 15.73, $P = 0.0298$) and qPCR (fold-change = 4.197, $P = 0.007$) results. The expression of this secreted cement protein was also characterized by qPCR in response to blood feeding, indicating high up-regulation (fold-change = 47.4, $P < 0.0001$) in accordance with its role in the feeding process. Lastly, an uncharacterized gene designated as *glycine rich* that encodes a putative glycine rich protein was selected from the catalog associated with infection response, and it was up-regulated based on the results of both RNA-Seq (fold-change = 14.76, $P = 0.0382$) and qPCR (fold-change = 2.931, $P = 0.016$) analyses.

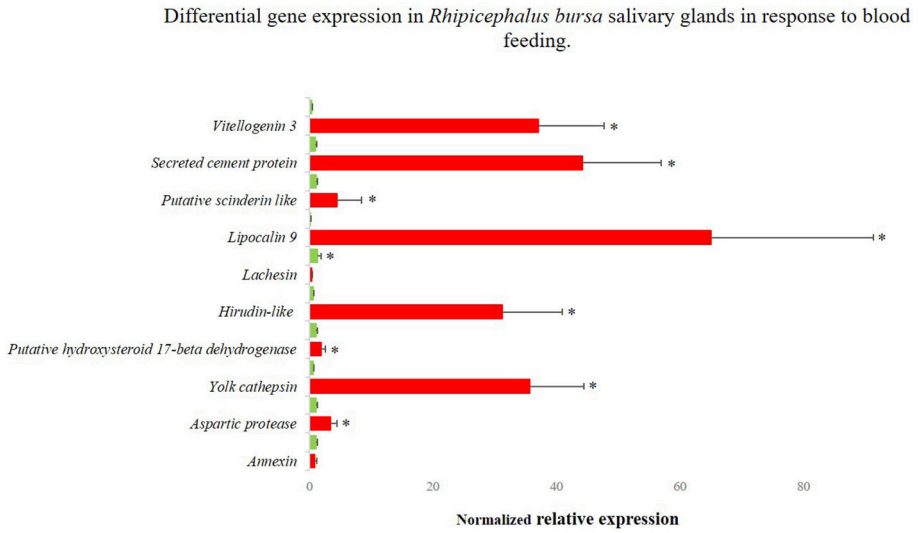

FIGURE 4 | Differentially gene expression of *Rhipicephalus bursa* SG in response to blood feeding evaluated by qPCR. Red bars represent SG from fed *R. bursa* ticks and green bars represent the SG from unfed *R. bursa* ticks. *$P < 0.05$.

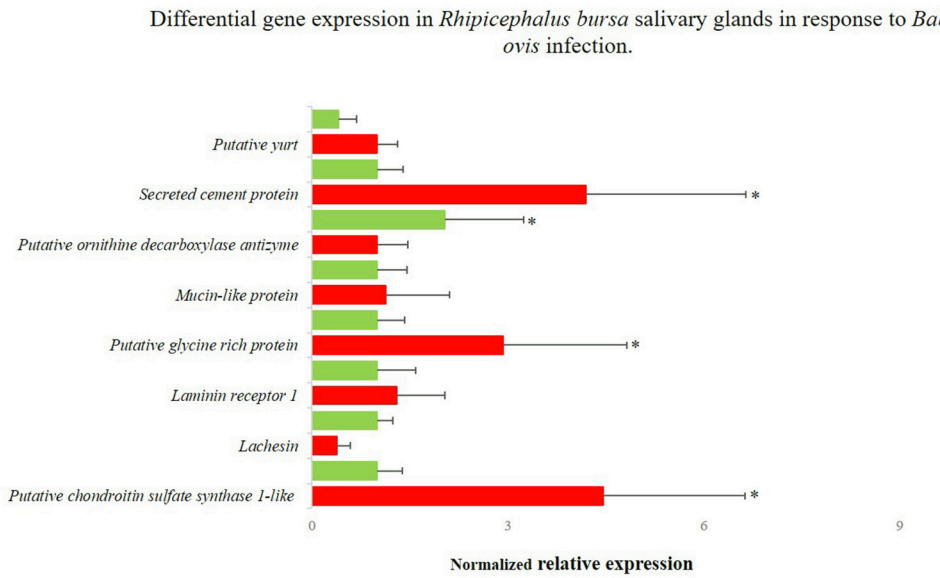

FIGURE 5 | Differentially gene expression of *Rhipicephalus bursa* SG in response to *Babesia ovis* infection evaluation by qPCR. Red bars represent the *B. ovis* infected SG and green bars represent the SG from uninfected *R. bursa* ticks. *$P < 0.05$.

Functional Analyses of Differentially Expressed Tick Genes in Response to Feeding and *B. ovis* Infection

Tick Attachment, Weight and Survival Rate After RNAi

After dsRNA injection, biological parameters such as tick mortality, attachment, and weight were determined and statistically analyzed (**Table 2**).

RNAi assays indicated that tick survival was significantly affected in dsRNA-injected ticks, in both ds*vitellogenin* (7/30; Chi-square, $P < 0.001$) and ds*lachesin* (9/30; Chi-square, $P < 0.001$) groups compared to controls (25/30), suggesting that these genes may play an important role in tick survival. The ds*vitellogenin* group was most affected with the highest mortality rate (76.67%). As represented in **Table 2**, the ds*cement* group was the most significantly affected by RNAi ($P = 0.008$), as 45.8% of the ticks were not able to correctly attach to the

TABLE 2 | Evaluation of tick mortality, attachment, and weight after dsRNA injection in *Rhipicephalus bursa* ticks.

Group	% of mortality rate (N)	% of the ticks completing feeding (N)	*R. bursa* weight (mean ± s.d.; mg/tick)
vitellogenin-3	76.67 (23)*	57.14(4)[b]	40 ± 19[b]
lachesin	70.00 (21)*	88.89(8)	149 ± 108
glycine-rich	36.37 (11)	57.90(11)[a]	73 ± 72[a]
secreted cement	20.00 (6)	54.20(13)*	52 ± 46*
Control	16.67 (5)	88.00(22)	136 ± 163

*Thirty female ticks per group were injected with dsRNA or unrelated dsRNA. Ticks were allowed to feed in three separated patches on a lamb experimentally infected with Babesia ovis. All attached ticks were removed after seven days of feeding, weighed, and held in a humidity chamber for four days to allow ticks to digest the blood meal. Tick mortality was evaluated as the ratio of dead ticks to the total number of ticks placed on the lamb using Chi-square test. (*P < 0.05). Female tick weight after feeding was compared between dsRNA and unrelated dsRNA ticks by Student's t-test. ds, double-stranded. [a]No statistical analysis was performed due to no gene knockdown. [b]No statistical analysis was performed because of the insufficient number of samples.*

TABLE 3 | Efficiency of gene knockdown by RNA interference and its influence on *B. ovis* infection levels in *Rhipicephalus bursa* ticks SG.

Group	Gene silencing (% Ave ± *S.D.*)	*B. ovis* infection levels (Ave ± *S.D.*)	Infection rate (Ave ± *S.D.*) (N)
vitellogenin-3	92 ± 2*	$4.67e^{-04} \pm 3.05e^{-04}$	314 ± 255.17(4)[a]
lachesin	51 ± 9*	$4.48e^{-07} \pm 1.20e^{-07}$	0.30 ± 0.09(8)*
glycine-rich	ND	–	–
secreted cement	65± 11**	$2.97e^{-06} \pm 2.68e^{-06}$	1.99 ± 2.18
Control	–	$1.49e^{-06} \pm 1.09e^{-06}$	–

*Thirty female ticks per group were injected with dsRNA or unrelated dsRNA. Ticks were allowed to feed in six separated patches on a lamb experimentally infected with Babesia ovis. All attached ticks (n = 4–22) were removed after seven days of feeding and held in a humidity chamber for four days to allow ticks to digest the blood meal. Gene knockdown was analyzed by qPCR by comparing mRNA levels between specific dsRNA-injected and control ticks using the CFX Manager™ Software by means of the Pfaff method, *P < 0.05. The B. ovis infection levels were determined by qPCR of the pathogen 18S rRNA gene and normalized against tick 16S rRNA using the ddCq method ($2^{-Cq\ -Cq}_{target\ control}$). Infection rate was calculated by the ratio of silenced per control groups. The mRNA levels and B. ovis infection in ticks were compared between specific dsRNA injected and control ticks by a Student's t-test (*P < 0.05; **P < 0.01). ds, double-stranded; ND, not demonstrated. [a]No statistical analysis was made due to the insufficient number of samples.*

vertebrate host to complete blood meal. The ds*lachesin* injected population mimicked the control group's ability to attach to the host and feed. The average body weight was also measured, and it was significantly higher in the control group (133 ± 119 mg) than the *Vg-3*-silenced group (40 ± 19 mg); however, no statistical study was conducted because of the low number of ticks (N = 4). *Lachesin* knockdown did not affect tick weight (149 ± 108 mg) ($P > 0.05$). The knockdown of the gene encoding the cement protein significantly reduced female weight (52 ± 46 mg, $P = 0.021$) and only 13 ticks were able to attach to the host.

Gene Silencing Efficiency and *Babesia* Infection Evaluation

Under the studied conditions, dsRNA-mediated gene knockdown efficiency and its effect on *B. ovis* infection was assessed (**Table 3**).

The injection of dsRNA molecules in *R. bursa* ticks led to a significant reduction of *vitellogenin*, *lachesin*, and *secreted cement* mRNA levels in SG by 92% ($P = 0.040$), 51% ($P = 0.047$), and 65% ($P = 0.018$), respectively. Regarding the levels of infection acquired after feeding on an experimentally *B. ovis*-infected lamb, the results indicated that the knockdown of *lachesin* significantly reduced *B. ovis* infection levels by 70% ($P = 0.00251$) in *R. bursa* SG (**Table 3**). The remaining groups exhibited increased infection levels.

Antigenicity of vitellogenin-3, lachesin, and secreted cement proteins were predicted by VaxiJen tool selecting parasite as the target organism. The three proteins showed to be probable antigens.

DISCUSSION

Babesiosis is one of the most important diseases transmitted by ticks that affect a wide range of vertebrates, considered an emerging zoonose (Hunfeld et al., 2008; Ord and Lobo, 2015; Antunes et al., 2017). *B. ovis* is a potentially lethal pathogen that is normally found in small ruminants, and it is primarily transmitted by *R. bursa*, a tick species that is widely distributed in the Mediterranean region (Walker et al., 2000; Ferrolho et al., 2016a). Despite the importance of the *R. bursa*-*B. ovis*-vertebrate host interactome, no studies have examined these molecular relationships. Although it is recognized that transcripts and protein levels in ticks do not always correlate because of post-transcriptional and post-translational modifications (Ayllón et al., 2015; Villar et al., 2015), transcriptomic analysis is essential for a proper understanding of the molecular constituents of cells and tissues and the interactions and relationship between parasites and disease development (Li and Biggin, 2015; Rokyta et al., 2015). The integration of different omics analyses have allowed the detailed characterization of tick-pathogen molecular pathways (Ayllón et al., 2015; Cabezas-Cruz et al., 2017a,b). Herein, to elucidate the cellular mechanisms behind blood feeding and *Babesia* infection, three sialotranscriptomes of *R. bursa* females were analyzed and SG genes were selected for further characterization with RNAi to assess their potential as tick protective antigens.

Overall Characteristics of the *R. bursa* Sialome in Response to Blood Feeding and *Babesia* Infection

A strong transcriptional response was induced after tick feeding and during *B. ovis* infection, since a higher and more diverse number of transcripts were detected in the fed-uninfected sample, and even more diverse transcripts were detected in the fed-infected samples (**Table 1** and **Figure 1A**) in comparison with the unfed-uninfected SG samples. This type of response was previously described in other systems (Heekin et al., 2013; Tirloni et al., 2014; Ayllón et al., 2015; Villar et al., 2015; de Castro et al., 2016, 2017; Kim et al., 2016; Perner et al., 2016; Valdés et al., 2016; Schroeder et al., 2017), thus indicating that

different tick biological processes or statuses stimulate different gene expression regulatory strategies.

Functional annotation indicated that in all transcriptomes, ion binding molecular function was the most represented category, and its representation nearly doubled in response to feeding (**Figure 1A**). Being obligatory hematophagous ectoparasites, ticks must deal with the iron and heme resulting from blood catabolism. Ticks are known to express iron and heme binding proteins that sequester excess iron or heme, preventing cell damage for physiologically normal cells (Galay et al., 2015; Kim et al., 2016).

Structural molecule activity is the only class more represented in the unfed-uninfected SG transcriptome, while other molecular function categories such as structural constituent of ribosome or enzyme regulator activity are exclusive to the fed-uninfected and fed-infected populations that exhibit high cellular activity (Villar et al., 2014).

The most represented biological process in all sialomes was the cellular protein modification. The transcript abundance of transcripts belonging to this biological process doubled in the fed-uninfected and fed-infected SG samples in comparison to the unfed-uninfected ones. The anatomical structure development process was only represented in the fed populations, and this possibly reflected SG enlargement during feeding as the majority of acinar cells undergo marked hypertrophy in Ixodid females (Šimo et al., 2017). Furthermore, some pathogens induce cytoskeletal rearrangement by affecting the regulation of specific mRNAs (Ayllón et al., 2013, 2015; Ireton, 2013; Cotté et al., 2014; de la Fuente et al., 2017). As expected, metabolism-related processes were markedly represented in the transcriptomes of fed samples. The response to stress was only identified in the fed-uninfected and fed-infected SG samples, and this was in accordance with previous studies that indicated high regulation of such pathways in ticks and cells infected with *Anaplasma* spp. (Villar et al., 2010, 2014) and during feeding (oxidative stress response) (Kim et al., 2016). The unfed sialotranscriptome profile revealed the maintenance of basal cellular metabolism (**Figure 1B**). Lipid metabolic processes were exclusively represented in the fed-uninfected and fed-infected samples, thus correlating with higher cellular energy requirements and saliva production (Denardi et al., 2011). Being a cellular energy source, lipids in tick SG are implied in cement cone formation, thus explaining the high representation of such metabolic activity (Denardi et al., 2011). A comparable result was obtained in *Ixodes ricinus* and *Rhipicephalus appendiculatus* SG after feeding (Kotsyfakis et al., 2015; de Castro et al., 2016). Salivary lipid interacting proteins were up-regulated in *I. ricinus* infected with *Borrelia burgdorferi* (Cotté et al., 2014) suggesting that certain pathogens can manipulate vector lipid metabolism to facilitate infection and multiplication (Perera et al., 2012; Grabowski et al., 2017).

Specific *R. bursa* Sialome Response to Blood Feeding

Few studies have focused on the sialotranscriptomic response to tick feeding (McNally et al., 2012; Kotsyfakis et al., 2015; Yu et al., 2015; de Castro et al., 2016, 2017; Maruyama et al., 2017), but all demonstrated that transcription was highly affected in SG. Kotsyfakis et al. (2015) showed that fed *I. ricinus*, SG exhibit 10 times more overexpression compared to the midgut. Herein, genes that were highly differentially expressed in response to blood meals indicated up-regulation at rates of 75.0% ($P < 0.05$) to 83.8% ($P < 0.1$). GO analyses revealed that expression of secreted proteins was induced during tick feeding, including 14 lipocalins, four metalloproteases, two glycine rich proteins, and three microplusins (Supplementary Datasheet 1, Supplementary Figure 2). Such transcriptional regulation differs throughout tick feeding, thus reflecting the necessity of the tick to first attach to the host, evade and modulate host immune defenses, and maintain this status during the prolonged feeding period (McNally et al., 2012; Kotsyfakis et al., 2015; Chmelar et al., 2016; de Castro et al., 2017). Furthermore, fatty-acid related transcripts were highly represented in the up-regulated SG genes, suggesting a significant investment in carbohydrate metabolism. After tick attachment, SG differentiate and convert from an inactive to a metabolically active status with intense biosynthesis of molecules and ion transport, which increase cell energy requirements (McNally et al., 2012). The most up-regulated transcripts identified herein using RNA-Seq analyses encoded a fatty acid synthase (fold-change = 17.67), followed by vitellogenin-3 (fold-change = 17.51) and a glycine-rich cell wall structural protein (fold-change = 17.51). Two uncharacterized proteins (fold-changes = −17.66 and −16.63) and two glycine rich proteins (fold-changes = −16.63 and −15.71) encoded transcripts were highly down-regulated (Supplementary Datasheet 1). These results suggested that in the late stage of feeding, female ticks switch the regulation of specific proteins related to the production of cement cone, thus driving drop-off in accordance with previous reports (McNally et al., 2012; Kotsyfakis et al., 2015; de Castro et al., 2017).

Specific *R. bursa* Sialome Response to *B. ovis* Infection

The sialotranscriptomes of fed-infected and fed-uninfected female *R. bursa* were compared to characterize SG transcriptional regulation in response to pathogen infection. As all of the SG samples belonged to fed ticks, the effect of the feeding process can be annulled. Some studies aimed to understand the effects of pathogens on tick SG at transcriptomic, proteomic, and metabolomic levels (Nene et al., 2004; Zivkovic et al., 2010; Mercado-Curiel et al., 2011; McNally et al., 2012; Cotté et al., 2014; Ayllón et al., 2015; Villar et al., 2015; Valdés et al., 2016). Because of their medical importance, many of these studies were dedicated to *Anaplasma* spp./*Borrelia* spp.-*Ixodes* spp. interactions This is the first study that specifically focused on the *Rhipicephalus* SG transcriptomic response to *Babesia* infection. Pathogens highly adapted to the vector such as *Anaplasma-R. microplus* do not induce great effects on SG, while pathogens that pose a higher threat to vector fitness would lead to a greater gene modulation (Cen-Aguilar et al., 1998; Zivkovic et al., 2010; Mercado-Curiel et al., 2011; Chmelar et al., 2016; de la Fuente et al., 2016; Šimo et al., 2017). In *Babesia* infections,

tick development tends to be impaired, but adaptive parasite tolerance has been described in *R. microplus* (Cen-Aguilar et al., 1998; Antunes et al., 2017). Furthermore, a small number of genes were considered differentially expressed (36 genes at $P < 0.05$ and 260 genes at $P < 0.1$), suggesting the long co-evolution of *R. bursa* and *B. ovis*. In both analyses an up-regulation of 63–64% of the genes occurred. Our results showed that during *Babesia* invasion, cellular metabolism tended to increase, whereas biosynthesis and protein processing were the most represented categories (Supplementary Datasheet 2, Supplementary Figure 2). This metabolism induction was previously demonstrated in other vector-pathogen systems (Mercado-Curiel et al., 2011; Heekin et al., 2012; Ayllón et al., 2015; Villar et al., 2015). The most up-regulated genes found were related to glycine-rich proteins (GRPs), including uncharacterized protein (fold-change = 17.53), glycine rich proteins (fold-change = 16.45 and 15.65), and secreted cement protein (fold-change = 15.73). Glycine rich proteins have been identified as upregulated in response to infection and cement proteins (Nene et al., 2004; Zivkovic et al., 2010). With rare exceptions, the role of such proteins during pathogen infection/dissemination have not been investigated (Trimnell et al., 2002). Lipocalins and defensins were identified as up-regulated in our dataset, showing an investment of the tick in the immune response, as expected. To validate the RNA-Seq results, qPCR was employed targeting putatively down-regulated *ornithine decarboxylase antizyme, lachesin*, and *chondroitin sulfate synthase* genes and putative upregulated *laminin receptor*, *yurt*, *glycine rich*, *secreted cement*, and *mucin*. *Chondroitin sulfate synthase* and *lachesin* expression trends were not confirmed, indicating up-regulation in infected SG. Chondroitin's are known to be involved in *Plasmodium* spp. adhesion to cells (Dinglasan et al., 2007; Couto et al., 2017), so the up-regulation of related molecules in infected tick SG suggests that *Babesia* spp. (considered a *Plasmodium*-like parasite) may use similar strategies to invade cells.

Functional Studies for the Identification of Tick Protective Antigens

Vitellogenin-3

Multiple vitellogenins (Vgs) have been described in ticks (Thompson et al., 2007; Boldbaatar et al., 2010; Khalil et al., 2011; Taheri et al., 2014; Rodriguez et al., 2016), and they are involved in detoxification and oxidative molecular processes (Galay et al., 2015). In the sialotranscriptome obtained in response to blood feeding, the translation of one of the assembled transcripts showed high similarity to *R. microplus* putative Vg-3 protein (UniProt ID: A0A034WWF8). An up-regulation of the expression of the correspondent gene in the SG of fed *R. bursa* was demonstrated by both RNA-Seq and qPCR (RNA-Seq: fold-change = 17.509, $P = 0.025$; and qPCR fold-change = 98.05, $P < 0.001$), and the results were in accordance with those of previous studies (Horigane et al., 2010; Yang et al., 2015). Vgs are thought to be absent from SG, whereas heme transport and storage are thought to be dependent of the hemelipoglyco-carrier protein (CP) (Donohue et al., 2009). In ticks, both Vg proteins and CP bind heme (Logullo et al., 2002), which is a functional component of many hemoproteins, but it is cytotoxic in larger amounts (Ferrolho et al., 2016b; Hajdusek et al., 2016). The similarities between CPs and Vgs in ticks, as well as their common evolutionary origin, greatly complicate their differentiation and function assignments (Gudderra et al., 2002; Donohue et al., 2009; Boldbaatar et al., 2010). The present study showed that *R. bursa* possesses a gene very similar to *Vgs* in SG, and it shares several molecular features with CPs. Further studies are necessary to clarify Vgs classification in ticks as well as the function and localization of *Vg-3* in *R. bursa* species as these *Vgs* are expressed in a tissue-specific manner in ticks (Rodriguez et al., 2016). *Vg-3* knockdown experiments resulted in increased tick mortality. No statistical analyses were performed regarding feeding behaviors, body weight and *Babesia* infection, because of the low number of samples; however, decreased blood-uptake and increased *Babesia* infection was observed. Based on the principal functions associated to this type of molecule, we can suggest that a decrease in the expression of putative *Vg-3* reduces heme and lipid binding and storage (**Figure 6A**). A deficient heme seizure may increase cellular toxicity, thus contributing for the formation of reactive oxygen species (ROS). Also, the role of Vgs on lipid transport is compromised, and this may unbalance normal energy production. Vgs have been consistently discovered has highly immunogenic molecules in *Rhipicephalus* ticks (Boldbaatar et al., 2008, 2010; Smith and Kaufman, 2013; Taheri et al., 2014; Rodriguez et al., 2016), and the results of the present study stimulates future research.

Putative Secreted Cement Protein and Glycine-Rich Protein

The genes encoding putative cement protein and GRP were found to be significantly up-regulated in response to *B. ovis* infection in *R. bursa* SG, in accordance with a previous study (Nene et al., 2004). The cement cone is composed of several molecules that are embedded in a proteinaceous matrix, presenting several GRPs (Bishop et al., 2002; Trimnell et al., 2005; Maruyama et al., 2010). Different species of ticks rely on different types and amounts of GRPs in order to attach and feed on their hosts. Briefly, ticks with short mouthparts need higher amounts of GRPs than those with long mouthparts. Moreover, one-host ticks present a greater variety of these proteins than ticks that feed on several hosts (Maruyama et al., 2010). A successfully knockdown was observed in *cement*-silenced ticks, but no silencing was demonstrated in *glycine-rich* dsRNA-injected ticks, suggesting that a higher concentration may be needed to reduce the expression of this gene. The *cement*-silenced ticks significantly affected tick attachment, feeding, and body weight (**Figure 6B**). The ds*glycine-rich* RNA inoculated group exhibited a slight decrease in these two parameters, reflecting its potential in tick feeding capacity and attachment to the host. Curiously, in both dsRNA-injected groups, an increase of *Babesia* levels was detected. Previous studies concerning cement cone proteins showed that immunization with these proteins significantly affected tick attachment to the host (Trimnell et al., 2005) and it reduced pathogen transmission (Labuda et al., 2006). Therefore, these two proteins are attractive targets for vaccine development.

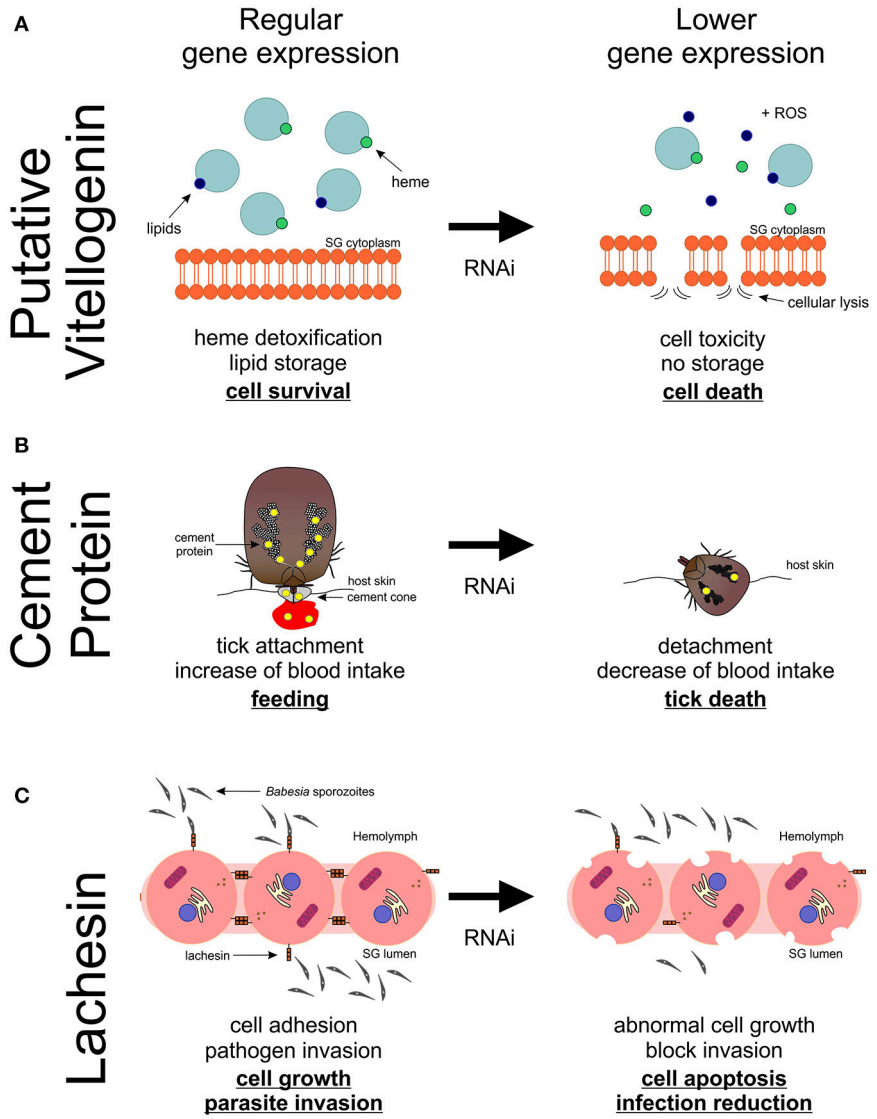

FIGURE 6 | Proposed model of putative vitellogenin-3, cement protein and lachesin functions and its impact on *Rhipicephalus bursa* SG during feeding and *Babesia ovis* infection. **(A)** Vitellogenin-3 described function relates to heme detoxification and lipid storage contributing for cell survival. A decrease of the expression of putative *vitellogenin-3* leads to deficient heme seizure, increasing the formation of reactive oxygen species (ROS) as well as cellular toxicity. Lipid storage is also compromised leading to an unbalance in the production of energy. **(B)** Putative cement protein is a component of the cement cone, which facilitates the tick attachment and feed on the host. An impact in the production of cement proteins leads to an incapacity of ticks to correctly attach and subsequently feed on the host, resulting in tick death and reduced blood ingestion. **(C)** Lachesin is a cell surface protein that as a potential role in cell adhesion, maintaining apical-basal polarity, vesicle trafficking, cell growth and survival, as well as parasite invasion. A negative manipulation of the expression of *lachesin* results in an abnormal cell growth and ultimately cell apoptosis, and also a decrease of *Babesia* spp. infection.

Lachesin

Lachesin is a cell surface protein of the immunoglobulin superfamily (Karlstrom et al., 1993; Llimargas et al., 2004) that regulates organ size by influencing cell length and cell detachments, suggesting a role in cell adhesion and connection (Llimargas et al., 2004). In ticks, the gene encoding lachesin was first identified in the genome of *Ixodes scapularis* (Gulia-Nuss et al., 2016) and more recently in the sialotranscriptome of *Amblyomma cajennense* (Garcia et al., 2014), *R. pulchellus* (Tan et al., 2015), and *R. appendiculatus* (de Castro et al., 2016). However, no studies that focus on this molecule in ticks have been performed. In the present study, an assembled transcript translated to a protein highly similar to lachesin (UniProt ID: L7M018). A highly dynamic expression profile of *lachesin* in response to infection and feeding was found in the present study, and this observation aligned to its presumed role on cell-adhesion led to its selection for RNAi studies. Tick inoculation with ds*lachesin* resulted

in 51% gene knockdown that led to a significantly high tick mortality. Lachesin accumulates in specific invertebrate cell junctions, and it is responsible for establishing and/or maintaining cell polarity, cell adhesion, and cell-cell interactions (Tepass et al., 2001). Apical-basal polarity is subjected to tight regulation, as it is crucial during tissue formation, including vesicle trafficking machinery, morphogenesis, and modulation of epithelial cell growth and survival (Bonazzi and Cossart, 2011). Moreover, adhesive contacts between cells and the extracellular matrix appear as important landmarks for polarity. Therefore, manipulating the expression of genes involved in this processes can induce abnormal cell growth and cell apoptosis (Tepass, 2012). In addition, the *lachesin* knockdown resulted in lower pathogen infection in the SG. No statistical effect was demonstrated in the other biological parameters studied. Despite the tight organization of the epithelium barrier and its interactions with cellular factors that are crucial to cell-pathogen defense, a large number of pathogens have developed strategies to target host proteins involved in cell adhesion, to colonize epithelia, invade host cells, or even disrupt host barriers to facilitate access to other tissues (Bonazzi and Cossart, 2011). Thus, our results suggest that lachesin plays an important role in tick survival and also that *B. ovis* may require this molecule for tissue invasion (**Figure 6C**). This molecule appears to be good candidate for future vaccination assays, as it demonstrates a dual-effect targeting both tick and pathogen.

CONCLUSIONS

Tick and tick-borne diseases constitute a growing burden for human and animal health, stressing the urgency in the development of new effective tools to control this global threat. Due to the important role of tick SG in tick biology and pathogen transmission, the main objective of the present study was the identification and functional characterization of *R. bursa* SG genes involved in tick feeding and *B. ovis* infection. Quantitative transcriptome analysis showed *lachesin* and putative *vitellogenin-3* has highly upregulated in response to blood meal and the genes encoding for a putative secreted cement and GRPs highly upregulated in response to *B. ovis* infection. RNAi studies suggest that *lachesin* and putative *vitellogenin-3* affect tick survival while the putative cement protein has an impact in tick attachment to the host and tick weight after feeding. Moreover, *B. ovis* infection levels in tick SG were reduced, subsequently to *lachesin* knockdown. Overall the results of the present study endorse the inclusion of these proteins in vaccination trials.

AUTHOR CONTRIBUTIONS

SA, JdlF, MMS-S, and AD designed the study; ASS and MMS-S were responsible for tick rearing and tick inoculation methodology. SA established and maintained *B. ovis* cultures. SA, JC, and JF performed transcriptomic analyses; SA and FR performed qPCR assays; SA, FR, JF, and JN performed the RNA interference studies; SA, JC, JdlF, and AD performed data analysis and wrote the manuscript. All authors edited and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00116/full#supplementary-material

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REVIEW
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The Tick Microbiome: Why Non-pathogenic Microorganisms Matter in Tick Biology and Pathogen Transmission

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Ticks are among the most important vectors of pathogens affecting humans and other animals worldwide. They do not only carry pathogens however, as a diverse group of commensal and symbiotic microorganisms are also present in ticks. Unlike pathogens, their biology and their effect on ticks remain largely unexplored, and are in fact often neglected. Nonetheless, they can confer multiple detrimental, neutral, or beneficial effects to their tick hosts, and can play various roles in fitness, nutritional adaptation, development, reproduction, defense against environmental stress, and immunity. Non-pathogenic microorganisms may also play a role in driving transmission of tick-borne pathogens (TBP), with many potential implications for both human and animal health. In addition, the genetic proximity of some pathogens to mutualistic symbionts hosted by ticks is evident when studying phylogenies of several bacterial genera. The best examples are found within members of the *Rickettsia*, *Francisella*, and *Coxiella* genera: while in medical and veterinary research these bacteria are traditionally recognized as highly virulent vertebrate pathogens, it is now clear to evolutionary ecologists that many (if not most) *Coxiella*, *Francisella*, and *Rickettsia* bacteria are actually non-pathogenic microorganisms exhibiting alternative lifestyles as mutualistic ticks symbionts. Consequently, ticks represent a compelling yet challenging system in which to study microbiomes and microbial interactions, and to investigate the composition, functional, and ecological implications of bacterial communities. Ultimately, deciphering the relationships between tick microorganisms as well as tick symbiont interactions will garner invaluable information, which may aid in the future development of arthropod pest and vector-borne pathogen transmission control strategies.

Keywords: tick, tick symbionts, tick borne pathogens, microbiome, microbial interactions

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INTRODUCTION

Over the last few decades, considerable research efforts have focused on the diversity, distribution, and impact of tick-borne pathogens (TBP). The list of known or potential TBP is constantly evolving, and includes a variety of viruses, bacteria, and parasites afflicting humans and many other animals worldwide (de la Fuente et al., 2008; Heyman et al., 2010; Dantas-Torres et al., 2012; Rizzoli et al., 2014). Less well studied or understood are whole microbial communities hosted by

ticks, which attract much less attention, yet are equally important. These communities include TBP, but also non-pathogenic microorganisms such as commensal and mutualistic microbes that are also abundant in ticks (Andreotti et al., 2011; Carpi et al., 2011; Williams-Newkirk et al., 2014; Duron et al., 2015a, 2017). Until recently, these non-pathogenic microorganisms were generally overlooked by scientists working with ticks and TBP. Before 1990, their existence was largely ignored and all bacteria found in ticks were usually considered to be potential TBP without necessarily undergoing rigorous health risk assessment. Toward the end of the 1990s, the advent of simple PCR assays led to a growing understanding that a few intracellular bacteria, such as the *Coxiella*-like endosymbiont and the *Francisella*-like endosymbiont (*Coxiella*-LE and *Francisella*-LE hereafter), are actually non-pathogenic microorganisms hosted by ticks (Niebylski et al., 1997a; Noda et al., 1997). Deeper investigation of microbial biodiversity through high-throughput sequencing and DNA barcoding led to another leap in understanding: non-pathogenic microorganisms from many different clades are present in ticks, and can generally coexist with TBP (Clay et al., 2008; Andreotti et al., 2011; Carpi et al., 2011; Lalzar et al., 2012; Vayssier-Taussat et al., 2013; Qiu et al., 2014; Williams-Newkirk et al., 2014; Narasimhan and Fikrig, 2015; Abraham et al., 2017). In addition, current available data on tick microbiomes suggest that non-pathogenic microorganisms exhibit higher taxonomic diversity than TBP since they encompass most major bacterial and Archaea groups (Andreotti et al., 2011; Carpi et al., 2011; Nakao et al., 2013; Qiu et al., 2014; Williams-Newkirk et al., 2014). Altogether, it is now clear that ticks carry complex microbial communities that are largely dominated by non-pathogenic microorganisms. Most importantly, this implies that both ticks and TBP are commonly engaged in interactions with non-pathogenic microorganisms.

The composition of these microbial communities is highly variable: environmental constraints are key drivers of their structure as shown by differences in bacterial diversity observed between laboratory-reared and wild ticks (Heise et al., 2010; Zolnik et al., 2016). It was further reported that bacterial community structures could vary depending on the examined tick species (Lalzar et al., 2012), the season during which ticks were collected (Lalzar et al., 2012), the examined geographical regions (van Overbeek et al., 2008; Carpi et al., 2011; Williams-Newkirk et al., 2014), the examined tick life stage (Moreno et al., 2006; Clay et al., 2008; Williams-Newkirk et al., 2014; Zolnik et al., 2016), and between different feeding statuses (Heise et al., 2010; Menchaca et al., 2013; Zhang et al., 2014). Furthermore, bacterial community structures may differ depending of the presence of pathogens (Steiner et al., 2008; Abraham et al., 2017). Overall, the quantity of potential variations highlights the lability of microbial communities hosted by ticks, and future studies should focus on understanding how these variations impact tick biology. Below, we will discuss the interesting hypothesis that the inherent flexibility of microbial communities may help ticks adapt to environmental stresses, such as TBP presence.

Microorganisms inhabiting ticks are not only taxonomically diverse, they are also ecologically diverse. This diversity is clearly illustrated by the large panel of lifestyle strategies that microorganisms use to infect and persist within tick populations (**Figure 1**). As vertebrate pathogens, TBP normally spread via infectious (horizontal) transmission through tick bite and blood feeding. A few TBP can also be vertically transmitted in ticks, and thus be maintained throughout each generation as observed for *Babesia* species (Chauvin et al., 2009), *Rickettsia rickettsii* (Burgdorfer et al., 1981), or viruses (Xia et al., 2016). Other tick microorganisms are highly specialized intracellular symbionts depending almost exclusively on maternal (transovarial) transmission to ensure their persistence in tick populations (Niebylski et al., 1997b; Lo et al., 2006; Sassera et al., 2006; Klyachko et al., 2007; Felsheim et al., 2009; Machado-Ferreira et al., 2011; Lalzar et al., 2014; Duron et al., 2015a; Kurtti et al., 2015). Tick microorganism diversity is further augmented due to the fact that environmental microorganisms can also colonize ticks: microbes present on vertebrate skin surfaces may colonize ticks during blood feeding, while those present in the soil or vegetation can colonize ticks on the ground, once they have dropped off their vertebrate hosts (Narasimhan and Fikrig, 2015). Overall, the diverse range of microbial lifestyle strategies creates a complex web of interactions offering excellent opportunities to tackle questions about the impact of whole microbial communities on tick biology and TBP transmission. In spite of this, the direct effects of pathogens and other microbes on tick physiology and activity has received much less attention than their effects on vertebrate hosts. In most cases, the function of tick endosymbionts in relation to their host has not been determined. Many of these endosymbionts have obligate intracellular life cycles or are difficult to cultivate, which may explain the gaps in current knowledge (Tully et al., 1995; Kurtti et al., 1996; Niebylski et al., 1997a; Duron et al., 2015a). However, for some bacteria, tissue-specific localization has been defined, which may aid us to understand bacterial impact on both tick biology and pathogen transmission (Noda et al., 1997; Klyachko et al., 2007; Lalzar et al., 2014; Narasimhan and Fikrig, 2015). Similarly, the use of microarray or RNASeq technologies to analyze induced tick microbiome expression patterns and varying composition following a variety of conditions, may also further elucidate their roles (Rodriguez-Valle et al., 2010). This

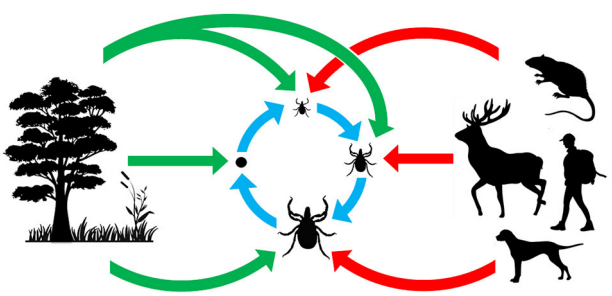

FIGURE 1 | Origin and acquisition of tick microorganisms. Red arrows: vertebrate pathogens acquired from tick bites; blue arrows: maternally inherited tick symbionts acquired via transovarial and transtadial transmission; green arrows: microorganisms acquired from the environment.

knowledge is of both medical and veterinary interest since it may enable the reassessment of tick-associated health risks, but also of ecological and evolutionary importance by highlighting co-evolutionary processes acting between ticks and their microbes. Indeed, some symbionts, but not all (Weller et al., 1998), have a joint evolutionary history of several million years with their tick hosts (Almeida et al., 2012; Duron et al., 2017), suggesting that complex interactions may have evolved in these associations. If biologists aim to fully understand the ecological and evolutionary processes involved in tick biology and the emergence of tick-borne diseases, a thorough examination of non-pathogenic microorganisms is also required.

Maternally-inherited symbionts are well-known to use specific adaptive strategies to spread and persist within arthropod populations, either providing fitness benefits to female hosts or manipulating host reproduction (Moran et al., 2008; Ferrari and Vavre, 2011). Two categories of widespread endosymbionts are usually recognized in arthropods, although intermediates and transition forms are also frequent:

– The first category consists of obligate (primary) mutualistic symbionts required to support normal host development, thus assisting their host in various essential functions. This includes nutritional diet upgrades by providing biosynthetic pathways absent from their hosts (Moran et al., 2008; Wernegreen, 2012). Indeed, most blood-feeding insects such as bed bugs, lice, and tsetse flies harbor obligate symbionts that provide B vitamins and cofactors not readily obtainable in sufficient quantities from a uniquely blood-based diet (Akman et al., 2002; Hosokawa et al., 2010; Boyd et al., 2013; Nikoh et al., 2014).
– The second category consists of facultative (secondary) symbionts not required for host survival. Some are defensive symbionts conferring protection against natural enemies or heat (Oliver et al., 2010; Ferrari and Vavre, 2011), while others are reproductive parasites that manipulate host reproduction through the induction of parthenogenesis, feminization of genetic males, male-killing, and cytoplasmic incompatibility (conditional sterility between infected and uninfected specimens) (Engelstadter and Hurst, 2009; Cordaux et al., 2011).

In this article we review four major biological aspects where our views on tick microbes have undergone substantial change over the last decade. Firstly, we must emphasize that non-pathogenic microorganisms have much more complex effects on ticks than previously thought. Indeed, it is now evident that several maternally-inherited symbionts are required for tick survival and reproduction, while other symbionts can have multiple effects on tick life history traits. Secondly, whilst tick TBP transmission modes have been studied for decades, we now understand that certain non-pathogenic microorganisms may also interfere with TBP transmission. Thirdly, although microorganisms are often categorized as "TBP," "commensals," or "maternally-inherited symbionts," both intermediate and transitional states frequently occur. In this context, it thus appears vital to not overlook the full range of potential effects, as have been recently described in microbiome studies. And finally, bacterial phylogeny demonstrates that several infectious agents have close genetic proximity with mutualistic tick symbionts. This indicates that some bacterial genera (eg. *Rickettsia*, *Francisella*, and *Coxiella*) have the capacity to frequently undergo evolutionary shifts between pathogenic and non-pathogenic forms, a process that may lead to the emergence of novel infectious diseases.

THE EFFECT OF NON-PATHOGENIC MICROORGANISMS ON TICK BIOLOGY

Perhaps the most remarkable observation of recent times is the pivotal role of symbiotic interactions in normal tick biology, including ecological specialization to an exclusive blood diet. Symbionts—i.e., microorganisms engaged in close and long-term interactions with their tick hosts—are exceptionally diverse in ticks: at least 10 distinct genera of maternally-inherited bacteria have been reported in ticks over the last decade (listed in **Table 1** and **Figure 2**) (Duron et al., 2017). Three of these symbionts are only found in ticks: *Coxiella*-LE, which infects at least two thirds of tick species, *Midichloria*, which inhabits the mitochondria of some tick species, and *Francisella*-LE, which has only been reported in a few tick species (**Table 1**). The seven remaining symbiont genera are more- or less-frequently found in other arthropod groups, including several well-studied insects. Five symbionts, *Wolbachia*, *Cardinium*, *Arsenophonus*, *Spiroplasma*, and *Rickettsia*, are commonly identified in some arthropod groups, while two others, *Rickettsiella* and *Lariskella*, have only been reported in a few other arthropod taxa in addition to ticks (**Table 1**).

Coxiella-LE has been reported as essential for tick survival and reproduction in the *Amblyomma americanum* lone star tick (Zhong et al., 2007). As an obligate symbiont, *Coxiella*-LE is, by definition, present in most individuals of a given tick species (Clay et al., 2008; Machado-Ferreira et al., 2011; Lalzar et al., 2012; Duron et al., 2015b, 2017): thus their mutualistic relationship is required for the survival of both organisms. Remarkably, some *Coxiella*-LE may form evolutionarily stable associations with their tick hosts that last for millions of years (Duron et al., 2017). These associations typically exhibit strict co-cladogenesis, resulting in congruent host-symbiont phylogenies as recently observed between members of the *Rhipicephalus* genus and their associated *Coxiella*-LE (Duron et al., 2017). The discovery of *Coxiella*-LE in numerous other tick groups (Jasinskas et al., 2007; Clay et al., 2008; Machado-Ferreira et al., 2011; Almeida et al., 2012; Lalzar et al., 2012; Duron et al., 2014, 2015a, 2017), indicates that it is the most widespread and biologically relevant tick symbiont. An examination of *Coxiella*-LE intra-host localization revealed pronounced tissue tropism in all examined tick species. This symbiont typically infects the ovaries (to ensure maternal transmission) and the distal part of Malpighian tubules, suggesting a possible role in nutrition, osmoregulation, or excretion (Klyachko et al., 2007; Machado-Ferreira et al., 2011; Lalzar et al., 2014). Examination of eggs from several tick species confirmed that *Coxiella*-LE is transmitted to >99% of tick progeny, demonstrating highly efficient maternal transmission (Machado-Ferreira et al., 2011; Lalzar et al., 2014;

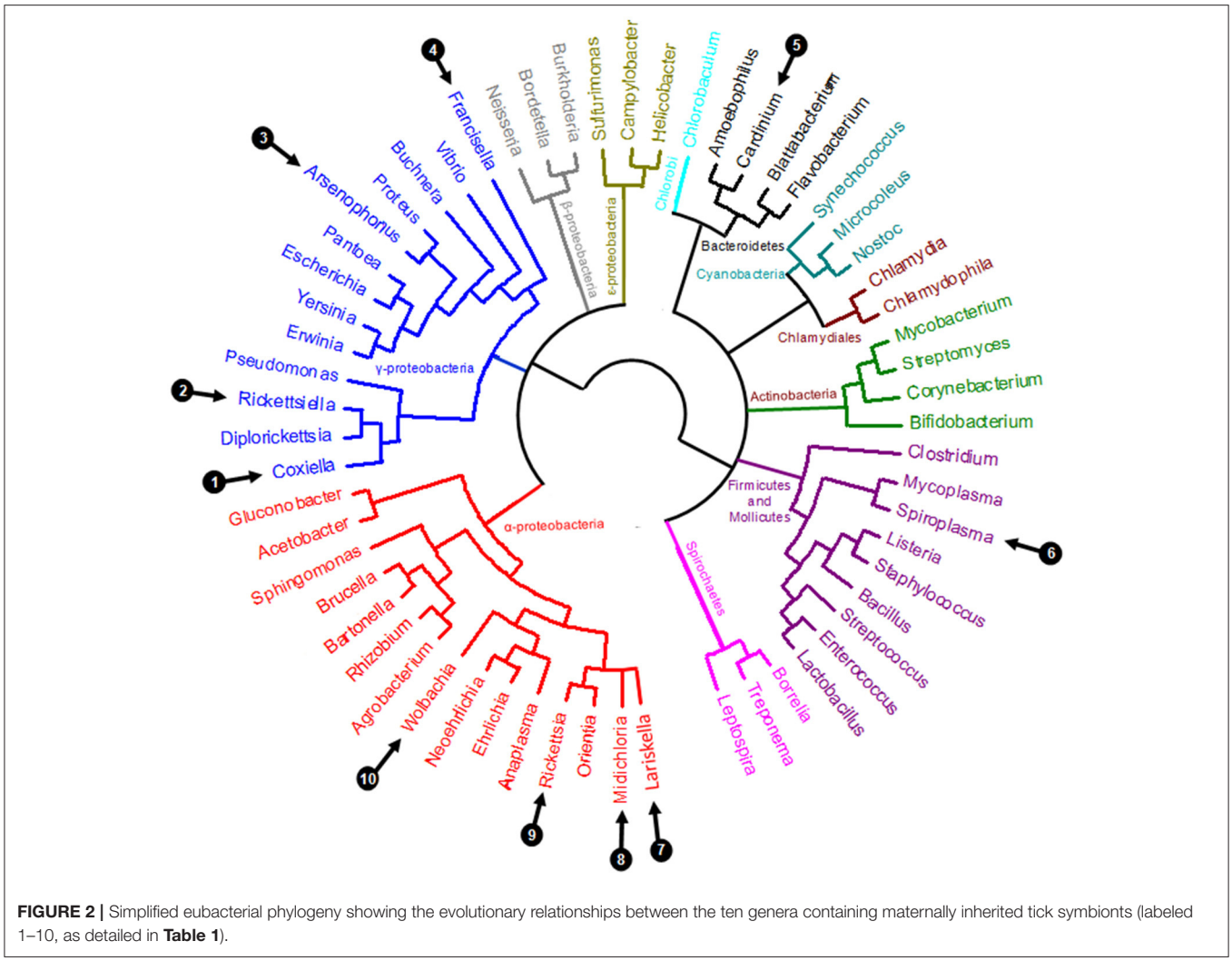

FIGURE 2 | Simplified eubacterial phylogeny showing the evolutionary relationships between the ten genera containing maternally inherited tick symbionts (labeled 1–10, as detailed in **Table 1**).

Duron et al., 2015a). Remarkably, the *Coxiella*-LE genome was shown to encode major B vitamin synthesizing pathways such as biotin (B7 vitamin), folic acid (B9), riboflavin (B2), and their cofactors, that are not usually obtainable in sufficient quantities from a uniquely blood-based diet (Gottlieb et al., 2015; Smith et al., 2015). By ensuring nutritional upgrading of the blood diet, *Coxiella*-LE has enabled ticks to utilize an unbalanced dietary resource and thus become hematophagy specialists.

Recent studies have suggested that alternative obligate symbionts other than *Coxiella*-LE may also exist. Around one third of examined tick species lack *Coxiella*-LE or harbor *Coxiella*-LE at much lower frequencies than expected for an obligate symbiont (Duron et al., 2014, 2015a, 2017). A large survey of 81 tick species showed that in almost all tick species without *Coxiella*-LE infection, another maternally-inherited symbiont was usually present (Duron et al., 2017). Among these alternative obligate symbionts were *Francisella*-LE, *Rickettsia*, and *Rickettsiella,* which are often present in all specimens of the infected tick species (Duron et al., 2017). Although formal testing with nutritional and physiological experiments is now required to validate their role as alternative obligate symbionts, recent bacterial genome data suggest that these bacteria have highly-evolved adaptive mechanisms enabling tick survival. Indeed, their genomes encode functions suggesting that they have—at least partially as for *Coxiella*-LE—a genetic capability for *de novo* B vitamin synthesis. Indeed, the *Francisella*-LE genomes from the fowl tick *Argas persicus* and the Gulf Coast tick *Amblyomma maculatum* contain complete genetic pathways for biotin, folic acid, and riboflavin biosynthesis (Sjodin et al., 2012; Gerhart et al., 2016). Similarly, recent metabolic reconstructions of *Rickettsia* genomes indicated that all genes required for folic acid biosynthesis are present in *Rickettsia* symbiont genomes obtained from both the black-legged tick *Ixodes scapularis* and the Western black-legged tick *I. pacificus* (Hunter et al., 2015). Worthy of note here is that laboratory findings directly corroborate the existence of beneficial *Rickettsia* symbionts since they exert a significant effect on larval motility of *A. americanum*, *Dermacentor variabilis*, and *I. scapularis* ticks (Kagemann and Clay, 2013). Overall, these maternally-inherited symbionts are thus of ecological and evolutionary importance to the tick species they infect, and potentially mediate tick adaptation to hematophagy. In addition, it should be notice

TABLE 1 | List of the ten maternally inherited bacteria found in ticks and relevant (non-exhaustive) references.

Maternally inherited bacteria	Distribution in ticks and other arthropods	Major properties (non-exhaustive references)
1-*Coxiella*-LE	Very common in ticks, not found in other arthropods (Noda et al., 1997; Jasinskas et al., 2007; Clay et al., 2008; Carpi et al., 2011; Machado-Ferreira et al., 2011; Almeida et al., 2012; Lalzar et al., 2012; Duron et al., 2014, 2015a, 2017)	Obligate symbiont in most tick species (Zhong et al., 2007; Gottlieb et al., 2015; Smith et al., 2015). Closely related to the agent of Q fever, *C. burnetii* (Duron et al., 2015a)
2-*Rickettsiella*	Scattered distribution in arthropods (Tsuchida et al., 2010, 2014; Bouchon et al., 2012; Iasur-Kruh et al., 2013), common in ticks (Kurtti et al., 2002; Vilcins et al., 2009; Anstead and Chilton, 2014; Duron et al., 2015a, 2016, 2017)	Unknown effect in ticks. Facultative mutualist in aphids (Tsuchida et al., 2010, 2014) and likely in other insects (Iasur-Kruh et al., 2013). Some strains are entomopathogenic (Bouchon et al., 2012)
3-*Arsenophonus*	Common in arthropods (Duron et al., 2008a; Novakova et al., 2009), present in ticks (Clay et al., 2008; Dergousoff and Chilton, 2010; Reis et al., 2011; Clayton et al., 2015; Duron et al., 2017)	Male-killer in parasitoid wasps (Werren et al., 1986; Duron et al., 2010), putative obligate symbiont in bat flies and louse flies (Duron et al., 2014), facultative symbiont in other insects (Novakova et al., 2009; Jousselin et al., 2013). In the sheep tick, *Ixodes ricinus*, detection of *Arsenophonus* may be due to contamination by a hymenopteran parasitoid (Bohacsova et al., 2016)
4-*Francisella*-LE	Rare in ticks, not found in other arthropods (Niebylski et al., 1997a; Noda et al., 1997; Scoles, 2004; Goethert and Telford, 2005; Clayton et al., 2015; Gerhart et al., 2016; Duron et al., 2017)	Unknown effect in most cases but alternative obligate symbiont in some tick species (Gerhart et al., 2016; Duron et al., 2017); closely related to the agent of tularaemia (*F. tularensis*) (Sjodin et al., 2012)
5-*Cardinium*	Common in arthropods (Zchori-Fein and Perlman, 2004; Duron et al., 2008a,b), present in ticks (Kurtti et al., 1996; Benson et al., 2004; Duron et al., 2017)	Unknown effect in ticks. Reproductive manipulator in diverse insect species (Engelstadter and Hurst, 2009)
6-*Spiroplasma*	Common in arthropods (Weinert et al., 2007; Duron et al., 2008a), present in ticks (Tully et al., 1981, 1995; Henning et al., 2006; Duron et al., 2017)	Unknown effect in ticks. Male-killer in diverse insect species (Engelstadter and Hurst, 2009)
7-*Lariskella*	Rare and with a scattered distribution in arthropods (Matsuura et al., 2012; Toju et al., 2013), rarely reported in ticks (Qiu et al., 2014; Duron et al., 2017)	Unknown effect
8-*Midichloria*	Present in ticks, not found in other arthropods (Lo et al., 2006; Epis et al., 2008; Venzal et al., 2008; Reis et al., 2011; Najm et al., 2012; Qiu et al., 2014; Williams-Newkirk et al., 2014; Paul et al., 2016; Bonnet et al., 2017; Duron et al., 2017)	Unknown effect; inhabits tick mitochondria (Epis et al., 2013)
9-*Rickettsia*	Common in arthropods (Perlman et al., 2006; Weinert et al., 2009), present in ticks (Niebylski et al., 1997b; Clayton et al., 2015; Kurtti et al., 2015; Duron et al., 2017)	Unknown effect in ticks. Reproductive manipulator in diverse insect species (Engelstadter and Hurst, 2009) and defensive symbiont in other insects (Lukasik et al., 2013) closely related to pathogenic strains, often tick-borne, infecting vertebrates (Perlman et al., 2006; Darby et al., 2007; Weinert et al., 2009)
10-*Wolbachia*	Very common in arthropods (Duron et al., 2008b; Zug and Hammerstein, 2012), present in ticks (Andreotti et al., 2011; Carpi et al., 2011; Reis et al., 2011; Duron et al., 2017)	Unknown effect in ticks. Reproductive manipulation in many arthropods (Engelstadter and Hurst, 2009), facultative mutualist (defensive symbiosis) in others such as mosquitoes (Brownlie and Johnson, 2009; Hamilton and Perlman, 2013), obligate symbiont in bed bugs (Hosokawa et al., 2010; Nikoh et al., 2014). At least in the case of the sheep tick, *Ixodes ricinus*, it has been demonstrated that the detection of *Wolbachia* was due to a contamination by a hymenopteran parasitoid (Plantard et al., 2012)

Adapted from Duron et al. (2017).

that some tick-borne pathogenic Anaplasmataceae bacteria (including *Anaplasma phagocytophilum, Ehrlichia chaffeensis,* and *Neorickettsia sennetsu*) are also able to synthetize all major vitamins, suggesting that they may also confer a beneficial role in ticks when present (Dunning Hotopp et al., 2006).

Many *Rickettsia* species are well-known TBP, therefore a comment on the true ecological diversity existing within the *Rickettsia* genus is appropriate. Indeed, most of the novel *Rickettsia* species or strains discovered in recent years are found exclusively in arthropods and never in vertebrates (Perlman et al., 2006; Darby et al., 2007; Weinert et al., 2009). In ticks, as for many other arthropods, these *Rickettsia* are not pathogenic but are actually maternally-inherited symbionts with poorly known effects on tick biology. This is the case for the *Rickettsia*

species identified in the black-legged tick *I. scapularis* (*Rickettsia buchneri*, formerly known as *Rickettsia* REIS; Kurtti et al., 2015), the American dog tick *Dermacentor variabilis* (*Rickettsia peacockii*; Felsheim et al., 2009), and likely the tree-hole tick *Ixodes arboricola* (*Rickettsia vini*; Duron et al., 2017). The fact that ticks carry both pathogenic and non-pathogenic *Rickettsia* that may interact (as early reported by Burgdorfer et al., 1981), underscores the need to be able to clearly distinguish between the two in further studies on these bacteria.

Along with obligate symbionts, ticks commonly harbor facultative symbionts belonging to a variety of bacterial genera (listed in **Table 1**). Examination of a representative collection of 81 tick species (i.e., approximately 10% of known tick species and including both soft and hard ticks) illustrated facultative symbiont diversity, since it revealed the presence of maternally-inherited bacteria in almost all species (79 of 81) (Duron et al., 2017). Remarkably, many of these tick species (44) hosted more than one symbiont. In multi-infected tick species, symbionts assembled in communities which could reach high levels of complexity. Indeed, six distinct genera of maternally-inherited symbionts co-exist in sheep tick *I. ricinus* populations (*Midichloria*, *Spiroplasma*, *Coxiella*-LE, *Rickettsia*, *Wolbachia*, and *Rickettsiella*) and in African blue tick *Rhipicephalus decoloratus* populations (*Midichloria*, *Coxiella*-LE, *Francisella*-LE, *Rickettsia*, *Cardinium*, and *Spiroplasma*) (Duron et al., 2017). It should be noted that detecting a heritable bacterium can sometimes be due to cross-contamination as for several *I. ricinus* studies. Some recorded *Wolbachia* and *Arsenophonus* infections were actually due to the cryptic presence of a *Wolbachia*- and *Arsenophonus*-infected endoparasitoid wasp, *Ixodiphagus hookeri*, within tick tissues (Plantard et al., 2012; Bohacsova et al., 2016). As a result, the presence of at least some of these tick symbionts must be treated with caution.

Although the role of these facultative symbionts is not yet clearly elucidated, one study suggested that *Arsenophonus* sp. can affect host-seeking success by decreasing *A. americanum, I. scapularis,* and *D. variabilis* tick motility when *Rickettsia* symbionts increased such mobility (Kagemann and Clay, 2013). Recent sequencing and analysis of the *Midichloria mitochondrii* genome led to the hypothesis that the bacteria could serve as an additional ATP source for the host cell during oogenesis (Sassera et al., 2011). In addition, this symbiont has been ascribed a possible helper role in tick molting processes (Zchori-Fein and Bourtzis, 2011). As mentioned above, there is no evidence to date showing that the *Wolbachia* detected in ticks are "true" tick symbionts (Plantard et al., 2012). Interestingly, in insects, *Wolbachia* is known to act as defensive endosymbionts (reviewed by Brownlie and Johnson, 2009), or as manipulator of host reproduction (review in Engelstadter and Hurst, 2009; Cordaux et al., 2011), suggesting that similar effect may exist in ticks.

The same questioning are required regarding *Arsenophonus nasoniae*: known to be responsible for sex-ratio distortion in diverse arthropod species (Werren et al., 1986; Duron et al., 2010) but of unknown effect in ticks. Finally and interestingly, a *Rickettsiella* symbiont has been observed in a parthenogenetic laboratory colony of the tick *I. woodi* (Kurtti et al., 2002). This tick species is generally known to be bisexual, suggesting that *Rickettsiella* infection may induce asexuality which represents to date the only demonstration of sex ratio distortion in ticks possibly due to a symbiont.

High symbiont infection frequency is rarely observed within each tick species, and an intermediate infection frequency is much more common (Noda et al., 1997; Clay et al., 2008; Lalzar et al., 2012; Duron et al., 2017). Interestingly, infection frequencies of each maternally-inherited symbiont are often variable between geographic populations of a given tick species (Clay et al., 2008; Lalzar et al., 2012; Duron et al., 2017). This is the case for the soft tick *Ornithodoros sonrai*, with *Midichloria* and *Rickettsia* reaching high infection frequencies in some populations, but remaining absent from others (Duron et al., 2017). Similar geographical patterns were observed for many other tick species and for several symbionts such as *Rickettsiella* and *Spiroplasma* in the tree-hole tick *I. arboricola* and the polar seabird tick *I. uriae* (Duron et al., 2016, 2017). These patterns strongly suggest that maternally-inherited symbiont infection dynamics are variable across tick populations and species. Such infection frequency variations may be driven by costs and benefits associated with harboring maternally-inherited symbionts, and may maintain intermediate frequencies in tick populations, as is commonly observed in other arthropod species (Oliver et al., 2010). However, even if the nature of these costs and benefits has been well-studied in insects, they remain to be determined in ticks. Another important parameter in our understanding of this infection dynamics may be variation of biological features between tick males and females: indeed, adult males from *Ixodes* species do not blood-feed, contrary to females, which may imply that adult males do not need nutritional symbionts. The importance of this "sex" parameter to symbiont infection dynamics remains also to be demonstrated. Interestingly, this pattern was observed for *Midichloria* with males less commonly infected than females in *I. ricinus* (Lo et al., 2006). This suggests that *Midichloria* may be an important nutritional symbiont for *I. ricinus*, as recently proposed (Duron et al., 2017).

Along with maternally-inherited symbionts, other non-pathogenic microbes are present in ticks (Andreotti et al., 2011; Carpi et al., 2011; Narasimhan et al., 2014; Qiu et al., 2014; Williams-Newkirk et al., 2014; Abraham et al., 2017). Most are likely to inhabit the tick gut, while others may also colonize the tick surface cuticle. Overall, the biological effects of these non-pathogenic microbes on ticks remain entirely unknown, but it is likely that those inhabiting the tick gut could participate in blood meal digestion (Narasimhan and Fikrig, 2015) and complex interactions with TBP as we further detailed.

NON-PATHOGENIC MICROORGANISMS INTERACT WITH TBP IN DIFFERENT WAYS

An alternative fascinating aspect is that non-pathogenic microorganisms can also interfere with TBP replication and transmission by influencing TBP abundance and diversity in tick populations, as well as their transmission to vertebrate hosts. All aforementioned non-pathogenic bacteria present in ticks could have the potential to impact TBP infection processes in different ways. One can assume that TBP and non-pathogenic

microorganisms may neutralize each other because they are in direct competition for limited resources, such as a particular nutrient or tissue, or because they stimulate the same immune system function. Alternatively, non-pathogenic microorganisms may excrete molecules directly inhibiting the growth of a TBP competitor, or, inversely, facilitate TBP development by immunosuppressing the tick host. As a result, non-pathogenic microorganisms may facilitate, limit, or block TBP transmission, depending on the nature of tick microbial interactions.

Equally pertinent in this regard is the role of the microbiota inhabiting the tick gut (Narasimhan et al., 2014; Abraham et al., 2017). The tick gut represents the TBP entry point, therefore gut microbiota can directly mediate TBP colonization and influence their early survival (Narasimhan and Fikrig, 2015). This finding has been perfectly illustrated in a recent study which manipulated the gut microbiota of the black-legged tick *I. scapularis*: specimens reared in sterile containers (i.e., thus preventing preventing external bacterial contamination) showed increased engorgement weights, and decreased colonization by the causative agent of Lyme disease, *Borrelia burgdorferi*, when compared to normal specimens (Narasimhan et al., 2014). Similarly, ticks fed on antibiotic-treated mice exhibited modified gut microbiota that also resulted in increased feeding and low *B. burgdorferi* colonization rates (Narasimhan et al., 2014). Altering the gut microbiota was actually found to decrease production of a glycoprotein from the tick peritrophic matrix, which separates the gut lumen from the epithelium. This peritrophic matrix is pivotal for *Borrelia* colonization success, as it protects *B. burgdorferi* colonizing the gut epithelial cells from toxic gut lumen compounds. Compromised peritrophic matrix due to altered gut microbiota will thus impede *B. burgdorferi* colonization. However, the reverse is true for another TBP, the anaplasmosis agent, *A. phagocytophilum* (Abraham et al., 2017). Remarkably, this bacterium manipulates the gut microbiota of *I. scapularis* to favor its establishment. By inducing tick glycoprotein production, *A. phagocytophilum* partially blocks bacterial biofilm formation, and thus reduces peritrophic matrix integrity, rendering the tick more susceptible to infection (Abraham et al., 2017). Altogether, these observations have uncovered a "Dr. Jekyll and Mr. Hyde"-like role of the tick gut microbiota, so that an unaltered gut microbiota will favor colonization by some TBP, such as *Borrelia*, whereas it may also block colonization by other TBP, such as *Anaplasma*. This antagonistic effect of tick gut microbiota on TBP may explain the rarity of *Borrelia-Anaplasma* co-infections in ticks collected from the field (Abraham et al., 2017).

Other interaction mechanisms may also exist. In well-studied animals, such as insects, antagonistic interactions arise when horizontally-transmitted parasites and vertically-transmitted microorganisms co-infecting the same host have conflicting evolutionary interests (Haine et al., 2005; reviewed by Haine, 2008; Ben-Ami et al., 2011; Hamilton and Perlman, 2013). Vertically-transmitted microorganisms, such as maternally-inherited symbionts, are under strong selection pressure to enhance the reproductive success of the hosts they infect (Moran et al., 2008). Conversely, parasites are typically transmitted between unrelated hosts and are therefore not directly affected by altered host fecundity. This conflict of interest has favored the emergence of defensive symbionts in insects, including maternally-inherited symbionts that protect their insect host against a variety of pathogens (reviewed by Haine, 2008; Brownlie and Johnson, 2009). For instance, some maternally-inherited symbionts such as *Wolbachia* may interfere with the replication and transmission of a wide range of pathogens (including viruses, bacteria, protozoa, nematodes, and parasitoids), and protect insects from parasite-induced mortality, possibly by up-regulating the insect's immune system (Brownlie and Johnson, 2009; Gross et al., 2009). Recently, some of these findings have been applied to the development of parasite control methods, where *Wolbachia* infection has been used to limit the vector competence of mosquitoes (Hoffmann et al., 2011). In comparison, very little was known about the existence of defensive symbionts in ticks. However, Burgdorfer et al. were the first to report the presence of defensive symbionts in the Rocky Mountain wood tick *Dermacentor andersoni*. In this tick, a maternally-inherited symbiont, *Rickettsia peacockii*, hampered the multiplication and transovarial transmission of the spotted fever agent, *Rickettsia rickettsii* (Burgdorfer et al., 1981). Similarly, resistance of the ovaries of *D. variabilis* to co-infection with *Ricketisia montana* and *Rickettsia rhipicephali* has been reported (Macaluso et al., 2002). Other experiments on *D. andersoni* further showed that *A. marginale* infection density was negatively correlated to the infection density of another maternally-inherited symbiont, *Rickettsia belli* (Gall et al., 2016). In *I. scapularis*, it has also been reported that male ticks infected by the maternally-inherited symbionts *Rickettsia buchneri* have significantly lower rates of *B. burgdorferi* infection than symbiont-free males (Steiner et al., 2008). Overall, these observations suggested that the maternally-inherited *Rickettsia* symbionts may be major defensive symbionts protecting ticks against TBP colonization. As a result, *Rickettsia* symbionts may be a key factor influencing TBP abundance and diversity in tick populations.

Conversely, maternally-inherited symbionts may not always protect ticks against pathogens: the presence of one maternally-inherited symbiont, *Francisella*-LE, in *D. andersoni* was positively associated with pathogenic *Francisella novicida* infection (Gall et al., 2016). Because these results were only obtained following laboratory manipulations, they should be treated with caution as *F. novicida* is not considered to be a TBP, as the majority of people infected with *F. novicida* contract the pathogen after ingesting infected water or ice, and not via tick bites. This study thus relies on an artificial *F. novicida* tick infection that is unlikely to happen in the field, and most importantly, using a pathogen that has not co-evolved with tick symbionts. This naturally raises the question of whether *Francisella*-LE can actually protect *D. andersoni* against TBPs that naturally occur in this tick species.

TICK SYMBIONTS CAN BE OPPORTUNISTIC VERTEBRATE PATHOGENS

Although biologists often classify host-microbe relationships as either "mutualism," "commensalism," or "parasitism," there are difficulties in defining the boundaries of these definitions. Rather,

host-microbe relationships should be best described as a broad continuum, as intermediate states and transitions between states occur frequently. Several maternally-inherited tick symbionts are remarkable examples of this continuum, as recent literature has reported that certain symbionts may be transmitted to vertebrates following tick bite, as will be detailed further in this section (Shivaprasad et al., 2008; Woc-Colburn et al., 2008; Vapniarsky et al., 2012; Bazzocchi et al., 2013; Edouard et al., 2013; Angelakis et al., 2016; Seo et al., 2016a; Bonnet et al., 2017). Most importantly, some of these symbionts have the potential to opportunistically infect vertebrate hosts, including humans.

Maternally-inherited arthropod symbionts are commonly thought to be exclusively domesticated by their arthropod hosts: they cannot invade naïve hosts and have evolved to be dependent on arthropod-based transmission mechanisms through transovarial inheritance (Moran et al., 2008; Wernegreen, 2012). However, some tick symbionts, such as certain *Coxiella*, *Midichloria* and *Arsenophonus* strains, are not actually completely dependent on ticks. Rather than strictly maternal, their transmission may be partially horizontal, i.e., infectious, thus presenting a substantial infection risk to vertebrates (Shivaprasad et al., 2008; Woc-Colburn et al., 2008; Vapniarsky et al., 2012; Bazzocchi et al., 2013; Edouard et al., 2013; Angelakis et al., 2016; Seo et al., 2016a). Among these symbionts, *Coxiella*-LE are the most commonly found microorganisms in vertebrates. Indeed, tick-transmitted *Coxiella*-LE has recently been reported to cause mild infectious symptoms in humans from Europe (Angelakis et al., 2016). These microorganisms were notably detected in human skin biopsy samples and may be a common causative agent of scalp eschar and neck lymphadenopathy. *Coxiella*-LE infections have also been occasionally reported in pet birds such as psittacines and toucans reared in North America (Shivaprasad et al., 2008; Woc-Colburn et al., 2008; Vapniarsky et al., 2012). These latter *Coxiella*-LE can cause fatal disease: infected birds exhibited lethargy, weakness, emaciation, and progressive neurologic signs for several days prior to death. Conversely, another *Coxiella*-LE was identified in several South Korean horse blood samples, but none of the horses showed apparent symptoms of infection (Seo et al., 2016b).

The ability of *Coxiella*-LE to infect vertebrates through tick biting is at least partially explained by their tissue tropism within the tick body. Aside from tick ovaries and Malpighian tubules, examination of tick internal organs also revealed substantial *Coxiella*-LE concentrations within the salivary glands of some tick species (Klyachko et al., 2007; Machado-Ferreira et al., 2011; Qiu et al., 2014) but not in others (Liu et al., 2013; Lalzar et al., 2014). This tissue tropism may enable *Coxiella*-LE release into the vertebrate during tick bite, thus favoring opportunistic infections (Duron et al., 2015a). The overall likelihood of such *Coxiella*-LE tick-to-vertebrate transfers seems high since (i) ticks are found worldwide and feed on many different vertebrate species, (ii) at least two thirds of tick species are infected by *Coxiella*-LE, and (iii) when present in a given tick species, *Coxiella*-LE are usually present in almost all specimens (Duron et al., 2015a). Overall, these observations suggest that, through tick parasitism, vertebrates are often exposed to *Coxiella*-LE, and probably at a higher rate than TBP. However, despite this, *Coxiella*-LE infections are very rare in vertebrates, and most strains described to date have only been identified from ticks (Duron et al., 2015a). It is thus thought that these bacteria pose a low infection risk to vertebrates because their genome seems to be extremely reduced and is devoid of known virulence genes (Gottlieb et al., 2015; Smith et al., 2015). Nonetheless, *Coxiella*-LE have the potential to cause rare infections in vertebrates and should always be considered in future studies on tick-borne diseases.

In comparison, vertebrate infections by symbionts other than *Coxiella*-LE are clearly less common. This includes the maternally-inherited symbiont *Arsenophonus*, present in approximately 5% of terrestrial arthropods, including some tick species (Duron et al., 2008a, 2017). *Arsenophonus* is actually unique among maternally-inherited symbionts because it is able to grow outside arthropod cells, in extracellular environments (Huger et al., 1985; Werren et al., 1986). This ability enhances the likelihood of successful opportunistic *Arsenophonus* infection, as was recently observed in a woman who was bitten by a tick during a trip to Southeast Asia. This patient presenting with a rash and an eschar developed a co-infection with *Arsenophonus* and *Orientia tsutsugamushi* (the causative agent of scrub typhus) (Edouard et al., 2013). In this context, it is likely that rash and eschar development following *Orientia* infection may have favored a secondary, opportunistic, *Arsenophonus* infection.

In other cases, the identification of symbionts as opportunistic vertebrate pathogens is more difficult and remains speculative. This is the case for *Midichloria*, an intra-mitochondrial symbiont of the sheep tick *I. ricinus* and a few other tick species. Several lines of evidence have recently suggested that vertebrate hosts can be inoculated with *Midichloria* during a tick bite. Indeed, most *Midichloria* are localized in the tick ovaries, where they are transmitted to the progeny, but some have also been detected in the salivary glands and saliva of *I. ricinus* (Di Venere et al., 2015). In addition, *Midichloria* DNA, as well as antibodies against a *Midichloria* antigen, were detected in the blood of vertebrates exposed to tick bites (Bazzocchi et al., 2013). However, whether *Midichloria* can cause a true infection and pathological alteration in mammalian hosts remains to be determined.

PATHOGENS AND TICK SYMBIONTS ARE OFTEN PHYLOGENETICALLY RELATED

The vast range of intracellular bacteria in ticks is particularly illustrative of their propensity to evolve extreme and contrasting phenotypes. Certain species, such as *Rickettsia* spp. and *Coxiella* spp., have taken eukaryote associations to the extreme by completely abandoning any semblance of a free-living phase and replicating solely within the host cell. However, they do use a large panel of lifestyle strategies to spread and persist within host populations: while some are extremely virulent pathogens, others behave as subtle mutualistic symbionts (Perlman et al., 2006; Darby et al., 2007; Weinert et al., 2009; Sjodin et al., 2012; Duron et al., 2015a, 2017; Gerhart et al., 2016). Although both strategies require high degrees of lifestyle specialization, they are not fixed endpoints along the bacterium-eukaryote interaction spectrum;

rather, parasitism and mutualism may shift through repeated evolutionary transitions. This explains why both pathogenic and mutualistic forms of several major bacterial genera commonly hosted by ticks are abundantly represented.

The foremost examples of these transitions are found in three major intracellular bacteria genera: *Coxiella*, *Francisella*, and *Rickettsia* (**Figure 2**), which are all commonly identified in ticks. In medical and veterinary research, these intracellular bacteria are traditionally recognized as highly virulent vertebrate pathogens, as they have evolved specific mechanisms to penetrate into the host cytosol, appropriate nutrients for replication, subvert host immune responses, and ultimately enable infectious transmission to a new host individual (Darby et al., 2007; Celli and Zahrt, 2013; van Schaik et al., 2013; Jones et al., 2014). In humans, major intracellular pathogens have been identified from these bacterial groups, as exemplified by the agent of Q fever, *Coxiella burnetii*, the agent of tularaemia, *Francisella tularensis*, the agent of epidemic typhus, *Rickettsia prowazekii,* the agent of Rocky Mountain spotted fever, *Rickettsia rickettsia*, or the causative agent of Mediterranean spotted fever, *Rickettsia conorii* (**Figure 2**). All of these organisms are extremely infectious and some are currently classified as potential weapons for biological warfare (Darby et al., 2007; Celli and Zahrt, 2013; van Schaik et al., 2013; Jones et al., 2014). In addition, several species of tick-borne bacteria as typified by rickettsiae that were considered non-pathogenic for decades are now associated with human infections (Parola et al., 2013; Bonnet et al., 2017). However, as we have detailed above, novel intracellular bacteria engaged in endosymbiotic associations with arthropod hosts have also recently been discovered within each of these groups (**Figure 2**).

Phylogenetic investigations have revealed rapid and repeated evolutionary shifts within these three genera between pathogenic (associated with vertebrates and, in some cases, vectored by arthropods) and endosymbiotic forms (specifically linked to arthropods). However, the evolutionary shifting pattern varies among genera (**Figure 2**). In *Coxiella*, complementary lines of argument indicate a recent emergence of the Q fever agent, *C. burnetii*, from a *Coxiella*-LE strain hosted by soft ticks (Duron et al., 2015a). The *Coxiella* genus displays extensive genetic diversity, with at least four highly divergent clades (Duron et al., 2015a). While *Coxiella*-LE strains hosted by ticks are found in all these clades, all *C. burnetii* strains cluster within one of these clades, delineating an embedded group among soft tick *Coxiella*-LE (**Figure 3A**). This phylogenetic pattern indicates that the ancestor of *C. burnetii* was a tick-associated *Coxiella* which succeeded in infecting vertebrate cells (Duron et al., 2015a). The remarkably low genetic diversity of *C. burnetii* indicates unique and recent emergence of this highly infectious vertebrate pathogen (Duron et al., 2015a). Interestingly, this hypothesis was initially raised a decade ago from observations of the profound differences in *C. burnetii* genome content relative to other pathogenic intracellular bacteria (Seshadri et al., 2003).

Similarly, in *Rickettsia* spp., recent evidence revealed that human pathogens—vectored by blood feeding arthropods such as ticks—emerged relatively late in the evolution of this genus (**Figure 3C**; Perlman et al., 2006; Darby et al., 2007; Weinert et al., 2009). Phylogenetic investigations taking into account the entire *Rickettsial* diversity (i.e., including pathogenic and non-pathogenic forms) clearly indicate that switching between hosts (invertebrates, vertebrates, and even plants) has been a common feature of *Rickettsia* evolution (Perlman et al., 2006; Darby et al., 2007; Weinert et al., 2009). Based on current data, it is difficult to estimate how often vertebrate pathogenesis has evolved within *Rickettsia*. But as intracellular adaptation to arthropods is a feature of all current *Rickettsia*, it suggests that their most common recent ancestor was adapted to arthropod endosymbiosis. Surprisingly, comparing human pathogens with closely related non-pathogens showed no relationships between pathogenicity and the acquisition of novel virulence genes: vertebrate virulence seems to occur rather as result of lost or malfunctioning replication systems (Darby et al., 2007).

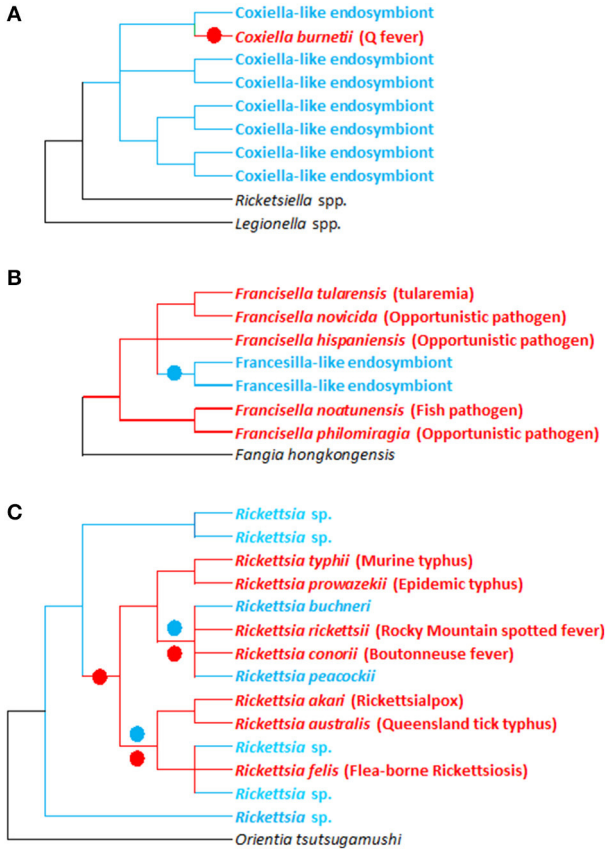

FIGURE 3 | Evolutionary relationships between pathogenic and non-pathogenic (symbiotic) forms within the *Francisella*, *Coxiellai,* and *Rickettsia* bacterial genera. **(A–C)** Simplified phylogenies of *Coxiella*, *Francisella*, and *Rickettsia*, respectively, adapted from Perlman et al. (2006), Weinert et al. (2009), Duron et al. (2015a), and Sjodin et al. (2012). Red: pathogenic forms; blue: endosymbiotic forms associated with arthropods (ticks for *Francisella* and *Coxiella*; ticks and other arthropods for *Rickettsia*); black: bacterial outgroups. Colored circles on tree branches indicate major evolutionary transitions from symbiotic ancestors to pathogenic descendants (red circles) and from pathogenic ancestors to symbiotic descendants (blue circles).

Conversely, in *Francisella*, the evolutionary pattern is substantially different, since most of the diversity found in this genus is due to pathogenic or opportunistic species (Sjodin et al., 2012). Very little is known about the evolution and origin of tick *Francisella*-LE (Michelet et al., 2013; Gerhart et al., 2016; Duron et al., 2017). However, the few *Francisella*-LE species identified to date delineate a unique monophyletic clade that clearly originates from pathogenic forms (**Figure 3B**; Duron et al., 2017). Interestingly, the *Francisella*-LE genome is similar in size to pathogenic *Francisella* species' genomes, but about one-third of the protein-coding genes are pseudogenized and are likely non-functional (Gerhart et al., 2016). This suggests that *Francisella*-LE is undergoing a global process of genome reduction, an evolutionary development typically observed in maternally-inherited symbionts (Moran et al., 2008). Interestingly, *Francisella*-LE has conserved intact most of its genes involved in B vitamin biosynthesis, highlighting the pivotal role these genes play in adaption to its current endosymbiotic lifestyle (Gerhart et al., 2016).

Overall, these two very different phenotypes (symbiosis vs. pathogenesis), along with two contrasting transmission modes (vertical vs. horizontal), and variable host specificity (ticks vs. vertebrates), make the *Coxiella*, *Francisella*, and *Rickettsia* genera especially fascinating. They thus offer an unusual opportunity to answer questions about the origins and mechanisms of symbiosis and pathogenesis. Further studies characterizing host range and infectivity of different genera members would be invaluable to obtaining such results, as would the characterization of tick symbiotic strain genomes. However, research efforts to date have invariably tended to concentrate on their medically important relatives, and so we know comparatively little about the biology of maternally-inherited symbionts. This neglect is unfortunate because fully understanding the whole scope of *Coxiella*, *Francisella*, and *Rickettsia* phenotypes linked to genome sequences, will provide an excellent system to test hypotheses on the importance of genome content and plasticity in the emergence and reversibility of extreme phenotypes such as symbiosis and pathogenesis.

CONCLUSION AND PERSPECTIVES

Extensive literature studies have now made it clear that TBP are not alone: an appreciable range of diverse non-pathogenic microorganisms has also been detected in almost all tick species examined so far. Perhaps the most important consideration for the future is not the incidence of these non-pathogenic microorganisms, but their phenotypes. The varied collection of non-pathogenic microorganisms includes intracellular maternally-inherited symbionts and microbes inhabiting the tick gut, and each could strongly influence—in very different ways—the biology of their tick hosts as well as TBP infection dynamics. Recent findings have shown that several maternally-inherited symbionts such as *Coxiella*-LE are important drivers of evolutionary change in ticks, as clearly shown by their role in driving their tick hosts to adapt to a strict hematophagous diet (Gottlieb et al., 2015; Smith et al., 2015; Duron et al., 2017). Other non-pathogenic microorganisms such as maternally-inherited *Rickettsia* symbionts and gut microbiota are also likely to substantially contribute to the acquisition of ecologically important traits, such as TBP resistance (Burgdorfer et al., 1981; Steiner et al., 2008; Narasimhan et al., 2014; Abraham et al., 2017). It is therefore vital to establish the nature of the interactions between non-pathogenic microorganisms, their tick hosts, and co-infecting TBP. To achieve this goal, it is essential to understand how ticks acquire their microbiota, and how microbial community structures are shaped by various environmental and host factors, and also by microbial interactions within these communities. This knowledge is a key step toward using non-pathogenic microorganisms to limit TBP transmission and persistence. Similarly, whether tick symbionts have the potential to opportunistically infect humans and other vertebrates should be investigated in depth. Lastly, we would like to emphasize that the study of some non-pathogenic microorganisms, such as members of the *Coxiella*, *Francisella*, and *Rickettsia* genera, can advance our understanding of many infectious diseases including Q fever, tularemia, and rickettsial diseases. The broad phenotypic diversity present in these three bacterial genera make them perfect models to study the evolutionary emergence of pathogenicity and adaptations to living in vertebrate cells. Owing to their medical importance, the pathogenic species of these genera have been the targets of several genome sequencing projects, which have provided insights into the mechanisms and consequences of their specialized lifestyles (Darby et al., 2007; van Schaik et al., 2013). Conversely, the symbiotic forms adapted to tick hosts have received much less attention and a lot of things remain to be elucidated (but see Gillespie et al., 2012, 2015; Clark et al., 2015 for genomics insights about *Rickettsia*). In this context, comparative genomic approaches will be highly valuable in enhancing our understanding of the evolutionary ecology of both pathogenic and non-pathogenic intracellular bacteria, and in identifying novel candidate genes contributing to virulence and persistence in vertebrate cells.

AUTHOR CONTRIBUTIONS

SB, AH, FB, and OD conducted the literature research, wrote the paper and prepared the figures and tables. All authors provided critical review and revisions.

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frontiers in Cellular and Infection Microbiology

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Microbial Invasion vs. Tick Immune Regulation

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Ticks transmit a greater variety of pathogenic agents that cause disease in humans and animals than any other haematophagous arthropod, including Lyme disease, Rocky Mountain spotted fever, human granulocytic anaplasmosis, babesiosis, tick-borne encephalitis, Crimean Congo haemorhagic fever, and many others (Gulia-Nuss et al., 2016). Although diverse explanations have been proposed to explain their remarkable vectorial capacity, among the most important are their blood feeding habit, their long term off-host survival, the diverse array of bioactive molecules that disrupt the host's natural hemostatic mechanisms, facilitate blood flow, pain inhibitors, and minimize inflammation to prevent immune rejection (Hajdušek et al., 2013). Moreover, the tick's unique intracellular digestive processes allow the midgut to provide a relatively permissive microenvironment for survival of invading microbes. Although tick-host-pathogen interactions have evolved over more than 300 million years (Barker and Murrell, 2008), few microbes have been able to overcome the tick's innate immune system, comprising both humoral and cellular processes that reject them. Similar to most eukaryotes, the signaling pathways that regulate the innate immune response, i.e., the Toll, IMD (Immunodeficiency) and JAK-STAT (Janus Kinase/ Signal Transducers and Activators of Transcription) also occur in ticks (Gulia-Nuss et al., 2016). Recognition of pathogen-associated molecular patterns (PAMPs) on the microbial surface triggers one or the other of these pathways. Consequently, ticks are able to mount an impressive array of humoral and cellular responses to microbial challenge, including anti-microbial peptides (AMPs), e.g., defensins, lysozymes, microplusins, etc., that directly kill, entrap or inhibit the invaders. Equally important are cellular processes, primarily phagocytosis, that capture, ingest, or encapsulate invading microbes, regulated by a primordial system of thioester-containing proteins, fibrinogen-related lectins and convertase factors (Hajdušek et al., 2013). Ticks also express reactive oxygen species (ROS) as well as glutathione-S-transferase, superoxide dismutase, heat shock proteins and even protease inhibitors that kill or inhibit microbes. Nevertheless, many tick-borne microorganisms are able to evade the tick's innate immune system and survive within the tick's body. The examples that follow describe some of the many different strategies that have evolved to enable ticks to transmit the agents of human and/or animal disease.

Keywords: pathobiology, Arp 23, caveolae, clathrin, salp 16, P11, JAKSTAT

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BORRELIA BURGDORFERI

Borrelia burgdorferi (sensu latu), the causative agent of Lyme disease, is a spirochete, a Gram-negative helically coiled bacterium approximately 0.5 μm wide by 20 μm long, with a flagellum below the outer membrane that controls its whiplash-like movements.

These bacteria are transmitted by ticks of the genus Ixodes, especially *I. scapularis* in North America and *I. ricinus* and *I. persulcatus* in Europe and Asia. In its tick host, *B. burgdorferi* is an intercellular pathogen, i.e., it survives in the midgut lumen, then migrates between the midgut's epithelial cells into the hemolymph and then into the salivary gland ducts. Bacteria are acquired by the tick host during blood feeding. Larval ticks ingest spirochetes while feeding on small mammals, especially white-footed mice. During and after feeding, the spirochetes remain within the midgut lumen. Those spirochetes trapped outside of the rapidly developing peritrophic membrane are unlikely to survive. Others between the membrane and midgut epithelial cells use an external surface lipoprotein, OspA, to bind to a species-specific receptor, TROSPA, located on the luminal surfaces of the midgut epithelial cells (Pal et al., 2004). Although surviving spirochetes may undergo an initial phase of multiplication, these populations decline during the tick's post-feeding molt cycle, perhaps due to antimicrobial effects of by-products of hemoglobin digestion, competition for nutrients and other unknown factors. Following molting to the nymphal stage, feeding by the recently molted nymph stimulates surviving spirochetes to begin prolific multiplication and attempt migration out of the midgut lumen (reviewed by Ogden et al., 2014) During this initial phase, the spirochetes form a complex network that moves between the epithelial cells toward their basolateral surfaces. OspA expression is reduced, allowing spirochetes to detach. Subsequently, they transition to the second phase of midgut migration, in which the spirochetes become motile, separate and escape through the basement membranes into the hemocoel (Dunham-Ems et al., 2009). Some host derived factors also play a role in the process, e.g., host-derived plasminogen, which protects the bacteria against phagocytosis and possibly even enhances their ability to penetrate the basement membrane (Coleman et al., 1997). Bacterial enolase was reported as the surface receptor that binds to the midgut receptor, Tre31, which facilitates migration out of the midgut (Zhang et al., 2011). It also binds host-derived plasminogen in the midgut and degrades it to plasmin (Noguiera et al., 2012). Borreliae upregulate OspC, which also binds and immobilizes plasmin, the enzymatically active form, which further enhances degrading intercellular matrices and other barriers, such as basement membranes (Önder et al., 2012). Nevertheless, the vast majority of spirochetes migrating into the hemolymph are destroyed, mostly by phagocytic hemocytes (Coleman et al., 1997). Upon contact with the salivary glands, borreliae bind to SALP15 (Ramamurthy et al., 2011), an immunosuppressive factor that protects these spirochetes from antibody-mediated killing, as well as other salivary gland proteins e.g., tick salivary lectin pathway (Schuijt et al., 2011) and tick histamine release factors (Dai et al., 2010) that also protect these spirochetes from host immune reactions (de Silva et al., 2009; Hajdušek et al., 2013). Exploitation of host-derived factors that enable *B. burgdorferi* to multiply and evade innate immune attack suggests that ticks tolerate these pathogens in the midgut but not in the hemolymph and other body tissues. Nevertheless, many questions remain, especially how the spirochetes are able to penetrate between the tightly bound midgut epithelial cells, avoid triggering expression or upregulation of antimicrobial peptides, and how they the penetrate salivary gland acini for dissemination into vertebrate hosts. Overarching factors directed by the tick microbiome (Narasimhan and Fikrig, 2015) will also be important when examining the infection of and transmission by ticks.

RICKETTSIA RICKETTSII

Little is known about the specific infection mechanisms of spotted fever group (SFG) *Rickettsia* and their tick hosts (Munderloh and Kurtti, 1995), compared with the mammalian host cell. These bacteria, as well as other species of the Rickettsiales, invade host cells by binding to cellular receptors by means of their outer surface cell antigens (sca0 or rOmpAa and sca5 or rOmpB) and are internalized by receptor-mediated endocytosis via clathrin-coated vesicles, whereupon the microbes are incorporated into phagosomes (Chan et al., 2010). A similar sca5-mediated invasion mechanism used for vertebrate cells is employed by rickettsiae for invasion of tick cells (Thepparit et al., 2010). Upon invasion, rickettsiae quickly lyse these inclusions to escape into the cytosol. Once in the cytosol, rickettsiae replicate and then hijack the host cell's actin cytoskeleton and attach to it via actin tails (Gouin et al., 2005). The actin protein complex Arp2/3 is essential for the internalization of *R. rickettsii*, as well as other known SFG rickettsiae (Petchampai et al., 2014). In the vertebrate host cell, the bacteria express rickA, which promotes the activation of the host cell actin complex. This enables these bacteria to be propelled throughout the host cells as well as into protrusions that mediate cell to cell infection, thereby spreading the infection throughout the surrounding tissues (Gouin et al., 2004; Jeng et al., 2004). These actions are also effected by other cell proteins—profilin, fimbrin/T-plastin, capping protein, and cofilin, essential to actin assembly (Serio et al., 2010). In contrast to *R. rickettsii* which spread by means of actin bridges, *R. parkeri* and perhaps other rickettsial species, manipulate the intercellular tensions and mechano-transduction between host cells to facilitate their spread (Lampson et al., 2016). The roles of specific Sca molecules in facilitating rickettsial dissemination within the vector are under investigation. However, the host cell is not without a defense, as it is appreciated that ticks respond to rickettsiae (Macaluso et al., 2003; Mulenga et al., 2003). Using a tick cell culture (ISE6), investigators observed that pathogen infection led to decreased glucose metabolism but increased subolesin and heat shock protein expression, limiting rickettsial infection (Gillespie et al., 2012).

ANAPLASMA PHAGOCYTOPHILUM

These bacteria employ a novel strategy for invading their host cells, evading cellular killing actions, manipulating the host cell's

molecular machinery, and creating protected enclosures for their development and multiplication. In the tick, as well as in their vertebrate hosts, these bacteria avoid recognition by the host's innate immune system because they lack either peptidoglycans or lipopolysaccharides in their cell walls (Rikihisa, 2010). *A. phagocytophilum* bacteria also avoid the clathrin-receptor mediated endocytosis and phagolysosome typically used by host cells to capture and destroy invasive microbes. Instead, these bacteria are internalized via caveolae-mediated endocytosis: bacteria interact with caveolae and glycosylphosphatidylinositol-anchored proteins which enables them to bypass conventional phagolysosomes and form specialized endosomes. Bacterial outer surface protein MSP2 induces host intracellular signaling via an extracellular stimulation membrane receptor which induces recruitment of endocytic machinery at the binding site. This response leads to "zippering" around the pathogen and internalizing the microbe inside the host cell (Ireton, 2013). In tick cell cultures, *A. phagocytophilum* adhere to the cell membranes within 40 min post-infection. After binding, bacteria invade the host cells and form the specialized membrane-bound enclosures known as morulae. A mutant form of O-methyltransferase, identified as Msp4, also facilitates infection of the tick cells by *A. phagocytophilum* (Oliva Chávez et al., 2015). Within morulae, bacteria downregulate NADPH expression in enclosures, thereby minimizing reactive oxygen species (ROS) by suppressing expression of glutathione-s-transferase, superoxide dismutase and heat shock proteins (IJdo and Mueller, 2004). Like other rickettsiae, *A. phagocytophilum* alters cell gene expression (spectrin, fodrin) to control actin synthesis and remodel the host cell's cytoskeleton (de la Fuente et al., 2016). In mammalian cells, *A. phagocytophilum* hijacks host cholesterol to use in building the membrane surrounding the morulae (Xiong et al., 2009); however, whether this also occurs in tick cells is not known. In addition to these common strategies for infecting their hosts, *A. phagocytophilum* also exhibit more selective responses to different tissues. To infect the tick's midgut epithelial cells, these bacteria express genes that upregulate the JAK/STAT pathway, thereby inhibiting cell apoptosis (Ayllón et al., 2015). In addition, Cabezas-Cruz et al. (2016) suggest that *A. phagocytophilum* manipulates the host cell's epigenetics, increasing expression of histone deacetylase, Sirtuin and other molecules, which inhibits apoptosis and facilitates the microbe's multiplication. In addition, *A. phagocytophilum* infection can increase the levels of the tick's histone-modifying enzymes which makes it possible to regulate transcription and apoptosis selectively in different tissues, thereby not only facilitating both the pathogen's and the tick's survival. After escaping from the midgut, a salivary protein, P11, enables the microbes to infect circulating hemocytes, thereby enabling their migration to the salivary glands (Liu et al., 2011). Upon contact of infected hemocytes with the salivary gland cells, they induce expression of the salivary gland gene salp16 (Sukumaran et al., 2006), facilitating binding to the target cells. Following invasion of these host cells, the bacteria suppress the apoptotic mechanism by downregulating host cell porin expression, resulting in inhibition of cytochrome C release and thereby enabling their survival in the salivary glands. By upregulating this gene, the bacteria are able to selectively regulate transcription of this gene in association with RNAPII and the TATA-binding protein. However, the tick host is not without defenses. Recent work by Shaw et al. (2017), demonstrates a role for the tick IMD pathway in restricting *A. phagocytophilum* colonization of *I. scapularis*. Likewise, to control infection, tick salivary gland cells may limit *A. phagocytophilum* infection by inducing apoptosis via the extrinsic apoptosis pathway. Thus, in contrast to the midgut, these bacteria had considerably lower impact on salivary gland cells, presumably because they do not develop or multiply in that organ (Ayllón et al., 2015). Understanding the immune-related factors coordinating the balance between restriction and colonization of the vector is central to understanding vector competence. An illustration of how these pathogenic bacteria invade the tick host cells, multiply and prepare for transmission to their vertebrate hosts is shown in **Figure 1**.

BABESIA MICROTI

This eukaryotic microbe is the causative agent of human babesiosis, and is closely related to similar protozoans that cause deadly febrile disease in cattle and other livestock throughout the world. *B. microti* parasites are transmitted to humans during blood feeding of its vector, *I. scapularis*. After inoculation into the human host, the parasites (in the form of sporozoites) invade and undergo their development in the erythrocytes. These apicomplexan parasites express a membrane protein, apical membrane antigen 1 (AMA1), located near its apical end of its cell body that binds to the surfaces of the red cells, facilitating invasion (Moitra et al., 2015). Within the erythrocytes, the parasites transform into trophozoites (vegetative stage), divide into 2–4 merozoites. The merozoites lyse the host cells, escape into the blood plasma and invade other red blood cells. In their new host red blood cells, some mature into gametocytes. During tick blood feeding, the ingested erythrocytes are lysed in the tick's midgut lumen, liberating the *B. microti* gametocytes. The latter give rise to gametes, some of which develop a spike-like arrowhead organelle. These arrowhead-bearing gametes are known as strahlenkorpers. These gametes fuse to become zygotes. Zygotes metamorphose into elongated, motile 8–10 μm parasites, which proceed to invade the tick's midgut epithelial cells. To penetrate the peritrophic membrane, *B. microti* use their spike-like arrowhead organelles to rupture the membrane and allow these microbes to cross the membrane and access the lining epithelial cells. Upon contact with the midgut cells, contact by the arrowheads induces the membranes to invaginate around the babesias and allows them to invade the host cells (Rudzinska et al., 1979). Once inside the midgut cells, the arrowhead organelle is lysed and disappears (Rudzinska et al., 1983). Little is known about the life cycle of the parasite within the tick tissues, especially how they suppress or evade recognition by the tick's immune system in order to develop within the midgut cells. Ultimately, the parasites emerge from the midgut epithelium, transform into motile kinetes that escape into the hemolymph and invade the tick's salivary glands. Following invasion of the salivary glands, the microbes transform into sporoblasts. Development is arrested until the tick, usually a nymph, feeds again, whereupon thousands of sporozoites are produced from each sporoblast.

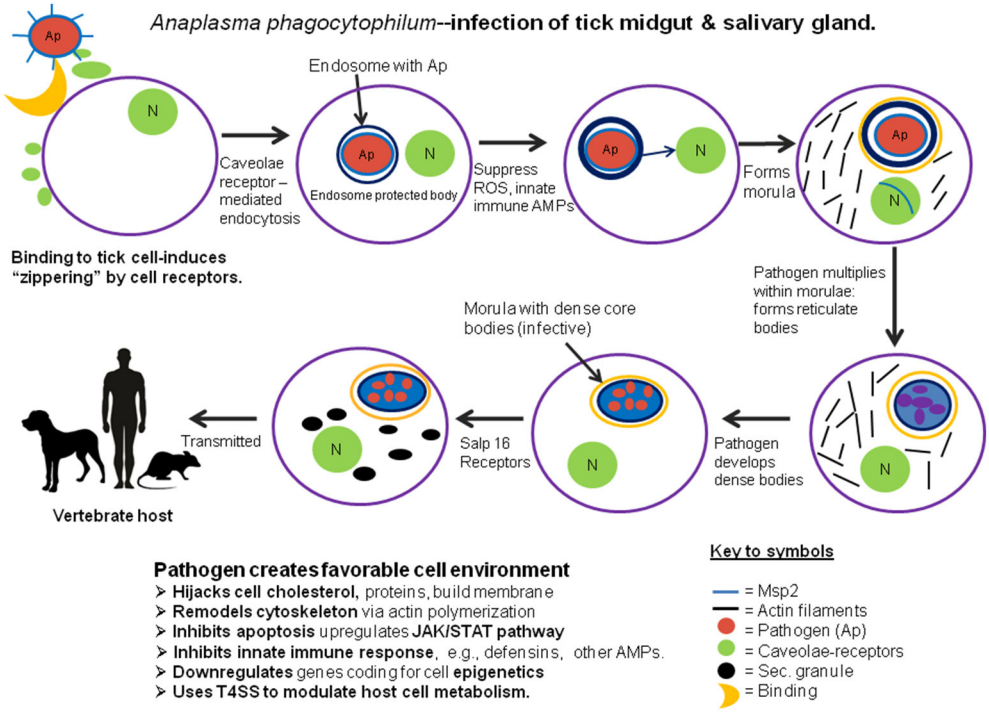

FIGURE 1 | Diagrammatic representation of the process of invasion and development of the pathogenic microbe, *Anaplasma phagocytophilum* in the midgut and salivary glands of its tick host.

The sporozoites are the infectious stage for the vertebrate host. Sporozoites are transmitted to the new host during tick feeding, whereupon they invade and develop within the host's erythrocytes.

AUTHOR CONTRIBUTIONS

DS wrote the preliminary draft. KM edited the draft and contributed additional information and related references. DS designed the original figure. KM revised the figure to emphasize clarity of the infection process and progress toward transmission, as well as improving the image quality. Both authors edited and accepted the final draft.

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Characterization of *Ixodes ricinus* Fibrinogen-Related Proteins (Ixoderins) Discloses Their Function in the Tick Innate Immunity

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Ticks are important vectors of serious human and animal disease-causing organisms, but their innate immunity can fight invading pathogens and may have the ability to reduce or block transmission to mammalian hosts. Lectins, sugar-binding proteins, can distinguish between self and non-self-oligosaccharide motifs on pathogen surfaces. Although tick hemolymph possesses strong lectin activity, and several lectins have already been isolated and characterized, little is known about the implementation of these molecules in tick immunity. Here, we have described and functionally characterized fibrinogen-related protein (FReP) lectins in Ixodes ticks. We have shown that the FReP family contains at least 27 genes (*ixoderins*, *ixo*) that could, based on phylogenetic and expression analyses, be divided into three groups with differing degrees of expansion. By using RNA interference-mediated gene silencing (RNAi) we demonstrated that IXO-A was the main lectin in tick hemolymph. Further, we found that ixoderins were important for phagocytosis of Gram-negative bacteria and yeasts by tick hemocytes and that their expression was upregulated upon injection of microbes, wounding, or after blood feeding. However, although the tick hemocytes could swiftly phagocytose *Borrelia afzelii* spirochetes, their transmission and burst of infection in mice was not altered. Our results demonstrate that tick ixoderins are crucial immune proteins that work as opsonins in the tick hemolymph, targeting microbes for phagocytosis or lysis.

Keywords: fibrinogen-related protein, ixoderin, lectin, complement, tick, Ixodes, RNAi, *Borrelia*

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INTRODUCTION

Ticks belong to the family of blood-feeding chelicerates (Arachnids). They transmit a broad spectrum of viruses, bacteria, protozoa, fungi, and nematodes, causing serious health problems to humans and animals (Jongejan and Uilenberg, 2004). The three-host tick, *Ixodes ricinus,* is the most common tick in Europe and is responsible for transmission of human Lyme borreliosis (caused by spirochetes of *Borrelia burgdorferi* sensu lato) and tick-borne encephalitis virus (TBEV). The evolutionarily closely related tick, *Ixodes scapularis,* is widely spread in the USA and is implicated in the transmission of local *Borrelia* species. The fact that ticks are susceptible vectors and reservoirs for such a broad variety of pathogens is largely due to their adaptation to prolonged feeding and the ability to survive long periods of starvation (years). An enormous amount of blood taken during

each feeding fully satisfies the tick's requirements for reproduction and molting, but simultaneously allows pathogen colonization of the tick body and transmission to the next host without markedly depleting tick energy resources.

Nevertheless, ticks possess several mechanisms to stop those pathogens that reduce tick fitness and reproduction. Although our knowledge of the tick immune system is rather limited when compared to model arthropod organisms, a wide spectrum of immune proteins (e.g., defensins, lysozymes, antimicrobial peptides, complement-like components, adapted host-blood proteins), pathways (Toll, Imd, JAK-STAT), and their interactions with pathogens, have been described (Hajdušek et al., 2013; Sonenshine and Macaluso, 2017). Therefore, fine-tuning of the tick immune system could help facilitate the fight against pathogens.

Tick fibrinogen-related proteins (FRePs) are immune molecules (lectins) most likely involved in the process of self/non-self-recognition within the tick hemolymph and interactions with carbohydrates (N-acetyl-D-hexosamines, sialic acids, and glycoconjugates) associated with pathogen-associated molecular patterns (PAMPs) of invading microbes. Tick FRePs are closely related to the horseshoe crab tachylectins 5A,B (Gokudan et al., 1999) and vertebrate ficolins (Kovár et al., 2000; Rego et al., 2006). Unlike the ficolins, invertebrate FRePs lack the typical N-terminal collagen-like domain (Kovár et al., 2000). Vertebrate ficolins, similar to horseshoe crab carcinolectin 5 (a homolog of tachylectin 5) (Zhu et al., 2005), are important immune factors involved in the pathways of the complement system (Endo et al., 2011). Importantly, mosquito and snail FRePs are involved in the immune defense reaction against *Plasmodium* parasites and schistosomes, respectively (Dong et al., 2006; Dong and Dimopoulos, 2009; Hanington and Zhang, 2011). We performed series of functional studies on the tick FRePs to characterize their biological functions and interactions with pathogens.

Here, we show that the tick genome contains at least 27 genes encoding single-domain FRePs (ixoderins, IXO), which can be divided into three main groups with various degrees of expansion. We have also used RNA-mediated gene silencing (RNAi) to show that ixoderins are immune molecules involved in the phagocytosis of *Escherichia coli* and *Candida albicans* and that IXO-A is the main lectin of tick plasma. Although we did not observe any significant effect of gene silencing on phagocytosis and transmission of *Borrelia* spirochetes we nevertheless believe that these proteins may play an important role in the tick immune system and defense against microbes invading tick hemolymph.

MATERIALS AND METHODS

Biological Material

Adult *Ixodes ricinus* males and females were collected by flagging in Ceske Budejovice, the Czech Republic. Adult females were fed on laboratory guinea pigs and engorged ticks were kept in glass vials in wet chambers at 24°C until oviposition and hatching. All laboratory animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb., ethics approval No. 102/2016.

Quantitative Real-Time PCR Profiling

Material used for tick tissue and stage profiling and for the analysis of gene expression after injection of pathogens was obtained as described previously (Urbanova et al., 2015). For the pathogen feeding assay, unfed females were infected with a suspension of Gram-negative bacteria *E. coli* (1106), Gram-positive bacteria *Micrococcus luteus* (CIP A270), spirochetes *B. afzelii* (CB43), yeast *C. albicans* (MDM8), or PBS (control) using glass capillaries placed over the tick hypostomes (each tick absorbed 1–3 μl). The RNA was extracted using a NucleoSpin RNA II kit (Macherey-Nagel) and its integrity was checked on an agarose gel. The RNA was reverse transcribed (0.5 μg per reaction) into cDNA using the Transcriptor High-Fidelity cDNA Synthesis Kit (Roche) and diluted 20-times in sterile water. Gene expression was determined by quantitative real-time PCR (qRT-PCR) using a LightCycler 480 (Roche) and SYBR green chemistry as described previously (Urbanova et al., 2015) using primers listed in Supplementary Table 1. Relative expression was normalized to *I. ricinus actin* (AJ889837) or *elongation factor* (GU074769) using the mathematical model of Pfaffl (Pfaffl, 2001). The differences between individual groups were calculated from the average means.

Database Search and Phylogenetic Analysis

The search for tick fibrinogen-related proteins (FReP) was performed using the *I. scapularis* genome database (www.vectorbase.org) or GenBank (http://www.ncbi.nlm.nih.gov). The primary amino acid sequence used for phylogenetic analysis comprised a conserved part of the fibrinogen-related domain (FReD, 64 amino acids residues). The sequences were aligned and manually checked using BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Alignment and sequence descriptions are provided as Supplementary Data Sheets 1, 2. Tree reconstruction employed the Neighbor Joining (NJ) method in the program MEGA 4 (http://www.megasoftware.net/). Nodal supports were calculated with 1000 replications.

RNA Silencing

A 243-bp fragment of *I. ricinus ixo-a* (position 1-243 of AY341424), a 249-bp fragment of *I. ricinus ixo-b* (position 1-249 of AY643518), and a 268-bp fragment of *I. scapularis ixo-c* (position 1256-1523 of ISCW009412) were amplified from *I. ricinus* cDNA and cloned into pll10 vector with two T7 promoters in reverse orientations (Levashina et al., 2001), using primers listed in Supplementary Table 1 containing additional restriction sites ApaI and XbaI. The dsRNA was synthesized as described previously (Hajdusek et al., 2009). The dsRNA (3 μg/μl) was injected through coxa of the third pair of legs into the hemocoel of adult females (345 nl) or nymphs (32.2 nl) using Nanoinject II (Drummond). After 1 (adults) or 3 (nymphs) days of rest in a humid chamber at room temperature, ticks were fed on guinea pigs or C3H/HeN mice (Charles River, GER), respectively. The level of gene silencing was checked by qRT-PCR.

Borrelia-Transmission Experiment

To prepare *Borrelia*-infected nymphs for the transmission experiment, C3H/HeN mice were intra-dermally injected with 10^5 of *Borrelia afzelii* CB43 (Štepánová-Tresová et al., 2000) spirochetes. After 4 weeks, pathogen-free larvae were fed on the infected mice and after repletion, were kept in wet chambers at 24°C until hatching. The infection of mice and nymphs was checked by PCR. Next, the infected nymphs (60 per group) were injected with a mix of *ixoderin a+b+c* or *gfp* (control) dsRNAs (3 μg/μl, 64.4 nl), rested for 3 days, and fed (10 nymphs per mouse) on naïve 6-weeks old C3H/HeN mice (5 mice per group) using plastic cylinders attached to the murine back. Detached engorged nymphs were weighed. The DNA from each nymph was extracted using NucleoSpin Tissue kit (Macherey-Nagel) and checked by PCR (tick *actin*) using primers listed in Supplementary Table 1. The level of knock-down was measured by qRT-PCR in an independent feeding experiment using cDNA prepared from five fully-engorged nymphs. The number of *Borrelia* and bacteria per nymph was measured by qRT-PCR using primers described in Supplementary Table 1. The level of *Borrelia* infection in each mouse was measured weekly by qRT-PCR using DNA isolated from an ear biopsy and normalized to the number of mouse genomes (*actin*). Four weeks after tick detachment, mice were sacrificed and the numbers of *Borrelia* in bladder and heart tissue were measured.

Hemagglutination Assay

The hemagglutination assay was carried out as described previously (Kovár et al., 2000). Briefly, hemolymph from a single semi-engorged adult female, uninjected or injected before feeding with *ixo-a, b, c* or *gfp* dsRNA, was suspended in 10 μl of hemaglutination buffer (20 mM TRIS–HCl, 150 mM NaCl pH 7.2). The volume of hemolymph was measured by pipetting. In a 96-well U-shaped microtitration plate, the hemolymph suspension (serial two-fold sample dilutions in TBS) was mixed with 10 μl of a 2% (v/v) suspension of native mouse erythrocytes (kept in sterile 3.8% (w/v) Na_3-citrate and prior to use washed three times in 0.15 M NaCl). Hemagglutination activity (HA) was determined after 2 h of incubation at room temperature and expressed as the reciprocal of the last sample dilution causing visible agglutination. HA in the last test well with positive hemagglutination was defined as 1 HA unit. The volume of hemolymph was taken into account. The differences between individual groups were calculated from the average means.

Phagocytic Assay

The *in vitro* phagocytic assays with *Chryseobacterium indologenes*, *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* and the methylamine (MA) pre-treatment assay were carried out as described previously (Buresova et al., 2011; Urbanova et al., 2014). For the phagocytic assay with *Borrelia* (modified from Urbanova et al., 2017), spirochetes of *B. afzelii* CB43 were cultivated in BSK-H complete medium (Sigma) at 33°C for 5–7 days to a concentration of 10^8 spirochetes per ml. The hemocytes ($\sim 4 \times 10^4$) from *ixoderin* or *gfp* dsRNA-injected semi-engorged females, collected in 240 μl of L15-BOFES medium, were incubated with 10 μl of *B. afzelii* CB43 (10^6 spirochetes) for 120 min at 28°C. The slides were fixed with 4% paraformaldehyde, washed three times with PBS, and the primary anti-*Borrelia burgdorferi* antibody (Thermo Scientific), at a dilution of 1:200, was applied to the slides and incubated on a horizontal shaker at room temperature (RT) for 1 h. The slides were washed three times with PBS and incubated with Alexa 488 (Molecular Probes) 1:500 s antibody in PBS. After 1 h of incubation at RT, the slides were washed three times with PBS and the cell membranes were permeabilized by incubation with 1% BSA in PBS containing 1% TritonX-100 on a horizontal shaker at 4°C for overnight. The next day, the slides were re-incubated with the primary anti-*B. burgdorferi* antibody (Thermo Scientific) 1:200 in PBS with 0.1% TritonX-100 (PBS-TX) on a horizontal shaker at room temperature for 1 h. After that, the slides were washed three times with PBS-TX and incubated with Alexa 594 (Molecular Probes) 1:500 s antibody in PBS-TX. Finally, the slides were washed twice with PBS-TX, the cell nuclei were counterstained with DAPI, and washed twice with PBS. After mounting in DABCO (Sigma), phagocytic hemocytes were counted using a 488/594 (FITC/Texas red) dual filter and a BX51 (Olympus) fluorescent microscope. For each group, 100 hemocytes were counted on each of at least 15 slides representing three independent biological replicates. Relative phagocytosis was calculated in relation to the number of phagocytic hemocytes in the control group injected with *gfp* dsRNA, taken as 100% for each respective experiment. The phagocytic index was determined as the number of hemocytes with ingested *Borrelia* counted for a total 100 hemocytes in the microscopic field.

Statistical Analysis

Statistical significance of differences was analyzed using GraphPad Prism 4.0 (GraphPad Software, CA) employing One-way ANOVA Kruskal-Wallis test or non-parametric Mann-Whitney test (*Borrelia*-transmission experiment only) and $P < 0.05$ (*) or $P < 0.001$ (**) were considered as significant. If not further specified, all results are expressed as the mean ± standard error (SEM). Data showed in **Figures 2**, **4** (qRT-PCR) were not analyzed by statistical methods as they represent the mean ± SEM of three biological replicates (each with a number of ticks as described above).

RESULTS

The *Ixodes Scapularis* Genome Contains Three Types of Fibrinogen-Related Proteins

To identify variability in fibrinogen-related proteins (FRePs) in ticks we performed *in silico* screening of the *I. scapularis* genome database (www.vectorbase.org) using available FReP sequences from *I. ricinus* (AAQ93650) and *Ornithodoros moubata* (AAP93589) as matrices. We identified 27 genes encoding proteins containing a single fibrinogen-related domain (FReD), which we designated as ixoderins (Rego et al., 2005). None of the genes encoded other domains than FReD. Using phylogenetic analysis we further showed that the tick ixoderins

could be divided into three groups (**Figure 1**). The first group (Ixoderin A) contained the following sequences: *ixo-a* from *I. ricinus*; five ixoderins from *I. scapularis*; DorinM and OMFREP from the soft tick *O. moubata*; and Tachylectins 5A and 5B from the horseshoe crab *Tachypleus tridentatus*. The second, clearly expanded group (Ixoderin B) was clustered around 15 *I. scapularis* ixoderins and the previously sequenced *ixo-b* from *I. ricinus*. The last group, designated as Ixoderin C, constituted a distinct group of ixoderins and contained *I. ricinus* and *I. scapularis* single sequences related to FBN39 from the mosquito *A. gambiae*. Sequences homologous to the most of the *I. scapularis* genome sequences can be identified in the NCBI Transcriptome Shotgun Assembly TSA database of *I. ricinus* (Supplementary Table 2). In conclusion, we identified 27 genes

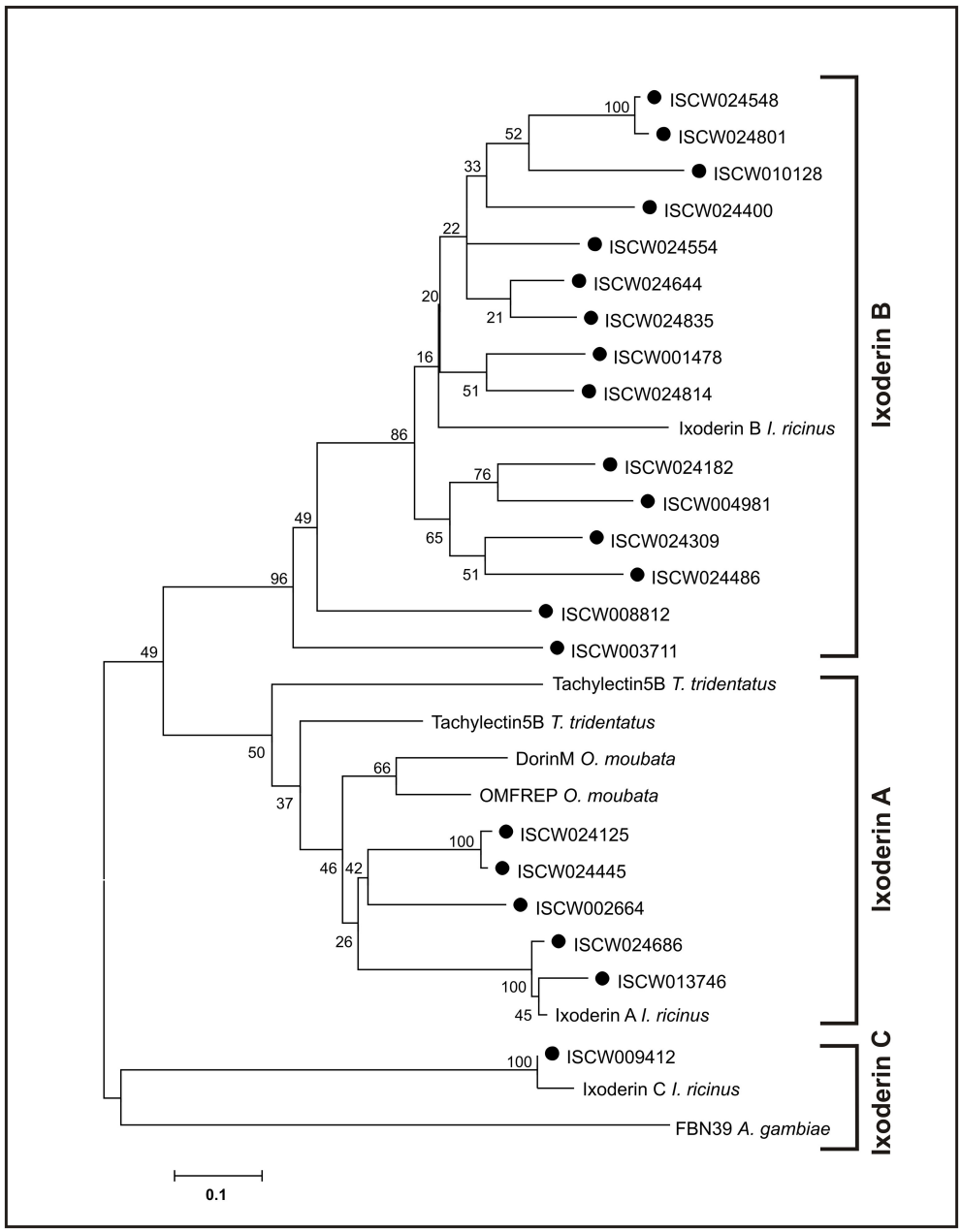

FIGURE 1 | Tick FRePs cluster into three groups designated as Ixoderin A, B, and C. An unrooted phylogenetic tree of the tick and related invertebrate FReP amino acid sequences, reconstructed using the Neighbor Joining (NJ) method and based on alignment using ClustalX. Full circles indicate genomic ixoderin sequences of *I. scapularis*. Numbers at branches represent bootstrap support using NJ criterion with 1,000 replicates each. Bar: 0.1 substitutions per site. Ixoderin A *I. ricinus*: AAQ93650 (AY341424), Ixoderin B *I. ricinus*: AAV41827 (AY643518), Ixoderin C *I. ricinus*: GCJO01000224. Alignment and full sequence descriptions are provided as Supplementary Data Sheet 1 and 2.

encoding single-domain ixoderins in the genome of *I. scapularis*, and these could be divided into three groups with various degrees of expansion.

Ixoderins Show Distinct Tissue and Stage Expressions

To verify the phylogenetic diversification of tick ixoderins into three groups and to reveal their possible functional variations, we performed a gene-specific qRT-PCR profiling using sets of *I. ricinus* cDNA prepared from tissues of semi-engorged females and different stages of tick development. The primers (Supplementary Table 1) were designed for one representative sequence from each ixoderin group: AY341424 (*I. ricinus*) from IXO-A group, AY643518 (*I. ricinus*) from IXO-B group, and ISCW009412 (*I. scapularis*) from IXO-C group. The analysis was performed on three independent biological replicates. Our data show that *ixo-a* was expressed mainly in hemocytes and Malpighian tubules (**Figure 2A**). Importantly, expression of *ixo-a* was 15.4, 10.6, and 29.3 times upregulated after blood feeding in larvae, nymphs, and females, respectively (**Figure 2B**). On the contrary, *ixo-b* was solely expressed in salivary glands, while *ixo-c* was ubiquitously expressed in all tissues with notably higher transcription in the gut and trachea. Expression of *ixo-b* and *c* was independent of feeding. The relatively high expression of *ixo-c* in tick eggs (developing embryos) was notable. In summary, *ixo-a*, *b*, and *c* show distinct tissue and stage-specific expression profiles, confirming the previous segregation of ixoderins into three groups and implying different functions in tick immunity or development.

Ixoderin A Is Indispensable for the Lectin Activity of Tick Hemolymph

Previously purified DorinM from the hemolymph of the soft tick *O. moubata*, and also pure hemolymph taken from the hard tick *I. ricinus*, possessed strong lectin activities against N-acetyl-D-hexosamines, sialoglycoproteins and sialic acid (Kovár et al., 2000; Grubhoffer et al., 2004; Sterba et al., 2011). We therefore questioned which ixoderins were responsible for hemolymph lectin activity in *I. ricinus*. By measuring hemagglutination activity of tick hemolymph using mouse red blood cells (RBC) we observed a two-fold increase in the hemagglutination titer between uninjected and dsGFP-injected (wounded) semi-engorged *I. ricinus* females (**Figure 3**). Further, we performed gene-specific knockdown (KD) of *ixo-a*, *b*, and *c* and compared the hemagglutination activities with control *gfp* dsRNA-injected females. Efficacies of the KDs in tissues with the most abundant gene expressions reached levels of 63.9–96.6% (Supplementary Table 3). Based on sequence homologies between available *I. scapularis* and *I. ricinus* sequences we believe that dsRNA targeted against *ixo-a* would have also silenced expression of *I. ricinus* homologs of *I. scapularis* genes ISCW024686 and ISCW013746 (three other genes from the Ixoderin A group were

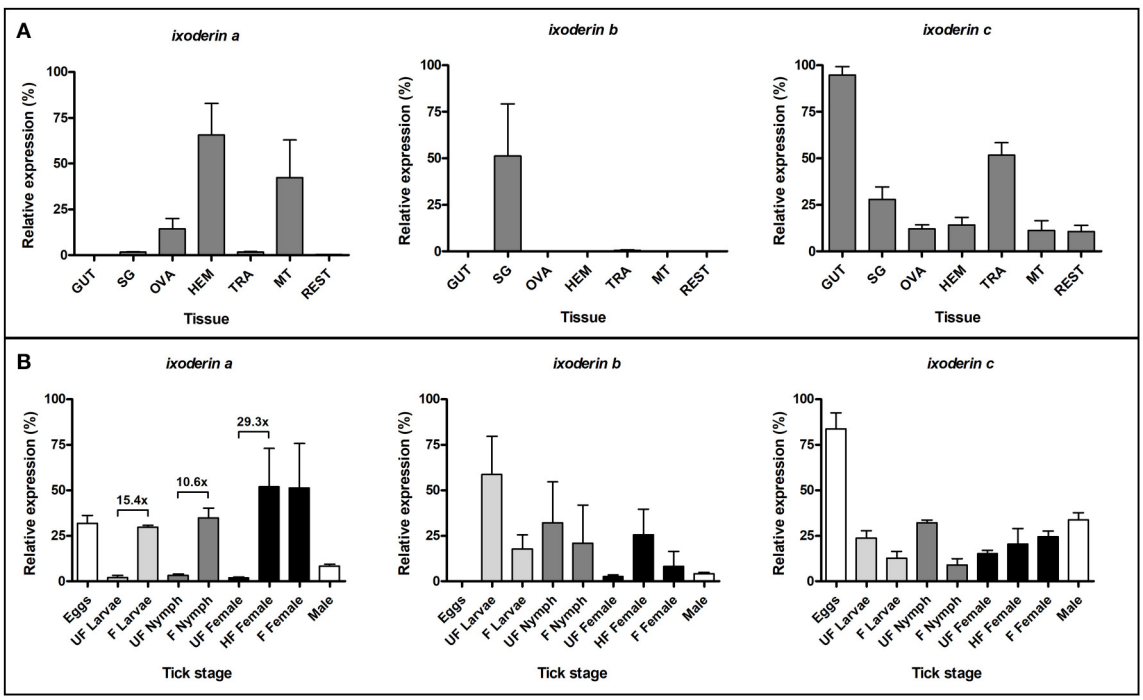

FIGURE 2 | *Ixoderins a*, *b*, and *c* show distinct tissue and stage-specific expression profiles. qRT-PCR analysis was performed on tissues of semi-engorged *I. ricinus* females **(A)** and a mixture of ticks from each developmental stage before and after blood feeding **(B)**. The samples were prepared in three biological replicates. In each graph, cDNA with the highest expression was set as 100% (relative expression). Tick *actin* and *elongation factor* were used as housekeeping genes for the tissue and stage-specific profiling, respectively. (GUT) midgut, (SG) salivary glands, (OVA) ovaries, (HEM) hemolymph, (TRA) trachea and fat body, (MT) Malpighian tubules, (REST) rest of the body, (UF) unfed ticks, (HF) half-fed (semi-engorged) adult females fed for 5 days, (F) fully-fed ticks.

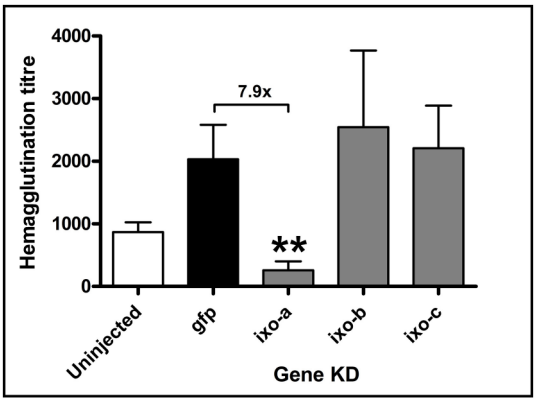

FIGURE 3 | Silencing of *ixoderin a* abolishes lectin activity of the tick hemolymph. The effect of injection (wounding) and *ixoderin* KDs on the haemagglutination activity of semi-engorged tick hemolymph was measured using 2% mouse RBC. The hemagglutination titer was calculated as hemagglutination activity per 1 μl of tick hemolymph. Hemagglutination titer in the dsGFP group served as an injection control. Two asterisks indicate *p*-value < 0.001.

probably not silenced). Because of the missing 5′ prime ends of sequences, a similar prediction was more difficult for *ixo-b*. However, although the dsRNA directed against *ixo-b* had the capability to silence four *I. ricinus ixo-b* genes available in the GenBank database (AY643518 and EF063561-4) it is probable that many *ixo-b* genes were not affected. KD of *ixo-a* significantly (7.9 times) decreased lectin activity of the tick hemolymph compared to the *gfp* control group, whereas KD of *ixo-b* and *c* did not display any significant effect (**Figure 3**). Thus, IXO-A is the lectin most likely responsible for hemagglutination activity of tick hemolymph, which itself notably increased upon tick wounding.

Expression of *Ixoderins* Is Stimulated by Wounding and Immune Challenge

To determine whether expression of *ixoderins* alters after wounding or exposure to microbes, we injected or capillary fed adult *I. ricinus* females with Gram-negative bacteria *E. coli*, Gram-positive bacteria *Micrococcus luteus*, spirochetes *B. afzelii*, or yeast *C. albicans* and subsequently measured *ixoderin* expression levels by qRT-PCR 12 h after the challenge. The analysis was performed as three independent biological replicates. Untreated and PBS-injected or fed ticks were used as controls. Injection of sterile PBS increased expression of *ixo-a* and *c* 3.4 and 5.4 times respectively (**Figure 4A**), indicating a wounding response. Injection of *B. afzelii* slightly increased expression of *ixo-a* and expression of *ixo-b* was 4.6 times higher in *E. coli*-injected groups compared to the PBS control. Furthermore, capillary feeding of PBS increased expression of *ixo-a* and *c*, 2.6 and 9.1 times respectively (**Figure 4B**). Interestingly, feeding of pathogens decreased expression of *ixo-b* in all experimental groups and feeding of *E. coli* increased by 2.8 times expression of *ixo-c* compared to the PBS control. In conclusion, wounding or capillary feeding stimulate expression of *ixo-a* and *c*, while *ixo b* and *c* specifically react to the presence of *E. coli* in the hemolymph and midgut, respectively, implicating involvement of these genes in pathogen sensing.

Ixoderin KD Impairs Phagocytosis of Bacteria and Yeasts by Tick Hemocytes

Vertebrate and invertebrate FREPs have been shown to function as opsonins capable of binding to pathogens and to cause their phagocytosis and/or lysis (Cerenius and Soderhall, 2013). To assess biological functions of tick ixoderins, we performed an *in vitro* phagocytic assay with various bacteria and yeasts, employing tick hemocytes of semi-engorged tick females after KD of particular *ixoderin*. At least five slides with hemocytes were analyzed for each of the biological triplicates. Dashed lines in the graphs (**Figure 5**) indicated the level of phagocytosis obtained after methylamine (MA) pre-treatment of the hemolymph, which specifically reacted with thioester groups and inactivated tick thioester proteins important for phagocytosis of Gram-negative bacteria and yeasts (Buresova et al., 2011; Urbanova et al., 2015). As a result, KD of *ixo-a* significantly decreased phagocytosis of *E. coli* to the level of MA pre-treatment, indicating a simultaneous involvement of tick lectins and thioester proteins in common complement-like pathway (**Figure 5A**). Silencing of *ixo-a* and *b* showed a strong effect on the phagocytosis of Gram-negative bacteria *Chryseobacterium indologenes* (**Figure 5B**), an effective pathogen of ticks (Burešová et al., 2006). Double KD of *ixo-a* and *b* reached *C. indologenes* phagocytosis levels of individual *ixo-a* and *b* KDs. This result implies that both proteins act in the same pathway (non-synergistic effect) or that the active protein is, in its native state, a heteromer composed of different ixoderin subunits. Furthermore, KD of *ixo-a* and *b* caused a significant decrease in the phagocytosis of the yeast *C. albicans* (**Figure 5C**). Consistent with ME pretreatment, none of the *ixoderin* KDs had a reducing effect on the phagocytosis of Gram-positive *S. aureus* (**Figure 5D**). In summary, ixoderins appear to be important opsonins involved in the phagocytosis of different Gram-negative bacteria and yeasts by tick hemocytes.

Ixoderins Do Not Affect Phagocytosis and Transmission of *Borrelia* Spirochetes

To reveal a possible role of ixoderins in the transmission of *Borrelia* spirochetes from the tick into the host, we employed (i) an *in vitro* phagocytic assay using tick hemocytes and (ii) a *Borrelia* transmission test on the background of *ixoderin* KDs. Tick hemocytes were suggested to phagocytose and kill *Borrelia* spirochetes in the hemolymph on their route from the midgut to salivary glands, although the number of *Borrelia* crossing the hemolymph seemed to be low (Dunham-Ems et al., 2009). By using the tick hemocytes of semi-engorged females we tested the effects of individual *ixoderin* KDs on the phagocytosis of *B. afzelii* CB43. For this purpose we used a phagocytic assay (modified from Urbanova et al., 2017) that can reliably distinguish between spirochetes located outside of the hemocytes, sticking to their surface, and those, which were certainly phagocytosed (**Figure 6**). The phagocytosis of *Borrelia* can be reduced by pre-incubation of the hemolymph with the thioester-blocking reagent methylamine (**Figure 7A**).

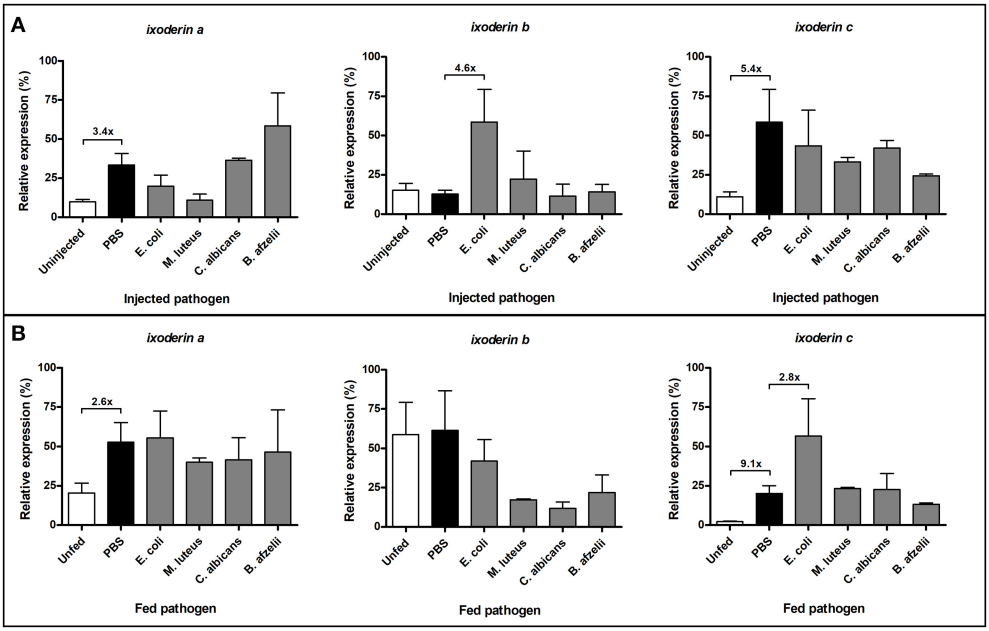

FIGURE 4 | Wounding and exposure to microbes alter expression of *ixoderins*. Gene expression was measured by qRT-PCR 12 hrs after pathogen injection or feeding. The analysis was performed using unfed adult females injected **(A)** or capillary fed **(B)** with pathogens. The samples were prepared in three biological replicates. In each graph, cDNA with the highest expression was set as 100% (relative expression). Tick *actin* was used as a housekeeping gene.

Further, we observed that *Borrelia* spirochetes were well phagocytosed (37% phagocytic hemocytes) and formed "coils" in the cytosol of hemocytes, remaining the coiling phagocytosis of *Borrelia* by vertebrate and invertebrate phagocytic cells (Rittig et al., 1996). However, individual KDs of i*xoderins* did not significantly decrease phagocytosis of *Borrelia* by tick hemocytes (**Figure 7B**). In summary, although *Borrelia* spirochetes, and other tested microbes, were substantially phagocytosed by tick hemocytes, ixoderins constituting the main lectin activity of tick hemolymph seem not to be involved in *Borrelia* engulfment.

Further, we tested if ixoderins were able to interfere with spirochetes in ways other than phagocytosis and affect *Borrelia* during the transmission cycle or, reversely, bind to *Borrelia* surfaces to support their survival in the tick or the vertebrate host. Therefore we utilized a mouse transmission model for *B. afzelii* CB43, employing feeding of naturally *Borrelia*-infected nymphs in combination with triple KD of *ixoderins*. Efficacy of the triple KD ranged from 64.9 to 97.1% (Supplementary Table 4). After feeding, no differences in the feeding success were noticed for the *ixoderins*-silenced *I. ricinus* nymphs comparing to dsGFP controls (**Figures 8A,B**). Weights of the fully engorged nymphs were in agreement with previously reported values (Dusbábek, 1996) and reflected differences between males and females. The number of total bacteria was about 15 times higher in the fully fed nymph females than in males, however no differences were observed between the ticks of the KD and control groups (**Figure 8C**). The differences between fed females and males disappeared when we determined the number of *Borrelia*. The ticks after *ixoderins* KD contained the same numbers of spirochetes as ticks in the control group (**Figure 8D**). Finally, we followed mice infection (*Borrelia* transmission) after feeding of the silenced and control ticks. The progress of infection was tracked for 4 weeks after the infestation by measuring the number of *Borrelia* spirochetes in ear biopsies. However, we did not detect any significant differences between the two groups (**Figure 8E**). The number of spirochetes in the destination tissues of *B. afzelii* (urinary bladder and heart) was also similar in all groups (**Figure 8F**). In conclusion, KD of *ixoderins* did not affect the number of *Borrelia* in the fed nymphs and/or burden of *Borrelia* in mice tissues.

DISCUSSION

The lectin pathway of the complement system constitutes an evolutionarily ancient branch of defense systems. Vertebrate ficolins (related to invertebrate FRePs) and mannose-binding lectins can activate the complement system and are critical to early defense against infection (DeFranco et al., 2007; Ricklin et al., 2010). Research carried out on the immune system of horseshoe crab, the "living fossil" of the Chelicerate lineage (reviewed in Iwanaga, 2002), revealed that the major agglutinating plasma FReP lectins CL5a and CL5b and the TE-containing C3-like molecule CrC3 are the dominant proteins bound to the surfaces of a wide range of microbes (Zhu et al., 2005). This binding initiates activation of the complement-like system, leading to the phagocytosis of pathogens. Different pools of CL5a and CL5b isoforms bind to bacteria and fungi,

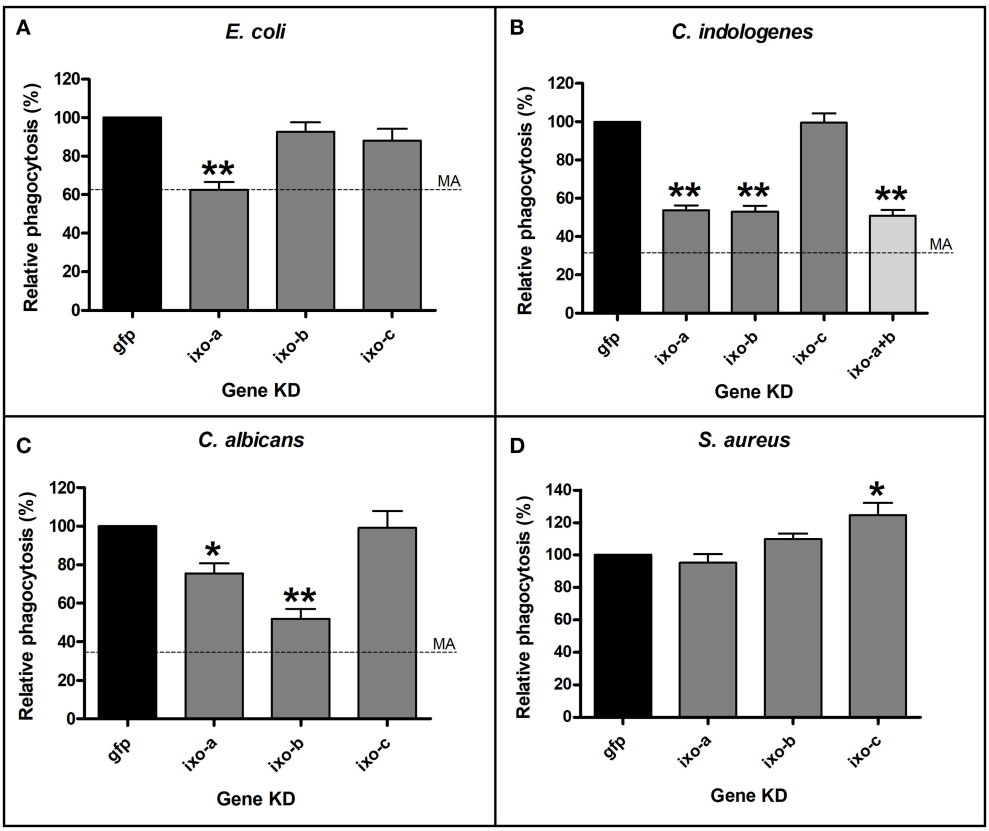

FIGURE 5 | Silencing of *ixoderins* impairs phagocytosis of microbes by tick hemocytes. Tick hemocytes acquired from the adult semi-engorged females after *ixoderin* KDs were incubated *in vitro* with *E. coli* **(A)**, *C. indologenes* **(B)**, *C. albicans* **(C)**, and *S. aureus* **(D)**. At least five independent slides with hemocytes were analyzed for each of biological triplicates. The level of phagocytosis in the dsGFP control was set as 100%. Dashed lines indicate phagocytosis after methylamine (MA) pre-treatment (no effect on *S. aureus*). One and two asterisks indicate *p*-value < 0.05 and <0.001, respectively.

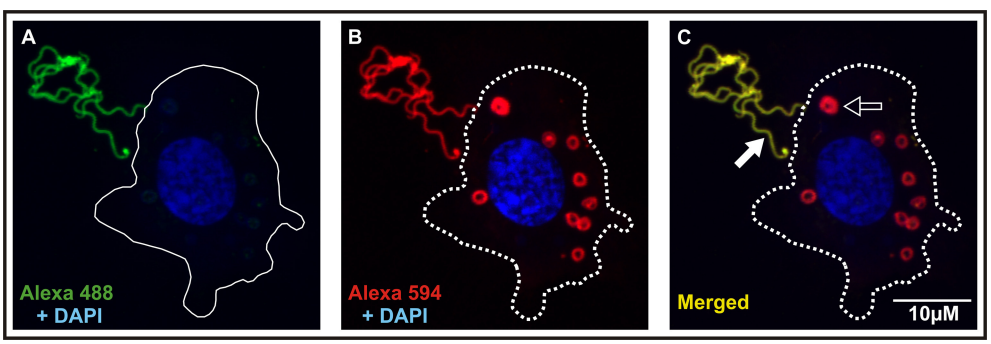

FIGURE 6 | Dual staining is necessary for proper interpretation of the *Borrelia* phagocytic assay. Hemocytes of adult semi-engorged females were mixed *in vitro* with *B. afzelii* CB43. The slides were then incubated with anti-*Borrelia* primary antibody and stained with Alexa 488 **(A)**. Finally, cell membranes of hemocytes were permeabilized, incubated again with the anti-*Borrelia* primary antibody, and stained with Alexa 594 and DAPI **(B)**. *Borrelia* spirochetes localized outside or on the surface of hemocytes are stained green and red, engulfed spirochetes are stained only red. The 488/594 (FITC/Texas red) dual filter can be used for rapid analysis of the slides **(C)** and can distinguish between phagocytosed (red; black arrow) and non-phagocytosed spirochetes (yellow; white arrow). Full and dashed lines indicate the hemocyte surface before and after permeabilization, respectively.

suggesting unique roles of these lectins in the recognition and differentiation of microbes (Zhu et al., 2006). Notably, horseshoe crab C-reactive protein (CRP) and galactose-binding protein (GBP), major hemolymph proteins forming bacteria-binding complexes (pattern-recognition receptor) on the surface of pathogens (Ng et al., 2007), are absent from the tick genomes.

FRePs are widely distributed among arthropods in different numbers and domain combinations, playing a fundamental

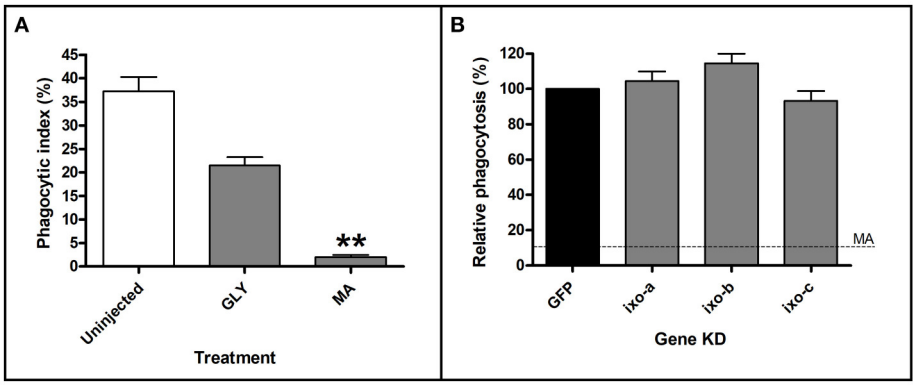

FIGURE 7 | Pre-treatment of hemolymph with methylamine (MA), but not *ixoderin* KDs affects phagocytosis of *Borrelia*. Tick hemocytes after MA pre-treatment **(A)** or *ixoderin* KD **(B)** were incubated *in vitro* with *B. afzelii* CB43. At least five independent slides with hemocytes were analyzed for each of three biological replicates. The phagocytic index in **(A)** was determined as the ratio of phagocytic vs. non-phagocytic hemocytes, and the level of phagocytosis in **(B)** was set as 100% in the dsGFP control. (Uninjected) untreated hemolymph, (GLY) glycine pre-treatment (control), (MA) MA pre-treatment. Dashed line in **(B)** indicates phagocytosis level after MA treatment. Two asterisks indicate p-value < 0.001.

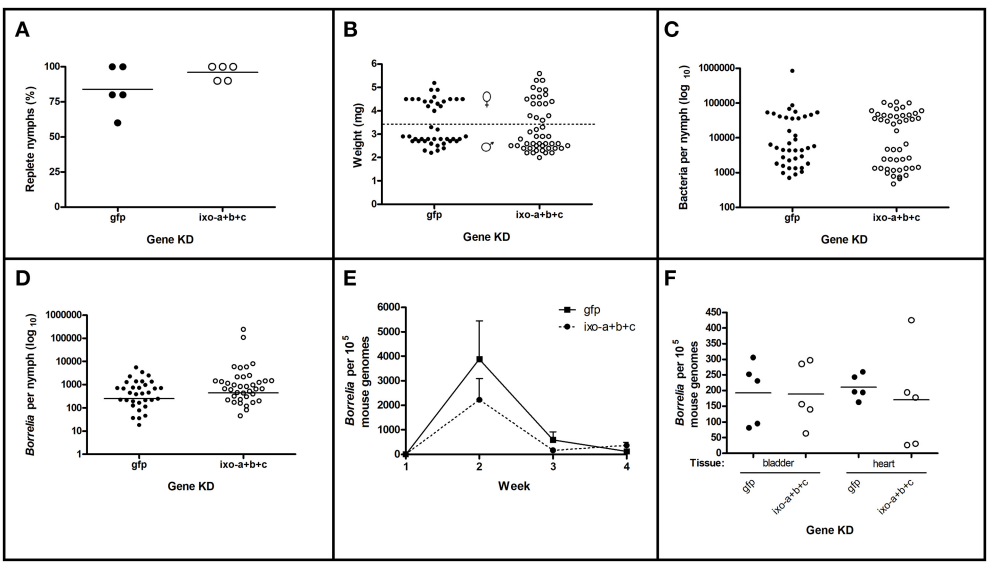

FIGURE 8 | Silencing of *ixoderins* does not affect *Borrelia* transmission. Transmission of *B. afzelii* CB43 from naturally infected nymphs to mice was tested after *ixoderins* triple KD. Five mice were infested with 10 nymphs each in individual groups. **(A)** Percent of nymphs replete from each mouse. **(B)** Weights of individual replete nymphs. Dashed line indicates suggested border between female and male nymphs. **(C)** Number of bacteria (universal primers) in the individual nymphs measured by qRT-PCR (log_{10} scale). **(D)** Number of *Borrelia* in the individual nymphs measured by qRT-PCR (log_{10} scale). **(E)** Number of *Borrelia* in the mice ear biopsies during 4 weeks of infection and **(F)** number of *Borrelia* in the destination tissues 4 weeks after infestation measured by qRT-PCR. The number of *Borrelia* was normalized to 10^5 mouse genomes.

role in anti-parasitic defense. Thus, mosquito *Anopheles gambiae* possesses 59 single-domain FReps important for anti-plasmodial and anti-bacterial defense (Dong and Dimopoulos, 2009). Mollusk *Lottia gigantea* contains 70 FReps with an immunoglobulin superfamily (IgSF) domain(s) additional to the FReP domain, and these have been shown to be active in resistance to digenean trematodes (schistosomes) (Hanington and Zhang, 2011). Considering the chelicerate lineage, FReps (single-domain only) have been found in relatively small numbers in horseshoe crabs (tachylectins homologous to *ixo-a*) and mites. The family has then expanded in scorpions and spiders, comprising 25 and 20 members, respectively (Palmer and Jiggins, 2015). We show, that the tick genome contains at least 27 single-domain FReps, which can be phylogenetically arranged into three groups comprising different numbers of members (**Figure 1**) and different tissue and developmental expression profiles (**Figure 2**).

Tick hemolymph possess strong lectin activity (measured by the hemagglutination assay) with a preferential specificity for N-acetyl-D-hexosamines and sialic acid, attributed mainly

to the presence of fibrinogen-related proteins (FRePs) (Kovár et al., 2000; Grubhoffer et al., 2004; Sterba et al., 2011). Here we have demonstrated that the *I. ricinus* FReP IXO-A is the protein that facilitates the lectin activity of tick plasma (**Figure 3**). We show that *ixo-a* is mainly expressed in tick hemocytes and is overexpressed after tick injury or feeding (**Figure 4**). Furthermore, we show that silencing of *ixo-a* and *ixo-b* by RNA interference inhibits tick hemocyte phagocytosis of Gram-negative bacteria and yeasts and that these FRePs are thus important defense molecules associated with the tick innate immune system (**Figure 5**). The tick FRePs probably function as homo- or heteromultimers, as they possess ability to haemagglutinate red blood cells (at least two red blood cells bound by one FReP multimer) and the double KD of *ixo-a* and *ixo-b* does not show a synergistic effect with phagocytosis of Gram-negative bacteria. This result is in agreement with our previous biochemical characterization of the tick FReP DorinM from *O. moubata*, which, in the native state, forms 640 kDa aggregates composed of 37 kDa monomers (Kovár et al., 2000). This multimerization has also been confirmed for FRePs of horseshoe crabs (Gokudan et al., 1999), mosquitoes (Dong and Dimopoulos, 2009), snails (Hanington and Zhang, 2011), as well as for vertebrate ficolins (Endo et al., 2011).

We have previously shown that the tick complement-like system possesses thioester-containing proteins [C3 proteins, α2-macroglobulins, insect-type thioester protein (TEP) and macroglobulin-related proteins (MCR)] (Buresova et al., 2011). Other molecules related to the components of vertebrate or invertebrate complement systems have been also described in ticks (Factor D, homologs of Limulus Factor C, and Factor C2/Bf (Simser et al., 2004; Urbanova et al., 2014, 2018). These proteins constitute an important defense mechanism in tick hemolymph and we assume that ixoderins function in the initial phase of its activation. A proposed model based on our previous data and the work published on the complement system of horseshoe crab (Le Saux et al., 2008; Tagawa et al., 2012) suggests that ixoderins act as non-self-recognition molecules via specific binding on the glycan structures of the pathogen associated molecular patterns (PAMPs) present on the surface of invading microbes. Together with putative C3 convertases (e.g., Factor C2/B or Limulus Factor C-like protease) they form a pattern recognition receptor (PRR) that enhance binding of additional C3 molecules on the surface of microbes (Gram-negative bacteria, yeasts), hereby leading to their elimination by enhanced phagocytosis or lysis.

Lyme borreliosis is an important human infection in temperate climates in North America and Eurasia, caused by *Borrelia* spp. (Hajdušek et al., 2013). The spirochetes are believed to migrate during tick feeding from the midgut through the salivary glands into the host. An alternative transmission route was proposed via regurgitation of spirochetes from the midgut into the feeding lesion (Burgdorfer, 1984). Once in the host, spirochetes can be attacked and destroyed by the complement system (de Taeye et al., 2013). However, killing efficacy differs between vertebrate hosts, as demonstrated by their different susceptibility to the infection. Interestingly, it has been shown that the tick salivary protein Salp15 can be bound by *Borrelia* to protect them from the host immune attack (Ramamoorthi et al., 2005). Similarly, the tick salivary protein TSLPI inhibits the host complement system and thus facilitates *Borrelia* transmission (Schuijt et al., 2011a,b). By using the *Borrelia*-phagocytic assay and *Borrelia*-transmission system (Urbanova et al., 2017) we tested whether tick ixoderins, expressed both in the hemolymph and salivary glands, are able to activate the tick complement-like system and kill the spirochetes or bind to the surface of pathogens to protect them in the tick vector or the vertebrate host. In these assays we used *in vitro* cultivated *Borrelia* (BSK-H complete medium, 33°C), which express similar surface proteins as the activated spirochetes in the tick hemolymph during tick feeding and are infectious to the vertebrate host (Obonyo et al., 1999; Ohnishi et al., 2001; Dunham-Ems et al., 2009). However, after KD of *ixoderins* we observed no phenotypic changes in the phagocytosis of *Borrelia* by tick hemocytes (**Figure 7**), the survival of *Borrelia* in ticks, nor the transmission of spirochetes and infection of the hosts (**Figure 8**). These results are in line with our previous data showing that KD of tick complement proteins (Urbanova et al., 2014, 2017, 2018), blocking of thioester proteins by methylamine (**Figure 7**), or depletion of phagocytosis by injection of latex beads (Urbanova et al., 2017) markedly reduced phagocytosis of spirochetes by tick hemocytes, but in no case had any (positive or negative) effect on the transmission of *Borrelia*. This suggests that defense mechanisms in the tick hemocoel based on the primordial complement system and/or phagocytosis are likely not capable to block or limit successful transmission of the Lyme borreliosis spirochetes from the tick midgut to the host. Nevertheless, other important tick-transmitted pathogens that come into contact with tick hemolymph during their transmission, e.g., protozoan malaria-like piroplasms (*Babesia* and *Theileria*) or intracellular rickettsial bacteria (*Anaplasma* and *Ehrlichia*), remain to be tested in our assays for interactions with ixoderins and the tick complement system, that may lead to the new ways of protection against these tick-transmitted infections.

AUTHOR CONTRIBUTIONS

OH, PK, LG, ROMR conceived the study and designed experiments. OH, VK, RS, VU, HHM, PK performed the experiments and analyzed data. OH, PK wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2017.00509/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Tick Thioester-Containing Proteins and Phagocytosis Do Not Affect Transmission of *Borrelia afzelii* from the Competent Vector *Ixodes ricinus*

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The present concept of the transmission of Lyme disease from *Borrelia*-infected *Ixodes* sp. ticks to the naïve host assumes that a low number of spirochetes that manage to penetrate the midgut epithelium migrate through the hemocoel to the salivary glands and subsequently infect the host with the aid of immunomodulatory compounds present in tick saliva. Therefore, humoral and/or cellular immune reactions within the tick hemocoel may play an important role in tick competence to act as a vector for borreliosis. To test this hypothesis we have examined complement-like reactions in the hemolymph of the hard tick *Ixodes ricinus* against *Borrelia afzelii* (the most common vector and causative agent of Lyme disease in Europe). We demonstrate that *I. ricinus* hemolymph does not exhibit borreliacidal effects comparable to complement-mediated lysis of bovine sera. However, after injection of *B. afzelii* into the tick hemocoel, the spirochetes were efficiently phagocytosed by tick hemocytes and this cellular defense was completely eliminated by pre-injection of latex beads. As tick thioester-containing proteins (T-TEPs) are components of the tick complement system, we performed RNAi-mediated silencing of all nine genes encoding individual T-TEPs followed by *in vitro* phagocytosis assays. Silencing of two molecules related to the C3 complement component (*Ir*C3-2 and *Ir*C3-3) significantly suppressed phagocytosis of *B. afzelii,* while knockdown of *Ir*Tep (insect type TEP) led to its stimulation. However, RNAi-mediated silencing of T-TEPs or elimination of phagocytosis by injection of latex beads in *B. afzelii*-infected *I. ricinus* nymphs had no obvious impact on the transmission of spirochetes to naïve mice, as determined by *B. afzelii* infection of murine tissues following tick infestation. This result supports the concept that *Borrelia* spirochetes are capable of avoiding complement-related reactions within the hemocoel of ticks competent to transmit Lyme disease.

Keywords: *Borrelia,* complement, *Ixodes,* phagocytosis, thioester-containing proteins

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INTRODUCTION

Ticks are obligatory blood-feeders capable of transmitting a wide variety of pathogens including viruses, bacteria, protozoa, fungi, or nematodes to their vertebrate hosts (Jongejan and Uilenberg, 2004; De La Fuente et al., 2008). Yet, the vector competence of different tick species is quite specific and is determined by the ability of any particular pathogen to overcome several barriers on its route

from the infected tick to the naïve host, including defense mechanisms within the tick midgut, hemocoel, and salivary glands; for review see (Hajdusek et al., 2013). One of the most thoroughly investigated tick-borne diseases of humans is Lyme disease, caused by spirochetes of the genus *Borrelia* (Burgdorfer et al., 1982; Stanek et al., 2012) transmitted mainly by *Ixodes scapularis* in the USA and *Ixodes ricinus* in Europe (Piesman and Gern, 2004; Radolf et al., 2012). Several tick molecules have been described to play important roles in the *Borrelia* transmission cycle; reviewed in Hajdusek et al. (2013) and Kung et al. (2013): Glutathione peroxidase Salp 25D facilitates spirochete acquisition from the infected host (Narasimhan et al., 2007); TROSPA, tick receptor for the outer surface protein A, plays a role in *Borrelia* long-term persistence within the tick gut (Pal et al., 2004); tre31 that binds to another outer *Borrelia* surface lipoprotein, BBE 31, allows crossing of the midgut barrier (Zhang et al., 2011). Salivary proteins Salp15 (Ramamoorthi et al., 2005), tick histamine release factors (Dai et al., 2010), and tick salivary lectin pathway inhibitor (Schuijt et al., 2011) protect *Borrelia* at the tick-host interface via modulation of the host immune response. In addition to tick molecules, transmission of spirochetes is also aided by proteins originating from the blood meal, such as host plasminogen that is bound and activated on the spirochete surface, facilitating *Borrelia* migration through the tick and dissemination in the host (Coleman et al., 1997).

A previous study suggested that the difference in capacity to transmit *Borrelia burgdorferi* between *I. scapularis* (competent tick) and *Dermacentor variabilis* (refractory tick) is likely to be due to higher borreliacidal and phagocytic activities in the hemolymph of *D. variabilis* (Johns et al., 2001). Despite this important observation, our current knowledge on tick-*Borrelia* interactions in the hemocoel of *Ixodes* sp. ticks is rather limited. Phagocytosis of *B. burgdorferi* by *I. scapularis* hemocytes upon spirochete penetration from the midgut to the hemocoel has been described in several studies (Coleman et al., 1997; Dunham-Ems et al., 2009) but whether this cellular defense reaction plays any role in *Borrelia* transmission in a competent vector remains unclear.

In vertebrate animals, including mammalian, avian or reptile hosts, the decisive role in susceptibility or resistance to infection by a certain genospecies of *B. burgdorferi* sensu lato complex, is most likely played by the serum complement system (Kurtenbach et al., 1998, 2002; Kuo et al., 2000; Bhide et al., 2005; De Taeye et al., 2013). A borreliacidal effect on spirochetes was reported to be exerted by the alternative pathway of mammalian complement (Kurtenbach et al., 1998; Kuo et al., 2000). Since ticks possess a primitive complement system, comprising thioester-containing proteins (TEPs), ficolin-like lectins or putative C3-convertases (Kopacek et al., 2012), we primarily asked whether or not tick complement plays a role in the competence of ticks to act as a vector for Lyme disease. In this study we focused on molecules of the TEP family, which, in *Ixodes* sp. ticks, involves representatives of four major classes of TEPs known in invertebrates: (i) three proteins related to C3-complement component; (ii) three different α_2-macroglobulins; (iii) one insect-type TEP, and (iv) two macroglobulin-complement-related (MCR) molecules (Buresova et al., 2011; Urbanova et al., 2015). Using RNAi-mediated silencing of individual genes encoding tick TEPs (T-TEPs), followed by *in vitro* phagocytosis assays, we have previously demonstrated that different T-TEPs are involved in phagocytosis of different model microbes (Gram-negative *Chryseobacterium indologenes*, *Escherichia coli* and yeast *Candida albicans*) by tick hemocytes (Buresova et al., 2009, 2011; Urbanova et al., 2015). However, a similar study focusing on *Borrelia* sp. spirochetes has not been performed yet, mainly due to the lack of a reliable phagocytic assay for this tick-borne pathogen. To overcome this problem, we have implemented a phagocytic assay for *Borrelia* sp. that exploits dual labeling, making it possible to clearly distinguish free and/or attached spirochetes from those being engulfed by tick hemocytes. Phagocytosis of different microbes by invertebrate hemocytes could be efficiently blocked by intra-hemocoelic injection of inert particles such as latex or polystyrene beads, as previously demonstrated for the fruit fly *Drosophila melanogaster* (Nehme et al., 2011) or the tick *I. scapularis* (Liu et al., 2011). With this experimental background, and using a recently established laboratory transmission model for *B. afzelii* (the most important agent of Lyme disease in Europe), we have examined the role of T-TEPs in phagocytosis of spirochetes by tick hemocytes and addressed the question of whether or not this cellular defense plays a role in spirochete transmission from *Borrelia*-infected *I. ricinus* nymphs to naïve mice. Our results collectively demonstrate that complement-like molecules are involved in tick phagocytic responses to *B. afzelii*, but do not prevent transmission of *B. afzelii*. These findings add to our understanding of the competence of this tick species to act as a vector for Lyme disease.

MATERIALS AND METHODS

Biological Material

Adult females and males of *I. ricinus* were collected by flagging in woodlands around České Budějovice, the Czech Republic. All developmental stages (eggs, larvae, nymphs, and adults) were maintained in wet chambers with a humidity of about 95%, temperature 24°C and day/night period set to 15/9 h. Females were fed naturally on laboratory guinea pigs. The larvae were fed on guinea pigs, allowed to molt to nymphs and, after 4–6 weeks, further fed on guinea pigs or rabbits. The nymphs (pathogen free or infected with *B. afzelii* CB 43) and adult females (pathogen free) were used for experiments described below. All laboratory animals were treated in accordance with the Animal Protection Laws of the Czech Republic No. 246/1992 Sb., ethics approval No. 095/2012.

Borreliacidal Assay

B. afzelii CB43 spirochetes were cultivated in BSK-H complete medium (Sigma-Aldrich) at 33°C for 5–7 days and for the assay, were diluted to a concentration of 5×10^6 cells/ml. Hemolymph samples from 50 semi-engorged females (6th day of feeding) were collected into a glass capillary from the cut front leg, immediately cooled on ice and the collected pool was centrifuged at 300 × g for 10 min. The supernatant was transferred to a fresh tube and centrifuged at 9500 × g for 10 min. The hemocyte-free

plasma so obtained was used for subsequent experiments. Bovine serum was prepared from manually defibrinated bovine blood as described previously (Perner et al., 2016) and inactivated bovine serum was prepared by heating at 56°C for 40 min.

The assay was performed in 96-well plates (Nunc) by adding 50 μl of *B. afzelii* (5×10^6/ml) to 10 μl of tested sample (tick plasma, bovine serum, inactivated bovine serum, or BSK-H medium as a control), and incubated for 24 h in a wet chamber at 33°C. The number of live *B. afzelii* was determined using dark-field microscopy as follows. The *Borrelia* culture (3.5 μl) was transferred onto a microscope slide and covered with 18 × 18 mm coverslip. Spirochetes present in the view field were counted and the average number, calculated from 10 fields counts, was multiplied by the coefficient 3.9×10^5 pre-determined for our microscope Olympus, model BX53 (total magnification—400x). The obtained results thus represent the number of *Borrelia* normalized for 1 ml culture volume.

In vivo Phagocytosis of *Borrelia afzelii* CB43

Pathogen free, adult *I. ricinus* females were fed naturally for 6 days on guinea pigs. Cultivated *B. afzelii* (5×10^4 spirochetes per tick) were injected into the hemocoel of semi-engorged females in a volume of 138 nl by microinjection (microinjector Drummond). Hemolymph samples from individual ticks were collected at defined time points (0, 1, 3, and 6 h) after injection of spirochetes and mixed with 10 μl of L15-BOFES medium supplemented with 10% fetal calf serum (PAA Laboratories) on microscope slides. Cells were fixed with 4% formaldehyde in phosphate saline buffer (PBS) for 20 min and washed 3 times with PBS. Spirochetes were stained with primary rabbit anti-*B. burgdorferi* (Thermo Scientific) antibody (1:200 in PBS), on the slides and incubated on a horizontal shaker at room temperature (RT) for 1 h. After washing with PBS (3 times/5 min), slides were stained with fluorescently labeled goat anti-rabbit secondary antibody (Alexa 594) (Molecular Probes) diluted 1:500 in PBS and incubated for 1 h at RT. Hemocytes were then permeabilized using 1% Triton X-100 in PBS with 1% BSA (Bovine serum albumin, Sigma-Aldrich) overnight at 4°C. The next day, spirochetes were restained with primary antibody (rabbit anti-*B. burgdorferi*) diluted 1:200 in 0.1% Triton in PBS (PBS-Tx) for 1 h at room temperature, and washed 3 × 5 min with 0.1 % PBS-Tx. Spirochetes were then stained with anti-rabbit secondary antibody (Alexa 488) (Molecular Probes), diluted 1:500 in PBS-Tx for 1 h at RT. Cells were then washed with PBS-Tx and nuclei were counterstained with DAPI for 10 min. After mounting in DABCO (Sigma), the number of phagocytic hemocytes was counted using a 488/594 (FITC/TexasRed) dual filter and BX51 fluorescent microscope (Olympus). For each sample, 100 hemocytes were counted.

For the experiment with latex beads (LtxB), 138 nl of 2x diluted surfactant-free red CML latex beads, 0.3 μm diameter (Interfacial Dynamics Corp.) were injected into semi-engorged fed females and, after 2 h, ticks were injected with *B. afzelii* CB43 (5×10^4). Hemolymph from individual ticks was collected 3 h after injection of *Borrelia* and phagocytic activity of hemocytes was analyzed (12 ticks for PBS control group and 15 ticks for latex beads group) as described above. The phagocytic index was determined as the number of hemocytes with ingested *Borrelia* counted for a total 100 hemocytes in the microscopic field.

Determination of Hemocyte Number after Injection of Latex Beads

Unfed *I. ricinus* females (25 per group) were injected with 138 nl of 2x diluted LtxB or PBS (control) and allowed to rest for 1 day. After that, females were fed naturally on guinea pigs for 6 days and then hemolymph samples from individual females were collected on microscope slides. Cells were fixed with 4% formaldehyde in PBS for 20 min and washed 3 times with PBS. Hemocyte nuclei were counterstained with DAPI. Hemocytes were counted on the whole slide using a BX51 Olympus fluorescence microscope.

RNAi-Mediated Silencing of Tick TEPs Linked with *In vitro* Phagocytosis Assay

dsRNAs of nine *t-teps* and *gfp* (control) were produced as described previously (Buresova et al., 2011). For each experiment, *t-teps*-specific dsRNA (0.5 μl; 3 μg/μl) was injected into the hemocoel of 25 unfed *I. ricinus* females through to the coxae using a microinjector (Drummond). Ticks were allowed to rest for 1 day and then fed for 6 days on guinea pigs. Hemolymph samples collected from 25 semi-engorged females were mixed with L15-BOFES medium supplemented with 10% fetal calf serum (PAA Laboratories) (Buresova et al., 2011; Urbanova et al., 2015). Hemocytes (4×10^4) in a volume of 150 μl were transferred onto round microscope cover slips in a 24-well culture plate, and then 10 μl of *B. afzelii* CB43 (10^8 cells/ml) were added and incubated for 2 h at 28°C. Cells were fixed with 4% formaldehyde in PBS for 20 min and washed 3 times with PBS. Spirochetes were detected by indirect immunofluorescence, using the method of double staining as described above. Phagocytosed spirochetes were counted using the BX51 Olympus fluorescence microscope. For each group, 100 hemocytes were counted on each of at least 14 slides. Relative phagocytosis was calculated in relation to the number of phagocytic hemocytes in the *gfp* dsRNA injected control group, taken as 100% for each respective experiment.

Expression of Genes Encoding *I. ricinus* Thioester-Containing Proteins in Response to Injection of *Borrelia* sp. Spirochetes

Different species of *Borrelia* (*B. burgdorferi* NE5264, *B. burgdorferi* CB26, *B. garinii* MSLB, *B. afzelii* CB43) were cultivated in BSK-H complete medium (Sigma) at 33°C for 5–7 days. All *Borrelia* species were diluted in PBS to contain 10^4 spirochetes in the injection dose. Unfed, pathogen free *I. ricinus* females were surface-sterilized by a subsequent immersion into 3% H_2O_2, 70% EtOH and sterile distilled water. A 69 nl volume of *Borrelia* suspension in sterile PBS, or BSK-H medium for controls, was injected into ticks using sterile glass capillaries and a microinjector (Drummond). After inoculation, ticks were allowed to rest for 12 h at room temperature, total RNA was

extracted from the whole body homogenates using TRI reagent® (Sigma), treated with DNAse (Ambion), and the integrity of RNA was checked by agarose gel electrophoresis. Single-stranded cDNA was reverse-transcribed from 0.5 μg of total RNA using the Transcriptor High-Fidelity cDNA Synthesis Kit (Roche). The resulting cDNA preparations served as templates for subsequent expression analysis by quantitative real-time PCR (qPCR) using a LightCycler 480 (Roche) and SYBR green chemistry. Reaction conditions and sequences of qPCR T-TEPs forward and reverse primers have been published previously (Urbanova et al., 2015). Relative expression of *t-teps* was normalized to elongation factor 1 (*ef-1*) using the mathematical model of Pfaffl (Pfaffl, 2001). For each experimental group, five *I. ricinus* females were injected in three independent biological triplicates.

Borrelia Transmission

B. afzelii CB43 spirochetes were cultivated as described above. To prepare *Borrelia*-infected nymphs for the transmission experiment, C3H/HeN mice were injected intra-dermally with 10^5 of *B. afzelii* spirochetes. After 4 weeks, pathogen-free larvae were fed on infected mice (∼100 larvae per mouse) and after repletion were kept in wet chambers at 26°C until molting. The infected nymphs (50 per group) were injected with mixed or individual dsRNAs (3 μg/μl, 64.4 nl) specifically silencing the group of IrAMs (*iram-1,2,3),* IrC3s (*irc3-1,2,3*), IrMCRs (*irmcr-1,2*), IrTep (*irtep*), *gfp* (control). For experiments with latex beads, 50 unfed *B. afzelii*-infected nymphs were micro-injected with LtxB (32 nl, 2x diluted) and 50 with sterile PBS as a control group. Following injection, nymphs were allowed to rest for 3 days, and then were fed until repletion on clean 6-weeks old C3H/HeN mice (10 nymphs per mouse, 5 mice per each experimental group) using plastic cylinders attached to the murine back. Infection of mice with *Borrelia* during the early phase following tick infestation was tested in ear tissue biopsies taken at 1 week intervals. After 4 weeks the mice were sacrificed and the *Borreli*a spirochetes were detected in the target tissues, namely the bladder and heart. The mice tissues were first tested for *Borrelia* positivity using sensitive PCR amplification of a 154 bp fragment of the *flagellin* gene. The PCR reactions contained 12.5 μl of FastStart PCR MasterMix (Roche), 4 μl of DNA extracted using Macherey-Nagel NucleoSpin®Tissue Kit (concentration in the range of 100–300 ng), 10 pmol of each primer FlaF1 (AAGCAAATTTAGGTGCTTTCCAA), FlaR1 (GCAATCATTGCCATTGCAGA) and PCR water up to 25 μl. The amplification program consisted of denaturation at 94°C for 10 min, then 40 cycles of: denaturation at 94°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 40 s. The program was finished by final extension at 72°C for 7 min. PCR products were visualized on a 1.5% agarose gel. Positive tissues were further analyzed by qPCR using a LightCycler 480 (Roche). qPCR was performed in a 25 μl reaction volume containing 12.5 μl of FastStart Universal Probe Master (Rox) (Roche), 5 μl of purified DNA, 10 pmol of each primer, FlaF1, FlaR1, and 5 pmol of TaqMan probe, FlaProbe1 (FAM-TGCTACAACCTCATCTGTCATTGTAGCATCTTTTATTTG-BHQ1) (Schwaiger et al., 2001). The remaining reaction volume was adjusted with PCR water. The qPCR program consisted of denaturation at 95°C for 10 min, followed by 50 cycles of: denaturation at 95°C for 15 s, annealing plus elongation at 60°C for 1 min. The number of spirochetes in tissues was normalized to the number of murine genomes as described previously (Dai et al., 2009).

Statistical Analysis

The appropriate statistical analyses (non-parametric Kruskal-Wallis test, non-parametric Mann-Whitney test or un-paired *t*-test) were selected for the specific data-sets and specified in the legends to the corresponding figures. All statistics was performed using GraphPadPrism (version 6.00 for Windows, GraphPad Software, San Diego, CA, USA). A *P*-value of < 0.05 was considered to be statistically significant.

RESULTS

Ixodes ricinus Plasma Did Not Exhibit Complement-Mediated Borreliacidal Activity Similar to Bovine Serum

Complement systems in sera of various vertebrate animals exhibit different borreliacidal effects against different *Borrelia* genospecies (Kurtenbach et al., 2002; Bhide et al., 2005; Ticha et al., 2016). Because *Ixodes* sp. ticks possess a primordial complement system involving three molecules related to the C3 complement component and putative convertases (Buresova et al., 2011; Kopacek et al., 2012; Urbanova et al., 2014) we tested for possible effects of tick hemolymph on *Borrelia* viability. Cultivated *B. afzelii* spirochete*s* were incubated with tick cell-free plasma, bovine serum, heat-inactivated bovine serum or BSK-H medium and tested for *Borrelia* survival after 24 h of incubation *in vitro*. Incubation of bovine serum with *B. afzelii* resulted in spirochete immobilization, lysis and cluster formation. This effect was markedly reduced by heat inactivation of serum complement. In contrast, no borreliacidal effect was observed upon incubation of spirochetes with tick plasma or BSK-H medium used as a negative control (**Figure 1**).

Tick Hemocytes Were Capable of Phagocytosing *Borrelia afzelii* Injected into the Hemocoel

As no humoral reaction against *B. afzelii* was observed in tick plasma, we further investigated phagocytic activity of tick hemocytes against spirochetes injected into the tick hemocoel. *B. afzelii* spirochetes were injected into semi-engorged females, and phagocytosis was examined in hemolymph collected at different time intervals post injection. In order to distinguish between *Borrelia* spirochetes that were ingested by tick hemocytes from attached or free spirochetes, a dual-labeling assay was exploited (**Figure 2A**). We observed that spirochetes were phagocytosed immediately after injection and a phagocytic rate of about 25% was reached after 1 h; this was maintained for at least 6 h (**Figure 2B**).

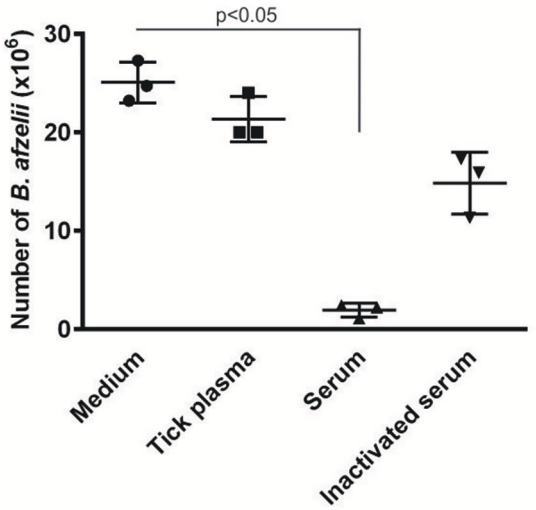

FIGURE 1 | Plasma of *I. ricinus* exhibits no borreliacidal activity against *B. afzelii* spirochetes. Spirochetes were incubated for 24 h in the presence of BSK-H medium (negative control), *I. ricinus* hemocyte-free plasma, bovine serum or heat-inactivated bovine serum. Numbers of live *Borrelia* were normalized to 1 ml volume. This result demonstrates that complement-like molecules in tick hemolymph do not exert borreliacidal effects similar to that of the complement system present in bovine serum. Data were analyzed by non-parametric Kruskal-Wallis test.

Phagocytosis of *B. afzelii* Was Eliminated by Injection of Latex Beads

Following the evidence that injection of latex or polystyrene beads suppresses phagocytosis in fruit fly (Nehme et al., 2011) or ticks (Liu et al., 2011), we performed an experiment whereby LtxB was injected into the hemocoel of semi-engorged *I. ricinus* females 2 h prior to injection of *B. afzelii*. Phagocytosis was evaluated as described above, 3 h after spirochete injection (**Figure 3A**). Pre-injection of LtxB resulted in a significant reduction in spirochete phagocytosis, where the phagocytic index decreased from 27% for the PBS control to 4% for LtxB pre-injection, while the numbers of hemocytes 5 h after LtxB or PBS injections were the same (**Figure 3B**).

Intriguingly, injection of LtxB into the hemocoel of unfed females that were further allowed to feed naturally for 6 days resulted in almost complete clearance of hemocytes from tick hemolymph compared to ticks pre-injected with PBS as a control (**Figure 4**). Despite this striking phenotype, the ticks were capable of feeding with no obvious impact on their fitness or fecundity.

Function of Tick TEPs in Phagocytosis of *B. afzelii*

In previous work, we demonstrated that different T-TEPs play non-redundant roles in the phagocytosis of Gram-negative bacteria (Buresova et al., 2011) or the yeast *C. albicans* (Urbanova et al., 2015). A similar experimental setup combining RNAi-mediated silencing of individual T-TEPs followed by an *in vitro* phagocytosis assay was used to identify T-TEPs playing a role in the phagocytosis of *Borrelia* spirochetes by tick hemocytes. Unfed *I. ricinus* females were injected with gene-specific *t-tep*s dsRNA or *gfp* dsRNA as a negative control. Ticks were allowed to feed naturally for 6 days, then the hemolymph was collected from semi-engorged females and used for an *in vitro* phagocytosis assay based on *B. afzelii* double immunostaining. Out of nine T-TEPs tested, only silencing of *irc3-2* and *irc3-3* significantly decreased phagocytosis of *B. afzelii*. In contrast, knockdown (KD) of the insect-type *irtep* led to a surprising increase in phagocytosis of spirochetes, by about 20% compared to the GFP control (**Figure 5**).

Expression Response of Genes Encoding *I. ricinus* Thioester-Containing Proteins to Injection of *Borrelia* sp. Spirochetes

We have previously shown that expression of genes encoding *I. ricinus* T-TEPs responds differentially to different model microbes (*E. coli, Micrococcus luteus, or C. albicans*) or to aseptic injury (Urbanova et al., 2015). Here we have analyzed the expression response of *t-teps* to injection of available species of the *B. burgdorferi* sensu lato complex. Surface-sterilized unfed *I. ricinus* females were injected with sterile PBS as an aseptic injection control (injury), four different cultivated *Borrelia* species, and BSK-H medium alone as a mock. Total RNA was isolated 12 h after injection from whole body homogenates and mRNA levels of the genes encoding *t-teps* were determined by qPCR. Gene *irc3-1* was the only *t-tep* for which, expression seemed to be up-regulated upon injection of the tested *Borrelia* genospecies (2–3 times, in relation to the PBS and BSK-H injection controls) (**Figure 6**). Expression of other *t-teps* did not change in response to any *Borrelia* species or injection injury (Figure S1).

Changes in *Borrelia* Phagocytosis in Ticks Had No Effect on Spirochete Burden in Murine Tissues

In order to examine whether or not immune reactions in the tick hemocoel affect *Borrelia* transmission to the host, we adapted the laboratory model for *Borrelia* transmission developed for *B. burgdorferi* sensu stricto and *I. scapularis* (Ramamoorthi et al., 2005; Dai et al., 2009) and applied it for *I. ricinus* nymphs infected with *B. afzelii* CB43 as described above. Groups of unfed, infected nymphs were injected with four combinations of dsRNAs, corresponding to the four classes of tick TEPs: (i) α_2-macroglobulins (IrA$_2$M-1,2,3); (ii) C3-complement component (IrC3-1,2,3); (iii) macroglobulin-complement-related (IrMcr-1,2), and (iv) insect-type IrTep, respectively. The efficiency of RNAi combinatorial KD was verified by qRT-PCR using cDNA prepared from a pool of five randomly selected nymphs. Additionally, we also tested whether the elimination of phagocytosis that followed pre-injection of LtxB (**Figure 3B**) affected transmission of *B. afzelii*. Mice infected with *Borrelia* during the early phase following tick infestation were tested in ear tissue biopsies taken at 1 week intervals. After 4 weeks, the mice were sacrificed and *Borrelia* spirochetes were detected in the bladder and heart (**Table 1**). The *Borrelia* numbers

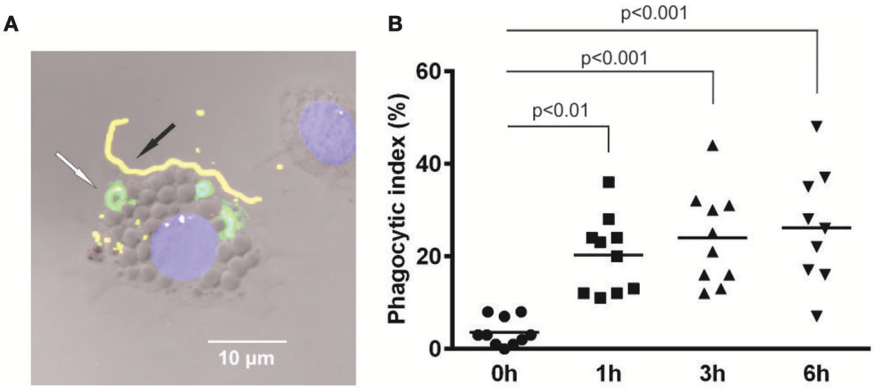

FIGURE 2 | *B. afzelii* spirochetes are actively phagocytosed by tick hemocytes upon inoculation into the tick hemocoel. (A) Double immunostaining of phagocytic *I. ricinus* hemocytes distinguished between ingested and attached *B. afzelii*. Green—ingested *Borrelia* inside the hemocyte (white arrow); Yellow—attached *Borrelia* outside the hemocyte (black arrow); Blue—nucleus of hemocyte-stained with DAPI; Nomarski contrast. **(B)** Semi-engorged females were injected with 5×10^4 of *B. afzelii* CB43. Hemolymph samples from different time points after injection were collected from individual ticks (10 ticks per time point) and the phagocytic index was determined as the number of hemocytes with ingested *Borrelia* per 100 hemocytes in the microscope field. Data were analyzed by non-parametric Kruskal-Wallis test.

in ear biopsies fluctuated, ranging from a few to several thousand spirochetes, usually reaching a maxima from the 2nd to 3rd week following infestation and then gradually decreasing (Table S1). The spirochete burdens in bladders and hearts in the 4th week after infestation were relatively stable, in the range of several hundreds of spirochetes per murine genome (**Figure 7**). The only statistically significant decrease of *Borrelia* load compared to the GFP control was observed in mice heart upon group silencing of *irc3-1,2,3* genes. Based on these results we conclude that T-TEPS and phagocytosis do not substantially affect the competence of *I. ricinus* to act as a vector for Lyme disease.

DISCUSSION

The concept of transmission of Lyme disease spirochetes from infected *Ixodes* sp. ticks to susceptible hosts via the salivary route, as proposed in the late eighties (Ribeiro et al., 1987), has been corroborated by a number of seminal studies published over the past three decades (De Silva and Fikrig, 1995; Coleman et al., 1997; Hojgaard et al., 2008; Dunham-Ems et al., 2009). Several tick proteins have been demonstrated to be involved in tick-*Borrelia* interactions and play roles in spirochete acquisition, midgut colonization, penetration of the midgut epithelium or shielding *Borrelia* against host immune and inflammatory responses at the tick-host interface (Pal et al., 2004; Ramamoorthi et al., 2005; Narasimhan et al., 2007; Dai et al., 2010; Schuijt et al., 2011; Zhang et al., 2011) or see (Hajdusek et al., 2013; Kung et al., 2013) for review. Except for the recently described antimicrobial peptide Dae2 (domesticated amidase effector), induced by tick GTPase (Chou et al., 2015; Smith et al., 2016), no other tick molecule has been described to limit *Borrelia* proliferation within a tick vector.

We have previously reported that ticks possess molecules related to components of the mammalian complement system (Buresova et al., 2011; Kopacek et al., 2012; Urbanova et al., 2015) and therefore we questioned whether tick cell-free plasma can exhibit lytic activity against *Borrelia,* as demonstrated for a variety of vertebrate animals (Kurtenbach et al., 1998, 2002; Kuo et al., 2000; Bhide et al., 2005; De Taeye et al., 2013; Ticha et al., 2016). The results shown in the **Figure 1** clearly demonstrate that there is almost no effect of *I. ricinus* plasma against cultivated spirochetes compared to bovine serum, which is known to have strong complement-mediated borreliacidal activity (Kurtenbach et al., 1998; Bhide et al., 2005). This result is in accord with previous work demonstrating that plasma from *I. scapularis*, (the competent *Lyme disease* vector in the USA) had no borreliacidal activity (Johns et al., 2001). In contrast, plasma from the refractory *D. variabilis* substantially reduced the survival of incubated *Borrelia*. In addition, the authors also reported that clearance of spirochetes inoculated into the hemocoel of both species was much faster in *D. variabilis* compared to *I. scapularis* (Johns et al., 2001).

Among cellular reactions, phagocytosis is believed to play the most important defense role against microbial infections of arthropods, including ticks, and have been reported to suppress *Borrelia* numbers migrating through the tick hemolymph toward the salivary glands (Munderloh and Kurtti, 1995; Coleman et al., 1997; Dunham-Ems et al., 2009). Two different mechanisms, namely conventional and coiling phagocytosis, were reported for engulfment of *B. burgdorferi* by *I. ricinus* hemocytes (plasmatocytes and granulocytes of type II) similar to that of mammalian phagocytic cells (Rittig et al., 1996). Upon direct inoculation of cultivated *B. afzelii* into the hemocoel of *I. ricinus,* we observed an immediate and potent phagocytic activity of tick hemocytes, reaching a phagocytic index of about 25% (**Figure 2**). The phagocytic activity of tick hemocytes could be almost completely abolished by pre-injection of latex beads into the hemocoel (**Figure 3**).

Our *in vitro* phagocytosis assays following specific gene knockdown of individual *t-teps* demonstrated that phagocytosis

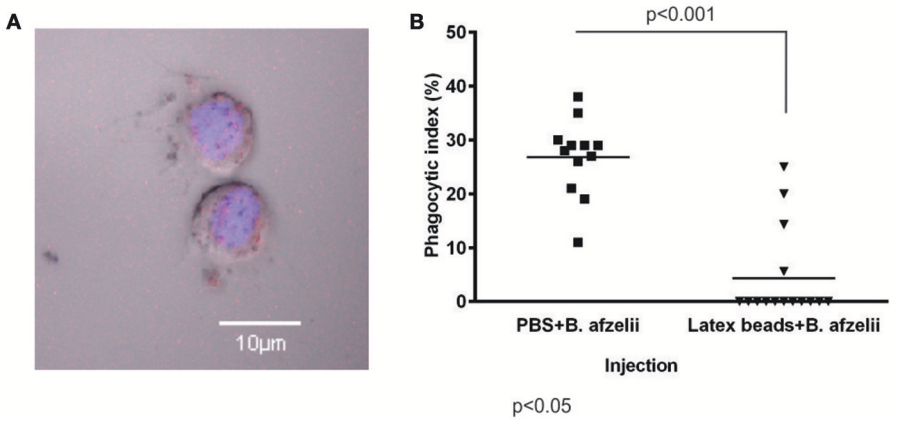

FIGURE 3 | Phagocytosis of *B. afzelii* by tick hemocytes is eliminated by pre-injection of latex beads into the hemocoel. (A) Tick hemocyte with ingested latex beads. Red—latex beads; Blue—nucleus of hemocytes-stained with DAPI; Nomarski contrast. **(B)** Pre-injection of latex beads (138 nl, 2x diluted) into the hemocoel significantly reduced phagocytosis of *B. afzelii* CB43 injected 2 h later. Hemolymph was collected from individual ticks (12 ticks for PBS and 15 ticks for latex beads) 3 h after injection of *Borrelia* and hemocytes were examined for phagocytosis of spirochetes. The phagocytic index was determined as the number of hemocytes with ingested *Borrelia* per 100 hemocytes in the microscope field. The number of hemocytes did not differ between experimental groups. Data were analyzed by non-parametric Mann-Whitney test.

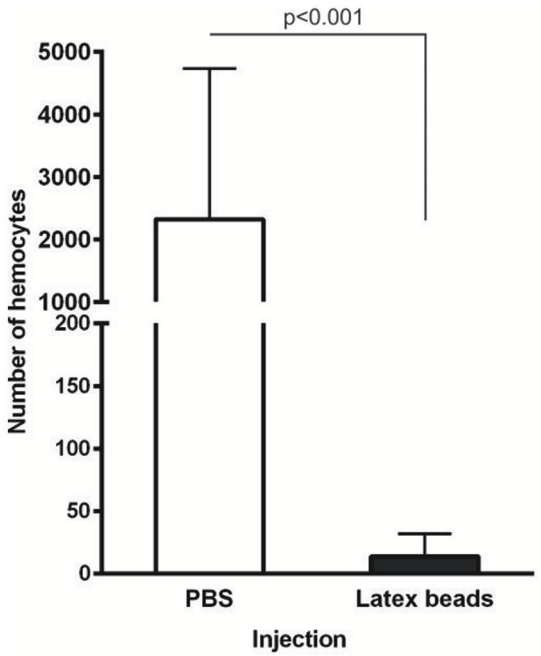

FIGURE 4 | Pre-injection of latex beads into unfed female *I. ricinus* results in hemocyte clearance from the tick hemocoel during feeding. Unfed *I. ricinus* females were injected with LtxB (7 and 9 ticks) or sterile PBS (6 and 6 ticks) in two independent biological experiments. The females were allowed to feed naturally for 6 days and the hemolymph was collected from individual semi-engorged ticks. Hemocytes were counted on the whole slide. Data were analyzed by unpaired *t*-test. The error bars represent standard deviations.

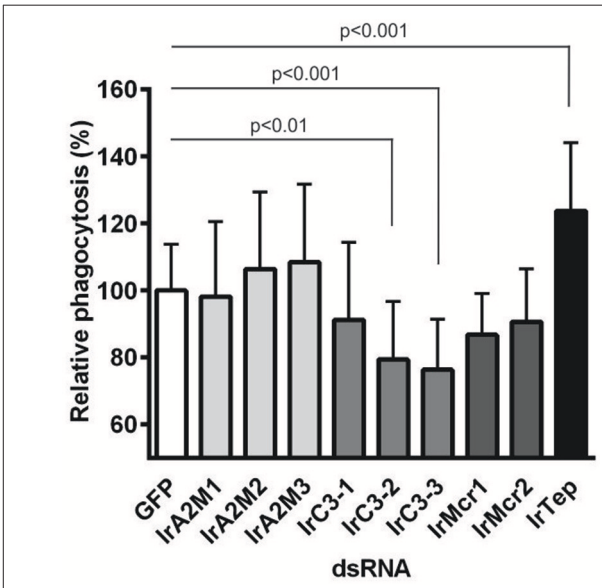

FIGURE 5 | The role of tick thioester-containing proteins in *Borrelia* phagocytosis. Groups of 25 unfed *I. ricinus* females were injected with individual *t-teps* dsRNA or *gfp* dsRNA for control. Hemolymph collected and pooled from semi-engorged ticks was incubated with *B. afzelii* CB43 spirochetes *in vitro*. The number of phagocytic hemocytes upon silencing of individual *t-teps* was related to that obtained for the *gfp* dsRNA injected control group taken as 100% in the respective experiment. Average results (bars) and standard deviations (error bars) from three independent experiments are shown. Data were analyzed by Kruskal-Wallis test, followed by Dunn's multiple comparisons test.

of *B. afzelii* CB43 was significantly reduced upon silencing of *irc3-2* and *irc3-3* and increased upon knockdown of *irtep* (**Figure 5**). For comparison, phagocytosis of Gram-negative bacteria *C. indologenes* (pathogenic to ticks) (Buresova et al., 2006) is mediated mainly by IrA2M-1,2 and IrC3-3. We proposed that involvement of α_2-macroglobulins in the cellular response against these bacteria was possible given the interaction

of these macromolecular protease inhibitors with the potent Zn^{2+}-dependent metalloprotease secreted by *C. indologenes* (Buresova et al., 2009). Phagocytosis of the model Gram-negative bacteria *E. coli* involves IrTep and IrC3-3 (Buresova et al., 2011), whereas for phagocytosis of the yeast *C. albicans,* the main role is carried out by IrC3-1 and IrMcr-2 (Urbanova et al., 2015). A similar non-redundant role of members of the TEP family has also been shown for the mosquito *Anopheles gambiae,* where silencing of *Ag*Tep1 and *Ag*Tep4 significantly inhibited phagocytosis of *E. coli* as well as the Gram-positive *S. aureus*, while RNAi silencing of mosquito *Ag*Tep3 only reduced phagocytosis of *E. coli* but not *S. aureus* (Moita et al., 2005). A study exploiting *D. melanogaster* S2 cells revealed that *Dm*Tep2, *Dm*Tep3 and *Dm*Tep6 were specifically required for phagocytosis of *E. coli*, *S. aureus,* and *C. albicans,* respectively (Stroschein-Stevenson et al., 2006). We also found that inoculation of different strains of the *B. burgdorferi* sensu lato complex up-regulated only *irc3-1* expression (**Figure 6**), while expression of other *t-teps* did not seem to be affected (Figure S1). A similar obvious up-regulation of *irc3-1* mRNA levels was observed upon injection of *C. albicans* (Urbanova et al., 2015), suggesting that the immune responses to *Borrelia* and yeast might be controlled by the same or related signaling pathways.

Earlier, it was elegantly demonstrated using fluorescent (GFP-expressing) *B. burgdorferi* that penetration of spirochetes from the midgut to the hemocoel was quite a rare event (Dunham-Ems et al., 2009), which agreed with other studies showing that the number of spirochetes that disseminate in tick hemolymph is very low compared to their massive presence in the tick midgut (Munderloh and Kurtti, 1995; Coleman et al., 1997; Zhang et al., 2011). The fact that the efficient phagocytic response of tick hemocytes is not capable of complete elimination the few *Borrelia* that migrate through the hemocoel toward the salivary glands could be possibly explained by an extremely fast movement of the motile spirochetes (Malawista and de Boisfleury Chevance, 2008; Dunham-Ems et al., 2009). Assuming that phagocytosis of *Borrelia* by tick hemocytes indeed reduces their number in the tick hemocoel and thereby negatively affects transmission of the spirochetes to the host via the salivary glands, RNAi-mediated silencing of *irc3-2* and *irc3-3* should result in a higher *Borrelia* number in tick salivary glands and subsequently in increased burden in murine tissues. Conversely, stimulation of phagocytosis by silencing of *irtep* should theoretically lead to reduced infections. However, the T-TEPs group-specific silencing in infected nymphs followed by their feeding on naïve mice did not confirm this view. RNAi-mediated silencing of *t-teps* did not result in any meaningful relationship with spirochete burden detected in ear biopsies during the early stages of infection. Only group silencing of *irc3-1,2,3* resulted in an apparently lower spirochete burden in the murine heart (**Figure 7**), however, more demanding transmission experiments should be performed either to reinforce or modify the statistical significance of this result for a reasonable interpretation.

Another scenario we can speculate about is that engulfment of spirochetes by tick hemocytes actually protects *Borrelia* against antimicrobial activity in tick plasma during their movement from the gut to the salivary glands. A "protective" role of tick hemocytes was proposed for the intracellular tick-borne pathogen, *A. phagocytophilum* (Liu et al., 2011). Secreted *I. scapularis* protein11 (P11) binds to bacteria and facilitates infection of tick hemocytes that serve as a vehicle for the internalized pathogen on its route toward the salivary glands. Silencing of P11 by RNAi or immunization of mice with anti-P11 antibodies significantly suppressed the *Anaplasma* burden in the tick hemolymph or salivary glands. The same effect was achieved by inhibition of *A. phagocytophilum* phagocytosis by injection of polystyrene beads into the *I. scapularis* hemocoel (Liu et al., 2011). Regarding the extracellular *Borrelia* spirochetes, the previous work by Johns et al. (2000, 2001) reported that *B. burgdorferi* inoculated into the *I. scapularis* hemocoel were eliminated much more slowly compared to the same experiment performed with the incompetent vector *D. variabilis.* Another *in vitro* study focused on phagocytosis of GFP-expressing *B. burgdorferi* by tick cell lines IDE12 and DAE15 derived from *I. scapularis* and *D. andersoni*, respectively, demonstrated that IDE12 cells required significantly more time to internalize and kill the spirochetes relative to DEA15 cells. Some intact coiled spirochetes (retaining GFP fluorescence) could be found in IDE12 cells as late as 7 days following their co-incubation (Mattila et al., 2007). However, our result showing that pre-injection of latex beads into infected nymphs had no apparent effect on *Borrelia* transmission (**Figure 7B**) suggests that tick

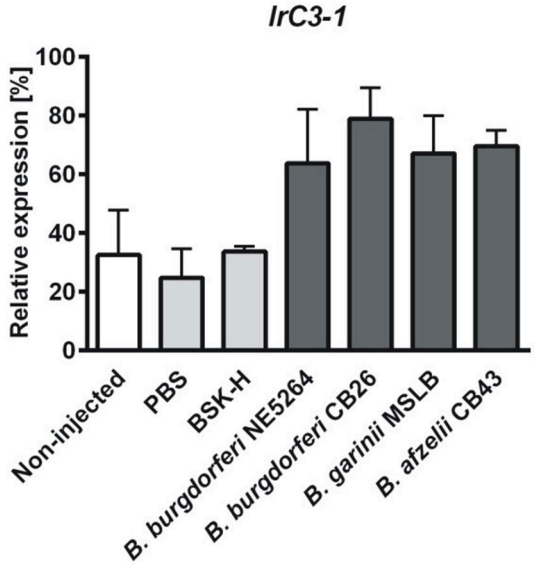

FIGURE 6 | Injection of *Borrelia* sp. affects expression of *irc3-1 gene*. Adult unfed *I. ricinus* females were injected with four different species of *Borrelia burgdorferi* s.l. complex (*B. burgdorferi* NE5264, *B. burgdorferi* CB26, *B. garinii* MSLB and *B. afzelii* CB43) or with sterile PBS, BSK-H medium as aseptic injection control and mock, respectively. Total RNA was isolated from the whole body homogenates, 12 h after inoculation, and transcribed into cDNA. Expression is shown in relation to *elongation factor-1* as a housekeeping gene. Expression of other *t-tep* genes were not responsive to *Borrelia* sp. injection (Figure S1). The error bars represent standard errors from three independent biological replicates. Data were analyzed by non-parametric Kruskal-Wallis test.

TABLE 1 | PCR detection of *Borrelia afzelii* CB43[a] in murine tissues after infestation with infected nymphs pre-injected with dsRNA or latex beads.

dsRNA KD/Injection	Ears 1st week	Ears 2nd week	Ears 3rd week	Ears 4th week	Bladder	Heart
GFP	0/5	5/5	5/5	5/5	5/5	5/5
IrA$_2$M-1,2,3	0/5	5/5	5/5	5/5	5/5	5/5
IrTep	0/5	5/5	5/5	5/5	5/5	5/5
IrC3-1,2,3	0/5	5/5	5/5	5/5	5/5	5/5
IrMcr-1,2	0/5	5/5	5/5	5/5	5/5	5/5
Latex beads	0/5	5/5	5/5	5/5	5/5	5/5

[a]*PCR detection based on amplification of flagellin B gene. Displayed are number of positive/number of examined mice.*

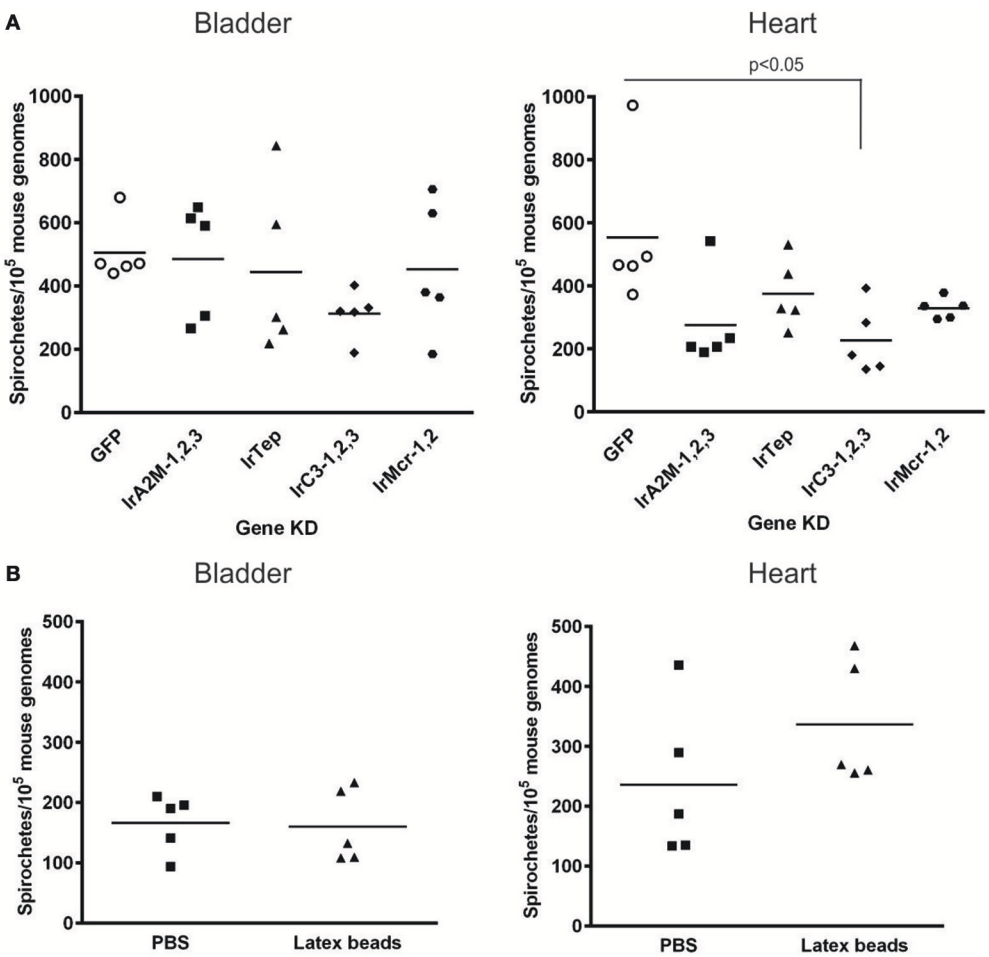

FIGURE 7 | Spirochete levels in murine bladder and heart biopsies in the 4th week after nymphal feeding. (A) Infection of naïve mice with *B. afzelii* CB43 transmitted from naturally infected nymphs pre-injected with different group combinations of *t-tep* dsRNAs or *gfp* dsRNA as a control. **(B)** Infection of naïve mice with *B. afzelii* CB43 from naturally infected nymphs pre-injected with LtxB or PBS as a control. For each individual group (*t-teps*, *gfp*, LtxB, or PBS), five mice were infested with 10 nymphs. The number of *Borrelia* in the target tissues (bladder and heart) was determined by qPCR 4 weeks after infestation. The number of *Borrelia* was normalized to 10^5 mouse genomes. **(A)** Data were analyzed by non-parametric Kruskal-Wallis test; **(B)** data were analyzed by unpaired *t*-test.

hemocytes do not protect spirochetes on their route toward the salivary glands.

Certainly, many other factors might be affected by manipulating the tick complement-like immune responses, such as an impaired balance between *Borrelia* spirochetes and the commensal microflora (Narasimhan and Fikrig, 2015), which makes an unequivocal interpretation of our results more difficult. Therefore, more detailed investigations of tick-*Borrelia* inter-relationships in the midgut, hemocoel and salivary glands of refractory vs. competent tick species (Johns et al., 2001; Soares et al., 2006; Mattila et al., 2007) might shed more light which tissue plays the decisive role

that determines the tick's capacity to act as a vector for Lyme disease.

AUTHOR CONTRIBUTIONS

VU, OH, RS, PK conceived the study and designed experiments. VU, OH, HH, RS performed the experiments and analyzed data. VU, PK wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00073/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Alternative Splicing of Differentiated Myeloid Cell Transcripts after Infection by *Anaplasma phagocytophilum* Impacts a Selective Group of Cellular Programs

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Eukaryotic proteome diversity exceeds that encoded within individual genes, and results in part from alternative splicing events of pre-messenger RNA. The diversity of these splicing events can shape the outcome in development and differentiation of normal tissues, and is important in pathogenic circumstances such as cancer and some heritable conditions. A role for alternative splicing of eukaryotic genes in response to viral and intracellular bacterial infections has only recently been recognized, and plays an important role in providing fitness for microbial survival, while potentially enhancing pathogenicity. *Anaplasma phagocytophilum* survives within mammalian neutrophils by reshaping transcriptional programs that govern cellular functions. We applied next generation RNAseq to ATRA-differentiated HL-60 cells established to possess transcriptional and functional responses similar to *A. phagocytophilum*-infected human neutrophils. This demonstrated an increase in transcripts with infection and high proportion of alternatively spliced transcript events (ASEs) for which predicted gene ontology processes were in part distinct from those identified by evaluation of single transcripts or gene-level analyses alone. The alternative isoforms are not on average shorter, and no alternative splicing in genes encoding spliceosome components is noted. Although not evident at gene-level analyses, individual spliceosome transcripts that impact nearly all spliceosome components were significantly upregulated. How the distinct GO processes predicted by ASEs are regulated by infection and whether they are relevant to fitness or pathogenicity of *A. phagocytophilum* should be addressed in more detailed studies.

Keywords: *Anaplasma phagocytophilum*, intracellular infection, RNA isoforms, RNAseq, spliceosome, gene ontology

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INTRODUCTION

The small genomes of many obligate intracellular bacteria provide a conundrum as to the limited genomic resources required to promote fitness in the environment of a host cell with a genome reservoir thousands times greater. Recent years have demonstrated the interplay between the genomic products of intracellular bacteria and those of their host cells, including an increasing

body of literature implicating direct action on the host genome by microbial effectors (Bierne and Cossart, 2012; Sinclair et al., 2014; Prokop et al., 2017). Such is the case with the tick-transmitted obligate intracellular rickettsia, *Anaplasma phagocytophilum* (Garcia-Garcia et al., 2009a,b; Rennoll-Bankert et al., 2015; Sinclair et al., 2015; Dumler et al., 2016). While its reserves of effectors are multiple and many have moonlighting functions (Lin et al., 2007; Truchan et al., 2013), the extent of neutrophil reprogramming that impacts bacterial fitness after infection is difficult to explain (Carlyon et al., 2002; Choi and Dumler, 2003; Choi et al., 2003, 2004a, 2005; Park et al., 2003; Garyu et al., 2005; Carlyon and Fikrig, 2006). Chromatin reconfiguration and transcriptional reprogramming under the control of microbial effectors, including AnkA, demonstrate that the extended genome of *A. phagocytophilum* includes those targets in the genome of the host cell as well.

A. phagocytophilum reprogramming of specific functions, such as respiratory burst driven by AnkA recruitment of HDAC-1 to the promoter of *CYBB* is an example of *cis*-gene silencing by restructuring of chromatin histone H3 at the gene promoter (Garcia-Garcia et al., 2009a,b; Rennoll-Bankert et al., 2015), yet global reprogramming becomes a greater challenge to explain. Although AnkA binds genomic sites broadly across every human chromosome in human model systems, a direct link between AnkA binding and transcriptional program changes is still not well-investigated (Park et al., 2004; Dumler et al., 2016). Stronger candidates for transcriptional and functional reprogramming include the sequestration of AnkA-bound DNA into the nuclear lamina that reshapes nuclear architecture and potentially cellular programs, and the magnitude of host cell DNA methylation after infection across all genomic features which provide opportunities for further exploration (Sinclair et al., 2015; Dumler et al., 2016). While transcriptional regulation is governed by many factors, the RNA landscape of cells plays a major role in events such as induced pluripotent stem cell reprogramming (Gamazon and Stranger, 2014), cellular differentiation (Fiszbein and Kornblihtt, 2017; Keightley and Lieschke, 2017), and oncogenesis (Narayanan et al., 2017). In fact, the RNA landscapes before and after infection by viruses and bacteria such as *Mycobacterium tuberculosis* and *Mycoplasma pneumoniae* demonstrate a role for alternative transcript splicing events as key fitness determinants that regulate intracellular survival and transmission (Akusjarvi, 2008; Boudreault et al., 2016; Hu et al., 2016; Graham and Faizo, 2017; Kalam et al., 2017; Wang et al., 2017). While methylated DNA in exons is well-known to play a role in alternative splicing events (ASEs), a role for this in infections has not been examined (Shukla et al., 2011; Maunakea et al., 2013; Lev Maor et al., 2015).

In this work, we interrogate a model of all-trans retinoic acid (ATRA)-differentiated HL-60 cells infected by *A. phagocytophilum* that we previously demonstrated to have transcriptional profiles most closely similar to *ex vivo* human neutrophils (Rennoll-Bankert et al., 2014), and demonstrate that ASEs occur in 18% of over 600 differentially expressed transcripts. Gene ontology processes enriched within this subset of genes that undergo alternative splicing map to unique pathways not identified by gene-level analyses. The lack of marked changes in alternative splicing among spliceosome genes as observed with *M. tuberculosis* infection of macrophages, and the lack of a significant change in overall transcript size among ASEs as observed with viral infection demonstrate that *A. phagocytophilum* infection is associated with a distinct profile of ASEs. These findings provide additional support for the role that alternative splicing plays in infection and microbial fitness within intracellular niches, and provides another example of complexity in how microbes regulate host gene expression via alternative splicing.

MATERIALS AND METHODS

A. phagocytophilum Infection in ATRA-Differentiated HL-60 Cell Model

We used the model as we previously described (Rennoll-Bankert et al., 2014). Briefly, the human promyelocytic HL-60 (ATCC CCL-240) cell line was purchased from American Type Culture Collection (Manassas, VA). HL-60 cells were differentiated 5 days with 1 μM ATRA prior to infection. Cells were grown in a humidified incubator at 37°C with 5% CO_2. Cell density was kept $<10^6$ cells mL^{-1} by diluting with fresh medium. Infection was established by inoculating low passage (<10 passages *in vitro*) *A. phagocytophilum* (Webster strain^T)-infected HL-60 cells into freshly prepared HL-60 cells to contain ∼20% infected cells. After infection was established, the proportion of infected cells was adjusted to 10–20% with uninfected HL-60 cells and ATRA was added to the medium. After 5 days, triplicate cultures that contained >90% infected cells and triplicate uninfected cultures were harvested. RNA was prepared using the Zymo Quick-RNA miniprep (Irvine, CA) kit. Control ATRA-differentiated HL-60 cells were maintained in parallel but uninfected.

TruSeq RNA-Seq Libraries, and Illumina HiSeq2000 Sequencing

Illumina RNA-Seq libraries were prepared with the TruSeq RNA Sample Prep kit (Illumina, San Diego, CA) per manufacturer's protocol. Adapters containing six nucleotide indexes were ligated to the double-stranded cDNA. The DNA was purified between enzymatic reactions and library size selection was performed with AMPure XT beads (Beckman Coulter Genomics, Danvers, MA). Libraries were multiplexed in two groups of three per flowcell lane using a 100 bp paired-end run.

RNAseq Alignment and Visualization

The RNAseq alignment and visualization pipeline used the FastX-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) for quality control and read trimming. Subsequently, short RNAseq reads were aligned using TopHat, a splice-aware aligner which is specifically built upon the Bowtie short read aligner for eukaryotic genomes (Trapnell et al., 2009; Langmead, 2010; Langmead and Salzberg, 2012) against the GRCh37 human genome.

RNAseq Differential Expression Analysis

The pipeline output was used to perform differential gene expression by fold-change calculations on normalized RPKM

(Mortazavi et al., 2008) (reads per kilobase per million mapped reads) values to measure gene level expression or FPKM (fragments per kilobase per million) values to measure isoform level expression. We used Cuffdiff (Trapnell et al., 2010) to identify differentially expressed genes or isoforms under infected and uninfected conditions. To compare results with previous expression profiling studies of *A. phagocytophilum* infection in human myeloid cells and primary neutrophils, differentially expressed genes from five other studies (Borjesson et al., 2005; de la Fuente et al., 2005; Pedra et al., 2005; Lee and Goodman, 2006; Lee et al., 2008), all conducted using microarray technologies, were compared for shared differentially regulated genes and for GO processes (Gene Ontology enRIchment anaLysis and visuaLizAtion tool [Gorilla] http://cbl-gorilla.cs.technion.ac.il/) (Eden et al., 2009) predicted based on those differentially regulated genes. Comparisons were visualized by creating Venn diagrams (http://bioinforx.com/free/bxarrays/venndiagram.php) using all differentially-expressed genes identified at p-values below 0.05 and absolute $\log_2$ fold change >1, and by comparing GO processes numbers identified when analyzing each of the sets of differentially expressed genes.

Analysis of Alternative Splicing

ASEs were identified by comparison of individual transcripts to gene records, and filtered to keep only isoform data with at least two replicates for both *A. phagocytophilum*-infected ATRA-differentiated HL-60 cells and uninfected cells. Events with a p-value < 0.05 were retained. To ensure higher stringency, the ASEs were further filtered with a cutoff Q-value < 0.05 and fold-change in isoform transcription between infected and uninfected conditions greater than 2 to obtain a total initial set. Further filtering to assure sufficient transcript number included only isoforms for which FPKM was >1; these were evaluated for percent spliced-in (PSI) metrics, as described previously (Wang et al., 2008; Boudreault et al., 2016). In brief, PSI estimates diversity of transcript isoforms (to characterize inclusion of exon, differential splice-site choice, intron retention, etc.) for a single gene as a function of the longest isoform/longest + shorter isoforms (ψ =L/(L+S)). From these events, only those with a difference higher than 7.5% in PSI among infected cells were considered biologically relevant.

Analysis of Changes in Spliceosome-Associated Isoform Expression

To analyze the participation of regulated expression of spliceosome components on alternative splicing events, a list of genes involved in splicing events was downloaded from the spliceosome database (http://spliceosomedb.ucsc.edu). This list included 135 genes involved in splicing that were used to query the differentially transcribed genes and identified by RNAseq. The spliceosome genes were then further queried to identify whether other isoform variants were differentially expressed in either infected of uninfected cells, and to determine whether the isoforms identified were considered the principal variant, or one of several alternative splicing events that could impact expression and therefore splicing events.

Gene Ontology Analysis of Gene-Level and Transcript Isoform-Level Expression

In order to discern specific pathways enriched with infection, Gene Ontology analysis was conducted using the gene-level differentially expressed genes, using genes identified by all isoforms, and genes with alternatively spliced isoforms that were significantly expressed. The GO analyses were conducted using Gorilla, as above, and for specific ASE isoforms, Panther GO was used (http://www.pantherdb.org/). For gene-level analyses, differentially expressed genes were compared with the GRCh37 list of genes as background; for transcript analyses, differentially expressed gene IDs derived from the transcript were examined using all transcripts identified by RNAseq that met quality control conditions as background; for transcripts with alternatively spliced isoforms, the list was ranked by absolute value of ΔPSI before GO process analysis compared with all differentially expressed transcripts, after assigning transcripts to specific gene IDs. GO Processes with p-values < 1.00E-03 were used for constructing graphical and Venn diagrams (http://bioinforx.com/free/bxarrays/venndiagram.php). GO Processes were ranked by $-\log_{10}$ p-value (pval) for display.

RESULTS

RNAseq Transcriptome of *A. phagocytophilum*-Infected ATRA-Differentiated HL-60 Cells

Read metrics indicated high quality results for RNAseq, with averages of > 81,000,000 and 89,000,000 reads for infected and uninfected cells, respectively, with >85 and 75% mapped, respectively, and >93.5% mapped to exons in both conditions (**Figures S1, S2**). A total of 1,740 differentially-expressed genes were identified after QC measures with *A. phagocytophilum* infection, including 968 upregulated and 772 downregulated more than 2-fold compared to uninfected ATRA-differentiated HL-60 cells. To assess the overall change in gene-level transcript reads between infected and uninfected cells, we examined the number of gene-level transcripts in RPKM infected and uninfected cells and found there was a marked increase in transcripts with infection (**Figure 1**). Similarly, when the number of gene transcripts in infected and uninfected cells was examined with respect to differential transcription, there was an increase in transcript quantities among upregulated genes with infection (**Figure 2**). To determine which biological pathways were most impacted by the changes in gene-level transcription, we conducted GO process analysis using a set of 1353 genes recognized within the GO gene term sets. A total of 64 GO processes were enriched with p-values ranging from <0.001 to 1.16×10^{-7}, and ranging in enrichment from 1.07 to 1353. As anticipated from prior *A. phagocytophilum* transcriptome studies in human myeloid cells, processes identified with GO analysis included cell surface

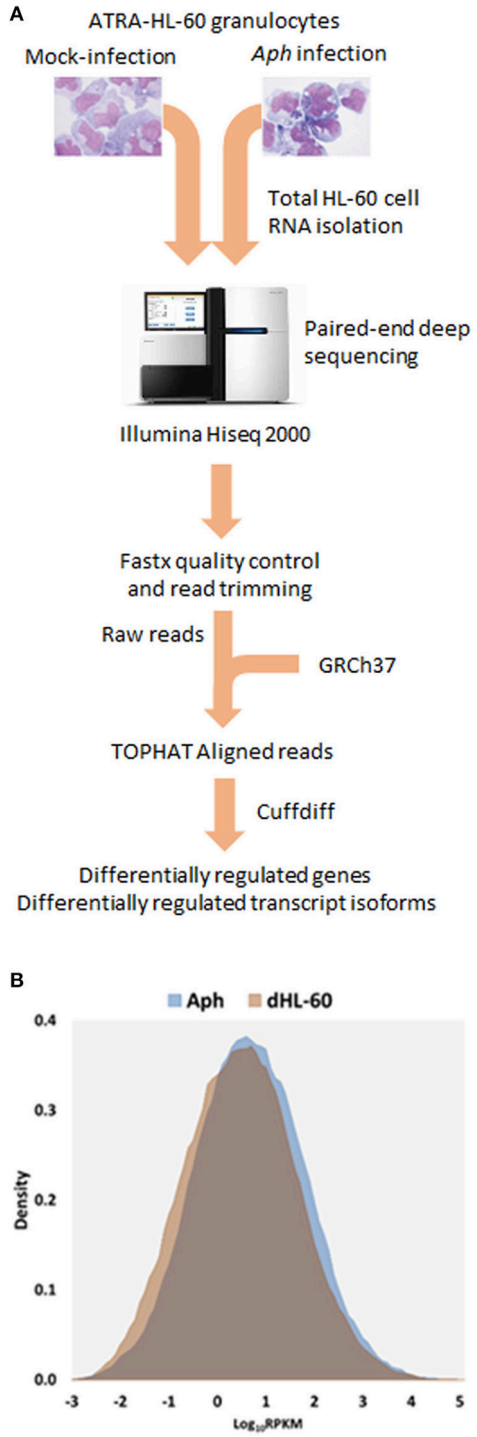


FIGURE 1 | (A) Flow-chart of the RNAseq experiments. *A. phagocytophilum*-infected and uninfected ATRA differentiated HL-60 granulocytes were propagated for 5 days until heavily infected; total host RNA was isolated and sent for RNAseq, and then raw reads were assembled after RNAseq, to obtain RPMK (gene level analyses) and FPMK (transcript-level analyses). **(B)** Density plots overlaying distribution of gene level RPKM in *A. phagocytophilum* infected and uninfected ATRA-differentiated HL-60 cells. Note the marked increase in overall gene-level transcript quantities, as noted by a shift in the density plot.

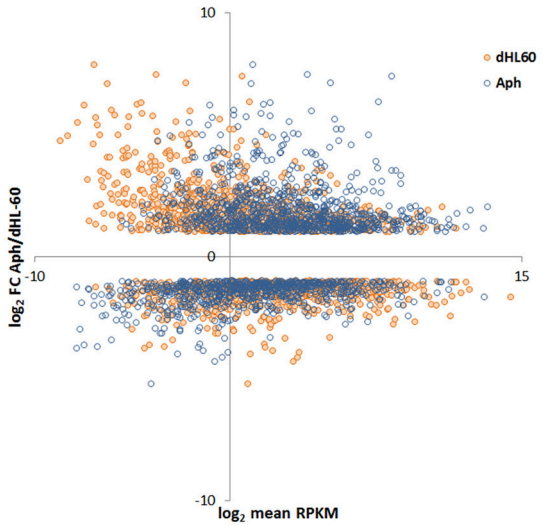
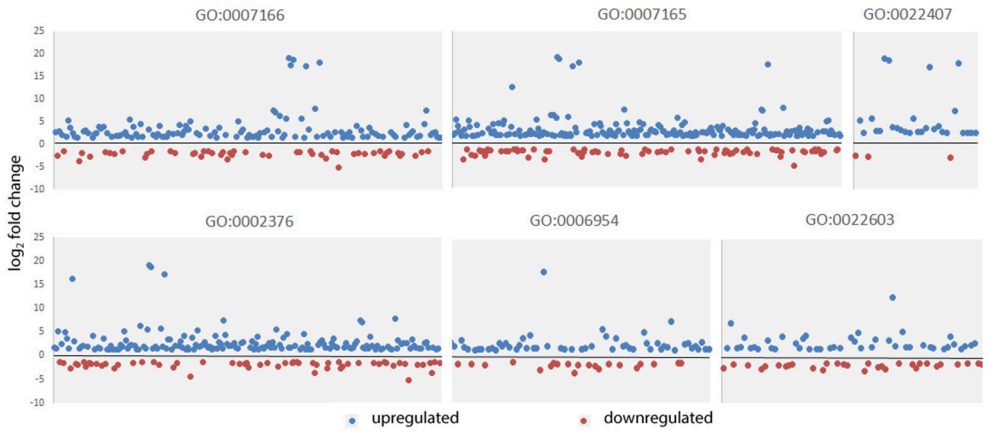


FIGURE 2 | MA-plot of HL-60 cell gene expression in *A. phagocytophilum*-infected vs. uninfected cells. Here, the RPKM values for infected and uninfected cells were plotted against the single $\log_2$ fold-change value of *A. phagocytophilum*-infected/uninfected cells; thus, each infected/uninfected $\log_2$ fold value will have two distinct RPKM values. The plot demonstrates a marked shift of upregulated genes to higher RPKM values for *A. phagocytophilum*-infected vs. uninfected cells.

receptor signaling pathways, processes related to immune system and interferon-gamma-mediated signaling, to antigen processing via MHC and regulation of apoptosis/cell death (**Figure 3**). Compared to other expression profiling studies that examined myeloid cell gene expression with *A. phagocytophilum* infection by microarray, there was only a small proportion of differentially expressed genes shared among any of the studies (**Figure 4A**), suggesting that a core set of genes are likely to regulate most functions in response to or controlled by *A. phagocytophilum*. However, since most studies identified similar altered transcriptional programs, GO analysis and comparison of enrich GO processes with each study showed a higher degree of similarity for all but the studies of de la Fuente et al. (2005) (**Figure 4B**).

A. phagocytophilum Infection Alters the Expression of Alternative Splicing Events in Cellular Transcripts

In order to examine whether an alternative cellular transcriptional landscape of infection by *A. phagocytophilum* occurs, the RNAseq data were evaluated for the presence of alternative transcript isoforms and compared with gene-level analyses. As with the gene-level analyses, only transcripts with absolute differential expression >2 fold between *A. phagocytophilum*-infected and uninfected ATRA-differentiated HL-60 cells were evaluated. Overall, 195,553 transcript reads were detected either in infected or uninfected cells, including transcripts from 59,747 unique genes or annotated gene features. After filtering for QC and significant



GO Term - Description
GO:0007166 - cell surface receptor signaling pathway
GO:0007165 - signal transduction
GO:0022407 - regulation of cell-cell adhesion
GO:0002376 - immune system process
GO:0006954 - inflammatory response
GO:0022603 - regulation of anatomical structure morphogenesis

FIGURE 3 | Gene Ontology processes significantly enriched in gene-level analysis of *A. phagocytophilum*-infected ATRA-differentiated HL-60 cells. Here, the absolute value of the $\log_2$ fold change in gene-level differential transcription was used to generate the list, and representative processes are shown here to demonstrate the range of differential gene transcription over several significantly-enriched processes known to be important for *A. phagocytophilum* survival and fitness.

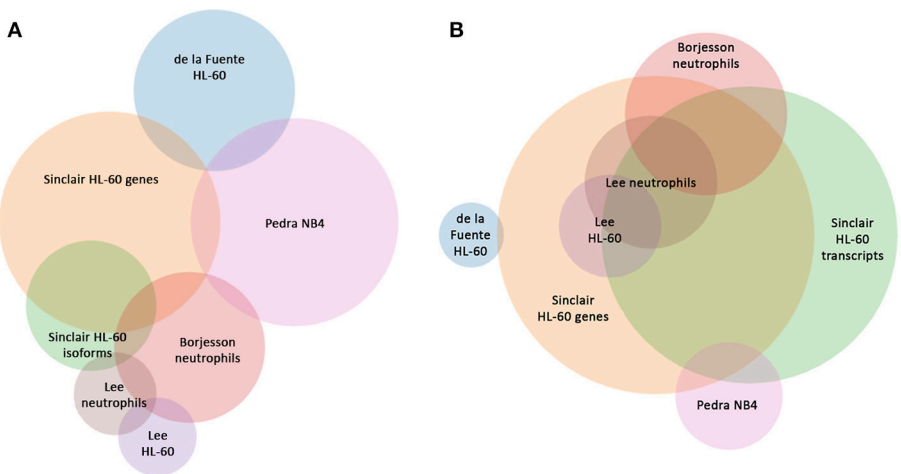


FIGURE 4 | Comparison of shared differentially-expressed genes and GO Processes for *A. phagocytophilum* expression profiling in human myeloid cells and *ex vivo* neutrophils. **(A)** is based upon gene identifiers in each study and demonstrates a lack of overlap in shared differentially-regulated genes in six distinct studies, including five based on microarray analyses, and this study that separately analyzes gene-level and transcript level differential expression in ATRA-differentiated HL-60 cells. **(B)** is based upon GO processes enriched for each study and demonstrates the much greater similarity between studies. Venn circles are labeled by the first author of each publication from which data was derived; here Sinclair represents the data in this manuscript.

fold change expression, but not FPKM cutoff, a list of 1,075 isoforms from 958 unique genes was identified, including 862 with one differentially expressed isoform, 77 genes with 2 differentially expressed isoforms, 17 genes with 3 differentially expressed isoforms, and 2 genes with 4 differentially expressed isoforms. Among the 96 genes with 2 or more differentially expressed isoforms, 60 genes were upregulated for all isoforms, 30 genes were downregulated for all isoforms, and 6 genes showed isoforms that were both up- and down-regulated. When the list was further filtered to include those with at least 1 FPKM, a total of 665 transcripts from 605 unique genes were identified, including 111 distinct transcripts

representing alternatively spliced isoforms from 51 distinct gene loci. Gene-level differential transcription was similar to that found with evaluation at the transcript level, when overall differential expression was calculated for isoforms found in those 51 gene loci (**Figure 5**). However, as in other studies of viruses and bacteria, RNAseq transcript isoforms yielded a distinct profile for transcriptional expression that supports the conceptual role of alternative splicing in control over host transcriptional programs and functions. Thus, PSI was calculated for each of the ASEs associated with specific genes, and examined in more detail for those with ΔPSI >7.5%. In this analysis, 51 genes had two distinct differentially expressed transcripts, 8 had 3 differentially expressed transcripts, and 1 had 4 differentially expressed transcripts (**Figures 6**, **7**).

Of all ASEs, differential transcription varied considerably from marked upregulation, e.g., with *CD74* and *FADS2* to moderate downregulation, as with *SRRT* and *CCR2*. Of the 51 genes with ASEs, for 34, all ASEs were upregulated and for 15, all ASEs were downregulated; however, 2 genes had both up- and downregulated splicing events—as much as a 20-fold difference in isoform expression for *SRRT*, for which the gene product is believed to be involved in microRNA processing and in transcript splicing events (**Figure 6**). Similarly, for each gene, alternative splicing events were examined to generate the PSI metric for comparison of infected and uninfected cells, where large deviations (defined as >7.5% deviation from uninfected cell PSI) could indicate impacted processes in part regulated with *A. phagocytophilum* infection (**Figure 7**). Among the 51 genes with ASE events, the PSI for 13 varied from that of uninfected HL-60 cells by >7.5%, including seven for which PSI was less with infection, and 6 for which PSI was greater. For five genes, PSI was more than 10% changed (*SRRT*, −53%; *PRKAG2*, −38%; *NFATC1*, +16%; *KCTD15*, +14%; and *FAM20C*, +12%). Among the 13 genes for which ΔPSI was > 7.5%, differential gene-level expression was significantly lower or higher in infected cells for 8, although these had ΔPSI values below 15%, whereas the three genes with the greatest ΔPSI did not demonstrate gene-level differential transcription; yet, for each of two detected isoforms, at least one demonstrated significant differential expression (**Figure 8**).

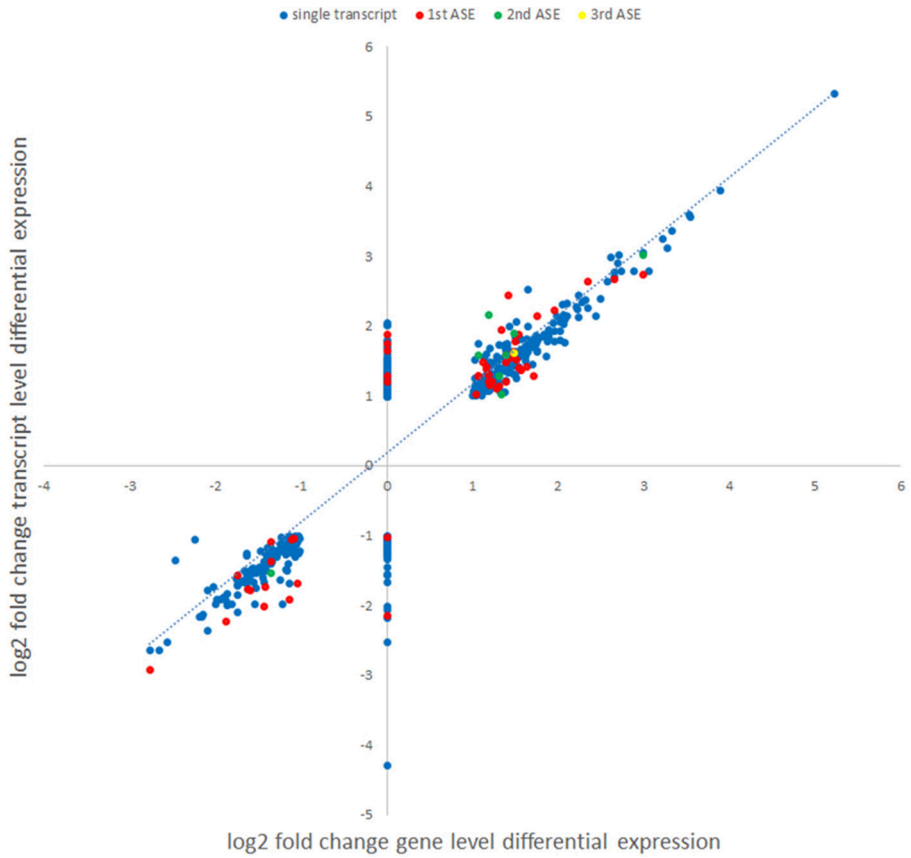


FIGURE 5 | Comparison of gene level and transcript-level changes in expression between *A. phagocytophilum*-infected and uninfected ATRA-differentiated HL-60 cells. Each identified differentially-expressed transcript $\log_2$ fold change value was used to map the $\log_2$ fold change value of expression identified in the gene-level analysis. Alternative splicing events identified in the isoform analyses are labeled in different colors (blue, single or main transcript; red, first ASE; green, second ASE; yellow, third ASE). Genes lacking identified significant differential gene expression (assigned $\log_2$ fold change value = 0) for which differential transcription was identified at the isoform-level analysis.

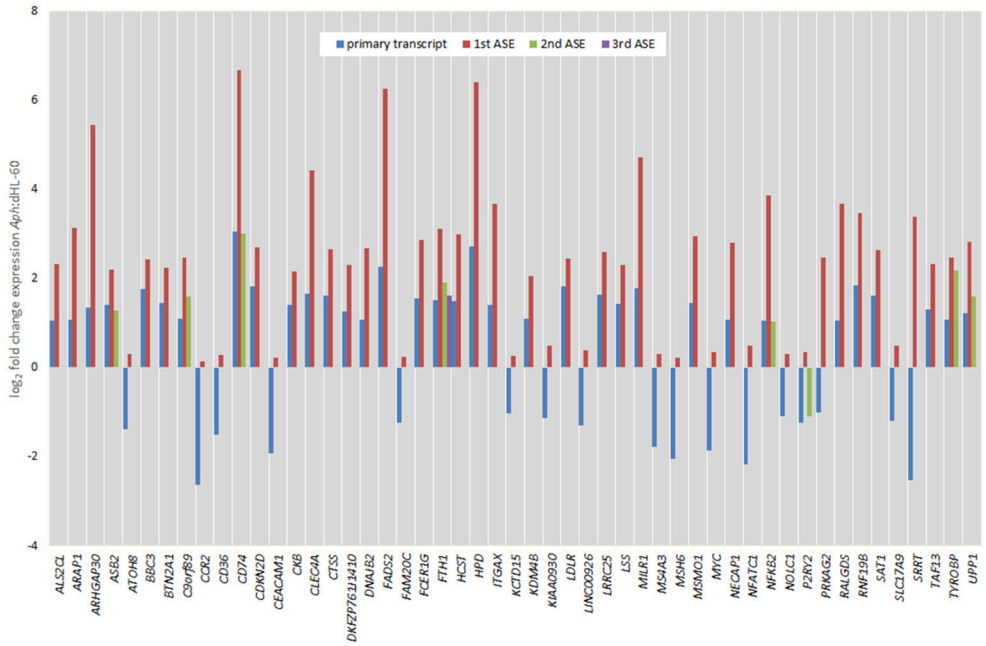


FIGURE 6 | Comparison of fold change expression levels among alternative splicing events for 51 genes identified by transcript-level RNAseq. The primary transcript is shown in red, with ASEs depicted by blue, green, or yellow bars.

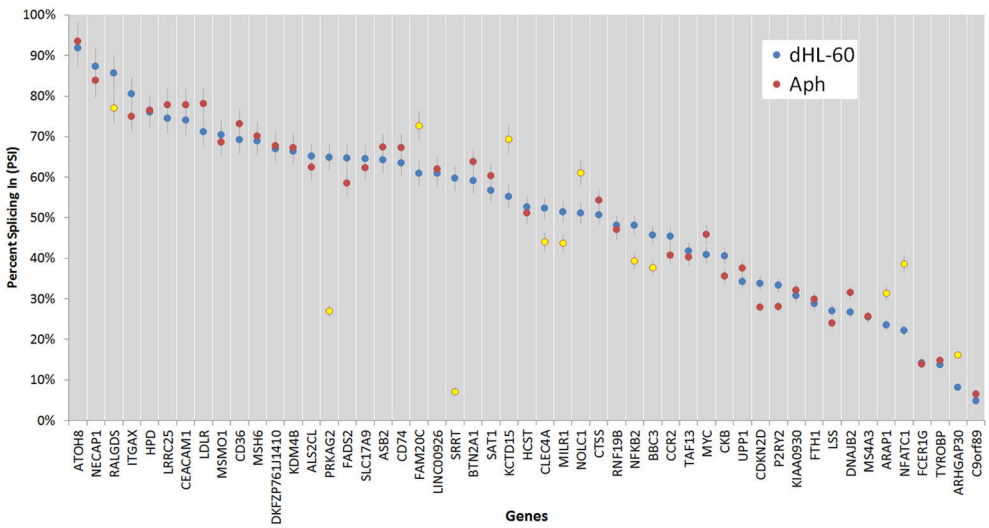


FIGURE 7 | Percent splicing in for the alternative spliced events was calculated as described in the methods. The PSI distribution is shown for all ASEs; blue markers represent PSI values for uninfected ATRA-differentiated HL-60 cells; red markers show the PSI values for *A. phagocytophilum*-infected cells, with those deviating from the HL-60 PSI values by more than 7.5% highlighted in yellow; error bars represent 7.5% change range by comparison with infected or uninfected cells.

Transcript Isoform Length and Spliceosome

Other investigators found that the average length of ASE transcripts decreases with infection. **Figure S1** shows the relationship between transcript length for those significantly transcribed isoforms with more than one isoform detected vs. those transcripts for which only a single isoform was identified. There were no significant differences identified in transcript length or in fold-change gene expression associated with transcript length ($p = 0.114$; Student's t-test) when ASEs were compared with single transcripts. Since 51 of 606 genes (8%) in gene-level RNAseq and 111 transcripts were identified

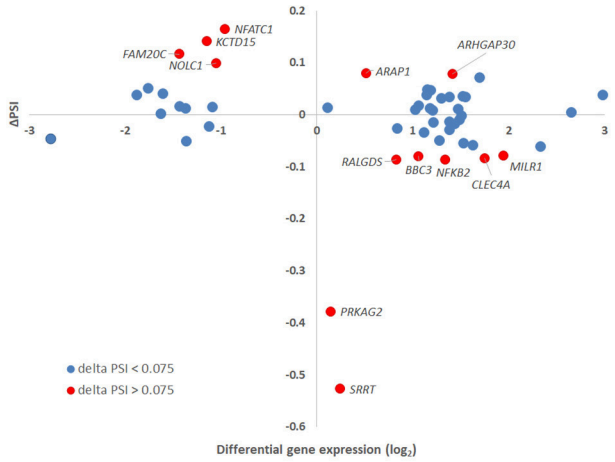


FIGURE 8 | Delta PSI and gene-level differential expression. The absolute values of PSI for isoforms assigned to a specific gene were plotted against the gene-level differential expression in *A. phagocytophilum*-infected vs. uninfected ATRA-differentiated HL-60 cells. Thirteen genes had ΔPSI greater than 7.5%, but only eight had gene-level differential expression greater or less than 2-fold. Of the three genes with the greatest ΔPSI, none had gene-level differential expression changes greater or less than 2-fold, but at least one isoform for each of these was expressed at 2-fold greater or 2-fold lower levels than in uninfected cells.

in transcript-level RNAseq from those 606 genes, it was evident that the spliceosome and its components were likely involved, as observed with viral and *M. tuberculosis* infections. Thus, expression of genes encoding components of the spliceosome were examined at gene- and isoform-levels to determine whether alternative splicing of transcripts encoding spliceosome components could also be impacted by this process. At the gene level, none of the 135 genes in the sliceosome list were identified as differentially regulated; however, when the list of differentially transcribed isoforms was examined, 9 isoforms were identified spanning all components of the sliceosome complex, and with the exception of *HNRNPC* (−3 $\log_2$-fold), all were upregulated with infection at an average of 4.7 $\log_2$-fold (range 3.2- to 6.1 $\log_2$-fold) (**Table S1**).

Gene Ontology Analysis of Genes, Transcripts, and ASEs

To determine whether separate functions could be regulated or directed by the ASEs promoted with infection, all gene-level identifications, transcripts and separately ASEs were examined for enrichment in GO process analysis. This identified 430 GO Processes associated with differentially expressed genes at p-values < 0.001. When differentially expressed transcript gene IDs were examined as a single list ranked by absolute value $\log_2$-fold change in expression, GO analysis identified 53 processes, which have relevance for *A. phagocytophilum* fitness (**Tables S2, S3**). Owing to the inclusion of background sets for some, GO process analysis focusing on all differentially expressed transcript isoforms (using all detected transcripts as background) and ASEs sorted according the absolute ΔPSI (using all differentially expressed transcript isoforms as background) revealed larger sets of GO processes: 393 for differentially expressed transcripts and 64 for differentially expressed ASEs; 84 GO processes were identified in both the differential transcript and ASE evaluations. For all differentially expressed transcripts, these focused on host defense, inflammatory response, respnose to external stimulus, cytokine-mediated signaling, signal transduction regulation, vesicle transport, programmed cell death regulation, chemotaxis, and related subprocesses; with ASE enrichment, GO analysis revealed shared pathways, including those specific for neutrophils (neutrophil activation in immune response, degranulation, regulated exocytosis, leukocyte migration, regulation of myeloid leukocyte differentiation) and pathways not shared (including cellular response to low-density lipoprotein particle stimulus, intestinal lipid absorption, intestinal cholesterol absorption, sterol import, cholesterol import, positive regulation of myeloid leukocyte cytokine production involved in immune response, positive regulation of macrophage cytokine production, positive regulation of cytokine production involved in immune response, regulation of blood coagulation, regulation of hemostasis, regulation of coagulation, among others) (**Figure 9A** and **Table S2**). In fact, owing to the related nature of GO processes in myeloid and bone marrow-derived cells, biological processes predicted by GO often share gene products that have overlapping or multiple functions (**Figures 9A,B**); thus, significant enrichment for similar processes is observed by GO processes identified separately by gene-, transcript-, and ASE-level analyses. Overall, many but not all GO Processes were shared among gene-, transcript-, and ASE-level analyses, and each analysis identified uniquely enriched GO Processes not significantly enriched otherwise (**Figure 9B**; **Table S2**; **Figure S2**).

DISCUSSION

In the post-genomic era, a major incongruency between the numbers of genes in the human genome and the number of protein products is widely recognized (Wang et al., 2008). There are two major processes by which the eukaryotic proteome is expanded from that encoded by single genes in the genome, including alternative splicing and alternative translation initiation signals (Miles et al., 2017). For the former, pre-messenger RNA processing in eukaryotes results in the production of several or many mRNA variants from a given gene (Baralle and Baralle, 2017). While this premise indicates that alternative splicing from individual genes generates isoforms and functional diversity, the overall impact of alternative splicing and its effects on regulating and tuning gene networks is now increasingly studied. Alternate splicing clearly plays a critical role in generating biological complexity and is thereby subject to dysregulation as observed with abnormalities in the spliceosome as associated with some human diseases (Barash et al., 2010), or in development (Keightley and Lieschke, 2017), cancer (Narayanan et al., 2017), and other inherited disease processes (Ramanouskaya and Grinev, 2017). The roles that such alternate splicing events play after exposure to or under the

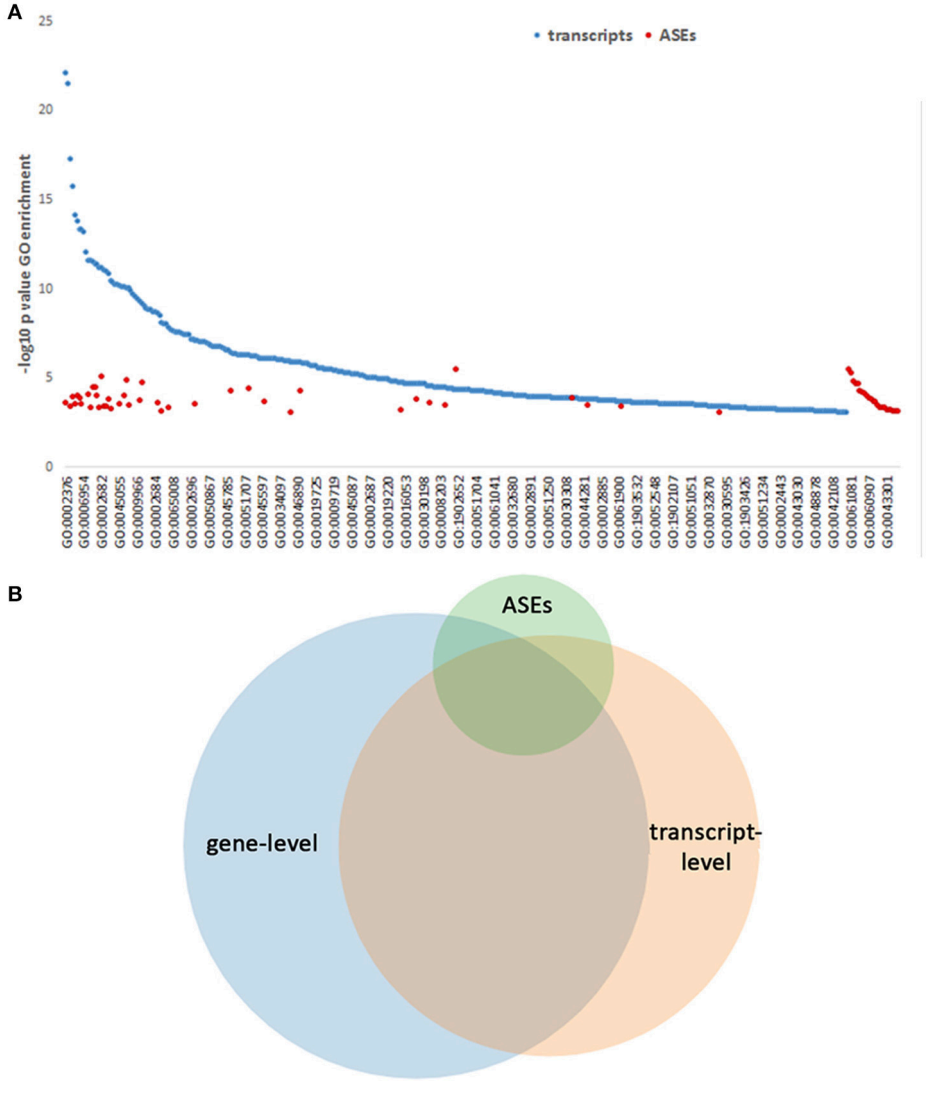


FIGURE 9 | GO Process predictions based on gene-level, transcript-level, and ASE analyses. GO Processes were queried and those with p-values < 0.001 for each of the above categories are displayed ranked by $-\log_{10}$ $p$val for display. **(A)** GO Processes identified by transcript analysis span a wide range, with some overlap of those GO Processes identified by ASE only analysis, which predicts additional pathways. The full list of GO processes represented on the x-axis is included in **Table S2**. **(B)** Venn diagram showing the overlapping GO Processes identified by gene-level, transcript-level, and ASE-level analyses, demonstrating a unique set of GO Processes associated with ASEs.

direct influence of infectious agents, in particular microbes and viruses for which the eukaryotic genome, transcriptome, and metabolome are extensions of the agent's genomic repertoire, is in particular underappreciated (Boudreault et al., 2016; Kalam et al., 2017; Wang et al., 2017).

While viral infection is well-known to promote alternative splicing as a mechanism for subversion of host processes, improvement of viral fitness, and in pathogenic outcomes, the extent to which alternative splicing occurs in both viruses, and more recently with intracellular bacterial infection, is at minimum abundant and of potentially great impact toward understanding pathogenicity (Akusjarvi, 2008; Boudreault et al., 2016; Hu et al., 2016; Graham and Faizo, 2017; Kalam et al., 2017; Wang et al., 2017). *M. tuberculosis* infection pathogenicity directly relates to alternative splicing events that vary with time after infection of cultured macrophages, leading to transcription of some isoforms for which premature stop codons are present that preclude translation and result in early degradation through nonsense mediated decay (Kalam et al., 2017). Other isoforms exclude or include one or more exons, providing new contributions to the proteome each with a potentially distinct functional outcome.

Here we demonstrate that by using next generation RNAseq methods that can identify the presence and quantity of mRNA

isoforms, not achievable with microarray analyses previously used to discern expression pattterns of human genes with *A. phagocytophilum* infection, that there is extensive global alternative splicing. The methodology we used has some advantages and disadvantages: infection of human neutrophils *ex vivo* by *A. phagocytophilum* is challenging and the mere manipulation of neutrophils *ex vivo* presents a variety of obstacles including achieving infection among even a small proportion of cells for which the whole population is used in average to estimate transcriptional responses to active infection (Borjesson et al., 2005; Lee et al., 2008; Rennoll-Bankert et al., 2014). To overcome this barrier, we and others have employed myeloid model cell lines that allow high levels of infection to be achieved, but suffer from potential confounding experimental outcomes owing to genomic rearrangements, deletions, or aneuploidy of cells like HL-60 and NB4 promyelocytic leukemia cells, PLB-985 acute myelomonoblastic leukemia cells, or THP-1 acute monocytic leukemia cells (Goodman et al., 1996; Pedra et al., 2005; Garcia-Garcia et al., 2009a; Rennoll-Bankert et al., 2014). We previously showed that the use of ATRA-differentiated HL-60 cells yields a transcriptional profile like that of infected neutrophils that are sorted to high proportion of infection than could be achieved with bulk culture alone (Rennoll-Bankert et al., 2014). Using this approach, we identified the transcriptional responses of *A. phagocytophilum*-infected ATRA-differentiated HL-60 cells compared to mock-infected cells as a basis for advanced studies of gene regulation in the context of infection, including the epigenome and how *A. phagocytophilum*-exported DNA-binding proteins impact gene transcription and nuclear architecture.

Through the process of RNAseq, we established both gene-level transcriptional responses, identifying 1,740 differentially expressed genes, and these genes predicted similar gene ontology processes as observed using microarray transcriptome analysis in primary human neutrophils and any of the myeloid cell types able to sustain infection. In contrast, the algorithms used to identify differentially expressed transcript isoforms yielded a smaller number overall-−605 gene IDs among a total of 665 total differentially expressed transcripts, including 51 ASEs with 111 unique transcripts, including 8 with 3 distinct isoforms, and 1 with 4 isoforms that were differentially expressed. That 18% of all differentially expressed genes identified at the transcript isoform level with FPKM >1 included alternative splicing events was a surprising finding, but consistent with the emerging literature with virus and *M. tuberculosis* studies. Our study was limited in that whether these differentially expressed ASEs were previously identified in neutrophils was not examined.

The *M. tuberculosis* and viral infection studies underscore several features of interest, which we pursued (Boudreault et al., 2016; Kalam et al., 2017). For *M. tuberculosis*, an increase in the expression of truncated and non-translatable isoforms was found, especially with more virulent infections, and included a significant regulation of the macrophage spliceosome components that presumably belie these events. This obervation is an example of how controlling key checkpoints or master regulators can have a very substantial effect on the biology of the infected cells and the microbe (Sinclair et al., 2014). For viral infections, a variety of global changes, of similar magnitude as seen here with *A. phagocytophilum* infection, also occur, but unlike for *M. tuberculosis*, there are only limited changes in expression or splicing patterns of splicing factors, except for the overexpression of an alternative transcript from the splicing regulator *ESRP1* (Boudreault et al., 2016). Another observation shows that *M. tuberculosis* infection of human macrophages yields overall shorter transcripts associated with ASEs and that this is associated with reduced translation into proteins. A similar phenomenon was not identified with *A. phagocytophilum*, where the average transcript size of the alterative isoforms did not vary significantly from uninfected to infected conditions. Similarly, of the 136 genes most involved in the human spliceosome, considerable alternative splicing events in this group were not noted with virus or with *A. phagocytophilum* infection. However, with *A. phagocytophilum* infection of differentiated HL-60 cells, in the transcript analysis (but not in the gene-level analysis), there was marked differential expression of spliceosome transcripts that potentially impact each of the critical spliceosome stages. Whether such differential expression impacts the high degree of alternative splicing observed is not known, but is under study.

Perhaps of greatest interest is how the differentially-expressed alternative spliced transcripts or transcript gene IDs not detected as differentially regulated with gene-level analysis, predict altered cellular processes that govern cell function. *A. phagocytophilum* infection has a profound impact on neutrophils including alterations at virtually every cardinal funtion (Dumler, 2012). Many of these changes can be documented to be in part regulated at the level of gene transcription (Sinclair et al., 2014). For some of these changes a direct regulatory role has been established for the *A. phagocyotphilum* type 4 secretion system effector AnkA, while other secreted effectors have a greater impact by protein-protein interactions in signaling, cytoskeletal alterations, or endosomal trafficking (Carlyon and Fikrig, 2006; Rikihisa, 2011; Truchan et al., 2013; Rennoll-Bankert et al., 2015). While no apparent direct association of AnkA with the ASEs was observed (data not shown), *A. phagocytophilum* significantly regulates DNA methylation, in particular at intron and exon junctions, and DNA methylation is posited as an important mark for slowing of RNA polymerase and induction of alternative splicing (Shukla et al., 2011; Maunakea et al., 2013; Sinclair et al., 2015). Likewise, no specific colocalization of meDNA and ASEs was established here.

Reprogramming of cellular functions can be a challenge to study, and surveys of process changes can be conducted using bioinformatic tools such as gene ontology (GO) analysis. While the GO analysis at the gene- and transcript-levels identified core shared processes enriched during *A. phagocytophilum* infection of differentiated HL-60 cells, there remained a unique set identified solely by one or the other method of differential gene expression analysis. Likewise, the analysis of the smaller 51 gene, 111 transcript set of alternatively spliced isoforms identifies both shared and unique sets of GO processes, the latter predominantly focused upon lipids, lipoproteins, and cholesterol/sterol metabolism, but also including regulation of cytokine production and neutrophil degranulation, all recognized as critical events for *A. phagocytophilum* fitness leading to enhanced intracellular survival and spread (Dumler, 2012). That the regulation of lipids

and lipoproteins was somewhat selectively enriched when ASE differentially expressed genes were examined suggests a more specific role in these processes, for which recent investigations have shown a critical role for cholesterol (Manzano-Roman et al., 2008; Xiong and Rikihisa, 2012), phospholipid and eicosanoids (Wang et al., 2016), as well as lipoproteins and polar lipids (Choi et al., 2004b; Choi and Dumler, 2007) which can directly or indirectly affect proinflammtory state in neutrophils that results in cytokine production and initiation of innate and adaptive immune response, including inflammasome triggering (Dumler et al., 2000, 2007; Martin et al., 2000; Wang et al., 2016). Of course, in silico analyses leave great overlaps and suggest cross-talk because the GO processes identified by each approach applied here share many genes. Although little investigation has been conducted of how infection affects gene regulation at the level of pre-messenger RNA processing and splicing, this work indicates that ASEs are a significant comoponent of the transcriptional response to *A. phagocytophilum* infection. Yet, those attributes of ASEs identified for viral and *M. tuberculosis* infections are not entirely shared with observations of ASEs in *A. phagocytophilum* infection. This provides evidence that a diversity of responses to infectious agents can further focus and refine the host cell proteome. How these events inform the separate aspects of microbial fitness in the unusual niche of a neutrophil vacuole, from the ability to sense microbial infection, such as via the inflammasome, to eliciting inflammatory injury and immune dysregulation will be clearly more complex than anticipated, and will be an area for intense investigation over time.

DATA AVAILABILITY

The datasets generated and analyzed for this study can be found in the Gene Expression Omnibus database with GEO Accession GSE107770 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE107770).

AUTHOR CONTRIBUTIONS

JSD helped to design the project, coordinated experimental and analytical approaches, analyzed data and wrote the manuscript. SS conceived the project, designed the experimental project, executed the experiments and coordinated the library preparations and the sequencing. AS conducted primary bioinformatics processing and quality control of the sequencing data, conducted the majority of the identification of RNA isoforms, and provided statistical analysis for quality control purposes. All authors contributed to writing and revisions of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00014/full#supplementary-material

Figure S1 | Among all isoform transcripts that met quality control measures for both infected and uninfected ATRA-differentiated HL-60 cells, isoform length in bases was plotted against differential isoform expression to test the hypothesis that alternative splicing events result in shorter isoforms. Among those genes with more than one isoform detected compared to single isoforms, there was a range of both isoform lengths and differential expression, but differential expression was not significantly linked with isoform length.

Figure S2 | Enrichment of GO Processes and the significance of the enrichment for all differentially expressed isoforms and for a set limited to alternatively-spliced isoforms. Enrichment and *p*-value were derived from the enrichment profile of the GO analyses.

Table S1 | Human spliceosome components examined for differential gene expression by gene-level and transcript isoform-level RNAseq.

Table S2 | GO Process enrichment for all transcripts and alternatively spliced isoforms (ASE).

Table S3 | Panther GO-Slim Biological Processes associated with alternatively spliced isoforms after A. phagocytophilum infection of ATRA-differentiated HL-60 cells.

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Transcriptional Immunoprofiling at the Tick-Virus-Host Interface during Early Stages of Tick-Borne Encephalitis Virus Transmission

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Emerging and re-emerging diseases transmitted by blood feeding arthropods are significant global public health problems. Ticks transmit the greatest variety of pathogenic microorganisms of any blood feeding arthropod. Infectious agents transmitted by ticks are delivered to the vertebrate host together with saliva at the bite site. Tick salivary glands produce complex cocktails of bioactive molecules that facilitate blood feeding and pathogen transmission by modulating host hemostasis, pain/itch responses, wound healing, and both innate and adaptive immunity. In this study, we utilized Illumina Next Generation Sequencing to characterize the transcriptional immunoprofile of cutaneous immune responses to *Ixodes ricinus* transmitted tick-borne encephalitis virus (TBEV). A comparative immune gene expression analysis of TBEV-infected and uninfected tick feeding sites was performed. Our analysis reveals that ticks create an inflammatory environment at the bite site during the first 3 h of feeding, and significant differences in host responses were observed between TBEV-infected and uninfected tick feeding. Gene-expression analysis reveals modulation of inflammatory genes after 1 and 3 h of TBEV-infected tick feeding. Transcriptional levels of genes specific to chemokines and cytokines indicated a neutrophil-dominated immune response. Immunohistochemistry of the tick feeding site revealed that mononuclear phagocytes and fibroblasts are the primary target cells for TBEV infection and did not detect TBEV antigens in neutrophils. Together, the transcriptional and immunohistochemistry results suggest that early cutaneous host responses to TBEV-infected tick feeding are more inflammatory than expected and highlight the importance of inflammatory chemokine and cytokine pathways in tick-borne flavivirus transmission.

Keywords: TBEV, flavivirus, tick, *Ixodes ricinus*, cutaneous, immune response

INTRODUCTION

Tick-borne encephalitis virus (TBEV) is a zoonotic tick-borne virus in the *Flaviviridae* family (genus *Flavivirus*). It is the causative agent of tick-borne encephalitis (TBE), a serious neurological disease in humans. During the last few decades, TBE has become a widespread public health concern in Eurasia with endemic regions extending from Western and Central Europe to Siberia and parts of Asia (Süss, 2011). The various strains of TBEV are subdivided into three main subtypes that are closely related genetically and antigenically: European (Eu), Siberian (Sib), and Far-Eastern (FE) (Gritsun et al., 2003a; Mansfield et al., 2009). TBEV-Eu is widely distributed in Europe, including the European regions of Russia, while TBEV-Sib is mainly found in Russia, the Baltic countries and Finland (Mansfield et al., 2009; Kovalev and Mukhacheva, 2014). TBEV-FE is present in Far-Eastern Russia and parts of China, Japan, and the Republic of Korea (Mansfield et al., 2009). Human infections with TBEV can range from mild flu-like symptoms to severe or fatal neuroinvasive disease, often with long-term neurological symptoms. There is a correlation between the TBEV subtype and severity of disease. TBEV-FE is associated with severe neurological disease and a case fatality rate of approximately 30–40%, while the case fatality rates for TBEV-Sib and TBEV-Eu are approximately 6–8% and 1–2%, respectively (Gritsun et al., 2003a; Tonteri et al., 2013). Although the incidence rates vary from year to year and between subtypes, several thousand human TBE cases are reported annually (CDC Tick-borne Encephalitis, 2017).

In nature, the *Ixodes ricinus* tick is the primary vector for TBEV-Eu while the *Ixodes persulcatus* tick is the main vector for the TBEV-Sib and TBEV-FE (Gritsun et al., 2003b). *I. ricinus* is widely distributed throughout Europe, extending to Turkey, and northern Iran, while *I. persulcatus* is distributed across the Urals, Siberia, Far-Eastern Russia, and parts of China and Japan (Gritsun et al., 2003a; Lindquist and Vapalahti, 2008). A sympatric zone exists in northern Baltics, western Finland and northwestern Russia where the habitats for *I. ricinus* and *I. persulcatus* overlap and multiple TBEV subtypes have been recorded (Lindquist and Vapalahti, 2008; Süss, 2011; Kovalev and Mukhacheva, 2014). TBEV is maintained in natural transmission cycles involving ixodid ticks and wild-living mammalian hosts. When infected with TBEV, a tick is supposed to remain infected throughout its life cycle (Gritsun et al., 2003a). Transovarial transmission of TBEV from an infected female tick to the egg mass can occur, but this route of tick infection is not entirely efficient at maintaining TBEV within the natural tick population (Danielová et al., 2002). During the tick feeding process, TBEV-infected ticks can transmit the virus to susceptible vertebrate hosts, but they can also transmit TBEV to uninfected ticks that are co-feeding on the same host (Mansfield et al., 2009; Randolph, 2011). During co-feeding, TBEV can be transmitted even non-viremically i.e., when the ticks feed on a non-viremic or virus-immune host (Labuda et al., 1993, 1997). The local skin site of tick feeding is understood to be an important focus for early TBEV replication, and immune cell infiltrates to this feeding site are believed to serve as vehicles for TBEV transmission between co-feeding ticks (Labuda et al., 1996).

Infectious agents transmitted by ticks are delivered to the vertebrate host together with saliva at the tick feeding site. Tick-borne viruses are transmitted to the host very early during the tick feeding process. TBEV can be transmitted from the saliva of an *I. ricinus* tick to the cement cone in the skin of a host as early as 1 h after the tick attaches and initiates feeding (Alekseev et al., 1996). As *I. ricinus* and *I. persulcatus* ticks feed, TBEV replicates to higher viral titers than in unfed ticks (Alekseev and Chunikhin, 1990; Belova et al., 2012; Slovák et al., 2014). The dynamic nature of TBEV replication in ticks has also been demonstrated in field-collected ticks. In partially engorged *I. ricinus* nymphs removed from humans, TBEV prevalence was higher than in questing, unfed nymphs collected in the same region (Süss et al., 2004). Experimental data suggest that in nature, ticks secrete repeated "pulses" of a few infectious virus particles over the course of feeding (Kaufman and Nuttall, 1996). Thus, virus transmission from an infected tick to a host is a very dynamic process that begins soon after the tick initiates feeding.

Ixodid ticks must remain attached to their hosts for several days to successfully acquire a bloodmeal and complete development, and have evolved salivary countermeasures directed against the host's immune and hemostatic defenses. Tick salivary glands produce complex cocktails of biologically active molecules that facilitate blood feeding and pathogen transmission by modulating host hemostasis, pain/itch responses, wound healing, and both innate and adaptive immunity. Bioactive tick salivary molecules include those with anti-pain/itch, antiplatelet, anticoagulation, vasodilatory, immunomodulatory, and anti-inflammatory activities (Ribeiro et al., 2006, Francischetti et al., 2009; Kazimírová and Štibrániová, 2013; Wikel, 2013; Šimo et al., 2017). As the course of tick feeding progresses, salivary gland genes are differentially expressed, reflecting the dynamic and complex composition of tick saliva (Ribeiro et al., 2006; Šimo et al., 2017).

The skin is the first host organ that tick saliva and a tick-borne pathogen contact during the tick feeding process. The cutaneous interface between the tick, pathogen, and host is crucial for influencing the initial host response to tick infestation and pathogen transmission (Kazimírová et al., 2017; Šimo et al., 2017). A prior study examined the tick-induced changes in cutaneous gene expression and histopathology during the early stages of uninfected *Ixodes scapularis* feeding. Early transcriptional and histopathological changes at the feeding site of uninfected *I. scapularis* nymphs are initially characterized by modulation of host responses in resident cells, followed by progression to a neutrophil-dominated immune response after 12 h of tick feeding (Heinze et al., 2012). Similarly, a complex proinflammatory environment was observed at the Powassan virus (POWV), a North American tick-borne flavivirus, infected tick feeding site. Together these findings from the cutaneous interface provide evidence of an immunologically privileged micro-environment at the tick feeding site that is established during the early stages of POWV-infected tick feeding (Hermance and Thangamani, 2014; Hermance et al., 2016).

In the present study, Illumina Next Generation Sequencing (NGS) and immunohistochemistry are utilized to understand host immunomodulation induced by TBEV-infected *I. ricinus* feeding at the earliest stages of TBEV transmission. By studying the interactions between the host immune response and tick-mediated immunomodulation during the early hours of infected tick feeding, we can begin to understand the immunologic processes that facilitate transmission of a tick-borne flavivirus to a host.

MATERIALS AND METHODS

Ethics Statement

All experiments involving mice were performed in accordance with the animal use protocol approved by the State Veterinary and Food Administration of the Slovak Republic (permit number 1335/12-221).

Animals

Five-week-old female BALB/c mice were purchased from Dobra Voda Breeding Station, Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences (SAS). The animals were housed at the Institute of Virology, Biomedical Research Center, SAS (Bratislava, Slovakia) under standard conditions. Food and water were provided *ad libitum*. Upon arrival, mice were allowed to adapt to the local environment before being incorporated into the study. Mice were 6 weeks old at the start of the study. At the end of the experiment animals were euthanized by cervical dislocation under anesthesia induced by carbon dioxide.

Virus and Tick Infection

I. ricinus ticks were obtained from a laboratory colony maintained at the Institute of Zoology SAS (Bratislava, Slovakia). The F1 generation of laboratory-bred *I. ricinus* females was used for virus inoculation. TBEV (Hypr strain prepared as a 10% mouse brain suspension of 1.1×10^9 PFU/ml in Leibovitz's L-15 medium) was provided by the Institute of Virology, Biomedical Research Center, SAS. Fasting *I. ricinus* females were inoculated into the haemocoel with TBEV (5.5×10^4 PFU per tick) through the coxal plate of the second pair of legs using a digital microinjector TM system (MINJ-D-CE; Tritech Research, Inc., USA) (Kazimírová et al., 2012). By this procedure, ∼100% of the ticks were found to acquire TBEV infection (Slovák et al., 2014). Inoculated ticks were incubated at room temperature and 85% relative humidity in a desiccator for 21 days prior to the infestation experiments.

Infestation of Mice by Ticks

Two groups of mice ($n = 6$ per group) were infested with either TBEV-infected or uninfected (control) *I. ricinus* females. Ticks were placed in small neoprene capsules glued on the shaved backs of the mice (two capsules per mouse, four tick females per capsule) (Kazimírová et al., 2012; Hermance and Thangamani, 2014). Ticks in each capsule were allowed to feed for either 1 or 3 h. After the allotted feeding time, skin biopsies were taken from euthanized mice, at each tick feeding site using a Premier Uni-Punch (Premier Products Co., Plymouth Meeting, PA). For immunohistochemical analysis, skin biopsies ($n = 3$) were harvested with attached ticks and placed in 4% formaldehyde. For RNA extraction, the attached ticks were removed from the skin biopsies and the biopsies ($n = 3$) were placed in RNALater (Ambion, Life Technologies, Carlsbad, CA). Control biopsies were taken from the shaved skin of naïve tick-free mice and stored in either 4% formaldehyde or RNALater.

Cutaneous Immune Response

Total RNA was extracted as previously described (Heinze et al., 2012; Hermance and Thangamani, 2014) and RNAseq analysis (Illumina deep sequencing / NGS) was performed on these samples. Briefly, 1 μg of total RNA from mouse skin biopsies ($n = 3$) was poly A+ selected and fragmented using divalent cations and heat (94°C, 8 min). Illumina TruSeq v2 sample preparation kits (Illumina Inc., San Diego, CA) were used for the RNA-Seq library construction. Each sample library was uniquely indexed to allow combining libraries during sequencing and subsequent separation post-sequencing. NGS was performed at the NGS core facility, Sealy Center for Molecular Medicine, The University of Texas Medical Branch (UTMB). Sample libraries were analyzed by the Illumina HiSeq 1500 using a 2×50 base paired end run protocol, with TruSeq v3 sequencing-by-synthesis chemistry. Reads were aligned to the mouse mm10 reference genome using TopHat version v2.0.4. Cuffdiff version 2.0.2 was used to estimate differential gene expression between TBEV-infected and uninfected feeding sites after 1 or 3 h of tick feeding. The total dataset of 23,000 genes was filtered for p-values ≤ 0.05 and a fold change ≤ -1.5 or $\geq +1.5$. The Log_2(fold change) and p-value data for each gene expression comparison (Supplement Table 1) were then uploaded to Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA) for further transcriptional analysis of the early cutaneous immune response, as previously described (Heinze et al., 2012; Hermance and Thangamani, 2014).

Real-Time PCR Validation

Real-time PCR was used to validate the NGS data (Supplement Table 1). Skin biopsies were harvested from the feeding sites of TBEV-infected and uninfected ticks as described above. At each time point, biopsies from three mice were used for the real-time PCR validation. Fifteen gene targets were selected for real-time PCR analysis. We selected these genes based our previous studies with POWV where these genes were shown to be modulated genes of interest during the host anti-tick response to POWV infection (Hermance and Thangamani, 2014, 2015). This list also includes genes that were not differentially modulated as per RNASeq analysis. Primers were purchased from Integrated DNA Technologies and the primer sequences are provided in Supplement Table 2. Primers were mixed with IQ SYBR green supermix (Bio-Rad) and loaded into iCycler IQ PCR 96-well plates (Bio-Rad) to create customized PCR arrays where each gene was measured in triplicate. For each PCR plate, 1 μg of total RNA extracted from skin biopsies was converted into cDNA using the RT^2 First Strand kit (Qiagen). cDNA was loaded onto the 96-well PCR plates, which were run on an iCycler iQ5

real-time PCR instrument (Bio-Rad) with the following cycling protocol: 10 min at 95°C; 15 s at 95°C, 1 min 60°C for 40 cycles, and an 80-cycle (+0.5°C /cycle) 55–95°C melt curve. Every array included GAPDH as an endogenous control gene and a no-template control. The iCycler's software was used to calculate the threshold cycle (C_T) values for all analyzed genes. The delta-delta C_T method was used to calculate fold-changes in gene expression between TBEV-infected and uninfected tick feeding sites. Data normalization was achieved by correcting all C_T values to the average C_T values of the GAPDH housekeeping gene. Statistically significant differences in gene expression between the 1 vs. 3 h tick feeding time points were determined by the Student's *t*-test. *P*-values less than 0.05 were considered significant. SPSS statistical software was used.

Immunohistochemistry

The skin biopsies with attached ticks were formalin-fixed for a minimum of 48 h in 4% formaldehyde. These biopsy samples were treated with Decal (Decal Chemical Corp, Tallman, NY) for 2 h, and then paraffin-embedded (Heinze et al., 2012). Five micron paraffin sections were taken from each sample and adhered to glass slides. The slides were deparaffinized in xylene and then rehydrated in decreasing concentrations of ethanol (Hermance et al., 2016). For antigen retrieval, the slides were treated with 10 mM Tris Base + 0.05% Tween 20 (pH 10) for 20 min with microwave heating. Upon returning to room temperature, endogenous peroxidase quenching was performed by incubating the slides in 4% H_2O_2 for 30 min. The primary antibody for TBEV detection used in this study was a Hyper Mouse Immune Ascitic Fluid (HMIAF) antibody against TBEV; therefore, the Mouse-On-Mouse kit (MOM, Vector labs, Burlingame, CA) was used in order to reduce the endogenous mouse Ig background staining generated when mouse primary antibodies are used on mouse tissue (Santos et al., 2016). Slides were incubated for 1 h at room temperature with the MOM mouse Ig blocking reagent. The HMIAF primary antibody against TBEV was diluted 1:250 in the MOM diluent and incubated for 30 min at room temperature. Secondary antibody consisted of MOM biotinylated horse anti-mouse IgG reagent, which was incubated for 30 min at room temperature. The biotinylated secondary antibody was detected with a streptavidin-peroxidase ultrasensitive polymer (Sigma-Aldrich, St. Louis, MO) followed by staining with the NovaRED HRP substrate kit (Vector Laboratories Inc., Burlingame, CA). Slides were counterstained with Harris hematoxylin and coverslips were mounted using Permount (ThermoFisher Scientific, Waltham, MA). Uninfected skin and tick biopsy sections generated from uninfected tick feeding sites were used as negative controls to verify the specificity of the MIAF anti-TBEV primary antibody. Secondary antibody only (no primary antibody) controls were used to confirm that the MOM biotinylated anti-mouse IgG reagent did not bind non-specifically to cellular components.

RESULTS AND DISCUSSION

In the present study, the host cutaneous immune response to TBEV-infected *I. ricinus* feeding after 1 and 3 h of tick attachment was investigated by Illumina NGS and immunohistochemistry. No prior studies have examined the early host immune response at the feeding site of a TBEV-infected tick. We also used the RNASeq data to check for the presence of TBEV at the infected tick feeding site by aligning the sequences against a TBEV reference genome (NM_001672). TBEV sequences were detected in the 3 hpi (hours post infection), but not in the 1 hpi samples (**Figure 1A**).

The differences in the total number of significantly up- and downregulated host genes ($p \leq 0.05$) between TBEV-infected and uninfected tick feeding sites as well as between TBEV-infected feeding sites 1 and 3 h post tick attachment are shown in **Figure 1B**. When the TBEV-infected and uninfected tick feeding sites were compared, the total number of significantly upregulated genes decreased from 1 to 3 h, while there was an overall increase in significantly downregulated genes from 1 to 3 h tick attachment. An online tool (Oliveros, 2007) was used to generate a Venn diagram showing the overlap of significantly modulated genes between the 1 and 3 h comparison of the TBEV-infected vs. uninfected tick feeding sites (**Figure 1C**). 10.2% of significantly modulated genes were shared between the three comparisons; however, the majority of significantly modulated genes were unique to either the 1 h TBEV-infected vs. 1 h uninfected tick feeding site (18.7%), the 3 h TBEV-infected vs. 3 h uninfected tick feeding site (25.7%), or the 3 h TBEV-infected vs. 1 h TBEV-infected tick feeding site (14%). Additionally, a list of all modulated genes at either time point in the study was used to generate a heat map (**Figure 1D**). This heat map suggests that after 1 h of TBEV-infected tick feeding, there was a pattern of mostly upregulated cutaneous genes; however, after 3 h, the pattern changed to downregulation. This pattern of gene expression was further validated by the real-time PCR data. Though we could not recapitulate the exact fold changes of the selected immune genes observed by RNASeq data, our data clearly concur with the expression pattern of the selected genes: upregulation at 1 hpi and downregulation at 3 hpi (**Figure 1E**). Together these data suggest that a distinctive cutaneous immune response profile exists after 1 h of TBEV-infected tick feeding, but it changes to reflect a new and unique profile after 3 h. This change in gene expression profile could be attributed to the dynamic salivary secretion and physical injury during tick attachment/feeding mechanisms.

Ingenuity Pathway Analysis

In total, 1,548 genes were analyzed by Ingenuity Pathway Analysis (IPA) software (Supplement Table 1). The focus of the present study is on the cutaneous immune response observed at the TBEV-infected vs. the uninfected tick feeding sites after 1 and 3 h of tick feeding. The networks generated from IPA analysis illustrate the interrelationships between genes and the temporal changes in gene modulation. The top IPA-generated networks are shown for the TBEV-infected vs. uninfected tick feeding sites after 1 h of tick feeding (**Figure 2A**), the TBEV-infected vs. uninfected tick feeding sites after 3 h of tick feeding (**Figure 2B**), and the TBEV-infected tick feeding site after 3 h of tick feeding vs. the TBEV-infected tick feeding site after 1 h of feeding (**Figure 2C**). The three bio-functions most associated

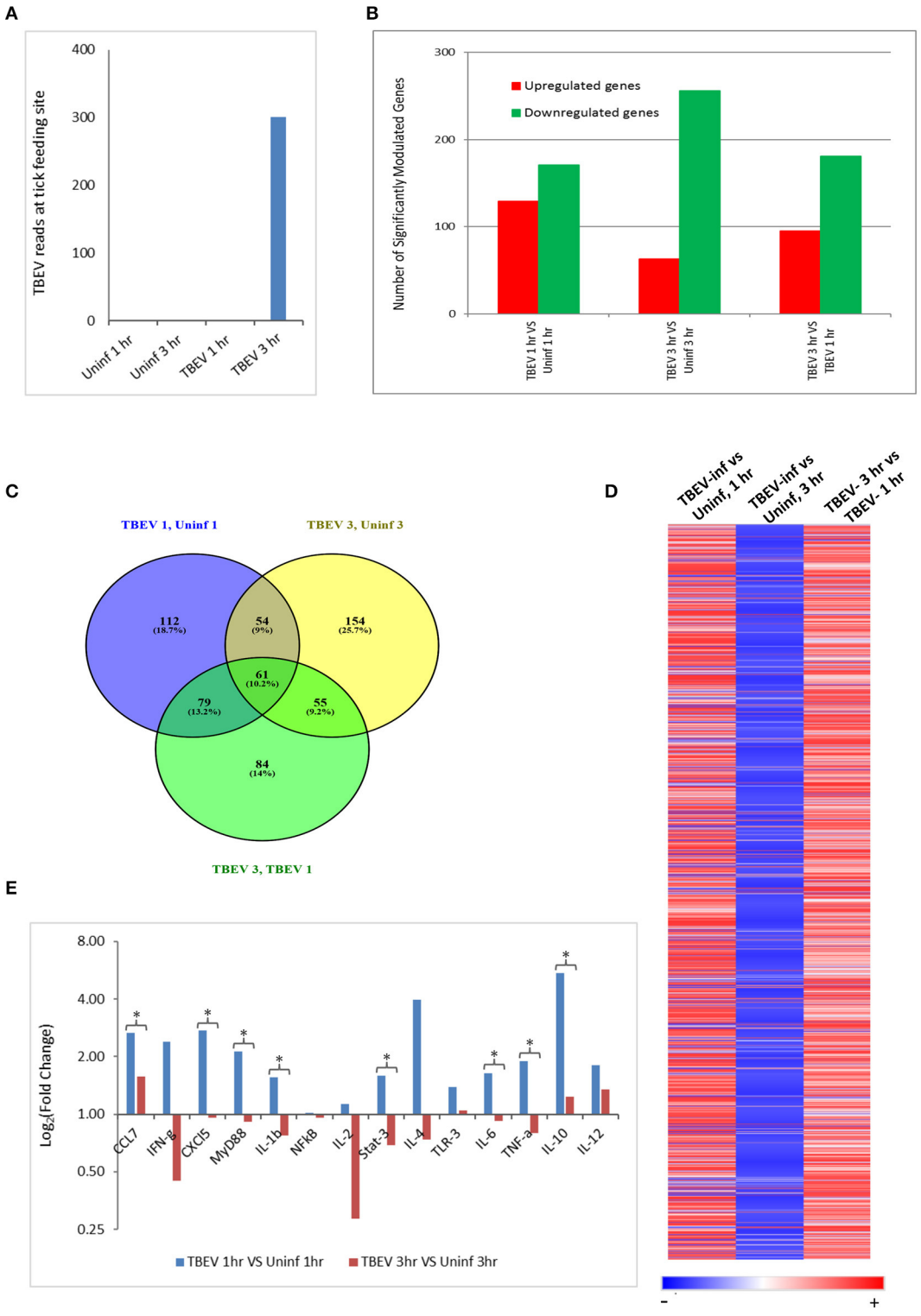

FIGURE 1 | Comparative transcriptional analysis of the TBEV-infected and uninfected *Ixodes ricinus* tick feeding loci. **(A)** The RNASeq data was screened for the presence of TBEV reads at the tick feeding site by aligning the sequences against a TBEV reference genome (MN_001672). The number of TBEV reads that match the TBEV reference genome is plotted for the uninfected tick feeding sites and the TBEV-infected tick feeding sites. **(B,C)** The following comparisons are depicted: TBEV-infected tick feeding loci at 1 h vs. uninfected tick feeding loci at 1 h, TBEV-infected tick feeding loci at 3 h vs. uninfected tick feeding loci at 3 h, TBEV-infected

(Continued)

FIGURE 1 | tick feeding loci at 3 h vs. TBEV-infected tick feeding loci at 1 h. **(B)** The total number of significantly up- or downregulated ($p \leq 0.05$) genes for each comparison. **(C)** Venn diagram showing overlap of significantly modulated genes for each of the three comparisons. **(D)** Heat map showing temporal changes in gene expression profiles. A list of all genes modulated at any time point in the study was used to generate a heatmap with Morpheus web server application (www.broadinstitute.org). **(E)** The immune genes selected for validation were shown to be modulated genes of interest during the host anti-tick response in previous studies. Pre-optimized primers were purchased from IDT and used for the real-time PCR validation. The delta-delta CT method was used to calculate fold-changes in gene expression between TBEV-infected and uninfected tick feeding sites as described in the methods section. GAPDH was used as an endogenous control gene. Statistically significant differences in gene expression between the 1 vs. 3 h tick feeding time points were determined by the Student's *t*-test. *P*-values less than 0.05 were considered significant. Significant differences are indicated by asterisks.

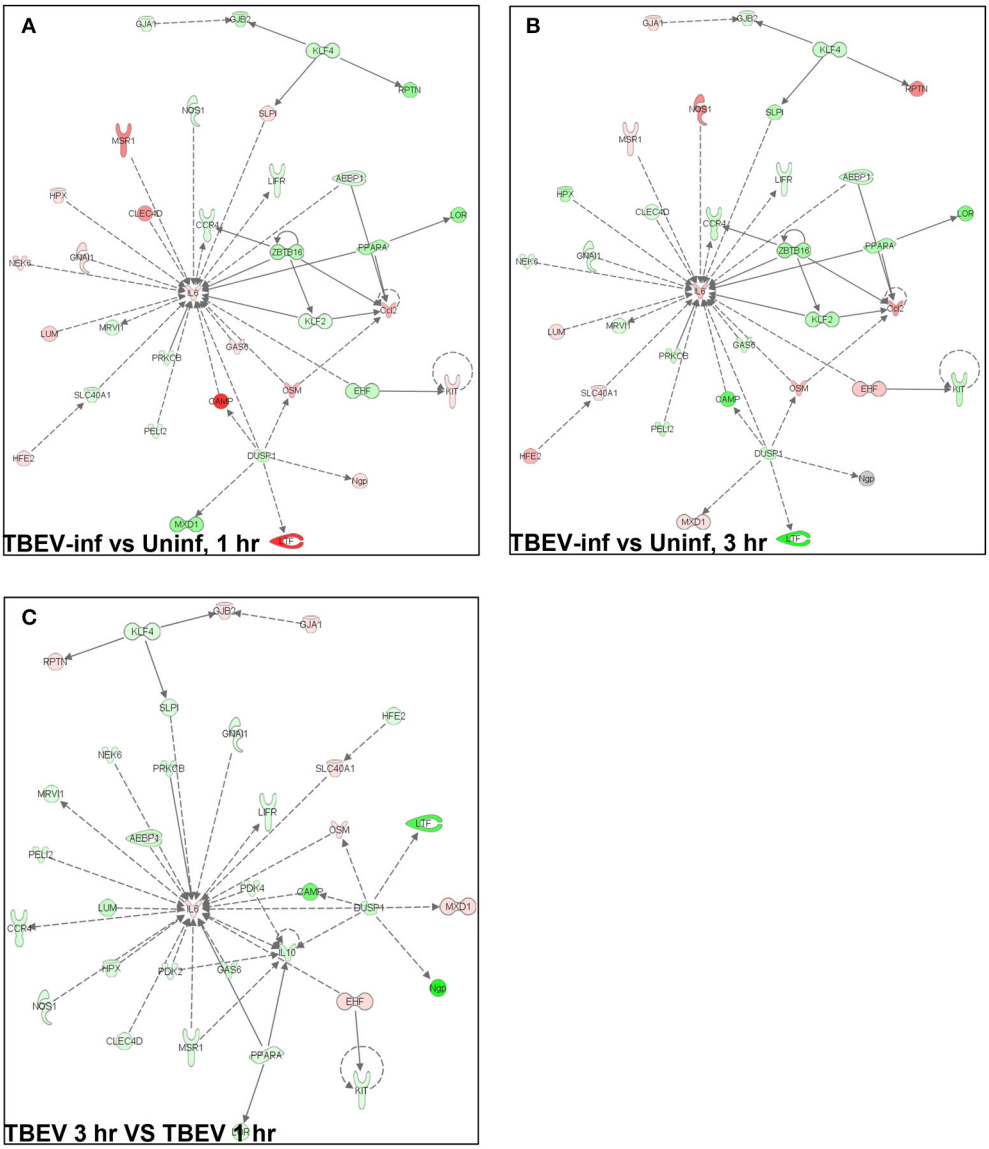

FIGURE 2 | An overview of host gene modulation after 1 and 3 h of TBEV-infected *Ixodes ricinus* tick feeding. **(A)** The top IPA-generated network is shown for the TBEV-infected tick feeding loci at 1 h vs. the uninfected tick feeding loci at 1 h **(B)** The top IPA-generated network is shown for the TBEV-infected tick feeding loci at 3 h vs. the uninfected tick feeding loci at 3 h. **(C)** The top-IPA-generated network is shown for the TBEV-infected tick feeding loci at 3 h vs. TBEV-infected tick feeding loci at 1 h. Note: Red/pink represents upregulated genes, green represents down-regulated genes, and gray represents unchanged or insignificant genes.

with the networks representing the TBEV-infected vs. uninfected comparisons (**Figures 2A,B**) are cellular movement, organismal injury and abnormalities, and hematological system development and function. After 1 h of TBEV-infected tick feeding, 19 host genes shown in the **Figure 2A** network were downregulated and 16 host genes were upregulated. After 3 h of TBEV-infected

tick feeding, 22 host genes in the **Figure 2B** network were downregulated, 12 were upregulated, and one gene (Ngp) had a fold change of zero. Thus, as 1 h progressed to 3 h of TBEV-infected tick feeding, the network displayed a slight shift toward cutaneous gene downregulation (**Figure 2**). The IPA-generated network for the TBEV-infected 3 h vs. the TBEV-infected 1 h tick feeding site (**Figure 2C**) is unique from the **Figures 2A,B** networks, as it lacks Ccl2, KLF2, and ZBTB16, but instead includes 20 unique genes which contribute to this network's association with bio-functions such as molecular transport, organismal injury and abnormalities, and cellular movement.

In both the 1 and 3 h comparisons between TBEV-infected vs. uninfected tick feeding sites, inflammatory response was the primary predicted host response. Based on the IPA transcriptional immunoprofiling, the inflammatory response was projected to be activated in the 1 h comparison of the TBEV-infected vs. uninfected tick feeding site (activation z-score = 1.549), and inhibited in the 3 h comparison (activation z-score = −1.26). Temporal changes in gene expression for all genes predicted to have direct correlative relationships with the inflammatory response were plotted after 1 or 3 h of TBEV-infected vs. uninfected tick feeding (**Figure 3**). Transcriptional levels of cytokines Ccl2, Ccl12, Cxcl1, Cxcl2, Cxcl5, IL6, and IL10 were all upregulated after 1 h of TBEV-infected tick feeding, thus contributing to the overall activation of the inflammatory response (**Figure 3**). At the 3 h time point, the majority of these cytokine transcriptional level were still upregulated, with the exception of Cxcl5 and IL10, which were both slightly downregulated. Real-time PCR validation of a few selected immune genes followed the general pattern observed in the

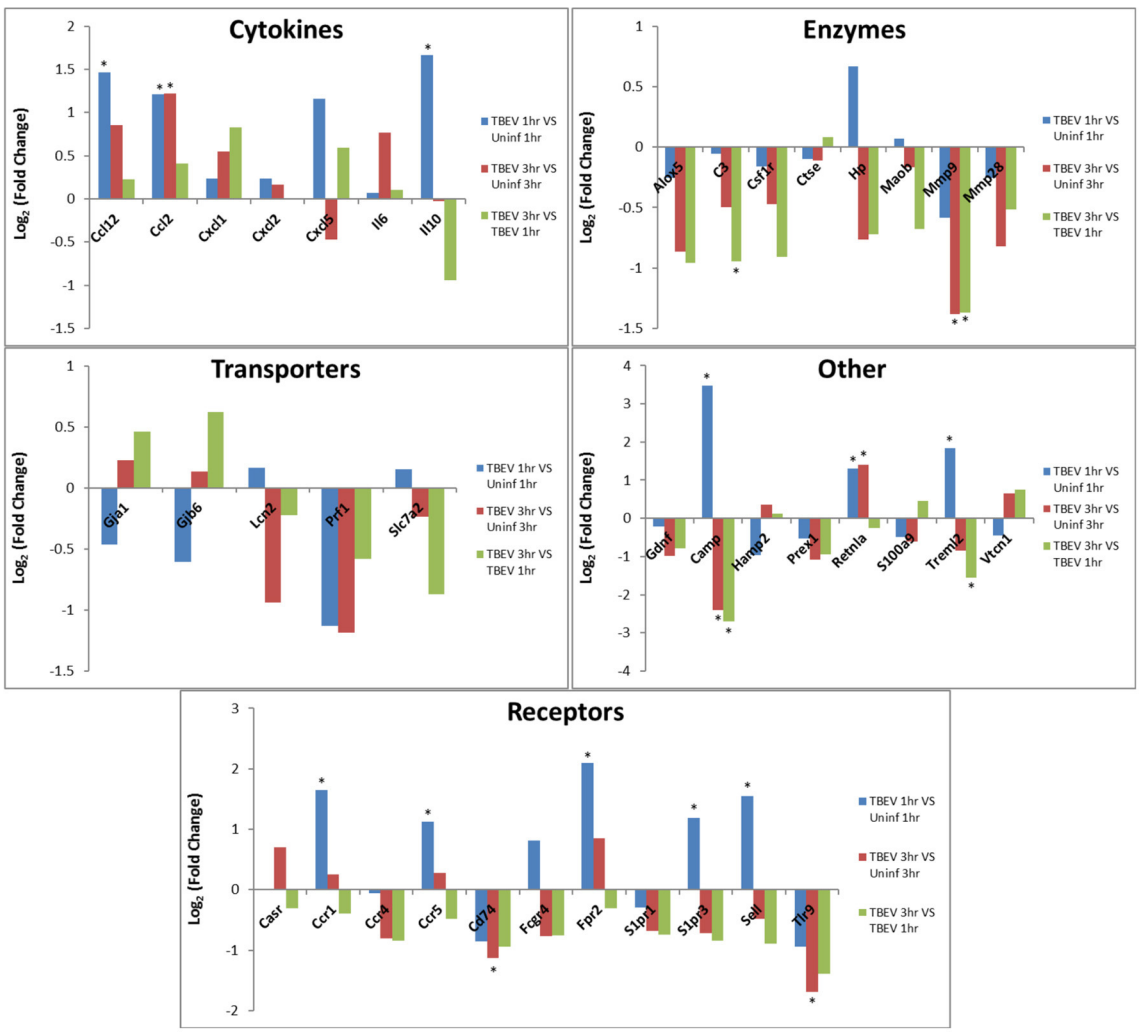

FIGURE 3 | Changes in gene expression related to the inflammatory response after 1 and 3 h of TBEV-infected *Ixodes ricinus* tick feeding. Temporal changes in gene expression data were analyzed by IPA software. Genes predicted to be directly involved in the "Inflammatory Response" were plotted to show temporal changes in gene expression for the following comparisons: TBEV-infected tick feeding loci at 1 h vs. uninfected tick feeding loci at 1 h, TBEV-infected tick feeding loci at 3 h vs. uninfected tick feeding loci at 3 h, TBEV-infected tick feeding loci at 3 h vs. TBEV-infected tick feeding loci at 1 h. Genes with significant modulation in expression ($p \leq 0.05$) are marked with an asterisk.

Illumina NGS analysis (**Figure 1E**). All of the enzymes projected to have a direct, correlative relationship with the inflammatory response were downregulated at 3 h, further contributing to the predicted inhibition of the inflammatory response at the feeding site of a TBEV-infected tick (**Figure 3**). Additionally, transcript expression of several receptors (CCR1, CCR5, and Sell) were all significantly upregulated after 1 h of TBEV-infected tick feeding (**Figure 3**), likely contributing to the overall accumulation of immune cells to the tick attachment site. In summary, the cutaneous gene expression analysis indicates that the inflammatory response is activated after only 1 h of TBEV-infected tick feeding, and that increased recruitment and accumulation of immune cells is expected at the 1 h time point. However, after 3 h of TBEV-infected tick feeding, the cutaneous inflammatory response is predicted to undergo slight inhibition, as demonstrated by the downregulated expression of over half of the genes shown to have a correlative relationship with the inflammatory response (**Figure 3**).

An IPA core comparison analysis was used to analyze changes in host cutaneous gene expression in response to TBEV-infected tick feeding vs. uninfected tick feeding observed across the two experimental time points (1 and 3 h). **Figure 4** shows activation status prediction networks for the top five immune responses or bio-functions generated from the IPA core comparison analysis. "Maintenance of leukocytes" was the top predicted bio-function in the core comparison analysis (**Figure 4**). This IPA category moniker refers to bio-functions associated with the normal cellular activities that maintain cellular homeostasis, including engulfment, phagocytosis, regulation, and stasis of cells. The maintenance of leukocytes was predicted to be inhibited at both the 1 and 3 h comparisons of TBEV-infected vs. uninfected tick feeding (activation z-score = −2 for both time points). At both the 1 and 3 h time points, GATA3 transcription was downregulated (**Figure 4**). GATA3 is a transcription factor required for both the maintenance and development of Th2 cells (Pai et al., 2004). Furthermore, IL34, which is highly expressed by keratinocytes in the epidermis and plays an important role in the maintenance and development of Langerhans cells (Greter et al., 2012), was also downregulated after 1 and 3 h of TBEV-infected tick feeding (**Figure 4**). CCR4 knockout ($CCR4^{-/-}$) bone marrow-derived dendritic cells are less efficient in the maintenance of Th17 responses compared to wild type dendritic cells (Poppensieker et al., 2012); therefore, the downregulation of CCR4 transcription in the present study contributes to the

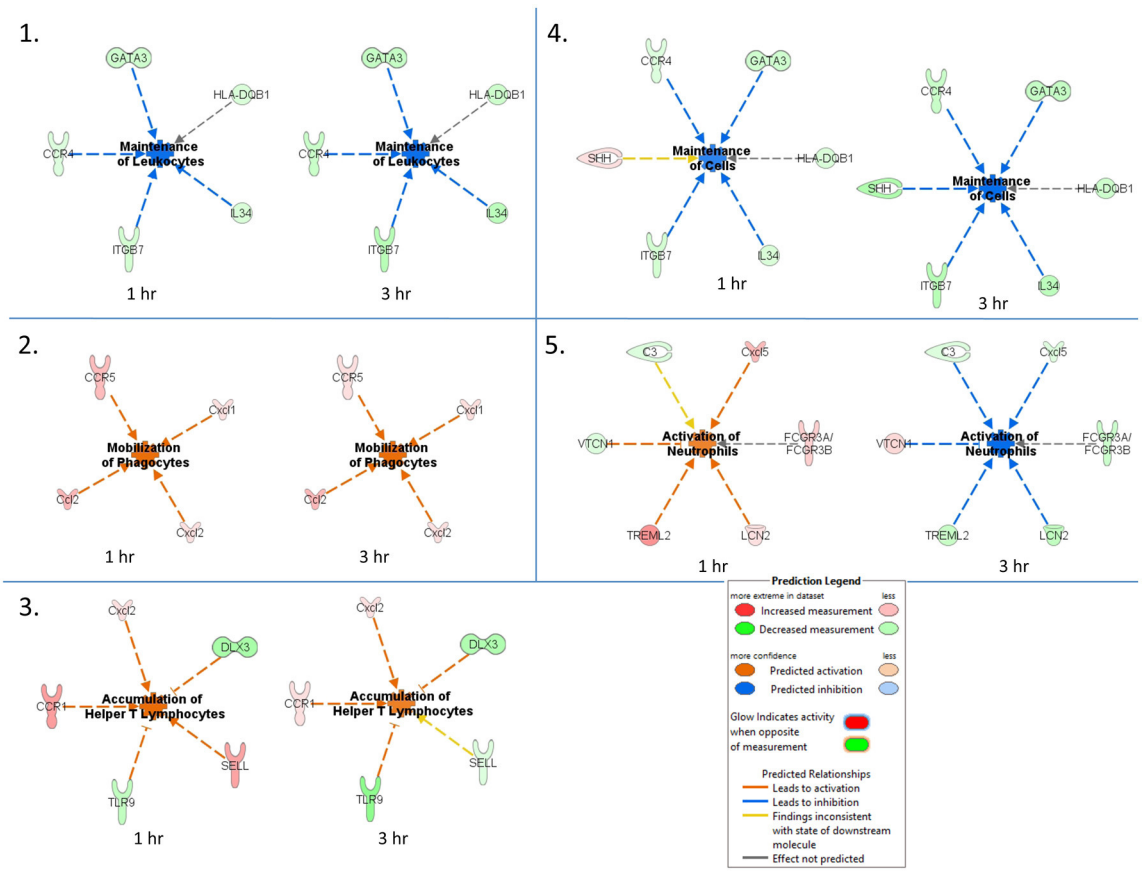

FIGURE 4 | Activation status prediction networks for the top five immune responses / bio-functions generated from the IPA core comparison analysis. An IPA core comparison analysis was used to analyze which biological processes are relevant at 1 and 3 h of TBEV-infected vs. uninfected tick feeding. The predicted activation status of each network is shown.

inhibition of leukocyte maintenance at the 1 and 3 h time points. Mouse integrin subunit beta 7 (ITGB7) is necessary for the maintenance of memory T lymphocytes expressing the CD4 protein (Yang et al., 2011). Since ITGB7 is downregulated in the present transcriptional immunoprofiling study, it likely contributes to the inhibited maintenance of memory CD4+ T cells after 1 and 3 h of TBEV-infected tick feeding.

"Mobilization of phagocytes" was the second predicted bio-function in the core comparison analysis. After 1 and 3 h of TBEV-infected vs. uninfected tick feeding, the mobilization of phagocytes was predicted to be activated (activation z-score = 1.982 for both time points) (**Figure 4**). Monocytes, macrophages, neutrophils, dendritic cells, and mast cells are several of the professional phagocytic cells. In mice, there are two Ccl2 homologs, Ccl2 and Ccl12, both of which are potent phagocyte chemoattractants. The transcript levels of these two chemokines are upregulated at both the 1 and 3 h time points, thereby increasing the mobilization and chemotaxis of phagocytes at the TBEV-infected tick feeding site (**Figures 3**, **4**). CCR5 is known to regulate accumulation of activated macrophages in the West Nile virus-infected mouse brain (Glass et al., 2005), and upregulated transcription of this chemokine receptor in the present study likely contributes to the activated mobilization of phagocytes. Mast cells and tissue resident macrophages release the potent neutrophil chemoattractants, Cxcl1 and Cxcl2 (De Filippo et al., 2008, 2013). In response to tissue inflammation, mast cells positioned near the vasculature initiate neutrophil infiltration from the circulation by releasing Cxcl1 and Cxcl2, while tissue resident macrophages also release Cxcl1 and Cxcl2, recruiting neutrophils deeper into the inflamed tissue (De Filippo et al., 2013). In the present study transcript levels of both these chemokines are both upregulated during TBEV-infected tick feeding, thus contributing to the activated mobilization of neutrophils that infiltrate the tick feeding site very early (**Figures 3**, **4**).

The third predicted bio-function generated in the IPA core comparison analysis was "accumulation of helper T lymphocytes." The activation z-score for this bio-function decreased from 2.19 at 1 h post-TBEV-infected tick feeding to 1.452 at the 3 h time point. Transcript levels of CCR1 and Cxcl2 are upregulated at both time points in the present study and these genes are associated with the recruitment and accumulation of helper T lymphocytes at the cutaneous site of TBEV-infected tick feeding (**Figure 4**). Selectin L (SELL) is a cellular adhesion molecule found on the surface of most lymphocytes that mediates lymphocyte capture and rolling at sites of inflammation (Tedder et al., 1995). In the present study, SELL transcription was upregulated at the 1 h time point, contributing to the IPA prediction that helper T lymphocytes are accumulating, but the downregulated transcription of SELL at 3 h is inconsistent with the predicted activation pattern, as depicted by the yellow arrow in **Figure 4**. TLR9, a receptor that preferentially binds unmethylated CpG sequences in bacterial and viral DNA, was downregulated in the present study (**Figure 4**). Here, the downregulation of TLR9 transcription is predicted to activate the accumulation of T lymphocytes at the skin feeding site of a TBEV-infected tick. This prediction is supported by a recently developed mouse model of cutaneous lupus that is dependent on the expression of TLR7 and the loss of TLR9, which ultimately results in extensive accumulation of IFNγ-producing T lymphocytes (Mande et al., 2017). Distal-less 3 (DLX3) is a transcription factor involved in the terminal differentiation of keratinocytes. In a

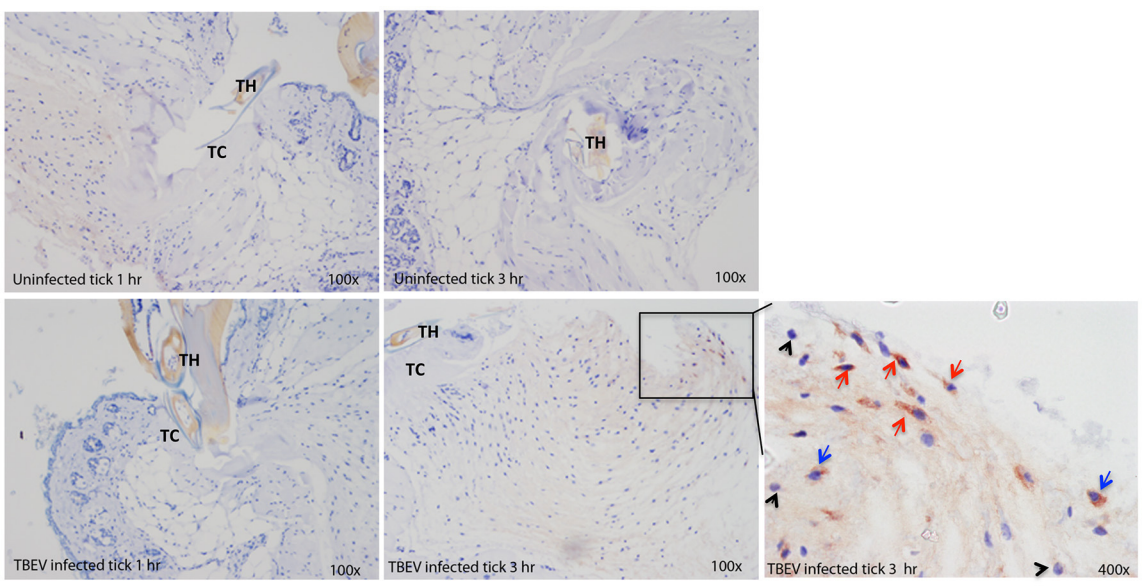

FIGURE 5 | Immunohistochemistry of the *Ixodes ricinus* feeding loci. Five micron sections from skin biopsies harvested at *I. ricinus* feeding sites were subjected to immunohistochemistry procedures to detect TBEV antigens. Red arrows point to TBEV infected fibroblasts; blue arrows point to TBEV infected mononuclear phagocytes, and black arrowheads point to uninfected neutrophils. TH, tick hypostome; TC, tick cement.

mouse model with epidermal ablation of DLX3, there was an increase of IL17-producing $CD4^+$ T cells, $CD8^+$ T cells, and γδ T cells in the skin and draining lymph nodes (Hwang et al., 2011); thus, the downregulated transcription of DLX3 in the skin biopsies from the present study likely contributes to the accumulation of helper T lymphocytes at the cutaneous feeding site of infected ticks (**Figure 4**).

"Maintenance of cells" was the fourth predicted bio-function in the IPA core comparison analysis. This bio-function was predicted to be inhibited at both experimental time points (1 h activation z-score = −1.342, 3 h activation z-score = −2.236). The "maintenance of cells" activation network is very similar to the top predicted network, "maintenance of leukocytes"; however, the only difference is that Sonic hedgehog (SHH) is present in the "maintenance of cells" network (**Figure 4**). The touch dome, a highly specialized mechanosensory epidermal structure composed of distinct keratinocytes in close association with innervated Merkel cells, is maintained by signaling with SHH to tissue-specific stem cells (Xiao et al., 2015). In the present study SHH transcription is upregulated after 1 h of TBEV-infected tick feeding, a finding that is inconsistent with the predicted pattern of inhibited cell maintenance (depicted by the yellow arrow in **Figure 4**); however, at the 3 h time point, SHH transcription was downregulated, contributing to the predicted inhibition of touch dome cell maintenance (**Figure 4**). Some damage to the perineural skin microenvironment is likely to occur because of the mechanical injury induced by tick feeding, and this could ultimately impact SHH signaling and the touch dome homeostasis.

The "activation of neutrophils" was the fifth predicted bio-function in the core comparison analysis. After 1 h of TBEV-infected vs. uninfected tick feeding, neutrophil activation was predicted to be activated (activation z-score = 1.342); however, after 3 h, neutrophils were predicted to be inhibited (activation z-score = −2.236) (**Figure 4**). The upregulated transcription of Cxcl5 and TREML2 at the 1 h time point, followed by the downregulation of these genes at the 3 h time point contribute to this phenomenon because Cxcl5 is a chemokine involved in the activation of neutrophils (Proost et al., 1993) and TREML2 potentiates neutrophil activation in response to G protein-coupled receptor signaling (Halpert et al., 2011). Likewise, Lipocalin-2 (LCN2) transcription was upregulated at 1 h post-TBEV-infected tick feeding and downregulated at 3 h. LCN2 is expressed in neutrophils and is a potent inducer of their chemotaxis and migration to sites of inflammation (Schroll et al., 2012); thus, its upregulation at the 1 h time point further contributes to the activation of neutrophils at the feeding site of TBEV-infected ticks. VTCN1 belongs to the B7 family of costimulatory proteins. VTCN1 inhibits the expansion of neutrophils from their progenitors, as demonstrated by VTCN1-deficient mice that display enhanced neutrophil-mediated innate immunity (Zhu et al., 2009); therefore, the downregulation of VTCN1 transcription after 1 h, and its upregulation after 3 h, contributes to the activation of neutrophils at the cutaneous feeding site of a TBEV-infected tick (**Figure 4**).

Immunohistochemistry

Immunohistochemical analysis was performed at the feeding sites of TBEV-infected and uninfected *I. ricinus* to determine if the gene expression data could be correlated to the tissue morphology and inflammation. The tick feeding site is characterized by extravasated erythrocytes and leucocytes, and with a steady influx of inflammatory cells. As early as 1 h post-TBEV-infected tick feeding, neutrophils were observed at both infected and uninfected tick feeding sites, with a marked increase at the infected tick feeding site. After 3 h of TBEV-infected tick feeding, increased recruitment of inflammatory cells was observed at the infected tick feeding site compared to the uninfected tick feeding site. Among these, TBEV antigens were localized in fibroblasts and mononuclear cells, but not in neutrophils (**Figure 5**). These immunohistochemistry observations support the cutaneous immune gene expression data, showing an inflammatory micro-environment at the feeding site of TBEV-infected ticks. The recruitment of inflammatory cells appears to be much more pronounced in the TBEV-infected tick feeding site than the uninfected tick feeding site.

CONCLUSIONS

The present study demonstrates that TBEV-infected *I. ricinus* adult ticks create an inflammatory environment at the murine cutaneous interface within 1 h of feeding. Significant differences in immune responses were observed between hosts infested with TBEV-infected vs. uninfected ticks. Furthermore, genes associated with neutrophil activation and mobilization were modulated in the presence of TBEV, suggesting that there is an influx of neutrophils and other phagocytic inflammatory cells to the tick feeding site after only 1 and 3 h of infected tick feeding. Immunohistochemistry further supported the cutaneous immune gene expression analysis, demonstrating pronounced recruitment of inflammatory cells, especially neutrophils, to the feeding site of TBEV-infected ticks. Taken together with our earlier study on POWV-infected tick feeding sites (Hermance and Thangamani, 2014), and the current study, it is clearly evident that during the earliest stages of flavivirus-infected tick feeding, a complex, inflammatory micro-environment is created in the host's skin. The present study serves to expand our understanding of the immunological events that occur at the cutaneous interface during the early stages of tick-borne flavivirus transmission to a host.

AUTHOR CONTRIBUTIONS

Conceived the idea: ST; Provided reagents and materials: ST and MK; Performed experiments: ST, MK, MH, RS, SW, MS, and DH; Data analysis: ST, MH, and SW; Wrote manuscript: ST and MH. All authors critically read and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2017.00494/full#supplementary-material

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Anaplasma phagocytophilum MSP4 and HSP70 Proteins Are Involved in Interactions with Host Cells during Pathogen Infection

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Anaplasma phagocytophilum transmembrane and surface proteins play a role during infection and multiplication in host neutrophils and tick vector cells. Recently, *A. phagocytophilum* Major surface protein 4 (MSP4) and Heat shock protein 70 (HSP70) were shown to be localized on the bacterial membrane, with a possible role during pathogen infection in ticks. In this study, we hypothesized that *A. phagocytophilum* MSP4 and HSP70 have similar functions in tick-pathogen and host-pathogen interactions. To address this hypothesis, herein we characterized the role of these bacterial proteins in interaction and infection of vertebrate host cells. The results showed that *A. phagocytophilum* MSP4 and HSP70 are involved in host-pathogen interactions, with a role for HSP70 during pathogen infection. The analysis of the potential protective capacity of MSP4 and MSP4-HSP70 antigens in immunized sheep showed that MSP4-HSP70 was only partially protective against pathogen infection. This limited protection may be associated with several factors, including the recognition of non-protective epitopes by IgG in immunized lambs. Nevertheless, these antigens may be combined with other candidate protective antigens for the development of vaccines for the control of human and animal granulocytic anaplasmosis. Focusing on the characterization of host protective immune mechanisms and protein-protein interactions at the host-pathogen interface may lead to the discovery and design of new effective protective antigens.

Keywords: anaplasmosis, immunology, HL60, tick, vaccine, sheep, *Anaplasma phagocytophilum*

INTRODUCTION

Anaplasma phagocytophilum (Rickettsiales: Anaplasmataceae) is an emerging tick-borne intracellular bacterial pathogen in many regions of the world, but vaccines are not available for prevention of transmission and infection in humans and animals (Dumler et al., 2001; Severo et al., 2013; Stuen et al., 2013, 2015; Bakken and Dumler, 2015). *Anaplasma phagocytophilum* causes

human granulocytic anaplasmosis (HGA), which has emerged as a tick-borne disease of humans in the United States, Europe and Asia (Severo et al., 2013). In Europe, *A. phagocytophilum* is an established pathogen of small ruminants, most notably in sheep, where it was first described as the etiologic agent of tick-borne fever (TBF; Gordon et al., 1932; Foggie, 1951; Dugat et al., 2015). Clinical presentation of *A. phagocytophilum* infection has been also documented in goats, cattle, horses, dogs, cats, roe deer, and reindeer (Severo et al., 2013). Although, *A. phagocytophilum* is recognized as a threat for human and animal health in Europe and the United States, its pathogenic and epidemic potential is neglected in tropical regions of the world (Heyman et al., 2010; Dugat et al., 2015). Prophylactic uses of tetracycline together with acaricide applications for tick control are the main measures to control *A. phagocytophilum* infection in endemic areas (Woldehiwet, 2006; Stuen et al., 2015). However, these control measures raise concerns about their impact on the environment and human health, and the selection of resistant pathogens and ticks (Woldehiwet, 2006; Stuen et al., 2015).

Results using next generation sequencing technologies have advanced our understanding of the mechanisms by which *A. phagocytophilum* infection affects gene expression, protein content and microbiota in the vertebrate host and tick vector (Ge and Rikihisa, 2006; Sukumaran et al., 2006; de la Fuente et al., 2010, 2016a,b,c,d, 2017, Neelakanta et al., 2010; Rikihisa, 2011; Severo et al., 2012; Ayllón et al., 2013, 2015; Hajdušek et al., 2013; Villar et al., 2015a; Cabezas-Cruz et al., 2016, 2017; Gulia-Nuss et al., 2016; Abraham et al., 2017; Mansfield et al., 2017). However, less information is available on the bacterial molecules involved in pathogen infection and multiplication (Ge and Rikihisa, 2007; Huang et al., 2010; Lin et al., 2011; Troese et al., 2011; Mastronunzio et al., 2012; Oliva Chávez et al., 2015; Seidman et al., 2015; Villar et al., 2015b; Truchan et al., 2016). Definition of bacterial proteins involved in host-pathogen and vector-pathogen interactions may provide target antigens for the development of vaccines and therapeutics that interfere with pathogen host infection and transmission by ticks (Gomes-Solecki, 2014; de la Fuente and Contreras, 2015).

Recently, Villar et al. (2015b) demonstrated that *A. phagocytophilum* activates a new mechanism associated with bacterial cell stress and membrane proteins to counteract tick cell response to infection and favor pathogen infection and multiplication. Their results showed that *A. phagocytophilum* proteins, Major surface protein 4 (MSP4) and Heat shock protein 70 (HSP70), are localized on the bacterial membrane where they interact with a possible role during pathogen infection in ticks (Villar et al., 2015b). Furthermore, antibodies against MSP4 and HSP70 inhibited pathogen infection of tick cells, supporting that these proteins are involved in tick-pathogen interactions (Villar et al., 2015b). They proposed that the inhibitory effect of anti-MSP4 and anti-HSP70 antibodies could be the result of the antibodies blocking the interaction between bacterial ligands (e.g., MSP4) and tick receptors or an effect on proteins functionally important for bacterial infection and/or multiplication in tick cells (e.g., HSP70 and those physically and/or functionally interacting with it; Villar et al., 2015b). The results of these experiments suggested that *A. phagocytophilum* MSP4 and HSP70 proteins constitute candidate protective antigens to interfere with pathogen infection in the tick vector, *Ixodes scapularis*.

The characterization of the *A. phagocytophilum* proteome demonstrated that chaperones, surface and stress response proteins are among the most abundant proteins found in *I. scapularis* tick salivary glands (Mastronunzio et al., 2012). HSP70 is a chaperone involved in protein folding and stress response (Johnson, 2012). This protein functions by protecting cells from stress-induced lethal damage and under physiological growth conditions by acting as carriers for immunogenic peptides, assisting in protein export or mediating adherence to host cells and may play an essential role during cell division (Scopio et al., 1994; Susin et al., 2006; Multhoff, 2007; Seydlová et al., 2012). The role of MSPs such as MSP4 in adhesion to tick cells for bacterial infection has been demonstrated in *A. marginale* (de la Fuente et al., 2001) and *A. phagocytophilum* (Villar et al., 2015b).

Anaplasma phagocytophilum infects vertebrate host neutrophils and various tick tissues, where it develops within membrane-bound inclusions in the cell cytoplasm (Severo et al., 2013). However, this pathogen has evolved common molecular mechanisms to establish infection in tick vectors and vertebrate hosts that collectively mediate pathogen infection, development, persistence, and survival (de la Fuente et al., 2016a). These strategies include, but are not limited to, remodeling of the cytoskeleton, inhibition of cell apoptosis, manipulation of the immune response, and the use of rickettsial proteins for infection and manipulation of tick and host gene expression (Cabezas-Cruz et al., 2016; de la Fuente et al., 2016a).

Based on these results, we hypothesized that the use of common strategies by *A. phagocytophilum* to establish infection in ticks and vertebrate hosts resulted in similar functions for MSP4 and HSP70 proteins in host-pathogen and tick-pathogen interactions. To address this hypothesis, in this study we characterized the role of these bacterial proteins in infection of vertebrate host cells and their potential protective capacity in immunized sheep. The results showed that *A. phagocytophilum* MSP4 and HSP70 are involved in host-pathogen interactions during pathogen infection, but were only partially protective against pathogen infection in sheep.

MATERIALS AND METHODS

Ethics Statement

The study was ethically approved by the local Animal Health and Welfare Authority (Diputación Foral de Alava) with reference No. 1820, 12th May 2015, following Spanish ethical guidelines and animal welfare regulations (Real Decreto 53/2013).

Production of Recombinant Proteins and Rabbit Antibodies

The His-tag recombinant *A. phagocytophilum* human NY18 isolate (Asanovich et al., 1997) proteins MSP4 (AFD54597) and HSP70 (KX891324) were produced in *Escherichia coli* BL21 cells (Champion pET101 Directional TOPO Expression kit, Carlsbad, CA, USA), after induction with IPTG and purified using the Ni-NTA affinity column chromatography system (Qiagen Inc.,

Valencia, CA, USA) as previously described (Villar et al., 2015b). Recombinant purified proteins showed purity higher than 85% of total proteins and were used to immunize rabbits to purify IgGs from preimmune and immunized animals (Montage Antibody Purification Kit and Spin Columns with PROSEP-A Media, Millipore, Billerica, MA, USA) for Western blot and antibody inhibition analyses as previously described (Villar et al., 2015b).

Surface Trypsin Digestion of *A. phagocytophilum* from Infected HL60 Human Cells

The *A. phagocytophilum* human NY18 isolate was propagated in cultured HL60 human promyelocytic cells as previously described (de la Fuente et al., 2005). The *A phagocytophilum*-infected cells ($\sim 1 \times 10^7$ cells) were collected when 70–80% of the cells were infected as determined by detection of intracellular morulae in stained cytospin cell smears. Host cell-free bacteria were isolated from cell lysates after five passages through a 27-gauge syringe, followed by differential centrifugation in Percoll gradients as previously described for *A. marginale* to separate bacteria from host cell debris (Lis et al., 2014). The pellet of purified *A. phagocytophilum* was resuspended in 200 µl of SPG buffer (0.25 mM sucrose, 10 mM sodium phosphate, 5 mM L-glutamic acid, pH 7.2), and 5 µl of sequencing-grade trypsin (Promega, Madison, WI, USA) was added to half of the cell reaction mixture. Bacteria were incubated at 37°C for 30 min and then centrifuged at 10,000 × g for 15 min and resuspended in Laemmli protein loading buffer, boiled for 5 min and loaded onto a 12% SDS-PAGE and analyzed by Western blot using rabbit antibodies against recombinant MSP4 and HSP70 proteins as previously described (Villar et al., 2015b).

Tertiary Models and Protein-Protein Docking

The active *A. phagocytophilum* HSP70 and MSP4 proteins were modeled using I-TASSER (Zhang, 2008), and the unbound (apo)-HSP70 protein was modeled using Robetta (Kim et al., 2004). All tertiary models were optimized with the Schrödinger's Maestro Protein Preparation Wizard (Li et al., 2007). All steric clashes were resolved via minimization with the default settings in the Schrodinger's Maestro package. For the tertiary models, the Protein Preparation Wizard clusters at the highest degree of hydrogen bonding in equilibrium were used. Monte Carlo orientations were performed (100,000) for each cluster. The optimized structure is based on electrostatic and geometric scoring functions. The membrane positioning for MSP4 was calculated by the OPM database (Lomize et al., 2006) and generated using the Desmond systems builder (Bowers et al., 2006) as part of the Schrodinger's Maestro package. The protein-protein docking was assessed using the SwarmDock server (Torchala et al., 2013) that incorporates flexible docking by exploring in proximity to the Cartesian center of mass of the target protein. Minimization steps are included for the whole system. The poses are calculated based on the most energy favorable poses, minimized once again and sent to the user. We chose to analyze the top 10 poses since these were highly energy favorable (−41 to −54 kcal/mol). The server also produces the residue contacts made between both proteins. All structures were visualized and analyzed using the Visual Molecular Dynamics (Humphrey et al., 1996).

Adhesion of Recombinant *E. coli* Strains to HL60 Human Cells

The adhesion of recombinant *E. coli* strains to HL60 human cells was characterized as previously reported (de la Fuente et al., 2001; Villar et al., 2015b). Briefly, *E. coli* strains producing *A. phagocytophilum* MSP4, HSP70, and mutant HSP70 with truncated peptide-binding domains that are involved in protein-protein interactions (Villar et al., 2015b) recombinant proteins were grown and induced as described before. The *E. coli* cells transformed with expression vector alone were used as negative control. Cell densities were determined and adjusted to 10^8 cells per ml in Luria Broth (LB). One hundred microliters (10^7 bacteria) culture were added to 900 µl of 10^6 cells per ml suspensions of HL60 human cells in LB. Human cells and bacteria were incubated for 30 min at 37°C with occasional agitation. Cells were then collected by centrifugation, washed two times in PBS and resuspended in 100 ml of PBS. Elimination of unbound bacteria from human cells with bound bacteria was performed by Percoll (Sigma, St. Louis, MI, USA) gradient separation (de la Fuente et al., 2001). The band containing human cells was removed with a pipette and washed in PBS. The final cell pellet was lysed in 1 ml of sterile water and 5 µl plated onto LB agar plates containing 100 µg of ampicillin per ml. Two replicates were done for each experiment. Adhesive bacteria were quantitated as the number of colony forming units (CFU) recovered from each test and compared to the control values by Chi2 test ($P = 0.001$; $N = 2$).

Transmission Electron Microscopy (TEM)

The HL60 human cells incubated as described above with *E. coli* strains producing *A. phagocytophilum* MSP4 and HSP70 recombinant proteins or transformed with expression vector alone as controls were pelleted and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h at room temperature. Fixed cells were washed three times in 0.1 M phosphate buffer with 4% glucose and embedded in 2% agar at 60°C. Samples were post-fixed using 2% OsO_4 for 2 h at room temperature, three times washed and dehydrated in a graded series of acetone (30–100%) solutions for 15 min at each step. Samples were infiltrated with 25, 50, and 75% solutions of Spi Pon Epoxy resin (Structure Probe, Inc. Supplies, West Chester, PA, USA) diluted in anhydrous acetone for 1 h at each step. Samples were left in 100% resin overnight, transferred to embedding molds and polymerized at 60°C for 48 h. Ultrathin sections were contrasted in ethanolic uranyl acetate and lead citrate, carbon coated and observed in a JEOL 1010 TEM (JEOL Ltd., Akishima, Tokio, Japan) at an accelerating voltage of 80 kV. Images were captured using a Mega View III camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Prediction of B-cell Epitopes

Anaplasma phagocytophilum MSP4 (AFD54597) and HSP70 (AAC31306) amino acid sequences were aligned using MAFFT version 7, applying a gap opening penalty of 3 (range 1–3, default 1.53; Katoh and Standley, 2013). Sequence homology was calculated using Clustal Omega (Sievers et al., 2011). The linear B-cell epitopes on *A. phagocytophilum* MSP4 and HSP70 proteins were predicted using the Bepipred Linear Epitope Prediction tool (http://tools.immuneepitope.org/bcell/; Haste Andersen et al., 2006; Larsen et al., 2006; Ponomarenko and Bourne, 2007). Subsequently, to search for epitope homology, each predicted epitope within each protein was aligned with the full sequence of the other protein.

Antibody Inhibition Assay

The inhibitory effect of rabbit IgG antibodies against MSP4 and HSP70 recombinant proteins on *A. phagocytophilum* human NY18 (Asanovich et al., 1997) and sheep (Alberdi et al., 2015) isolates infection of HL60 human cells was conducted as described previously for tick cells (Villar et al., 2015b). The inhibitory effect of IgG antibodies purified from MSP4 and MSP4-HSP70 immunized and control sheep at 0 and 94 days post-infection (dpi) on *A. phagocytophilum* human NY18 isolate infection of HL60 human cells was conducted using the same experimental approach as for rabbit IgG. The IgGs were purified from sheep sera using the NAb Protein G spin kit (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's recommendations. HL60 cells were pooled and used to seed 24-well plates for each assay. Each well received 1×10^6 cells in RPMI 1,640 medium (Gibco, Thermo Fisher, Madrid, Spain) 48 h prior to inoculation with *A. phagocytophilum*. Infected cultures for inoculum were harvested when infection reached 80% and host cells were mechanically disrupted with a syringe and 26-gauge needle. Rabbit or sheep purified IgGs (2.2–2.4 mg/ml) were mixed with inoculum (1:1) for 60 min before being placed on the cell monolayers. Each monolayer then received 100 µl of the inoculum plus IgG mix and plates were incubated at 37°C for 30 min. The inoculum was removed from the wells and cell monolayers washed three times with PBS. Complete medium (1 ml) was added to each well and the plates were incubated at 37°C. The control for each trial included inoculum incubated with rabbit pre-immune IgG or control sheep IgG. Four replicates were done for each treatment. After 7 days, cells from all wells were harvested, resuspended in 1 ml PBS and frozen at −70°C. Samples were thawed and solubilized with 1% Triton-X100 and processed for *A. phagocytophilum* detection by PCR after DNA extraction using TriReagent (Sigma) according to the manufacturer's recommendations. *Anaplasma phagocytophilum* infection levels were determined by *msp4* real-time PCR normalizing against human *β-actin* as described previously (de la Fuente et al., 2005) but using oligonucleotide primers MSP4-L (5′-CCTTGGCTGCAGCACCACCTG-3′) and MSP4-R (5′-TGCTGTGGGTCGTGACGCG3′), with PCR conditions of 5 min at 95°C and 35 cycles of 10 s at 95°C, 30 s at 55°C and 30 s at 60°C. Results were compared between treatments by the Student's *t*-test with unequal variance ($P = 0.05$; $N = 4$).

Protein Inhibition Assay

HL60 cells were incubated with 4 µM MSP4 and HSP70 recombinant proteins or their combination in culture media for 1 h at 37°C and 5% CO2 in a humidified atmosphere. For antigen combination, equal molar ratios of each protein, equivalent to one part of HSP70 and two parts of MSP4, were incubated at 4°C on a rotator overnight. HL-60 cells (4×10^5 cells/well) were fixed in 4% paraphormaldehyde (PFA) in PBS for 1 h at room temperature (RT), and then incubated with a 6x-His epitope tag monoclonal antibody (3 µg/ml mouse IgG1, Thermo Fisher 4A12E4) for 1 h at RT. After washing, cells were incubated with FITC-conjugated goat anti-mouse IgG (1/100, Sigma F2012) for 1 h at RT. Protein binding was assessed by flow cytometry using a FACScalibur® Flow Cytometer, equipped with the CellQuest Pro® software (BD-Biosciences, San Jose, CA, USA) as previously described (Seidman et al., 2015; Hebert et al., 2017). Incubation with PBS was used as negative control. The viable cell population was gated according to forward scatter and side scatter parameters. To determine the effect of MSP4 and HSP70 recombinant proteins on *A. phagocytophilum* infection, HL60 cells were incubated with 4 µM MSP4 and HSP70 recombinant proteins or their combination for 1 h, after which *A. phagocytophilum* human NY18 isolate bacteria purified as described above were added and incubated with the host cells in the continued presence of recombinant protein for 2 h. Unbound bacteria and proteins were removed and the infection was allowed to proceed for 48 h. Then, cells were harvested and infection levels determined by PCR and statistically analyzed as described above. The *Rhipicephalus microplus* Subolesin recombinant protein (SUB; Merino et al., 2011) and PBS were included as controls. Four replicates were done for each treatment.

Lamb Immunization and Infection with *A. phagocytophilum*

The recombinant MSP4 was formulated alone or combined with HSP70. Antigen combination was done as described above in Section Protein Inhibition Assay. Recombinant antigens or saline control were adjuvated in Montanide ISA 50 V2 (Seppic, Paris, France; Merino et al., 2013). Nine 3-month old lambs of the Latxa breed (Basque Country, Spain) were selected from the experimental sheep flock maintained at NEIKER and were kept indoor during the experiment. This flock has no known history of ticks or tick-borne diseases. However, blood of lambs and their dams were analyzed prior to the start of the study to check their status for hemoparasites *Theileria*, *Babesia,* and *Anaplasma* spp. as previously described (Hurtado et al., 2015). All animals were negative for these hemoparasites. Three groups of 3 lambs each with similar live weight were formed. Lambs from each group were injected subcutaneously in the loose skin of the axilla (armpit) using a sterile syringe with removable needle 20 G × 1″ (9.0 × 25 mm), and taking aseptic precautions. Lambs were immunized three times on days 55, 30, and 10 before

experimental infection with 1 ml doses of MSP4 (100 μg/dose), MSP4-HSP70 (100 μg of equal molar ratios of each protein/dose) or adjuvant/saline as control. The strain of *A. phagocytophilum* used for experimental infection originated from an infected lamb in Norway, which suffered TBF but was negative to other tick-borne pathogens (Alberdi et al., 2015; Stuen et al., 2015). The inoculum consisted of *A. phagocytophilum* infected heparinised blood that had been stored at −70°C with 10% dimethyl sulfoxide (DMSO). Once unfrozen, 1 ml of infected blood containing 1.8 × 10^6 infected neutrophils per ml was intravenously inoculated into each experimental lamb through the jugular vein using sterile winged infusion sets with needle 21 G × 3/4″ (0.8 × 19 mm).

Sheep Samples and Analysis

Whole blood and serum samples were collected from the jugular vein of each lamb previous to each immunization, daily starting on infection day during 10 days, and at weekly intervals until the end of the experiment at 94 dpi (Supplementary Table 1). Rectal temperatures were taken daily until 10 dpi and then weekly until 94 dpi (Supplementary Table 1). Lambs were also weighed periodically (Supplementary Table 1). Hematological analyses including leukocyte and erythrocyte cell counts, leukocyte cell differentiation (percent neutrophils, lymphocytes, monocytes and eosinophils), hemoglobin levels, hematocrit, mean cell volume (MCV), and mean corpuscular hemoglobin (MCH) were performed with an electronic counter (Hemavet 950, Drew, USA) in blood samples collected in EDTA-containing tubes (Supplementary Table 1). Blood smears stained with Giemsa stain were examined to investigate the presence of *A. phagocytophilum* in blood cells (Supplementary Table 1). At least 100 neutrophils were counted and examined to calculate the number of infected neutrophils per milliliter blood of each lamb throughout the experiment. The differential percent of *A. phagocytophilum*-infected neutrophils was calculated as the difference between values at different dpi and values at 3 dpi when infected neutrophils were first detected (Supplementary Table 1).

Analysis of the Antibody Response in Lambs

An indirect ELISA test was performed to detect IgG antibodies against MSP4 and HSP70 proteins in immunized and control lambs using serum samples collected before each immunization and at 0, 7, 10, and 94 dpi. High absorption capacity polystyrene microtiter plates were coated with 100 μl (0.01 μg/μl solution of purified recombinant MSP4 or HSP70 protein) per well in carbonate-bicarbonate buffer (Sigma). After an overnight incubation at 4°C, coated plates were blocked with 100 μl/well of blocking solution (5% skim milk in PBS). Serum samples or PBS as negative control were diluted (1:100, v/v) in blocking solution and 100 μl/well were added into duplicate wells of the antigen-coated plates. After an overnight incubation at 4°C, the plates were washed three times with a washing solution (PBS containing 0.05% Tween 20). A donkey anti-sheep IgG-peroxidase conjugate (Sigma) was added (diluted 1:1000 in blocking solution) and incubated at room temperature for 1 h. After three washes with washing solution, 100 μl/well of substrate solution (Fast OPD; Sigma) was added. Finally, the reaction was stopped with 50 μl/well of 3N H_2SO_4 and the optical density (OD) was measured in a spectrophotometer at 450 nm. Antibody titers were expressed as OD_{450nm} ($OD_{lambsera}$ $OD_{PBScontrol}$). The antigen-specific antibody response in immunized lambs was corroborated by ELISA using pooled sera collected at 0 dpi, but incubating sera with *A. phagocytophilum* purified from infected HL60 human cells as described above in Section Antibody Inhibition Assay. Results from rectal temperature and hematological analyses, differential percent of *A. phagocytophilum*-infected neutrophils and antibody titers were compared between immunized and control groups by two-way ANOVA test ($P = 0.05$; $N = 3$).

Analysis of *A. phagocytophilum* DNA Levels in Lambs

For the analysis of *A. phagocytophilum* infection in lambs during the trial, DNA was extracted from 200 μl of blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), including negative extraction controls every 9 samples. DNA was stored at −20°C until subsequent analysis. The presence of *Anaplasma* spp. was firstly determined using a real-time PCR assay for the screening of piroplasms and *Anaplasma* spp. (RTi-PCR1) that targets the 16S rRNA gene of *Anaplasma* spp. and the 18S rRNA gene of piroplasms of the genera *Theileria* and *Babesia* (Hurtado et al., 2015). The assay also includes an internal amplification control (IAC) to monitor for possible inhibition of the PCR reaction. All samples positive to *Anaplasma* spp. in the RTi-PCR1 were analyzed with a multiplex PCR assay that specifically amplifies the major surface protein 2 (*msp2*) gene of *A. phagocytophilum*, and the *msp4* gene of *Anaplasma ovis* (RTi-PCR2). Sequences of primers and probes, as well as details on cycling conditions were as reported previously (Hurtado et al., 2015). Analyses were performed in 20 μl volume reactions using an ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For the quantitative analysis of *A. phagocytophilum* infection levels, DNA was extracted from 200 μl blood samples using Nucleospin 96 Blood (Machery-Nagel, Düren Germany). A quatitative real-time PCR was conducted on DNA samples using a Quantitect SYBR Green RT-PCR Kit and a Rotor Gene Q thermocycler (Qiagen, Inc. Valencia, CA, USA) following manufacturer's recommendations. A dissociation curve was run at the end of the reaction to ensure that only one amplicon was formed and that the amplicon denatured consistently in the same temperature range for every sample (Ririe et al., 1997). The DNA levels were normalized against sheep *aldolase B* using primers Ovi-ALDOB-F: CCCATCTTGCTATCCAGGAA and Ovi-ALDOB-R: TACAGCAGCCAGGACCTTCT, and the genNorm method (ddCT method as implemented by Bio-Rad iQ5 Standard Edition, Version 2.0; Livak and Schmittgen, 2001). Normalized Ct values were compared between immunized and control groups by Student's *t*-test with unequal variance ($P = 0.05$; $N = 3$).

RESULTS

The *A. phagocytophilum* MSP4 and HSP70 Proteins are Localized on the Bacterial Membrane and Involved in Pathogen Infection of HL60 Human Cells

The subcellular localization of MSP4 and HSP70 proteins was characterized in *A. phagocytophilum* purified from infected HL60 human promyelocytic leukemia cells, mock treated or surface digested with trypsin and loaded onto polyacrylamide gels for Western blot analysis using rabbit antibodies specific against recombinant proteins. The results showed that as a transmembrane protein, MSP4 was partially resistant to trypsin digestion in *A. phagocytophilum* from HL60 cells, while HSP70 was extracellular and exposed to protease digestion (**Figure 1A**).

The apo and bound *A. phagocytophilum* HSP70 models showed that the major structural difference between the two HSP70 tertiary structures is at the C-terminus (residues 400–533), with a 40–90 Å α-carbon backbone root mean square deviation (RMSD; Supplementary Figure 1A). The *A. phagocytophilum* MSP4 showed a 25° ± 1° tilt from the membrane with its N/C-terminus oriented toward the cytosol, and the β-sheets buried within the membrane with remaining β-hairpin loops exposed extracellularly (Supplementary Figure 1B). The models suggested a limited number of possible MSP4-HSP70 binding positions due to the membrane orientation of MSP4 (Supplementary Figure 1C). The energy score of the optimum MSP4-HSP70 bound state (Supplementary Figure 1C) was calculated at −46 kcal/mol. Furthermore, although the residue map showed that the majority of protein-protein contacts are formed between the β-sheets of MSP4 buried within the membrane and the C-terminus of HSP70, several contacts between the β-hairpin loops of MSP4 and the N-terminus of HSP70 are exposed extracellularly, and therefore these residues may act as markers for mutational studies and antibody targeting (Supplementary Figure 1C). These models supported the interaction between *A. phagocytophilum* MSP4 and HSP70 proteins when localized on the bacterial membrane.

The binding of HSP70 and MSP4 to HL60 human cells was characterized using recombinant proteins and *E. coli* producing surface-exposed *A. phagocytophilum* proteins. The results demonstrated that MSP4 and HSP70 are involved in binding to human promyelocytic leukemia cells (**Figures 1B,C**). Furthermore, *E. coli* producing the mutant HSP70 with truncated peptide-binding domains that are involved in protein-protein interactions did not bind to human HL60 cells, thus supporting the role of this protein in interactions with host cells. The interaction of recombinant *E. coli* producing *A. phagocytophilum* MSP4 (**Figure 1D**) and HSP70 (**Figure 1E**) proteins with HL60 human cells was also characterized by electron microscopy in comparison with control *E. coli* cells to provide additional evidence for the role of these proteins in the interaction with vertebrate host cells.

To provide additional support for the role of *A. phagocytophilum* MSP4 and HSP70 proteins in the interaction with and infection of vertebrate host cells, recombinant proteins and antibodies against these proteins were used to evaluate their effect on pathogen infection of HL60 human cells. Anti-MSP4 and anti-HSP70 or recombinant MSP4 and HSP70 proteins were incubated with HL60 cells prior to infection with *A. phagocytophilum*. The results showed an inhibitory effect of anti-MSP4 and anti-HSP70 antibodies on infection of human cells with *A. phagocytophilum* human NY18 (**Figure 2A**) and sheep (**Figure 2B**) isolates when compared to cells treated with pre-immune serum. Furthermore, incubation with HSP70 and MSP4-HSP70 but not MSP4 recombinant proteins inhibited infection of human cells with *A. phagocytophilum* human NY18 isolate when compared to cells incubated with PBS or SUB controls (**Figure 2C**).

These results evidenced a role for MSP4 and HSP70 proteins in *A. phagocytophilum* adhesion to vertebrate host cells, and suggested a role for HSP70 during pathogen infection. These results also suggested that these proteins might constitute candidate protective antigens to prevent or control pathogen infection.

Experimental Infection with *A. phagocytophilum* Correlates with TBF in Lambs

To gain additional information on the role of *A. phagocytophilum* MSP4 and HSP70 proteins in host-pathogen interactions, sheep that are natural hosts for this pathogen were selected for immunization with recombinant proteins followed by experimental infection with *A. phagocytophilum*. Groups of three lambs each were immunized with recombinant MSP4, MSP4-HSP70 combination or adjuvant/saline control and infected with a sheep isolate of *A. phagocytophilum*. Then, several parameters including rectal temperature, animal weight, hemoglobin content, and hematological variables were evaluated in immunized and control *A. phagocytophilum*-infected lambs to correlate with TBF main clinical signs (Supplementary Table 1).

The results showed signs of TBF in lambs infected with *A. phagocytophilum*. Evidence of *A. phagocytophilum* in neutrophils was obtained for all animals (Supplementary Table 1). Fever was evident in animals from all groups, primarily between 3 and 9 dpi (**Figure 3A** and Supplementary Table 1). Although immunized lambs tend to gain more weight, differences with controls were not significant (Supplementary Table 1). Control sheep showed evidence of anemia at 4 dpi, and between 8 and 10 dpi with all animals being anemic at 9 dpi, a result that correlated with low erythrocyte counts at 8–10 dpi (**Figure 3B** and Supplementary Table 1). The percent neutrophils in the leukocyte population increased in all animals after *A. phagocytophilum* infection between 4 and 9 dpi (**Figure 3C** and Supplementary Table 1). A severe neutropenia was evident in all animals after 59 dpi and lasted until the end of the experiment at 94 dpi (**Figure 3C** and Supplementary Table 1). Although monocyte levels were within normal values throughout the experiment, an increase was observed in all animals after infection between 2–10, 38–45, and 59–94 dpi (**Figure 3D** and Supplementary Table 1).

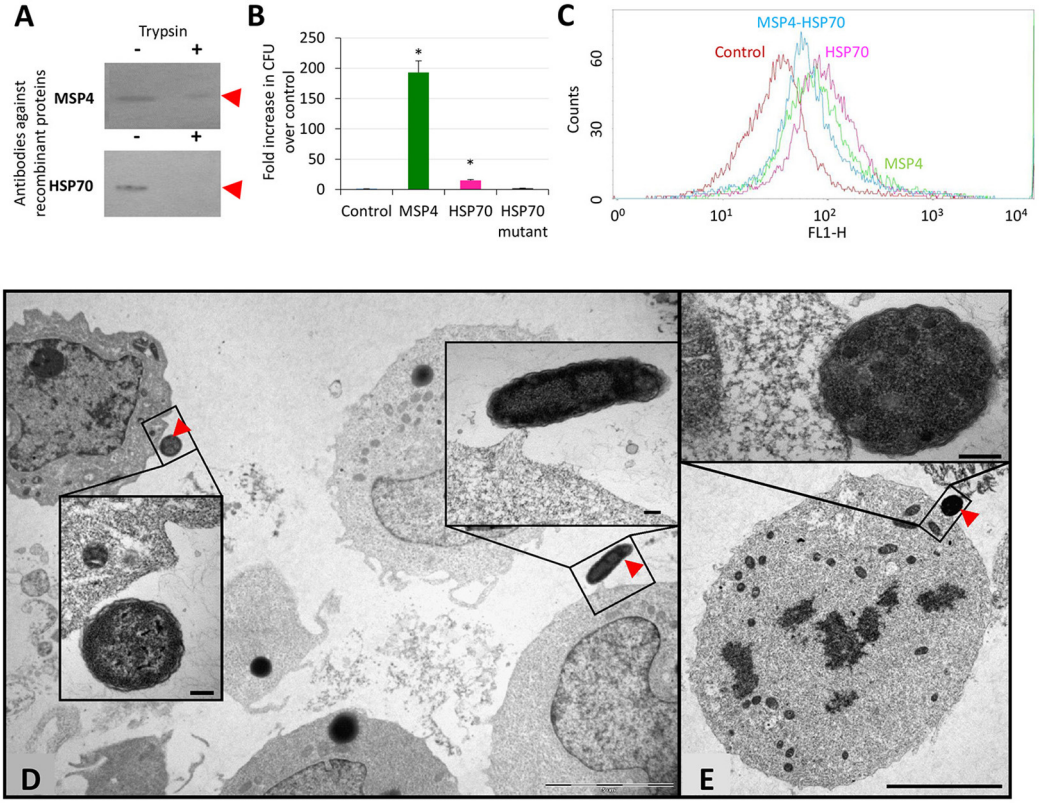

FIGURE 1 | Role of *A. phagocytophilum* MSP4 and HSP70 proteins in interactions with HL60 human cells. **(A)** *A. phagocytophilum* (NY18) purified from infected HL60 human cells were mock treated (–) or surface digested with trypsin (+) and 10 μg protein loaded onto polyacrylamide gels for Western blot analysis using rabbit antibodies produced against recombinant proteins. These experiments have been repeated three times with similar results. **(B)** *E. coli* strains were grown and induced for the production of recombinant proteins. *E. coli* cells transformed with expression vector alone were used as negative control (Control). Bacteria adhesion to human HL60 cells was quantitated as the number of colony forming units (CFU) recovered from each test and compared to the control values by Chi2 test. Asterisks denote statistical significant differences between CFU recovered from *E. coli* transformed with MSP4 or HSP70 and the control ($P < 0.001$; $N = 2$ replicates per treatment). **(C)** HL-60 cells were incubated with *A. phagocytophilum* human NY18 isolate in the presence of recombinant MSP4, HSP70 and their combination. HL60 cells incubated with PBS served as negative controls. After washing to remove unbound bacteria and proteins, host cells were incubated for 48 h and binding of recombinant proteins to human host cells was analyzed by flow cytometry. The viable cell population was gated according to forward scatter and side scatter parameters. **(D,E)** Representative images of the adhesion of recombinant *E. coli* producing membrane exposed *A. phagocytophilum* proteins to human HL60 cells. **(D)** *E. coli* producing MSP4 or **(E)** HSP70 were incubated with HL60 cells and revealed by TEM to show adhesion to human cells (arrows). Cells incubated with control bacteria did not show adhesion to HL60 cells. Details of both interacting cells are shown in insets. Scale bars: 5 μm **(D,E)** and 200 nm (insets).

The Antibody Response in Immunized Lambs Is Specific for *A. phagocytophilum* MSP4 and HSP70 Recombinant Proteins

The results showed that anti-MSP4 (**Figure 4A**) and anti-HSP70 (**Figure 4B**) IgG antibody titers were higher in MSP4-immunized than in control animals from second immunization until 94 dpi. In MSP4-HSP70 immunized lambs, anti-MSP4 IgG antibody titers were significantly higher until 10 dpi (**Figure 4A**), while anti-HSP70 IgG antibody titers remained higher then in control animals until 94 dpi (**Figure 4B**). The increase in the IgG antibody response to *A. phagocytophilum* MSP4 and HSP70 proteins after experimental infection was higher in immunized than in control animals (**Figures 4A,B**), suggesting an anamnestic response that may be protective against pathogen infection.

The IgG antibody response in MSP4 and MSP4-HSP70 immunized lambs was specific for MSP4 and HSP70 proteins as supported by the dilution effect observed after incubating sera collected before infection (0 dpi) with *A. phagocytophilum* purified from infected HL60 human cells (**Figure 4C**). However, the antibody response in MSP4-immunized lambs raised a question regarding the anti-HSP70 response in these animals. Possible explanations to this question are the production of polyreactive antibodies, and the existence of common B-cell epitopes between *A. phagocytophilum* MSP4 and HSP70 proteins. The MSP4 and HSP70 proteins were aligned and the linear B-cell epitopes were predicted and aligned to both protein sequences (Supplementary Figure 2A). A total of 14 and 32 linear B-cell epitopes were predicted for MSP4 and HSP70, respectively. Only epitopes longer than 8 amino acids were included in further analysis, resulting in 5 and 12 linear B-cell epitopes identified in MSP4 and HSP70, respectively. Only one epitope of MSP4 (DGATGYAI) aligned without gaps to a region of HSP70 (DGQTAVTI)

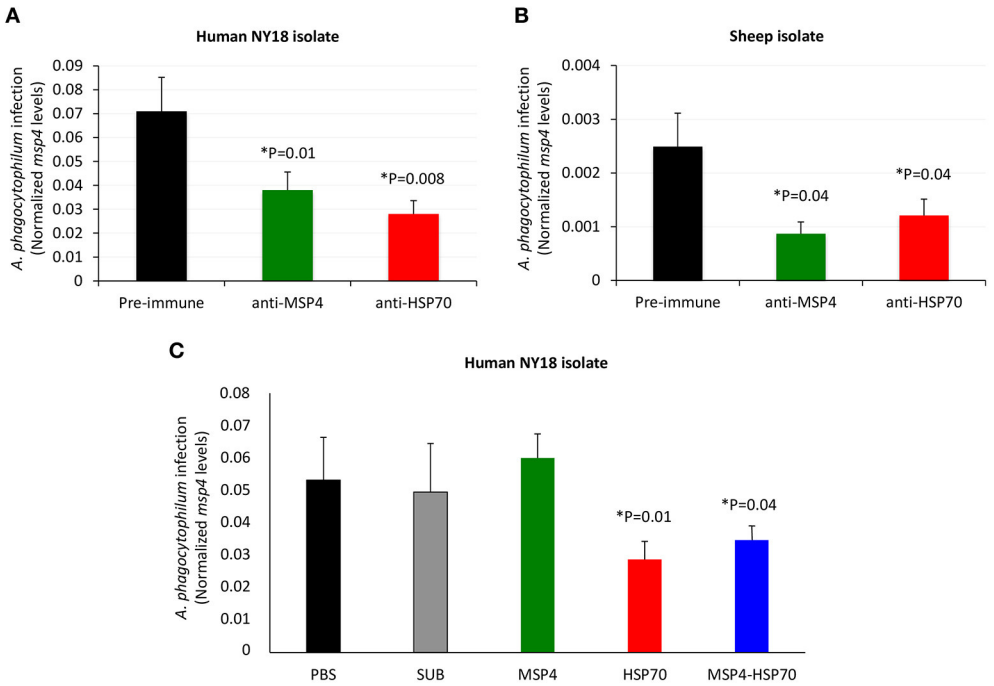

FIGURE 2 | Role of *A. phagocytophilum* MSP4 and HSP70 proteins in infection of HL60 human cells. **(A,B)** Rabbit antibodies against *A. phagocytophilum* MSP4 and HSP70 recombinant proteins or **(C)** MSP4, HSP70 and MSP4-HSP70 recombinant proteins were used to characterize the inhibition of pathogen infection of HL60 human cells. Rabbit purified IgGs or recombinant proteins were mixed with *A. phagocytophilum* inoculum of human NY18 or sheep isolates for 60 min before being placed on the cell monolayers. Treatments included rabbit pre-immune serum, PBS and SUB as negative controls. *A. phagocytophilum* infection levels were determined by *msp4* real-time PCR normalizing against human *β-actin*. Results were compared between groups treated with pre-immune and anti-MSP4/HSP70 antibodies **(A,B)** or between groups treated with PBS or SUB and recombinant proteins **(C)** by the Student's *t*-test with unequal variance (*$P < 0.05$; $N = 4$ replicates per treatment).

with 50% identity (Supplementary Figure 2A). Three epitopes from HSP70 (FNDAQRQATKDAGTI, AGIKDNSKV and SNCSTDTLQQ) aligned without gaps to regions of MSP4 (FVAVGRDATLTPDNF, AGIPASNRV and AVCACSLLIS), with 26, 44, and 20% identity, respectively. These results suggested that antibodies against MSP4 epitopes (i.e., DGATGYAI) could cross-react with a region of HSP70 (DGQTAVTI), thus explaining the anti-HSP70 response in MSP4-immunized lambs. Furthermore, these epitopes were highly conserved because *A. phagocytophilum* MSP4 and HSP70 protein sequences show a high homology between different strains (Supplementary Figures 2B–D). In 56 of the MSP4 sequences available containing this region, the B-cell epitope was conserved (Supplementary Figure 2D). This region was conserved in all HSP70 sequences available in GenBank (Supplementary Figure 2D).

Immunization of Lambs with *A. phagocytophilum* MSP4 and MSP4-HSP70 Recombinant Proteins Is Only Partially Protective against TBF

To address the role of *A. phagocytophilum* MSP4 and HSP70 proteins as potential targets for the development of vaccines for the control of pathogen infection in vertebrate hosts, their potential protective capacity was characterized in immunized lambs.

The IgG antibody levels to MSP4 and HSP70 antigens remained higher after infection in immunized animals when compared to controls (**Figures 4A,B**). Although all animals showed signs of fever after infection with similar fever relapses, rectal temperature decreased faster in lambs immunized with MSP4-HSP70 (**Figure 3A**). The anemia typical of TBF was evident in control sheep at 8–10 dpi, while in immunized animals it did not occur (MSP4 group) or was observed at 9 dpi only (MSP4-HSP70 group; **Figure 3B**). Erythrocyte counts were not affected in immunized animals (Supplementary Table 1). The analysis of leukocytes, lymphocytes and eosinophils showed lower values at various dpi in immunized animals when compared to controls (Supplementary Table 1). In contrast, neutrophil and monocyte levels were higher in immunized animals when compared to controls at different dpi (**Figures 3C,D**; Supplementary Table 1). These results showed that while immunized animals presented evidence of TBF such as fever and neutropenia, the response to immunization resulted in less severe anemia in response to infection.

Although the percent of infected neutrophils was apparently higher in immunized than in control animals at some

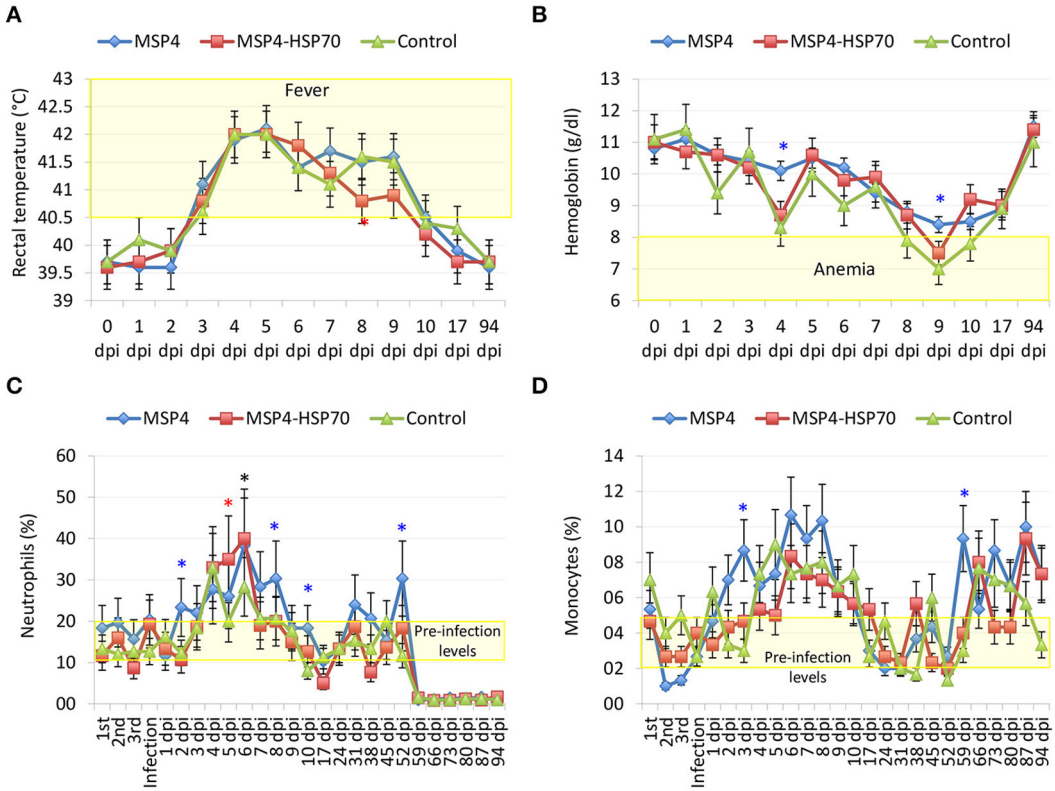

FIGURE 3 | Evidence of TBF in lambs experimentally infected with *A. phagocytophilum* sheep isolate. Groups of three lambs each were immunized with recombinant MSP4, MSP4-HSP70 combination or adjuvant/saline control and experimentally infected with a sheep isolate of *A. phagocytophilum*. **(A)** Rectal temperatures were taken daily until 10 dpi and then weekly until 94 dpi. **(B–D)** Whole blood was collected in EDTA-containing tubes from the jugular vein of each lamb at different time points for different hematological analyses including **(B)** hemoglobin, and percent **(C)** neutrophils and **(D)** monocytes for leukocyte cell differentiation using an electronic counter (Hemavet 950, Drew, USA). Results from rectal temperature and hematological analyses were compared between immunized and control groups by two-way ANOVA test (*$P < 0.05$; $N = 3$ replicates per treatment). Red and blue asterisks denote statistical significant differences between MSP4-HSP70 and MSP4 immunized animals and controls, respectively.

dpi, the results suggested differences in the initial infection rate despite the injection of the same amount of unfrozen infected blood (Supplementary Table 1). These differences could be explained by variations in cell viability between different inoculums, resulting in animal-to-animal variations in the initial infection rate. Therefore, the differential percent of infected neutrophils with respect to the initial value at 3 dpi was used to characterize the effect of vaccination to normalize for these differences. The results showed a significant decrease in *A. phagocytophilum*-infected neutrophils in animals immunized with the MSP4-HSP70, but not MSP4 antigen at 8–10 dpi when compared to controls (**Figure 5A**). Furthermore, the *A. phagocytophilum* normalized DNA levels were significantly lower in lambs immunized with MSP4 and MSP4-HSP70 antigens at 17 dpi (**Figure 5B**). Taken together, these results suggested that the number of infected neutrophils decreased at 8–10 dpi in response to immunization with MSP4-HSP70, while pathogen levels per cell were lower in immunized lambs when compared to controls at 17 dpi.

The Antibodies against Recombinant Proteins in Immunized Lambs Do Not Inhibit the *A. phagocytophilum* Infection of HL60 Human Cells

An antibody inhibition assay using IgG from immunized sheep at 0 and 94 dpi was conducted to further characterize the antibody response in immunized lambs in relation with the protective capacity of MSP4 and MSP4-HSP70 antigens (**Figure 6**). While rabbit IgG antibodies against *A. phagocytophilum* MSP4 and HSP70 recombinant proteins inhibited pathogen infection of HL60 human cells (**Figures 2A**, **7**), sheep IgG collected from immunized animals before infection (0 dpi) and after infection at the end of the experiment (94 dpi) did not affect pathogen infection (**Figure 6**). These results evidenced differences in the IgG response between immunized rabbits and lambs, and provided support for the limited protection against *A. phagocytophilum* infection observed in sheep immunized with MSP4 and MSP4-HSP70.

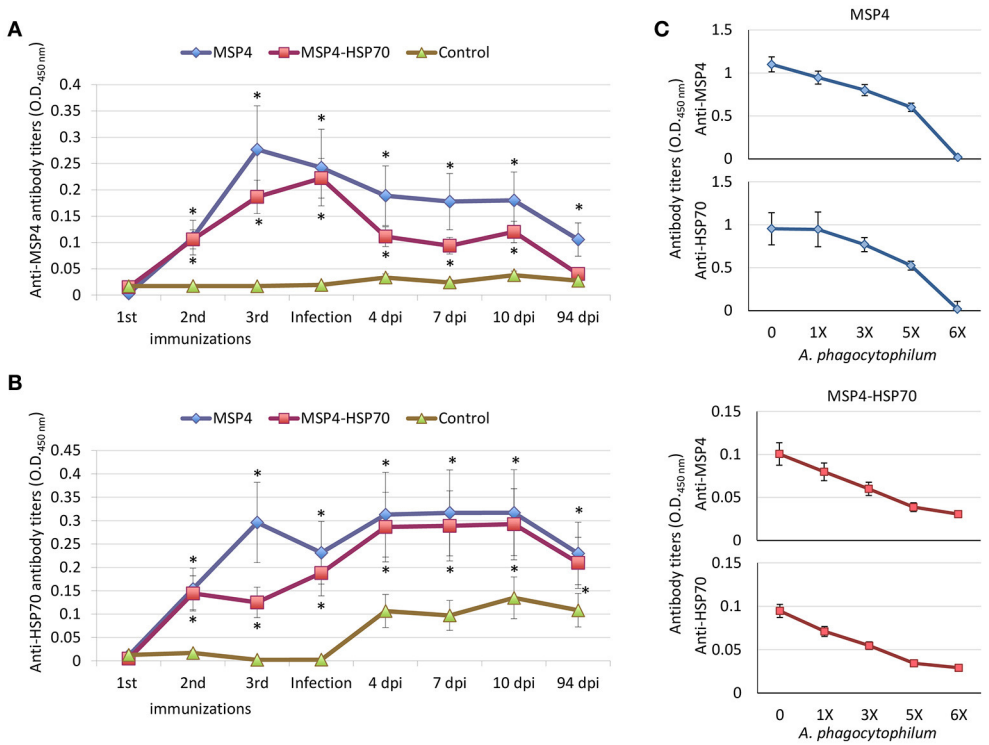

FIGURE 4 | Antibody response in lambs immunized with *A. phagocytophilum* MSP4 and MSP4-HSP70 proteins. Groups of three lambs each were immunized with recombinant MSP4, MSP4-HSP70 combination or adjuvant/saline control and experimentally infected with a sheep isolate of *A. phagocytophilum*. An indirect ELISA test was performed to detect IgG antibodies against **(A)** MSP4 and **(B)** HSP70 proteins in immunized and control lambs using serum samples collected before each immunization and at 0, 7, 10, and 94 dpi. Antibody titers were expressed as OD_{450nm} ($OD_{lambsera} - OD_{PBScontrol}$). The results were compared between immunized and control groups by two-way ANOVA test (*$P < 0.05$; $N = 3$ replicates per treatment). **(C)** The antigen-specific IgG antibody response in immunized lambs was corroborated by ELISA using pooled sera collected at 0 dpi, but incubating sera with different concentrations of *A. phagocytophilum* purified from infected HL60 human cells.

DISCUSSION

Anaplasma phagocytophilum transmembrane and surface proteins are involved in infection of vertebrate host cells (Seidman et al., 2015; Truchan et al., 2016). The *A. phagocytophilum* MSP4 and HSP70 proteins were previously shown to interact when localized on the bacterial membrane, with a possible role during pathogen infection of tick cells (Villar et al., 2015b). These results, together with the finding that *A. phagocytophilum* evolved common molecular mechanisms to establish infection in tick vectors and vertebrate hosts (de la Fuente et al., 2016a), suggested the hypothesis that MSP4 and HSP70 proteins have similar functions in host-pathogen and tick-pathogen interactions with possible implications as potential targets for the development of vaccines for the control of pathogen infection in both ticks and vertebrate hosts.

To address this hypothesis, we first characterized the role of these bacterial proteins in the infection of vertebrate host cells. The results using *A. phagocytophilum* derived from infected HL60 human cells corroborated those previously obtained with *A. phagocytophilum* derived from ISE6 tick cells (Villar et al., 2015b). The results showed that MSP4 is a transmembrane protein in *Anaplasma* spp. (de la Fuente et al., 2001), while HSP70 was probably translocated to the cell surface by still unknown mechanisms in which the bacterial type IV secretion system (T4SS) may be involved (Niu et al., 2006; Lin et al., 2007; Villar et al., 2015b). The binding of HSP70 and MSP4 to HL60 human cells was characterized using two alternative models based on recombinant proteins and *E. coli* producing surface-exposed *A. phagocytophilum* proteins with similar results, therefore supporting their role in the interaction with host cells. Although it is possible that the production of *A. phagocytophilum* proteins in *E. coli* may alter bacterial surface to cause binding to HL60 human cells not mediated by MSP4 and HSP70 proteins, previous results using this system with *A. marginale* MSP1a and MSP1b (de la Fuente et al., 2001) and with *A. phagocytophilum* proteins in tick cells (Villar et al., 2015b) makes this possibility unlikely. Furthermore, *E. coli* producing the mutant HSP70 with truncated peptide-binding domains that are involved in protein-protein interactions (Villar et al., 2015b) did not bind to human HL60 cells, therefore supporting the role of this protein in interactions with host cells.

Protein models supported the interaction between *A. phagocytophilum* MSP4 and HSP70 proteins when localized on the bacterial membrane, which was previously demonstrated in tick cells and may be functionally relevant for pathogen

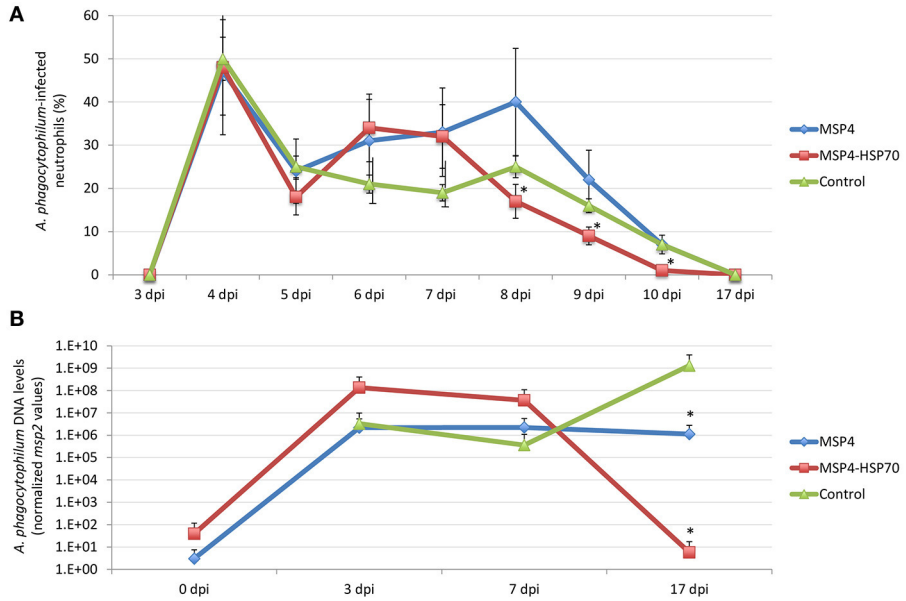

FIGURE 5 | *Anaplasma phagocytophilum* infection levels in immunized and control lambs. **(A)** Blood smears stained with Giemsa stain were examined to investigate the presence of *A. phagocytophilum* in blood cells. At least 100 neutrophils were counted and examined to calculate the number of infected neutrophils per milliliter blood of each lamb. The differential percent of *A. phagocytophilum*-infected neutrophils was calculated as the difference between values at different dpi and values at 3 dpi when infected neutrophils were first detected. The results were compared between immunized and control groups by two-way ANOVA test (*$P < 0.05$; $N = 3$ replicates per treatment). **(B)** For the quantitative analysis of *A. phagocytophilum* infection levels, a quatitative real-time PCR was conducted. The *A. phagocytophilum* DNA levels were normalized against sheep *aldolase B* using the genNorm method (ddCT method as implemented by Bio-Rad iQ5 Standard Edition, Version 2.0). Normalized Ct values were compared between immunized and control groups by Student's *t*-test with unequal variance (*$P < 0.05$; $N = 3$ replicates per treatment).

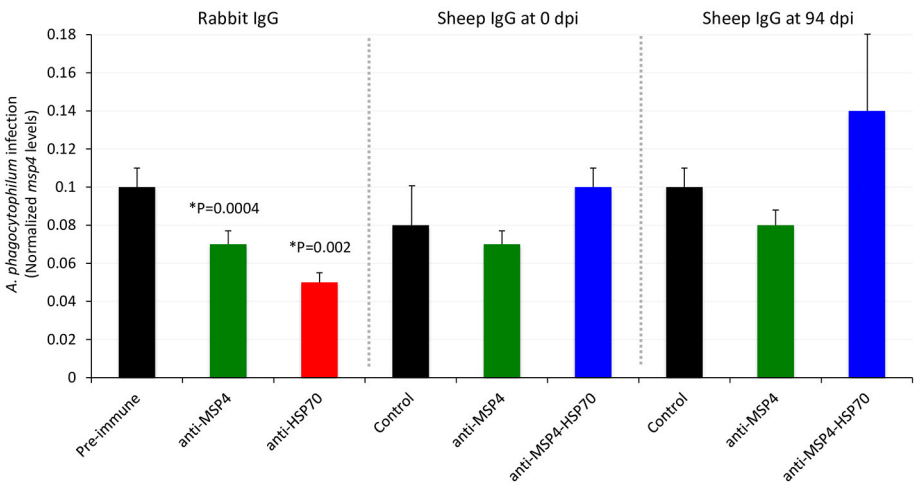

FIGURE 6 | Role of antibodies against recombinant proteins from immunized lambs in the inhibition of *A. phagocytophilum* infection of HL60 human cells. Sheep IgG antibodies against *A. phagocytophilum* MSP4 and MSP4-HSP70 recombinant proteins were obtained from control and immunized sheep at 0 and 94 dpi and used to characterize the inhibition of pathogen infection of HL60 human cells. Treatments included purified IgGs from rabbit pre-immune, anti-MSP4 and anti-HSP70 sera. Purified IgGs were mixed with *A. phagocytophilum* inoculum of human NY18 isolate for 60 min before being placed on the cell monolayers. *A. phagocytophilum* infection levels were determined by *msp4* real-time PCR normalizing against human *β-actin*. Results were compared between groups treated with pre-immune/control and anti-MSP4/HSP70 antibodies by the Student's *t*-test with unequal variance (*$P < 0.05$; $N = 4$ replicates per treatment).

infection of both tick and vertebrate host cells (Villar et al., 2015b). Antibody inhibition assays showed that as previously discussed in the experiments using ISE6 tick cells (Villar et al., 2015b), antibodies against MSP4 and HSP70 proteins could affect the interaction between bacterial ligands and tick receptors to interfere with infection or affect the interaction with

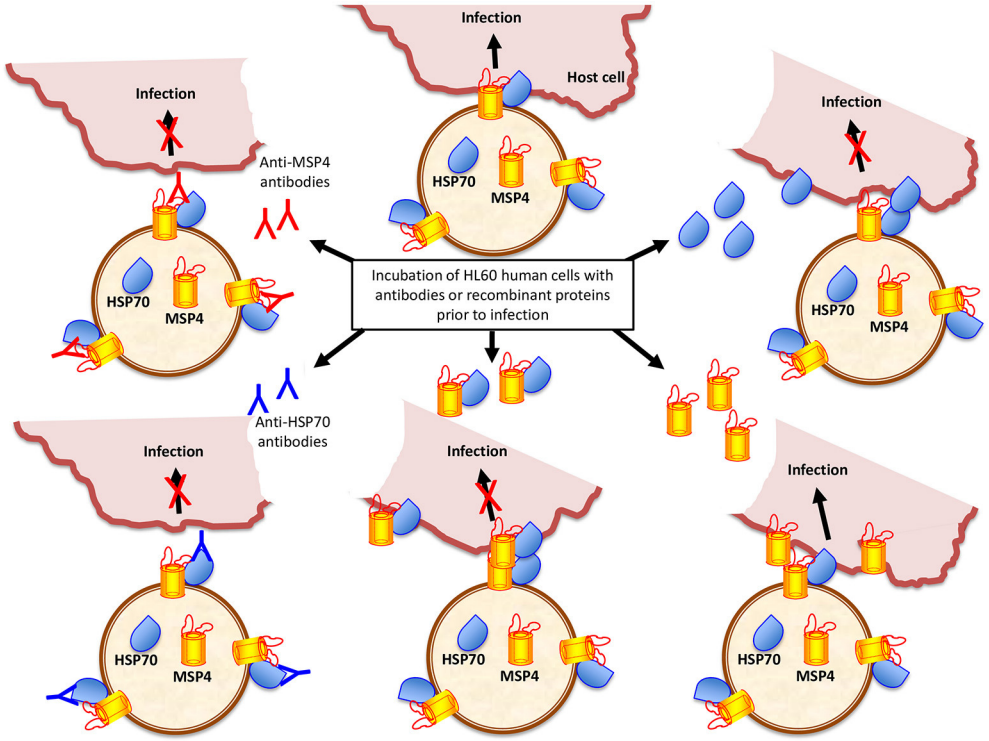

FIGURE 7 | *Anaplasma phagocytophilum* HSP70 and MSP4 are necessary for pathogen infection of host cells. Based on the results of this study, a model was developed to explain the role of HSP70 and MSP4 during pathogen infection of host cells. HSP70 and MSP4 form a complex on the bacterial membrane where MSP4 probably acts a doking protein for HSP70. The incubation of HL60 human cells with recombinant HSP70 or MSP4-HSP70 interacting proteins inhibits infection by interfering with pathogen interaction with host cells mediated by HSP70, which is necessary for infection. However, the addition of recombinant MSP4 does not affect infection because the interaction of bacterial HSP70 with host cells occurs and is sufficient for infection. The anti-MSP4 antibodies probably inhibit infection through binding to MSP4 at the MSP4-HSP70 interaction site, thus preventing HSP70 adhesion to host cells. The anti-HSP70 antibodies bind to HSP70 and prevent the interaction with host cells required for pathogen infection.

proteins functionally important for bacterial infection and/or multiplication in host cells. However, the inhibition assay using recombinant proteins suggested different roles for HSP70 and MSP4 during pathogen infection of host cells (**Figure 7**). While HSP70 seems to be directly involved in the pathogen interaction with host cells, MSP4 may acts as a doking protein for HSP70 to form the MSP4-HSP70 complex on the bacterial membrane (**Figure 7**). These results extended previous findings in tick cells (Villar et al., 2015b), supporting the role of MSP4 and HSP70 proteins in *A. phagocytophilum* infection and/or adhesion to vertebrate host cells.

The role of *Anaplasma* MSPs and other outer membrane proteins and invasins in adhesion to tick and vertebrate host cells for bacterial infection has been demonstrated in *A. marginale* and *A. phagocytophilum* (de la Fuente et al., 2001; Garcia-Garcia et al., 2004; Ge and Rikihisa, 2007; Rikihisa, 2011; Ojogun et al., 2012; Severo et al., 2012, 2013; Kahlon et al., 2013; Seidman et al., 2015; Truchan et al., 2016; Hebert et al., 2017). This mechanism appears to be conserved in other tick-borne pathogens, and in pathogen interactions with other arthropod vector species (de la Fuente et al., 2017). HSP70 was shown to relocate to the *Bacillus subtilis* membrane to restore membrane structure and function after ethanol stress (Seydlová et al., 2012), and to function in the molecular processing of *Borrelia burgdorferi* flagellin (Scopio et al., 1994). This protein may be functionally relevant at the *A. phagocytophilum*-host interface, and may interact with other membrane proteins for its function during pathogen infection (Susin et al., 2006; Multhoff, 2007).

To evaluate the potential protective capacity of these proteins, lambs that are natural hosts for this pathogen were immunized with recombinant MSP4, MSP4-HSP70 combination or adjuvant/saline control and infected with a sheep isolate of *A. phagocytophilum*. The MSP4-HSP70 combination was included based on evidence of protein-protein interactions, suggesting a physical and/or functional connection between these proteins (Villar et al., 2015b).

The results evidenced signs of TBF such as fever, anemia, and neutropenia in lambs infected with *A. phagocytophilum*, therefore validating the model for the comparative analysis between immunized and control animals. In sheep and dogs, *A. phagocytophilum* infection is accompanied by fever of approximate 7 days duration, which is the main clinical sign of TBF (Eberts et al., 2011; Stuen et al., 2011; Severo et al., 2013). The severe leukopenia and especially the prolonged neutropenia that

accompanies the disease are also evident with TBF (Eberts et al., 2011; Stuen et al., 2011; Severo et al., 2013). Immune suppression by impaired antibody and lymphocyte response and reduced oxidative burst, together with anemia and monocytosis have been also reported in animals infected with *A. phagocytophilum* (Whist et al., 2003; Eberts et al., 2011). Weaning weight is also affected in lambs infected with *A. phagocytophilum* (Grøva et al., 2011).

The immunized lambs raised an antibody response that was specific for *A. phagocytophilum* MSP4 and HSP70 recombinant proteins. However, MSP4-immunized lambs developed an anti-HSP70 response. One possible explanation to this finding was the production of polyreactive antibodies, which constitute a major component of the natural antibodies that bind with low affinity to structurally unrelated antigens with broad antibacterial activity (Gunti and Notkins, 2015). Additionally, the analysis of protein sequences showed the existence of common B-cell epitopes between *A. phagocytophilum* human isolate MSP4 and HSP70 proteins that may also contribute to serum cross-reactivity. The B-cell epitopes are protein regions that bind to antibodies. Most epitopes are composed of different parts of the polypeptide chain that are brought into spatial proximity by the three-dimensional structure of the protein. These discontinuous epitopes can also react with continuous peptide fragments (i.e., linear epitopes) within the protein (Larsen et al., 2006). Epitopes can be understood as "antigenic determinants" within proteins and homology between linear epitopes can results in antibody cross-reactivity (Terajima et al., 2013).

Despite the effect of *A. phagocytophilum* infection on the impairment of antibody response in sheep (Whist et al., 2003), the results showed that IgG antibody levels to MSP4 and HSP70 antigens remained higher after infection in immunized animals when compared to controls. In contrast to the results reported in lambs immunized with inactivated *A. phagocytophilum* (Stuen et al., 2015), the number of fever relapses was similar between immunized and control animals, supporting that antigen-specific response is different from that obtained with whole organisms. The immunization with MSP4-HSP70 resulted in a decrease in the percent of infected neutrophils and pathogen levels per cell, supporting that immunization with MSP4-HSP70 was only partially protective for the control of *A. phagocytophilum* infection of neutrophils.

A previous experiment using a crude *A. phagocytophilum* protein extract for immunization did not protect against pathogen infection in sheep, but immunized lambs had reduced levels of infection (Stuen et al., 2015). The authors discussed that the lack of protection was probably due to the presence of not protective dominant antigens in the vaccine preparation, stressing the need for the identification of protective antigens conserved among different strains (Stuen et al., 2015). The results obtained here were similar to those reported previously by Stuen et al. (2015), but using two proteins shown to be highly conserved and involved in pathogen infection and/or interaction with host cells. The failure to protect lambs from *A. phagocytophilum* infection after immunization with MSP4 and MSP-HSP70 antigens may be due to several factors. Although these proteins seem to be involved in host-pathogen interactions and infection, other proteins may be also necessary for infection within this mechanism or as part of alternative mechanisms of infection. The results showed that IgG antibodies rose in immunized lambs did not inhibit *A. phagocytophilum* infection of HL60 human cells, suggesting differences between rabbit and sheep IgG responses that may be associated with epitope recognition in MSP4 and HSP70 proteins. These differences in the immune response between rabbits and sheep could be used to identify candidate protective regions or epitopes in MSP4 and HSP70 proteins to increase vaccine efficacy. Additionally, the intravenous inoculation of infected blood is different from natural infection after tick bite, and may affect the evaluation of the protective response after immunization.

CONCLUSIONS

The *A. phagocytophilum* transmembrane and surface proteins play a crucial role during infection and multiplication in host neutrophils (Ge and Rikihisa, 2007; Rikihisa, 2011; Severo et al., 2012, 2013; Seidman et al., 2015; Truchan et al., 2016). However, the results reported here provided the first evidence for the role of *A. phagocytophilum* MSP4 and HSP70 proteins in this process. These results suggested that while membrane-localized MSP4 and HSP70 were involved in *A. phagocytophilum* interaction with host cells, HSP70 was directly implicated in pathogen infection. As for other intracellular pathogens, cellular immunity is essential for an effective protection against infection by *Anaplasma* spp. (Palmer et al., 1999; Hajdušek et al., 2013; de la Fuente et al., 2016a; Shaw et al., 2017). However, previous experiments have provided evidence that antibodies to bacterial proteins have a protective effect on infected hosts (Kaylor et al., 1991; Messick and Rikihisa, 1994; Sun et al., 1997; de la Fuente et al., 2003; Gomes-Solecki, 2014; Stuen et al., 2015). The results obtained here showed that the *A. phagocytophilum* MSP4-HSP70 antigen was only partially protective against pathogen infection in sheep. This limited protection may be associated with several factors, including the recognition of non-protective epitopes by IgG from immunized lambs. Nevertheless, these antigens may constitute candidate protective antigens for the development of vaccines against TBF in combination with other antigens. Focusing on the characterization of host protective immune mechanisms and protein-protein interactions at the host-pathogen interface may lead to the discovery and design of new protective antigens (de la Fuente et al., 2016c,d). Additionally, proteins involved in tick-pathogen and host-pathogen interactions such as *A. phagocytophilum* MSP4 and HSP70 may be used to develop double effect vaccines targeting infection in both vertebrate hosts and tick vectors (de la Fuente and Contreras, 2015).

AUTHOR CONTRIBUTIONS

Jd and CG conceived the study. MC, PA, LM, IF, MVa, MVi, and NA performed the experiments. MC, AG, and SS performed the vaccine trial. AC, MC, JV, and Jd performed

data analyses. Jd and MC wrote the paper, and other coauthors made additional suggestions and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00307/full#supplementary-material

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Comparative Transcriptome Profiling of Virulent and Attenuated *Ehrlichia ruminantium* Strains Highlighted Strong Regulation of *map1*- and Metabolism Related Genes

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The obligate intracellular pathogenic bacterium, *Ehrlichia ruminantium*, is the causal agent of heartwater, a fatal disease in ruminants transmitted by *Amblyomma* ticks. So far, three strains have been attenuated by successive passages in mammalian cells. The attenuated strains have improved capacity for growth *in vitro*, whereas they induced limited clinical signs *in vivo* and conferred strong protection against homologous challenge. However, the mechanisms of pathogenesis and attenuation remain unknown. In order to improve knowledge of *E. ruminantium* pathogenesis, we performed a comparative transcriptomic analysis of two distant strains of *E. ruminantium*, Gardel and Senegal, and their corresponding attenuated strains. Overall, our results showed an upregulation of gene expression encoding for the metabolism pathway in the attenuated strains compared to the virulent strains, which can probably be associated with higher *in vitro* replicative activity and a better fitness to the host cells. We also observed a significant differential expression of membrane protein-encoding genes between the virulent and attenuated strains. A major downregulation of *map1*-related genes was observed for the two attenuated strains, whereas upregulation of genes encoding for hypothetical membrane proteins was observed for the four strains. Moreover, CDS_05140, which encodes for a putative porin, displays the highest gene expression in both attenuated strains. For the attenuated strains, the significant downregulation of *map1*-related gene expression and upregulation of genes encoding other membrane proteins could be important in the implementation of efficient immune responses after vaccination with attenuated vaccines. Moreover, this study revealed an upregulation of gene expression for 8 genes encoding components of Type IV secretion system and 3 potential effectors, mainly in the virulent Gardel strain. Our transcriptomic study, supported by previous proteomic studies, provides and also confirms new information regarding the characterization of genes involved in *E. ruminantium* virulence and attenuation mechanisms.

Keywords: *Ehrlichia*, transcriptome, pathogenicity, attenuation, virulence

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INTRODUCTION

Ehrlichia ruminantium is the causal agent of heartwater, a fatal disease in wild and domestic ruminants (Allsopp, 2010). This disease represents a serious problem for livestock production in endemic areas such as sub-Saharan Africa and the Caribbean, threatening the American continent where indigenous competent ticks are present (Barré et al., 1987; Gondard et al., 2017). Heartwater is a major obstacle to large-scale production of ruminants in Africa, inducing high mortality within herds with susceptible animals. The economic impact has been evaluated at US $5.6 million per year for Zimbabwe and US $44.7 million per year for the SADC region (Southern Africa Development Community) (Mukhebi et al., 1999). Moreover, the US Department of Homeland Security has listed heartwater in the top-12 priority trans-boundary animal diseases. *E. ruminantium* belongs to the order Rickettsiales in the Anaplasmataceae family, which contains other genera such as *Anaplasma* and *Neorickettsia* as well as *Ehrlichia*. It is transmitted by at least ten species of *Amblyomma* ticks, mainly *A. variegatum*, present in Africa and the Caribbean islands, and *A. hebraeum*, present in Southern Africa (Allsopp, 2010).

Over the last 10 years, the development of *in vitro* models and significant technical progress in molecular biology using high throughput integrative methods have provided new knowledge on the global gene expression of Rickettsiales members and particularly on their pathogenesis. For example, transcriptomic studies have been conducted for some Rickettsiales members such as *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*, and *Rickettsia prowazekii* (Pruneau et al., 2014). These various transcriptomic studies of bacteria infecting its host or vector revealed the importance of groups of genes involved in Rickettsiales pathogenesis such as genes encoding for outer membrane proteins (OMPs), known to be involved in the interactions of pathogens with host and vector cells, the Type IV secretion system (T4SS) involved in delivering virulence factors inside infected cells, and the establishment of mechanisms for survival and development in the host or vector cells, such as escape in response to osmotic and oxidative stress (Moumène and Meyer, 2015).

In a preliminary transcriptomic study of *E. ruminantium*, the global transcriptome of the virulent Gardel strain (isolated in the Caribbean island of Guadeloupe) was compared during its developmental cycle. There are two main stages: the extracellular and infectious form (elementary body, or EB) and the vegetative intracellular non-infectious form (reticulate body, or RB) (Pruneau et al., 2012). A total of 54 genes showed a differential expression between the RB and EB stages. Several genes involved in metabolism, nutrient exchange, and defense mechanisms, including those involved in resistance to oxidative stress, were significantly induced in RB. This is consistent with the oxidative stress condition and nutrient starvation that seem to occur in *Ehrlichia*-containing vacuoles allowing their survival in this environment. During the EB stage, we showed the expression of the RNA polymerase-binding transcription factor *dksA*, which is also known to induce virulence in other pathogens. Our results suggested a possible role of these genes in promoting *E. ruminantium* development and pathogenicity (Pruneau et al., 2012).

In obligate intracellular bacteria, differential transcriptional and proteomic analysis of OMPs and secreted proteins between the virulent and attenuated strains is crucial to better understand their essential functions, such as host cell invasion, escape from the phagolysosome, intracellular motility and manipulation of the host response to infection.

The *E. ruminantium* genome contains several genes encoding for the Major antigenic protein 1 (Map1) family proteins, a family of OMPs. Previously, a study on the Map1 cluster showed that it was differentially expressed depending on the host and tick cell lines and *E. ruminantium* strains (Bekker et al., 2005). Even if the role of these proteins remains unclear in the pathogenesis, they are highly immunogenic, inducing a strong Map1 antibody response, which so far has not been associated to protection (Marcelino et al., 2007).

A comparative proteomic study between the virulent and attenuated Gardel strains at EB stage (Marcelino et al., 2015) was recently performed. This study revealed an upregulation of proteins involved in virulence, such as Ldp, AnkA, VirB9 and VirB10 for the virulent Gardel, and an upregulation of proteins involved in cell division, metabolism and transport and protein processing for the attenuated Gardel strain. For the virulent Gardel strain, there was also an upregulation of Map1-family proteins, Map1-2, Map1-3, Map1-4, Map1-8, and exclusive detection of Map1-12, whereas Map1-1 was upregulated in the attenuated Gardel. There was also a strong upregulation of a putative porin CDS_05140 with detection of four proteoforms in the attenuated Gardel strain (Marcelino et al., 2015).

To identify potential pathogenesis and mechanisms of attenuation, we investigated the global gene expression profiling of the virulent and attenuated strains of both Gardel and Senegal at infectious EB stage.

Both Senegal and Gardel *E. ruminantium* attenuated strains induced limited clinical signs after intravenous inoculation to naïve animals and conferred good protection against homologous and heterologous challenges (Faburay et al., 2007; Marcelino et al., 2015). They are still able to grow *in vitro* in host endothelial cells and induce cell lysis. However, Marcelino et al showed the reduction of the Gardel growth cycle *in vitro* for the attenuated strain, with cell lysis developing in 4 days compared to 5 days for the virulent one (Marcelino et al., 2015). The Gardel and Senegal strains are phylogenetically distant (Raliniaina et al., 2010; Pilet et al., 2012; Cangi et al., 2016) and need a different number of *in vitro* passages to be attenuated. Gardel is attenuated *in vitro* after ∼200 passages in bovine aortic endothelial cells (Marcelino et al., 2015), whereas only ∼15 passages were sufficient to attenuate the Senegal strain in bovine umbilical endothelial cells (Jongejan, 1991). Moreover, in terms of vaccine protection, there is no cross-protection between the Gardel and Senegal strains, underlining the presence of polymorphic protective antigens (D. Martinez, personal communication).

In our study, we identified a set of differentially expressed transcripts coding for Map1-family proteins in particular, upregulated in both virulent strains, as well as for hypothetical membrane proteins probably inducing significant modification

of membrane organization. Moreover, we showed an upregulation of 8 genes encoding T4SS components and 3 genes encoding for potential effectors only in the virulent Gardel strain, probably associated with virulence mechanisms.

Furthermore, the proportion of gene upregulation related to metabolism and energy production and conversion was higher in both attenuated strains than in the virulent strains. This result reflects a better fitness to cell culture conditions in the attenuated strains, as evidenced by a shorter bacteria growth cycle.

MATERIALS AND METHODS

Production of Biological Samples

Three biological replicates for each strain were used for the microarrays experiments. For virulent and attenuated Gardel, biological samples were produced at passage 38, 43, and 44 (Gp38, 43, and 44) and at passage 238, 243 and 251 (Gp238, 243, and 251) respectively. For virulent Senegal, the replicates were Senegal passage 7 (Sp7) and two passage 8 replicates (Sp8a and Sp8b). For attenuated Senegal, biological samples were Senegal passage 70, 75, and 77 (Sp70, 75, and 77). All the biological samples were produced in bovine aorta endothelial cells as previously described by Emboulé et al. (2009).

After infection with an appropriate inoculum, all cells were infected and the development of *E. ruminantium* growth was observed daily by light microscopy with detection of morula in each cell. *E. ruminantium* cell cultures used to produce biological samples were thus synchronized. When complete cell lysis was confirmed by light microscopy, samples containing supernatant and cellular debris were harvested and then ultra-centrifuged at 20,000 × g for 15 min at 4°C to collect EBs. The pellets were placed in sterile eppendorfs and homogenized in 2.5 mL of TRIzol reagent (Invitrogen). The samples were immediately stored at −80°C before RNA extraction.

It is noteworthy that the duration between cell infection and lysis was 4 days for the Gardel and Senegal attenuated strains and 5 and 6 days for the Gardel and Senegal virulent strains respectively (data not shown). The duration of lysis remained the same within biological replicates.

Extraction of Total RNA and Reverse Transcription of RNA Samples

The total RNA extraction procedure was carried out as described in Emboulé et al. (2009). Total RNA quantification was performed by Nanodrop 2000c (Thermo Scientific), and total RNA samples were pooled in RNase-free water at a final concentration of 0.5 μg/μL for further analysis.

E. ruminantium genomic DNA (gDNA) contaminant was evaluated in each RNA sample by performing the *pCS20* PCR using AB128-AB129 primers as described previously with a limited number of cycles (25 cycles) (Martinez et al., 2004). In the RNA samples, no signal was obtained by the *pCS20* PCR (data not shown), indicating limited *E. ruminantium* gDNA contaminant. Afterward, the RNA samples were reverse transcribed by random priming with Superscript II (Invitrogen) according to the manufacturer's instructions. Reverse transcription and PCR amplification of corresponding cDNA were done using *Kpn*I-primers as previously described (Emboulé et al., 2009).

Moreover, the efficiency of reverse transcription was checked using real-time PCR (qPCR) targeting the *E. ruminantium* 16S rRNA gene by processing the RNA and cDNA samples simultaneously. The primers used for qPCR were 16S-F: 5′ AGC GCAACCCTCATCCTTAG 3′ and 16S-R: 5′ AGCCCACCCTAT AAGGGCC 3′. The final concentration of these primers was 900nM. SyberGreen Master Mix was used according to the manufacturer's instructions (Applied Biosystems, France). The PCR program was 10 min at 95°C and 45 cycles with 30 s at 95°C, 30 s at 60°C and 1 min at 72°C. The difference of Ct between the RNA and cDNA samples was always higher than 5 cycles, indicating low contamination by gDNA in the RNA samples (data not shown).

Design of *E. Ruminantium* Microarrays

E. ruminantium microarrays (8 x 60 k) used in this study were manufactured by Agilent, France. The probes were designed with a modified version of the Oligoarray program (Rouillard et al., 2003). Probe design was carried out using the genomes of the three published strains of *E. ruminantium* (virulent Gardel, Welgevonden Erwe and Erwo) (Collins et al., 2005; Frutos et al., 2006) and using the genomes of the three newly sequenced but not yet published strains of *E. ruminantium* (attenuated Gardel, virulent and attenuated Senegal). There were probes common to the six strains and probes specific to one or more strains. Specific probes were designed for virulent and attenuated strains due to differences observed between genome sequences of virulent and attenuated strains (unpublished data). For data analyses, only probes that are specific for Senegal and Gardel strains were used.

The microarrays contained a total of 16,199 probes (60-mer), with three replicates per probe, including 83 bovine genes as negative controls. The selection of *E. ruminantium* probes gave 14,747 specific probes (91%), with Tm between 80 and 92°C containing 30 to 50% GC. These rules were progressively relaxed to get the additional 964 probes for a subset of missing sequences (Tm between 70 and 92°C and a GC % between 20 and 60). Analysis of specificity indicated that 46,696 oligos had 100% identity on 60 bases, 73,513 had 95% identity on 60 bases and 84,767 had 95% identity on at least 50 bases. Cross-reactivity of probes between the strains was assessed using this latter criterion (95% identity on at least 50 bases).

Forty-eight percent of ORF were covered by 10 or more probes, and 90% were covered by 3 or more probes. Experimental data and associated microarray design have been deposited in the NCBI Gene Expression Omnibus (GEO) http://www.ncbi.nlm.nih.gov/geo/ under series GSE55726 and platforms GPL18397.

Microarrays: cDNA Labeling, Scanning and Data Analyses

Two hundreds nanograms of cDNA were randomly amplified and labeled with Cy3-dUTP using the SureTag DNA Complete Labeling Kit (Invitrogen, France). Following purification with reaction purification columns (Invitrogen, France), quantification of Cy3-dUTP incorporation was performed by absorbance measurement at 550 nm. The yield of cDNA labeling

ranged between 3 and 5 μg, and the specific activity ranged between 20 and 25 pmol per μg of cDNA, according to the manufacturer's instructions (Invitrogen, France). Microarrays were incubated for 24 h at 65°C, with a rotation of 20 rpm, in a hybridization chamber. After hybridization, stringent washings were performed according to the manufacturer's instructions (Invitrogen, France). Arrays were scanned using an Agilent C microarray scanner and extracted with the Agilent Feature Extraction program (version 10.10). The obtained images were saved in TIFF format and data with the signal intensities of all spots on each image were saved as ".txt" files for further analysis.

Normalization of microarray data was performed using the Limma package (Smyth, 2004) in R (R Core Team, 2013), available from Bioconductor (http://www.bioconductor.org). Inter-slide normalization was performed using the quantile method. Mean $\log_2$ fold change (FC) between the virulent and attenuated strains was calculated for each probe and a *B* test was performed. Probes that had low or no signals were excluded from the analysis. For each probe, the expression values of the biological replicates for the virulent and attenuated strains were compiled. The average FC per probe was calculated by taking the average of the $\log_2$ values of the virulent strain from the average of the $\log_2$ values from the attenuated strain. The statistical significance of differential expression for each probe was calculated using a *t*-test in R 3.2.3 (R Core Team, 2013), and FDR correction was applied using the Benjamini-Hochberg (Benjamini and Hochberg, 1995) method in R.

Moreover, we checked the homogeneity of $\log_2$-transformed fluorescence intensities between biological replicates by computing linear correlation coefficients. For the virulent and attenuated Gardel and Senegal strains, linear correlation coefficients were >0.6. Accordingly, the mean intensity values of biological replicates could be calculated.

For genes covered by multiple probes, transcripts were considered differentially expressed between the virulent and attenuated strains for Gardel and Senegal when more than 50% of probes mapping the transcripts had an absolute value of $\log_2$-fold change (FC) ≥ 1 and a *p*-value < 0.1. A mean FC per CDS was then calculated.

The annotations of genes differentially expressed were checked on NCBI. For genes encoding for hypothetical functions, protein patterns were searched in the InterProt database (Quevillon et al., 2005) and alignments were performed using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/) in order to identify potential functional domains.

Quantitative Reverse Transcription PCR for *map1*-Related Genes

To validate the differential expression of *map1*-related genes, quantitative reverse transcription PCRs (qRTPCR) were done targeting 7 *map1*-related genes on Gp43 vs. Gp243 and Sp7 vs. Sp77. Briefly for each sample, 1.5 μg of RNA were reverse transcribed with the Superscript VILO cDNA synthesis kit (Invitrogen) according to manufacturer's instructions. The quantity of resulting cDNA was determined by qPCR as previously described (Pruneau et al., 2012) using the primers listed in Table S1.

The Fold Change (FC) between virulent and attenuated strains was measured as follows:

R = (number of cDNA for virulent strain) / (number of cDNA for attenuated strain).

The results were expressed in $\log_2$ FC between virulent and attenuated strains: comparing Gp43 vs. Gp243 and Sp7 vs. Sp77.

RESULTS

Transcriptome Profile Reproducibility

Global gene expression profiling of both virulent and attenuated Gardel and Senegal strains was performed using a home-made microarray containing more than 16,000 distinct probes covering all *E. ruminantium* CDS. The mean normalized signal intensities obtained were compared between the different biological replicates for each strain and indicated a good technical reproducibility with the linear correlation coefficients included between 0.6 and 0.94 (**Figure 1A**). Statistical analysis was then performed to identify the genes differentially expressed between the virulent and attenuated strains, and a clustering was then carried out to identify the 300 best probes discriminating between virulent and attenuated strains (**Figure 1B**). Clusters based on probe intensity allowed four groups to be identified, with each group including biological triplicates of each strain. The virulent Gardel group was clearly separated from the other groups formed by the attenuated Gardel and the attenuated and virulent Senegal strains (**Figure 1B**). Furthermore, we observed a clear separation between the virulent Senegal and the attenuated strains of both Gardel and Senegal. Volcano plots showing the global transcriptional changes in the virulent vs. attenuated strains were shown in **Figure 1C**. Globally, $\log_2$ FCs were higher for Gardel than for Senegal, with stronger *p*-values.

General Overview of Differentially Expressed Genes Between Virulent and Attenuated Strains

There were 114 and 133 out of 950 genes (12 and 14% of ORFs) differentially expressed between the virulent and attenuated Gardel and between the virulent and attenuated Senegal strains respectively. Fifty genes were found upregulated for the Gardel virulent strain compared to the attenuated strain, and conversely 64 genes were found upregulated for the attenuated Gardel strain compared to the virulent strain (**Table 1**, **Figure 2**). For Senegal, the number of upregulated genes was higher for the virulent strain (83 genes) than for the attenuated strain (50 genes) (**Table 2**, **Figure 2**). Common upregulation of expression for 10 genes was observed for both virulent strains and 12 for both attenuated strains (**Figure 2**, white numbers). The detailed list of upregulated genes for both Gardel and Senegal are shown in **Tables 1**, **2**. Interestingly, the common upregulated genes in the virulent strains included 8 *map-1* genes (*map1-3, map1-2, map1-4, map1-6, map1-9, map1-5, map1-7*, and *map1-12*) (**Figure 3**). The other transcripts coded for proteins involved in translation, ribosomal structure and biogenesis (*truA*) and for a hypothetical

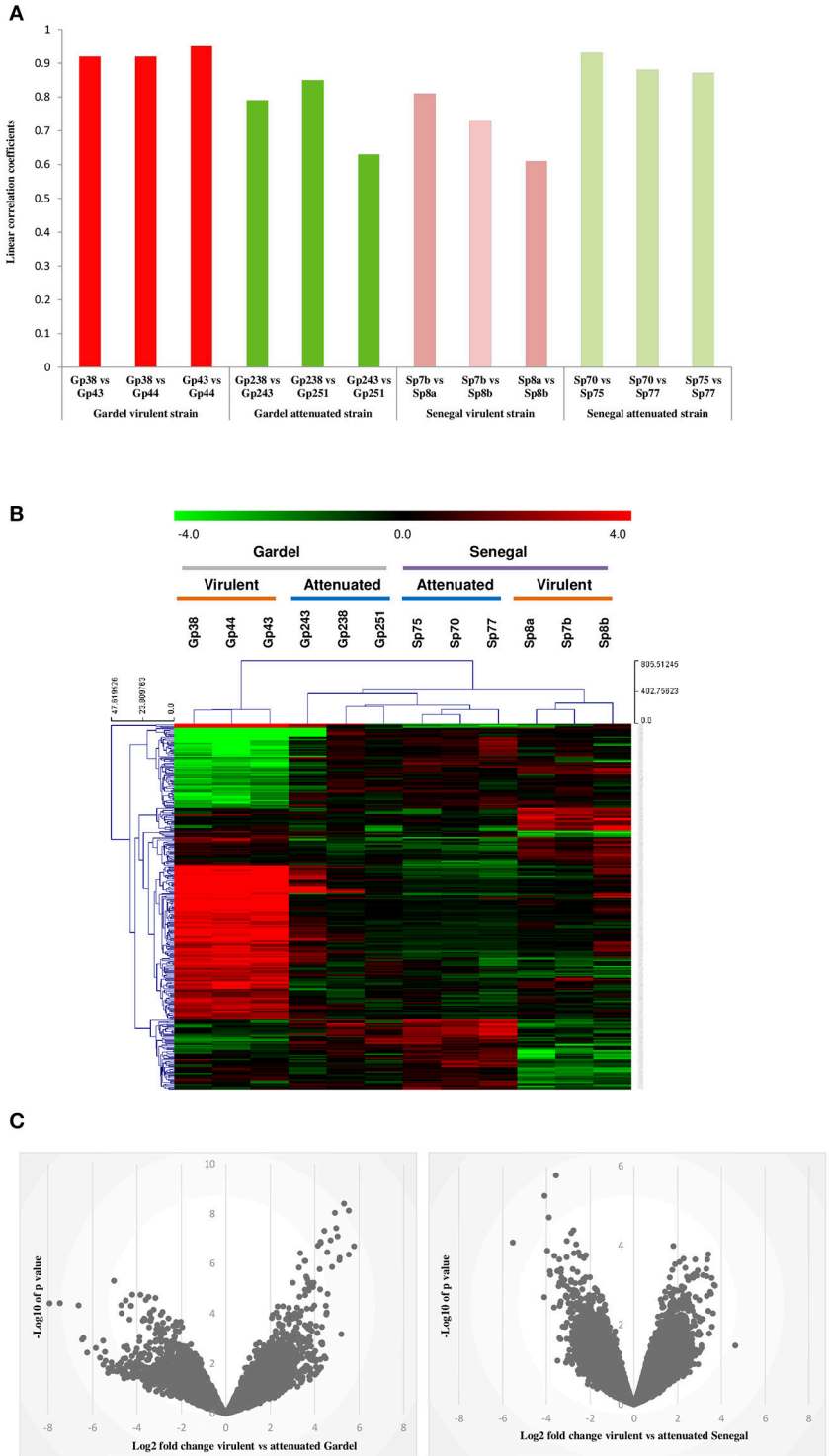

FIGURE 1 | Transcriptome profile reproducibility. **(A)** Linear correlation between the intensity values obtained from different biological replicates for each strain: virulent Senegal (Sp7b, Sp8a and b), attenuated Senegal (Sp70, p75, and 77), virulent Gardel (Gp38, p43, and p44), attenuated Gardel (Gp238, p243, and p251). **(B)** Heatplot showing the expression profile of the 300 most discriminating probes for the virulent and attenuated strains in each sample. Expression corresponds to $\log_2$ intensity followed by median centering, as visualized on the green to red gradient. Clustering was performed using a Manhattan distance metric and average linkage. **(C)** Volcano plots showing the global transcriptional changes in the virulent vs. attenuated Gardel and Senegal strains. All probes present on the microarray are plotted. Each circle represents one probe. The $\log_2$ FC is shown on the x-axis. The y-axis shows the log10 of the *p*-value.

TABLE 1 | Upregulated genes and their function for the Gardel strains (genome accession numbers: NC_006831.1).

Gene function	Virulent			Attenuated		
	Gene id	Name	log_2 FC	Gene id	Name	log_2 FC
Energy production and conversion	CDS_05160[c]	*petC*	1.19	**CDS_04300**	***fdxB***	**1.70**
				CDS_04070	***atpG***	**1.28**
				CDS_00110	*fdxA*	1.58
				CDS_08460	*ctaG*	1.31
				CDS_08790	*atpE*	1.10
Nucleotide transport and metabolism				**CDS_08980[a]**	***ndk***	**1.17**
				CDS_02940	*dnaX*	1.73
				CDS_07270[a]	*purC*	1.54
				CDS_07770	*tmk*	1.50
				CDS_07540	*pyrH*	1.32
Coenzyme transport and metabolism	**CDS_00530[c]**	***hemA***	**1.21**	**CDS_01790[c]**	***pdxH***	**1.64**
	CDS_01740	*coaX*	1.18	**CDS_07700**	***ribE***	**1.55**
Replication, recombination and DNA repair	CDS_03430[c]	*topA*	2.05	**CDS_02840[a]**	***ssb***	**1.11**
	CDS_00030	*ruvC*	1.75	CDS_02430	*nth*	1.21
	CDS_06660	*radC*	1.37	CDS_07870	*xseB*	1.12
Posttranslational modification, protein turnover, chaperones	CDS_04780	*clpA*	1.75	**CDS_03060**	***ccmE***	**1.14**
				CDS_05010[a]	*bcp*	1.64
Carbohydrate/AA/inorganic ion transport and metabolism	CDS_03250	*gdh2*	1.42	CDS_05860	*thio*	1.92
				CDS_04200	*rpiB*	1.30
Defense mechanisms				CDS_01120	*lolD*	1.52
Lipid transport and metabolism				CDS_02180	*pgpA*	1.62
				CDS_04850	*ispG*	1.42
				CDS_05330	*ispH*	1.14
				CDS_05660		1.13
Cell wall/ membrane/envelope biogenesis				CDS_08860	*lgt*	1.61
				CDS_08610[c]	*dacF*	1.33
				CDS_06490	*aprD*	1.30
				CDS_01510	*aprE*	1.14
				CDS_03590	*lepA*	1.06
Intracellular trafficking, secretion, and vesicular transport	**CDS_05400**	***virB4a***	**1.51**			
	CDS_08380	*virB2b*	3.72			
	CDS_08370	*virB2c*	2.62			
	CDS_00150[b]	*virB10*	2.08			
	CDS_05380	*virB6b*	1.77			
	CDS_05370	*virB6c*	1.59			
	CDS_00140[a]	*virB11*	1.60			
	CDS_05360	*virB6d*	1.31			
Map1-related protein	**CDS_09120[c]**	***map1-3***	**2.36**			
	CDS_09110[c]	***map1-4***	**2.14**			
	CDS_09090	***map1-6***	**1.84**			
	CDS_09130[c]	***map1-2***	**1.71**			
	CDS_09100[c]	***map1-5***	**1.44**			
	CDS_09060	***map1-9***	**1.36**			
	CDS_09030[b]	***map1-12***	**1.29**			
	CDS_09080	***map1-7***	**1.28**			

(Continued)

TABLE 1 | Continued

Gene function	Virulent			Attenuated		
	Gene id	Name	log_2 FC	Gene id	Name	log_2 FC
Translation, ribosomal structure and biogenesis	**CDS_04360[c]**	***truA***	**1.94**	**CDS_05070**	***rrmJ***	**2.50**
				CDS_05020	*rsmE*	1.66
				CDS_00280	*trmE*	1,46
				CDS_05240	*rpsB*	1,18
				CDS_06320	*spoU*	1,16
				CDS_03560	*rpsO*	1,10
				CDS_06190[c]	*rplN*	1,00
Hypothetical membrane protein	**CDS_04750**		**2.56**	**CDS_05140[b]**		**3.82**
	CDS_02390		2.62	**CDS_03690**		**1.81**
	CDS_07440		2.41	**CDS_07940[c]**		**1.59**
	CDS_07610		2.29	CDS_06520[c]	*T4SS Putative effector*	2.57
	CDS_06460		2.04	CDS_00970	*T4SS Putative effector*	2.30
	CDS_07370		1.80	CDS_07620		2.22
	CDS_05650[c]		1.40	CDS_02300		1.78
	CDS_03260		1.28	CDS_05100		1.75
	CDS_07630		1.21	CDS_02290		1.71
				CDS_02480		1.41
				CDS_07470		1.25
				CDS_05900		1.23
				CDS_02040		1.12
Hypothetical protein	CDS_01030		3.81	**CDS_09210[a]**		**2.46**
	CDS_05600		3.69	**CDS_03280**		**1.88**
	CDS_04510	*T4SS Putative Effector*	3.57	**CDS_04190**		**1.81**
	CDS_05770[c]		2.76	**CDS_08200[a]**	***LexA***	**1.76**
	CDS_08320		2.51	**CDS_03450**		**1.44**
	CDS_02410		2.51	**CDS_04560[c]**	***sspB***	**1.33**
	CDS_02370[b]		2.49	**CDS_04760**		**1.99**
	CDS_08310[a]	*T4SS Putative Effector*	2.31	CDS_07600		3.10
	CDS_05620		2.18	CDS_05350		2.24
	CDS_08100		1.82	CDS_06720		1.98
	CDS_03830[b]	*AnkA*	1.78	CDS_00080		1.45
	CDS_01350		1.56	CDS_01260		1.25
	CDS_00600		1.43	CDS_04680[c]		1.10
	CDS_02830		1.50	CDS_02440[a]		1.10
	CDS_04430		1.38	CDS_01780[c]		1.05
	CDS_09020		1.16			
Number of upregulated genes			50			64

Bold blue: common upregulated genes in the Gardel and Senegal strains.
Bold pink: conversely upregulated genes in the Gardel and Senegal strains.
[a]*gene encoding for proteins detected in a proteomic study comparing the virulent and attenuated Gardel strains (Marcelino et al., 2015).*
[b]*gene encoding for proteins differentially expressed in a proteomic study comparing the virulent and attenuated Gardel strains (Marcelino et al., 2015).*
[c]*gene encoding for strain-specific protein based on a proteomic study comparing the virulent and attenuated Gardel strains (Marcelino et al., 2015).*

membrane protein (hmp) (CDS_04750: hmp10) (**Tables 1**, **2**, **Figure 3**). The common upregulated genes for the attenuated strains belong to energy production and conversion (*fdxB* and *atpG*), nucleotide transport and metabolism (*ndk*), replication, recombination and DNA repair (*ssb*), hypothetical membrane proteins (CDS_03690: hmp9 and CDS_05140: hmp12) and

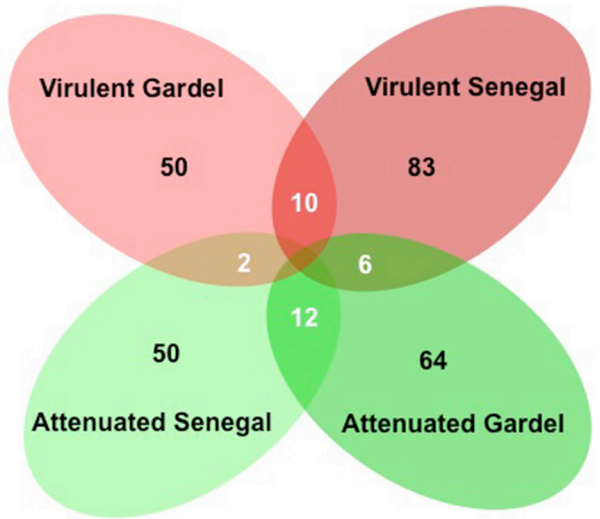

FIGURE 2 | Venn diagram of upregulated genes for the virulent and attenuated strains. Numbers of upregulated genes are indicated for each strain (in black). The numbers of common upregulated genes are indicated in circle intersections (in white).

hypothetical proteins (hp) (CDS_09210, CDS_3280, CDS_04190, CDS_08200, CDS_03450 and CDS_05460) (**Tables 1**, **2**).

The upregulated genes were classified according to their COG (clusters of orthologous groups of proteins) function. The proportion of upregulated genes in each COG for the virulent and attenuated Gardel and for the virulent and attenuated Senegal strains is shown in **Figure 4**. The genes encoding hypothetical proteins represented the highest proportion of upregulated genes with 32 and 23% for the virulent and attenuated Gardel and 29 and 34% for the virulent and attenuated Senegal strains respectively. The second highly upregulated gene COG corresponded to a "hypothetical membrane protein" for the virulent and attenuated Gardel strains. Genes belonging to several COGs were exclusively found to be upregulated only in a single strain: i.e., defense mechanisms upregulated in the attenuated Gardel strain, and cell cycle control, cell division, chromosome partitioning in the virulent Senegal strain (**Figure 4**). Nonetheless, the proportion of genes upregulated in strain-specific COGs was very low (<2%).

There was upregulation of genes belonging to nucleotide transport and metabolism and cell wall/membrane/envelope biogenesis in both virulent and attenuated Senegal and in the attenuated Gardel strain only (**Figure 4**).

Genes involved in metabolic functions such as energy production and conversion; translation, ribosomal structure and biogenesis; and posttranslational modification, protein turnover, chaperones were upregulated for both virulent and attenuated strains of Gardel and Senegal, but with more genes upregulated for the attenuated strains overall. Similarly, there was an upregulation for the Gardel and Senegal strains for genes involved in carbohydrate/Amino acid/Inorganic ion transport and metabolism, coenzyme transport and metabolism, but with more genes upregulated in the virulent Senegal strain (**Figure 4**).

Upregulation of the *map1* Gene Family in Virulent Strains

The percentage of upregulated genes belonging to the *map1*-related gene family was higher and more specific for the virulent strains than for the attenuated strains: 16 and 12% for the virulent Gardel and Senegal strains respectively (**Figure 4**). Eight out of 16 *map1*-family genes were upregulated in the virulent strains for both Gardel and Senegal as described above (**Tables 1**, **2**, **Figure 3**). Additionally, two genes were exclusively upregulated in the virulent Senegal strain: *map1-10* and *map1-11* (**Figure 3**).

Seven *map1*-related genes were chosen for a qPCR validation of the microarray results. The $\log_2$ FC obtained by qRTPCR for Gp 43 vs. Gp 243 and for Sp7 vs. Sp77 were compared to $\log_2$ FC obtained by microarrays (**Table 3**). The upregulation of 6 *map1*-related genes, observed by microarrays was confirmed by qRTPCR for both virulent Gardel and Senegal. For *map1-10* gene expression in Gardel strains, there was no significant differential expression obtained by microarrays which was confirmed by qRTPCR ($\log_2$ FC $= -0.6$) but we confirmed the upregulation of *map1-10* exclusively for virulent Senegal strain with a FC $= 4.1$ obtained by qRTPCR and 2.47 obtained by microarray (**Table 3**). The *map1-4* qRTPCR could not be optimized for Senegal strain, thus confirmation of microarray result on *map1-4* was not possible.

Differential Expression of Genes Encoding for Hypothetical Proteins

Genes encoding for hypothetical proteins (hp), including hypothetical membrane proteins (hmp), were found to be upregulated for both virulent and attenuated strains of Gardel and Senegal (**Tables 1**, **2**). There were 11 upregulated genes in common between the Gardel and Senegal strains. For hypothetical membrane proteins, CDS_03690 (hmp9) and CDS_05140 (hmp12) were upregulated in the attenuated strains of both Gardel and Senegal, whereas CDS_04750 (hmp10) was upregulated in the virulent strains. CDS_07940 (hmp13) and CDS_04760 (hmp11) were upregulated in attenuated Gardel and conversely upregulated in virulent Senegal (**Figure 3**). CDS_03690 (hmp9) and CDS_04750 (hmp10) encode for proteins that are unique to *E. ruminantium*. Some genes encoding hypothetical membrane proteins are located in the same chromosomal region and were identically modulated between the virulent and attenuated strains (**Figure 3**). For example, CDS_02240 (hmp1) and CDS_02250 (hmp2) or CDS_02320 (hmp5), CDS_02330 (hmp6) and CDS_02340 (hp1) were upregulated for virulent Senegal and CDS_02290 (hmp3) and CDS_02300 (hmp4) were upregulated for attenuated Gardel. CDS_02440 (hp4) associated with *nth* (CDS_2430) are also upregulated in attenuated Gardel (**Figure 3**).

Differential Expression of T4SS Components Between Virulent and Attenuated Gardel Strains

In *E. ruminantium*, genes encoding for T4SS are organized into five clusters comprising two major operons. Eight *vir* genes

TABLE 2 | Upregulated genes and their function for the Senegal strains (genome accession numbers: NC_006831.1).

Gene function	Virulent			Attenuated		
	Gene id	Name	log_2 FC	Gene id	Name	log_2 FC
Energy production and conversion	CDS_04540	*nuoE*	1.16	**CDS_04300**	***fdxB***	**1.76**
				CDS_04070	***atpG***	**1.41**
				CDS_04900	*nuoL*	1.90
				CDS_04410	*nuoH*	1.87
				CDS_06480	*qor2*	1.60
				CDS_04700	*atpC*	1.59
				CDS_08270	*acnA*	1.26
				CDS_03100	*nuoA*	1.10
				CDS_04910	*nuoK*	1.09
Nucleotide transport and metabolism	CDS_06810	*purM*	1.56	**CDS_08980**	***ndk***	**1.70**
	CDS_02460	*purB*	1.50	CDS_07060	*thyX*	1.72
	CDS_06330	*cmk*	1.33			
Coenzyme transport and metabolism	**CDS_07700**	***ribE***	**1.57**	**CDS_00530**	***hemA***	**2.87**
	CDS_01790	***pdxH***	**1.37**	CDS_04080	*folE*	1.89
	CDS_09450	*ubiF*	1.72	CDS_01680	*bioF*	1.58
	CDS_06870	*gshB*	1.63			
	CDS_08040	*ubiE*	1.52			
	CDS_02030	*thiE*	1.25			
	CDS_02910	*nadD*	1.06			
Replication, recombination and DNA repair	CDS_01880	*dnaA*	1.90	**CDS_02840**	***ssb***	**1.24**
	CDS_05050	*recO*	1.53			
	CDS_01110	*xerD*	1.32			
	CDS_00800	*holA*	1.31			
Posttranslational modification, protein turnover, chaperones	CDS_07310	*coxW*	1.82	CDS_06620	*clpB*	2.44
	CDS_03060	***ccmE***	**1.32**	CDS_07200	*grxC2*	1.66
	CDS_05090		1.29	CDS_08630	*dsbD*	1.43
Carbohydrate/AA/inorganic ion transport and metabolism	CDS_02740	*proP*	1.73	CDS_07110	*glyA*	1.03
	CDS_05960	*pstB*	1.73			
	CDS_05320	*carA*	1.66			
	CDS_00460	*rpe*	1.59			
	CDS_08830	*trkH*	1.44			
	CDS_02590	*znuC*	1.42			
	CDS_03380	*cutA*	1.22			
Lipid transport and metabolism	CDS_03460	*acpS*	1.33			
Cell cycle control, cell division, chromosome partitioning	CDS_08240	*parA*	1.48			
Cell wall/ membrane/envelope biogenesis	CDS_01150	*lnt*	1.47	CDS_07250	*wcaG*	1.45
				CDS_04980	*mraW*	1.36
Intracellular trafficking, secretion and vesicular transport	CDS_07740	*secB*	1.77	**CDS_05400**	***virB4a***	**1.20**
	CDS_04840	*tatC*	1.48	CDS_05410	*virB3*	2.43
Map1-related protein	**CDS_09130**	***map1-2***	**2.87**			
	CDS_09060	***map1-9***	**2.40**			
	CDS_09100	***map1-5***	**2.11**			
	CDS_09110	***map1-4***	**2.10**			

(Continued)

TABLE 2 | Continued

Gene function	Virulent			Attenuated		
	Gene id	Name	log_2 FC	Gene id	Name	log_2 FC
	CDS_09080	***map1-7***	**1.96**			
	CDS_09090	***map1-6***	**1.67**			
	CDS_09030	***map1-12***	**1.62**			
	CDS_09120	***map1-3***	**1.07**			
	CDS_09050	*map1-10*	2.47			
	CDS_09040	*map1-11*	1.10			
Translation, ribosomal structure and biogenesis	**CDS_04360**	***truA***	**2.42**	CDS_05500	*rpmB*	2.42
	CDS_05070	***rrmJ***	**1.45**	CDS_01760		2.58
	CDS_03220	*rluC*	1.53	CDS_02000	*fmt*	1.89
	CDS_08540	*hemK*	1.51	CDS_01290	*rplT*	1.51
	CDS_08260		1.42	CDS_09290	*rplS*	1.24
	CDS_00440		1.28	CDS_04050	*raiA*	1.08
	CDS_09490	*glyS*	1.13			
Hypothetical membrane protein	**CDS_07940**		**1.80**	**CDS_03690**		**1.49**
	CDS_04750		**1.43**	**CDS_05140**		**1.48**
	CDS_07410		2.00	CDS_07920	*surA*	1.14
	CDS_00760		1.84	CDS_03840		1.11
	CDS_02790		1.69			
	CDS_02320		1.67			
	CDS_02330		1.58			
	CDS_02240		1.51			
	CDS_07580		1.43			
	CDS_02250		1.43			
	CDS_04730		1.37			
	CDS_04790		1.11			
Hypothetical protein	**CDS_04760**		**1.43**	**CDS_04560**	***sspB***	**2.68**
	CDS_00540		2.38	**CDS_09210**		**2.32**
	CDS_02340		2.04	**CDS_03450**		**2.13**
	CDS_05530		1.93	**CDS_04190**		**2.10**
	CDS_00020		1.70	**CDS_08200**	***LexA***	**1.60**
	CDS_03330		1.65	**CDS_03280**		**1.07**
	CDS_07820		1.63	CDS_07190		2.30
	CDS_00620		1.60	CDS_06440		2.18
	CDS_08490		1.59	CDS_00430		1.92
	CDS_02800		1.51	CDS_05720		1.77
	CDS_07490		1.50	CDS_02070		1.56
	CDS_01720		1.50	CDS_05890		1.48
	CDS_01200		1.46	CDS_06760		1.44
	CDS_04010		1.46	CDS_03000		1.29
	CDS_07400		1.40	CDS_02360		1.25
	CDS_06420		1.35	CDS_04440		1.06
	CDS_01390		1.33	CDS_09350		1.06
	CDS_08340		1.31			
	CDS_06800		1.30			
	CDS_03870		1.28			
	CDS_08440		1.25			
	CDS_01210		1.20			

(Continued)

TABLE 2 | Continued

Gene function	Virulent			Attenuated		
	Gene id	Name	log_2 FC	Gene id	Name	log_2 FC
	CDS_02630		1.17			
	CDS_01930		1.11			
Number of upregulated genes			83			50

Bold blue: common upregulated genes in the Gardel and Senegal strains.
Bold pink: conversely upregulated genes in the Gardel and Senegal strains.

were upregulated only in virulent Gardel with a higher log_2-FC ranging from 3.72 to 1.31 (**Table 1**). There are *virB4a*, *virB6b*, *virB6c*, and *virB6d* localized on one operon (operon *virB3-B6*); *virB10* and *virB11* localized on a second operon (operon *virB8-11/D4*); and *virB2b* and *virB2c* localized on a third operon (operon *virB4b/B2a-d*), as shown in **Figure 3**. Moreover, CDS_04510 (hp5), CDS_08310 (hp6) and CDS_03830 (homolog of *ankA*) have been identified by S4TE software as potential effectors of T4SS and were also upregulated in virulent Gardel (Meyer et al., 2013). CDS_06520 and CDS_00970 are also potential effectors and were upregulated in attenuated Gardel.

For Senegal, there were only 2 *vir* genes (*virB3* and *virB4a*) differentially expressed between the virulent and attenuated strains, but contrary to Gardel, they were upregulated in the attenuated strain (**Table 2**, **Figure 3**).

E. ruminantium Defense Against Host Cell Immune Response

Among the genes differentially expressed between the virulent and attenuated Gardel and Senegal strains, several genes are involved in the subversion of host cell response. In fact, *grxC2*, which encodes for glutaredoxin, was upregulated in the attenuated Senegal strain whereas *bcp*, which encodes for the Bacterioferritin co-migratory protein, was upregulated in the attenuated Gardel strain. Only Senegal displayed differential gene expression for genes involved in counteracting osmotic stress. For Senegal, there were 8 genes differentially expressed between the virulent and attenuated strains. Genes encoding for proline-betaine transporter (*proP*), for NADH dehydrogenase subunit E (*nuoE*) and for a putative Na+/H+ antiporter subunit (CDS_01720) were upregulated for the virulent strain (**Table 2**). Genes *nuoH, nuoL, nuoA,* and *nuoK* encoding for NADH-quinone oxidoreductase and CDS_05720 (Putative Na+/H+ antiporter subunit) were upregulated for the attenuated strain (**Table 2**).

Other genes known to be induced during stress were found to be differentially expressed between the virulent and attenuated strains with a high log_2-FC. For example, gene encoding for protein ClpB (2.44) and CDS_07190 (2.30) encoding for BolA-like protein were upregulated in attenuated Senegal (**Table 2**).

DISCUSSION

In the Anaplasmataceae family, most studies have been conducted on virulent strains and have identified a small number of key components of pathogenicity (Pruneau et al., 2014). However, there are a few studies on attenuated strains, comparing them with virulent strains in order to identify mechanisms of attenuation. In *E. ruminantium*, three strains have been attenuated *in vitro* by successive passages in canine macrophages for the Welgevonden strain (Zweygarth et al., 2005) and in bovine endothelial cells for the Senegal (Jongejan, 1991) and Gardel strains (Marcelino et al., 2015). These attenuated strains confer strong and long-lasting protection against homologous and some heterologous challenges (Faburay et al., 2007). However, so far, the mechanisms of attenuation remain unknown and the use of attenuated vaccines is limited partly due to the possible reversion of virulence. In this study, to investigate both the virulence and attenuation mechanisms, we compared the global transcriptome profile of two distant virulent strains of *E. ruminantium*, Gardel and Senegal, with their corresponding attenuated strains *in vitro*. Moreover, three biological replicates per strain were used, and the transcriptome profiles obtained between the different replicates were at least 72% identical (main linear correlation coefficient), thus strengthening our microarray data.

Transcriptomic data for the Gardel strains obtained in this study were compared with previous proteomic data obtained by comparing the Gardel virulent and attenuated strains (Marcelino et al., 2015). Among the proteins upregulated or detected only in virulent Gardel in the proteomic study, 10 and 1 corresponding genes were found upregulated in our transcriptomic results for virulent and attenuated Gardel, respectively. From these 10 upregulated genes, there are 4 MAP1-related proteins (*map1-3, map1-4, map1-2*, and *map1-12*) and 2 genes encoding proteins related to virulence (*virB10* and *ankA*) (**Table 1**). Furthermore, among the proteins upregulated or detected only in attenuated Gardel by the proteomic analysis, 9 corresponding genes were upregulated for the attenuated strain and 4 for the virulent strain. Finally, CDS_05140 (hmp12), which displays the highest value of FC, was the second most abundant protein in the proteomic study (Marcelino et al., 2015). The transcriptomic and proteomic studies are consistent: for the virulent strain, an upregulation of Map1-family and virulence gene and protein expression, and for the attenuated strain, an upregulation of gene and protein expression related to metabolism.

The virulent Gardel strain showed a different transcriptomic profile compared to others strains (**Figure 1B**). This result correlates with the COG differences observed between the four

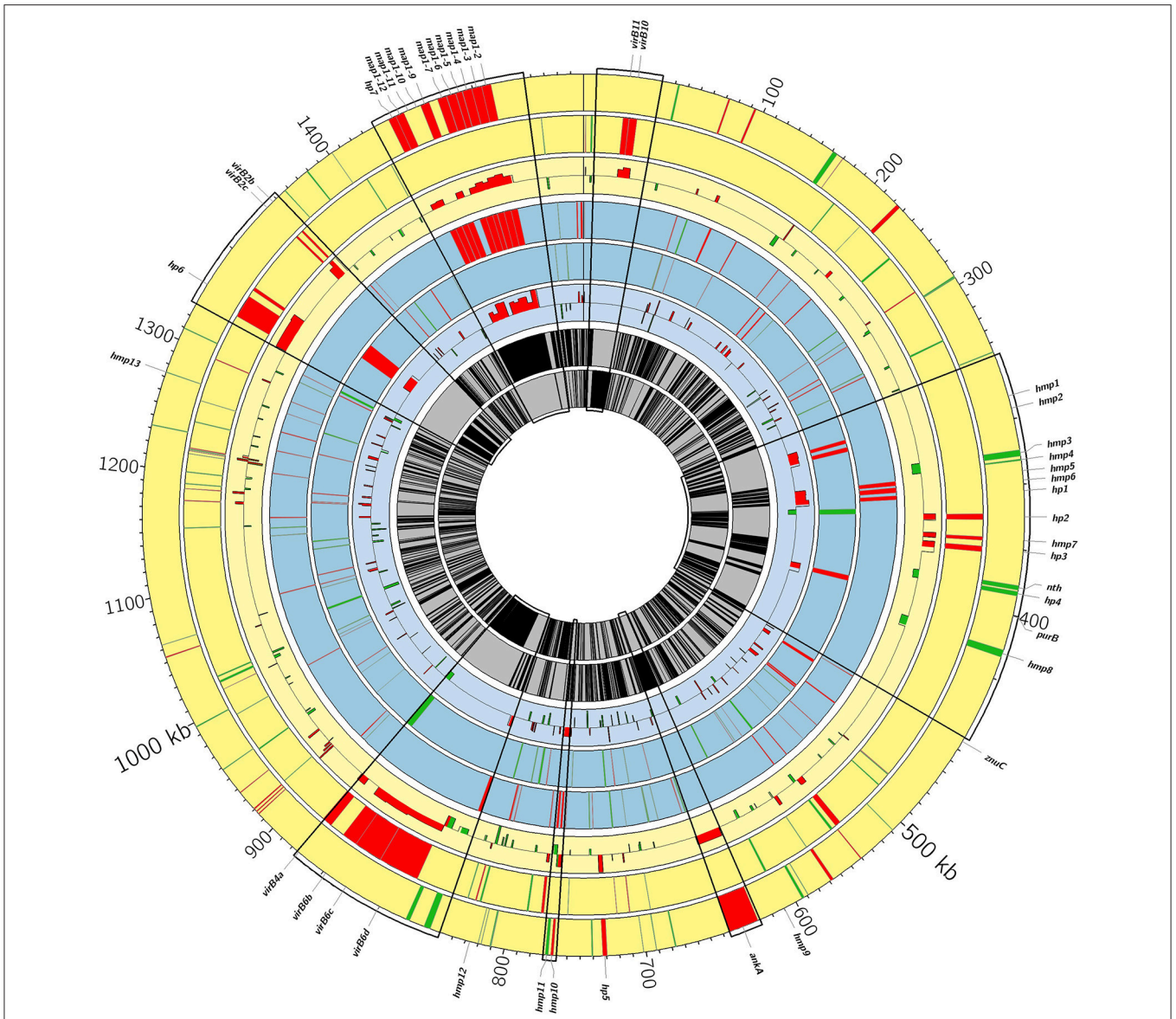

FIGURE 3 | Circos genome diagram showing differentially expressed genes between the virulent and attenuated strains. From outer to inner, positive and negative genome strands for Gardel (yellow) and for Senegal (blue) respectively and mean of $\log_2$ FC for each CDS are shown. Upregulated genes in the virulent strains are in red and upregulated genes in the attenuated strains are in green. Some areas are enlarged and correspond to *map1*-family genes (magnification 8-fold) and to *Vir* genes and genes encoding hypothetical membrane proteins (hmp) and hypothetical proteins (hp) (magnification 5-fold). Hmp1: CDS 02240; hmp2: CDS 02250; hmp3: CDS 02290; hmp4: CDS 02300; hmp5: CDS 02320; hmp6: CDS 02330; hmp7: CDS 02390; hmp8: CDS 02480; hmp9: CDS 03690; hmp 10: CDS 04750; hmp11: CDS 04760; hmp12: CDS 05140; hmp13: CDS 07940. Hp1: CDS 02340; hp2: CDS2370; hp3: CDS 02410; hp4: CDS 02440; hp5: CDS 04510; hp6: CDS 08310; hp7: CDS 09020.

strains. In fact, two COGs were absent in the virulent Gardel strain when compared to other strains (Nucleotide transport and metabolism; Cell wall/membrane/envelope biogenesis) and three others displayed the highest proportion of upregulated genes (**Figure 4**). This result suggests that the virulence phenotype may be associated with distinct gene subsets. In fact, these categories of COGs contain genes related to virulence, such as *vir* genes encoding components of T4SS. *Vir* genes upregulated in virulent Gardel encode for two major T4SS clusters (**Figure 3**). The T4SS is a protein complex that is important for the pathogenesis of intracellular bacteria because it permits translocation of virulence factors into the host cell (Voth et al., 2012). The crucial role of T4SS and its effector proteins such as AnkA has been shown for two others pathogens of the Anaplasmataceae family, *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* (Rikihisa and Lin, 2010). The upregulation of genes encoding for T4SS together with gene ERGA_CDS_03830, encoding for the AnkA homolog,

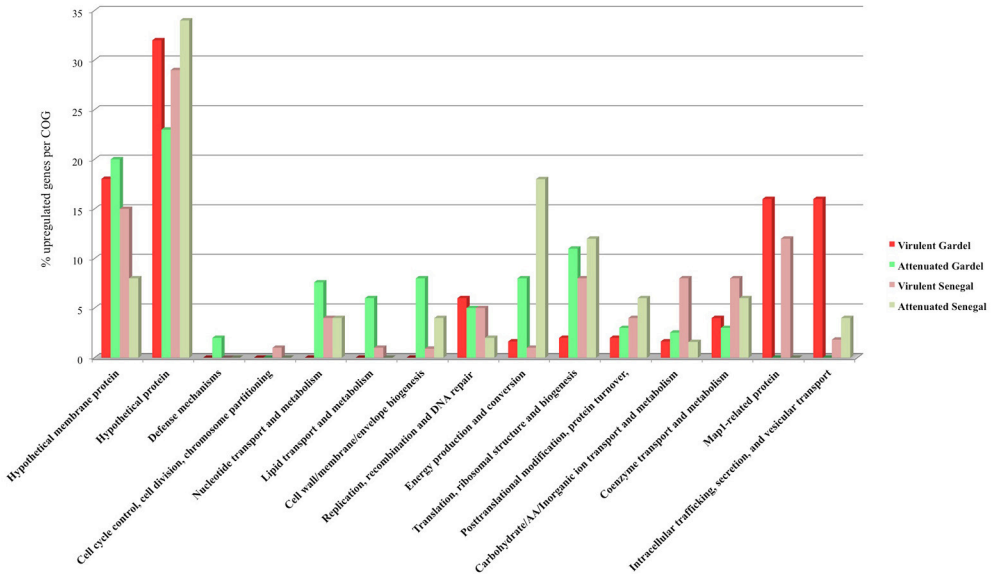

FIGURE 4 | Proportion of upregulated genes per COG for the virulent and attenuated strains.

TABLE 3 | Comparison of $\log_2$ FC for *map1*-related genes obtained by microarrays and qRTPCR.

Gene name	Gene Id	Gardel		Senegal	
		$\log_2$ FC microarrays	$\log_2$ FC qRTPCR	$\log_2$ FC microarrays	$\log_2$ FC qRTPCR
map1-12	CDS_09030	1.29	1.56	1.62	11.6
map1-10	CDS_09050	1*	−0.6	2.47	4.1
map1-9	CDS_09060	1.36	1.17	2.4	2.2
map1-6	CDS_09090	1.84	1.61	1.67	2.2
map1-4	CDS_09110	2.14	1.38	2.10	ND
map1-3	CDS_09120	2.36	2.57	1.07	3.69
map1-2	CDS_09130	1.71	9.27	2.87	2.8

ND, not detected.
**not significantly differentially expressed.*

suggests an important role in the virulence of the virulent Gardel strain of *E. ruminantium*.

Another gene ERGA_CDS_06440, encoding for the AnkB homolog, was found upregulated in attenuated Senegal, as well as two genes encoding for T4SS components (*virB3* and *virB4a*), whereas no upregulation of *vir* genes was observed in the virulent Senegal strain. Moreover, ERGA_CDS_03830 (homolog of *ankA*) and ERGA_CDS_06440 were predicted as potential T4SS effectors for *E. ruminantium* using S4TE software (Meyer et al., 2013). In a newly published transcriptomic study on the virulent Welgevonden strain of *E. ruminantium*, genes encoding for T4SS components and homologs of *ankA* and *ankB* were also upregulated in EBs in comparison to RBs (Tjale et al., 2018). Further studies of these genes and genes encoding for T4SS components are crucial for the understanding of *E. ruminantium* pathogenesis.

In our study, genes related to virulence display a differential expression between the virulent and attenuated Gardel strains, whereas limited differential expression was observed for the Senegal strains. The mechanism of attenuation between Gardel and Senegal is probably different because the speed of attenuation for Senegal is faster than that for Gardel. Some virulence factors could be affected by attenuation mechanisms leading to a lack of virulence.

Moreover, the attenuated strains seem to have a higher metabolic activity than the virulent strains. In fact, the proportion of genes involved in energy production and conversion, posttranslational modification, protein turnover, chaperones and translation, ribosomal structure and biogenesis was higher for the attenuated strains. Genes encoding for proteins of ATP synthase such as *atpC*, *atpE*, and *atpG* were found upregulated only in the attenuated strains. Also, another gene exclusively upregulated in both attenuated strains, *ndk*, encodes for a nucleoside diphosphate kinase, which catalyzes the formation of nucleoside triphosphate required for DNA synthesis, from nucleoside diphosphate and ATP (Mishra et al., 2015). This

activation of ATP production needed for energy, but also for DNA synthesis, could have contributed to the shorter infectious cycle observed *in vitro* for the attenuated strains (4 days after infection), with intensive DNA replication beginning quickly after infection of new host cells (Marcelino et al., 2015; this study for the Senegal strain). This result is consistent with the economic game theory proposed by Tago and Meyer to explain the attenuation mechanism of obligate intracellular bacteria (Tago and Meyer, 2016). In fact, the authors explain that, in *in vitro* conditions, bacteria decrease the expression of genes related to virulence and, in parallel, increase the expression of genes related to metabolism, which results in a better fitness and a shorter development cycle.

Interestingly, we observed a higher proportion of upregulated genes involved in coenzymes, carbohydrate, amino acid and inorganic ion transport and metabolism for the virulent Senegal strain. For this strain, lysis of host cells was observed at 6 days post-infection, whereas the virulent Gardel strain with a higher number of passages displays a lysis of host cells at 5 days post-infection. Replicates of virulent Senegal used in this study had a small number of passages *in vitro* (fewer than ten passages). This result reflects the progressive adaptation of *E. ruminantium* to the *in vitro* culture.

Like many intracellular pathogens, *E. ruminantium* seems to be able to fight efficiently against osmotic and oxidative stresses generated by host cells. Osmotic and oxidative stresses are key methods by which mammalian infected cells kill bacteria. Thioredoxin and Glutaredoxin are the key proteins that fight oxidative stress (Holmgren, 1989; Bjur et al., 2006). Genes encoding for glutaredoxin were found upregulated in attenuated Senegal. The upregulation of *grxC2* was also reported for *Rickettsia conorii* infected eschars (Renesto et al., 2008). It seems that attenuated strains activate a greater number of mechanisms against oxidative stress. To fight osmotic stress, intracellular bacteria have systems of transport or synthesis of osmoprotectants (or compatible solutes) that maintain homeostasis through their accumulation or rejection in the cytoplasm (Roessler and Müller, 2001). Both Senegal strains had differential expression of genes involved in fighting osmotic stress. Several genes were upregulated for each strain indicating a different activation of pathways between attenuated and virulent Senegal. For example, *proP*, which encodes for a proline/betaine transporter, was found upregulated for virulent Senegal. Proline and betaine are two osmoprotectants that work to overcome the inhibitory effects of hyperosmolarity. This gene was also found upregulated in *R. conorii* (Renesto et al., 2008).

For attenuated Senegal, four genes (*nuoA*, *nuoH, nuoK*, and *nuoL*) encoding for proteins of NADH-quinone oxidoreductase complex, were upregulated. This complex is usually involved in energy production, but it can help to maintain homeostasis as observed in *R. conorii* (Renesto et al., 2008). For the Gardel strains, there were no genes differentially expressed and involved in fighting osmotic stress. This result suggests that the virulent and attenuated Gardel strains could use common mechanisms to fight osmotic stress with the activation of similar genes.

Genes encoding for Map1-related proteins were exclusively upregulated in the virulent strains. However, *map* gene sequences were not different between the virulent and attenuated strains, which could explain the downregulation of *map1*-family gene expression (data not shown). The modulation of gene expression is probably due to mutations within unknown *map1* regulatory regions. These proteins are major antigenic proteins and in the case of Map1 induce a strong humoral response, which is not protective. Previous studies shown that *map1*-family genes were differentially expressed in host or vector cells suggesting that they are involved in the adaptation of *E. ruminantium* to its host or vector (Postigo et al., 2008). The upregulated expression of genes encoding for Map1-related proteins in the virulent strains suggests that they could induce a non-protective immune responses. We hypothesized that Map1-family proteins may play a role in luring the immune system. For the four strains, many genes encoding for hypothetical membrane proteins, as well as genes involved in posttranslational modifications and cell wall/membrane/envelope biogenesis, were upregulated. These results suggest membrane reorganization to escape immune response, as described previously for *Chlamydia trachomatis* (Nicholson et al., 2003) and *Rickettsia prowazekii* (Bechah et al., 2010). The lower expression of *map1*-family genes in the attenuated strains and the upregulation of other genes encoding for hypothetical membrane proteins could be important in inducing efficient immune responses. These hypothetical membrane proteins should be functionally characterized and could be potential candidates for vaccine development.

For example, CDS_05140 (hmp12) was one of the most upregulated genes in both attenuated strains. This gene encodes for a proteoform porin located in the outer membrane of *E. ruminantium* (Marcelino et al., 2015). Further studies are required to characterize the role of the protein encoded by CDS_05140 (hmp12), and it could be a potential candidate for vaccine development. Moreover, the function of two another hypothetical membrane proteins unique to *E. ruminantium* could also be studied: CDS_03690 (hmp9), which was upregulated for both attenuated strains, and CDS_04750 (hmp10), which was upregulated for both virulent strains.

In conclusion, the comparison of global transcriptomes of virulent and attenuated strains suggested a reorganization of the membrane of *E. ruminantium*. The upregulated membrane proteins expressed in the attenuated strains could be vaccine candidates against heartwater, and their function will be further studied.

In fact, genes encoding for Map1 proteins were found to be downregulated in both attenuated strains, suggesting that these proteins have a specific role in *E. ruminantium* pathogenesis. For both the virulent and attenuated strains, most of the upregulated genes encoding hypothetical membrane proteins were different between Gardel and Senegal, indicating the possible modification of membrane structures. These gene expression modifications could induce either unprotective or protective immune responses, highlighting the importance of studying these proteins.

AUTHOR CONTRIBUTIONS

BM, DM, NV, and TL: conceived and designed the experiments; LP and KL: performed the experiments; LP, BM, KL, DM, and NV: analyzed the data; LP and NV: wrote the paper. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00153/full#supplementary-material

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Functional Redundancy and Ecological Innovation Shape the Circulation of Tick-Transmitted Pathogens

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Ticks are vectors of pathogens affecting human and animal health worldwide. Nevertheless, the ecological and evolutionary interactions between ticks, hosts, and pathogens are largely unknown. Here, we integrated a framework to evaluate the associations of the tick *Ixodes ricinus* with its hosts and environmental niches that impact pathogen circulation. The analysis of tick-hosts association suggested that mammals and lizards were the ancestral hosts of this tick species, and that a leap to Aves occurred around 120 M years ago. The signature of the environmental variables over the host's phylogeny revealed the existence of two clades of vertebrates diverging along a temperature and vegetation split. This is a robust proof that the tick probably experienced a colonization of new niches by adapting to a large set of new hosts, Aves. Interestingly, the colonization of Aves as hosts did not increase significantly the ecological niche of *I. ricinus*, but remarkably Aves are super-spreaders of pathogens. The disparate contribution of Aves to the tick-host-pathogen networks revealed that *I. ricinus* evolved to maximize habitat overlap with some hosts that are super-spreaders of pathogens. These results supported the hypothesis that large host networks are not a requirement of tick survival but pathogen circulation. The biological cost of tick adaptation to non-optimal environmental conditions might be balanced by molecular mechanisms triggered by the pathogens that we have only begun to understand.

Keywords: networks, ticks, tick-borne pathogens, communities

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INTRODUCTION

Communities are fundamental units of ecological information. They elucidate the interactions among species cohabiting within a defined area and describe changes in species composition and resilience after a disturbance (Christian et al., 2015). Metacommunity theory is a relatively recent development in community ecology (Leibold et al., 2004; Holyoak et al., 2005). The basic postulates of this framework posit local communities are interconnected by the processes of dispersal and extinction (Leibold et al., 2004). The species composition of the local communities is determined by both these regional processes and the local interactions that determine habitat suitability (Holyoak et al., 2005; Leibold and McPeek, 2006; Mihaljevic, 2012).

The concepts of the ecological community have been scarcely applied to host-tick systems, in which acquisition of parasites occurs mostly via horizontal transmission (Krasnov et al., 2010). Ecological community approaches could dramatically enhance our understanding of complex pathogen circulation systems that include several vectors and hosts (Estrada-Peña et al., 2015). In addition, since colonization of new habitats is considered to be a frequent event in ectoparasites evolution (Krasnov et al., 2010), we hypothesize that new habitat colonization during tick evolution might have played a especial role in pathogen spread and circulation. The communities of vector-borne pathogens include a panoply of pathogenic microorganisms that circulate through the bite of a competent vector and remain in permanent foci due to the presence of reservoir hosts. The interest in these pathogenic organisms has raised in the last decade due to the increase in the incidence of human diseases related with them (de la Fuente and Estrada-Peña, 2012; Estrada-Peña and de la Fuente, 2014). Ticks are versatile arthropod vectors capable of transmitting the broadest spectrum of pathogens to vertebrates (Jongejan and Uilenberg, 2004). The community approach offers the potential to explore the interactive roles of joint evolutionary history between ticks and their hosts, the impact of abiotic environment, and the evolutionary pressure on associated pathogens. However, this approach is in its infancy, despite some studies focusing on interactions between ectoparasites and their hosts at the local or regional scale (Lindgren et al., 2000; Ostfeld and Keesing, 2000; Keesing et al., 2006, 2010; Jaenson and Lindgren, 2011). Tools such as phylogeography and evolutionary adaptations of the species relationships can be integrated into a framework to quantify environmental and biological factors governing the structure of complex communities of multiple hosts, vectors, and pathogens. These tools have never been jointly applied to the understanding of co-evolution and relationships among the members of a community of tick-transmitted pathogens.

Here, we elaborate on a prominent tick species, *Ixodes ricinus*, that displays complex ecological interactions. This tick species is an interesting model to study the association between tick-host-pathogen communities and the environment because it has a wide distribution in temperate Europe, infest hundreds of vertebrate host species, and support the circulation of several pathogens (Estrada-Peña et al., 2006; Medlock et al., 2013). We explicitly explored how the hosts exploited by this tick result in a functional redundancy that improves the circulation of pathogens. Our results showed that by accessing hosts that are pathogen super-spreaders, ticks occupied non-optimal environments. This is sustainable for the tick only if the cost associated with the colonization of non-optimal environments is balanced by benefits provided by pathogens infection (Cabezas-Cruz et al., 2017).

METHODS

Background

The methods below refer to the following steps: (i) the collection of association of the metacommunity of ticks, host, and pathogens for the literature, (ii) the construction of the phylogenetic relationships of the hosts of the tick or reservoirs of pathogens, (iii) the construction of the environmental niche of tick and hosts to check for environmental signature in the previous phylogenetic tree, and (iv) the building of the network of ticks and hosts to infer epidemiological relationships. Definitions of the most important terms of this framework are included in **Box 1**.

Literature Data Collection

Data on specific associations of *I. ricinus* and its hosts are the backbone of this study. Data on pairs of associations among ticks, vertebrates, and pathogens were compiled from a literature review focused on the Western Palearctic. A "record" is a pairwise combination of "pathogen and tick," "tick and vertebrate" or "pathogen and vertebrate" at one site. The pathogen can be associated to either the vertebrate or to the tick, while the tick is only associated with the vertebrate. The literature review was based on journals searchable in Thomson Reuters, Scopus, and PubMed.

We performed a deliberately relaxed query, including only the name "*I. ricinus*," to manually select the papers reporting ecological information after a critical evaluation of the abstract. The obtained papers were included in the dataset only if adequate information about the host, tick and/or pathogen was available. We purposely removed every report concerning livestock, because they are recognized as accidental hosts, generating spurious information that distorts the natural relationships of the metacommunity of tick-borne pathogens (Estrada-Peña et al., 2015). The literature review included reports published during the period 1970 to December, 2015. As far as possible, the scientific names of pathogens were updated to include the most recent and accepted ones. When not possible (i.e., the complex of species of *Borrelia burgdorferi*, whose specific names changed radically in the last years) it has been included as a generic name (in the example, *Borrelia burgdorferi* sensu lato) to reflect the relationships but without reliability of the name of the pathogen.

Phylogenetic Relationships of Hosts

A total of 168 species of vertebrates were recorded as hosts for *I. ricinus*. We used a previously published, dated supertree of Tetrapoda of the Western Palearctic (Roquet et al., 2014). The complete details for the construction of the supertree have been published and extensively contrasted (Roquet et al., 2014). The tree of Tetrapoda was pruned to accommodate only the hosts of *I. ricinus* using the package ape (Paradis et al., 2004) for the R programming environment (R Core Team, 2014). The complete phylogenetic tree of the hosts of *I. ricinus* is available in newick format as **Figure 1**, **Supplementary Table S1**, and **Supplementary Figure S1**.

Data on Environmental Variables and Calculation of Niche Dimensions of Ticks and Hosts

We wanted to track the environmental niche in which each species of hosts prevails and how the environmental niche of the tick and its hosts overlap. This is commonly captured by running models that measure the distribution of the tick

BOX 1 | Definitions of terms used in this paper.

Environmental niche: The range of abiotic conditions (mainly climate, also known as **environmental variables**) under which an organism can persist. In example, high and low temperature restrict the distribution of living beings. The plot of the recorded distribution of an organism against the main abiotic variables can help to describe its environmental niche. Using a phylogenetic tree of organisms, it is possible to track the gradient of environmental variables to which each organism is associated, known as **environmental signature**.

Pagel's λ: It is an index that allows to correlate the environmental variables (or other traits) to a phylogeny. When λ equals 1, the structure of the phylogeny alone can explain changes in environmental niche and therefore environmental variables show a strong phylogenetic signal. On the other hand, when λ equals 0, the phylogeny alone is not able to explain that evolution. The exploration of the phylogenetic signature of environmental variables on a phylogeny tracks if groups of genetically related hosts prefer the same portions of the environmental niche.

Metacommunity: An ecological metacommunity is a set of interacting communities of interacting organisms. In this study, we used the term to describe the communities of ticks, vertebrates and tick-transmitted pathogens. Vertebrates interact in the system being hosts of the ticks or reservoirs of the pathogens, ticks are the vectors of the circulating pathogens.

Network: An ecological network is a construct that represents biotic interactions between organisms. These organisms are commonly referred to as "nodes," which interact through "links." The relationships between interacting organisms provide indexes from which the strength of the associations or the role of organisms in the context of the network can be measured.

Authority: The nodes of a network have different indexes that give an idea of its importance in the context of the interacting organisms. The terms "authority" and "hub" are two of these indexes. Nodes with high Authority are nodes which are pointed to by important nodes, in fact, by nodes with high Hub scores. And the latter obtain their high Hub scores by pointing to good Authority nodes. In short: Hubs point, and Authorities are pointed to. The ecological meaning for our application is that i.e., a vertebrate that acts as reservoir of several species of pathogens, or on which the focal tick has been repeatedly recorded, while have a high Authority. It is a measure of the relative importance of the vertebrate (or other organisms) in the network. In the calculation of Authority, the importance of the Hubs is not considered.

PageRank: It is another measure of the importance of a single node of the network, that considers the relative importance of the nodes pointing to it. Therefore, a node with a high PageRank is not only linked by many nodes, but with nodes that have also a high relative importance in the network. The ecological meaning is that i.e., a vertebrate will have a high PageRank if nodes of prominent species of pathogens are tightly interacting with it. The difference with Authority is that the importance of the neighbor nodes is considered. Both indexes, Authority and PageRank are complementary.

Weighted Clustering Coefficient: Evidence suggests that in most networks, nodes tend to create tight groups characterized by a relatively high density of ties, which is greater than the average probability of a tie randomly established between two nodes. The Weighted Clustering Coefficient measures the degree to which nodes in a graph tend to cluster together. The meaning in our application is that nodes with a high value of centrality will have a tendency to tightly cluster with others, meaning for an ecological interaction that is higher than with other nodes of the network.

and each host species in the environmental niche. We used a previously developed global dataset of environmental variables (Estrada-Peña et al., 2014) based on the transformation by harmonic regression of monthly data derived from the MODIS series of satellites at a nominal resolution of 0.05°. The dataset includes day temperature (LSTD) and the Normalized Difference Vegetation Index (NDVI), an index of vegetation vigor, which were obtained for the period 2001–2015. We retained three variables for LSTD (LSTD1–LSTD3) and three for NDVI (NDVI1–NDVI3) that explain the annual average and the slope (seasonality) in spring and autumn, respectively. The ability of the dataset to capture the environmental niche of organisms has been already demonstrated (Estrada-Peña et al., 2014).

We obtained pairs of coordinates of every species of host for the tick querying the Global Biological Information Facility (GBIF) (Garcia-Rosello et al., 2014). Approximately 11 million records were obtained for 203 unique species. We retained a set of 142 hosts for which phylogenetic information and reliable distribution data (at least 100 geo-referenced records) were simultaneously available. We independently trained environmental suitability models of each species of host using the niche modeling program MaxEnt (Phillips et al., 2006). MaxEnt was chosen because it demonstrated robust model performance compared to other modeling algorithms when presence-only data is available. We used the lineal and quadratic features with a maximum number of 10,000 background points and 70% of points for training purposes, using cross-validation to compare the resulting models. The regularization multiplier was set to 1. Each model was replicated 100 times using the cross-validation function in MaxEnt to partition the data into replicate folds, with each fold being used in turn to test the model. The aims of model building were to: (i) identify the host's environmental niche (Elith et al., 2011), (ii) reconstruct the phylogenetic signature of the environmental variables in the phylogenetic tree of hosts, and (iii) calculate the niche overlap between the tick and each host, following published approaches (Warren et al., 2010). The niche of *I. ricinus* was calculated using the same methods and a set of more than 8,000 occurrence records of the species with reliable coordinates (Estrada-Peña and de La Fuente, 2016).

Inferring the Signature of Environmental Traits in the Host Phylogeny

We wanted to track the signature of the environmental variables on the phylogenetic tree of hosts for *I. ricinus* to check if the environmental niche is conserved along the phylogeny of hosts. We could therefore conclude that the tick exploits hosts that share environmental conditions because they are genetically related. We treated environmental niche of species as evolving traits whose evolutionary history can be reconstructed through phylogenetic analysis, calculating a potential niche occupancy for ranges of the environmental covariates. The original supertree of Tetrapoda (Roquet et al., 2014) was pruned to retain the species of vertebrates exploited as hosts by *I. ricinus* as obtained from the literature search mentioned above, resulting in a sub-phylogeny of 142 vertebrate species. We used Pagel's λ (see

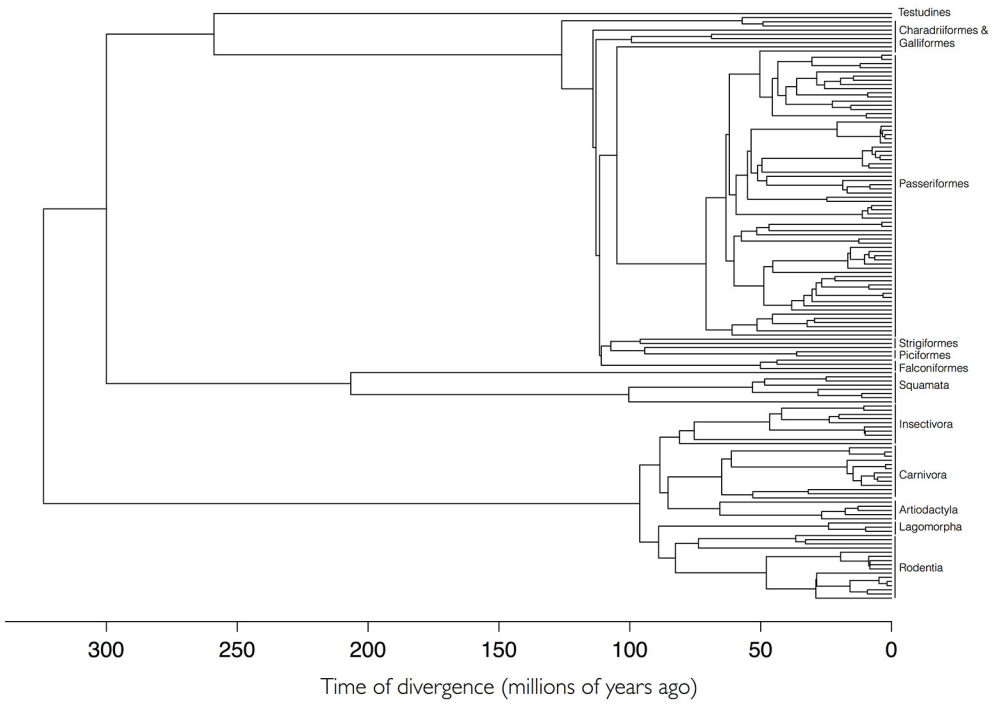

FIGURE 1 | The phylogenetic tree of 168 species of hosts of *Ixodes ricinus* based on a supertree of Tetrapoda of Western Palearctic. Higher taxonomical categories of hosts are included at the tips of the tree. **Supplementary Figure S1** provides a high-resolution version of this figure with the names of species at the tips of the tree.

Box 1) to measure the phylogenetic signal of the environmental variables along the phylogenetic tree (Munkemuller et al., 2012) as implemented in *phytools* (Revell, 2012) for R Core Team (2014). We calculated Pagel's λ separately for each of the six environmental variables (LSTD1-LSTD3 and NDVI1-NDVI3) on the phylogenetic tree of hosts for *I. ricinus*.

Building the Network of Hosts of *I. ricinus* and Transmitted Pathogens

The analyses before produced an estimation of the niche overlap between *I. ricinus* and its hosts as well as the correlation between the phylogenetic tree and the environmental variables. The last step of our framework aims to evaluate the impact of each host on the circulation of the pathogens.

We used a dataset on reported relationships between ticks, hosts, and pathogens to develop a network of biotic connections (Estrada-Peña and de La Fuente, 2016) in terms of "who is a parasite of whom" and then obtain conclusions on the effects of that structure on the circulation of pathogens. In host-parasite networks (see **Box 1**), nodes represent "cargos" that are linked to "carriers." Thus, the network is directed: each edge links a pathogen "to" a vertebrate or a tick, or a tick to a vertebrate. "Cargos" are thus the pathogens or the ticks, "carriers" are the ticks (for pathogens) or the vertebrates (as hosts for ticks or reservoirs for pathogens).

Centrality measures in ecological networks detect high-ranking nodes in the network (Blondel et al., 2008; Jacomy et al., 2014). To be central, a carrier is infected by many species of cargos that infect many other carriers in the network. The hosts with the greatest centrality are super-spreaders. Authority (**Box 1**) is an index that provides the importance of a node to which other nodes are connected (Holland and Leinhardt, 1971; Kourtellis et al., 2013). This is complimentary to PageRank (**Box 1**), an index that assigns a rank to nodes based on the importance of the other nodes to which it is linked (Holland and Leinhardt, 1971). The Weighted Clustering Coefficient is a measure of the degree to which nodes in a graph tend to cluster together (Holland and Leinhardt, 1971; Watts and Strogatz, 1998; Kourtellis et al., 2013). The Weighted Clustering Coefficient (**Box 1**) expresses the statistical level of cohesiveness measuring the global density of interconnected nodes in the network. Network computations were carried out using *igraph* (Csardi and Nepusz, 2006) for R Core Team (2014), the Louvaine clustering algorithm (Blondel et al., 2008) and the ForceAtlas2 algorithm for displaying the network (Jacomy et al., 2014).

RESULTS

Ixodes ricinus Uses Hosts with a Phylogenetic Signature of Environmental Niche

To test whether the hosts of *I. ricinus* Were significantly associated to environmental variables (i.e., LSTD and NDVI, see Methods), we built a host phylogenetic tree and measured the phylogenetic signal of the environmental signature (see **Box 1** for definitions). A strong signature was found along the phylogeny of *I. ricinus* hosts (**Figure 2**, **Supplementary Figure S2**). Pagel's

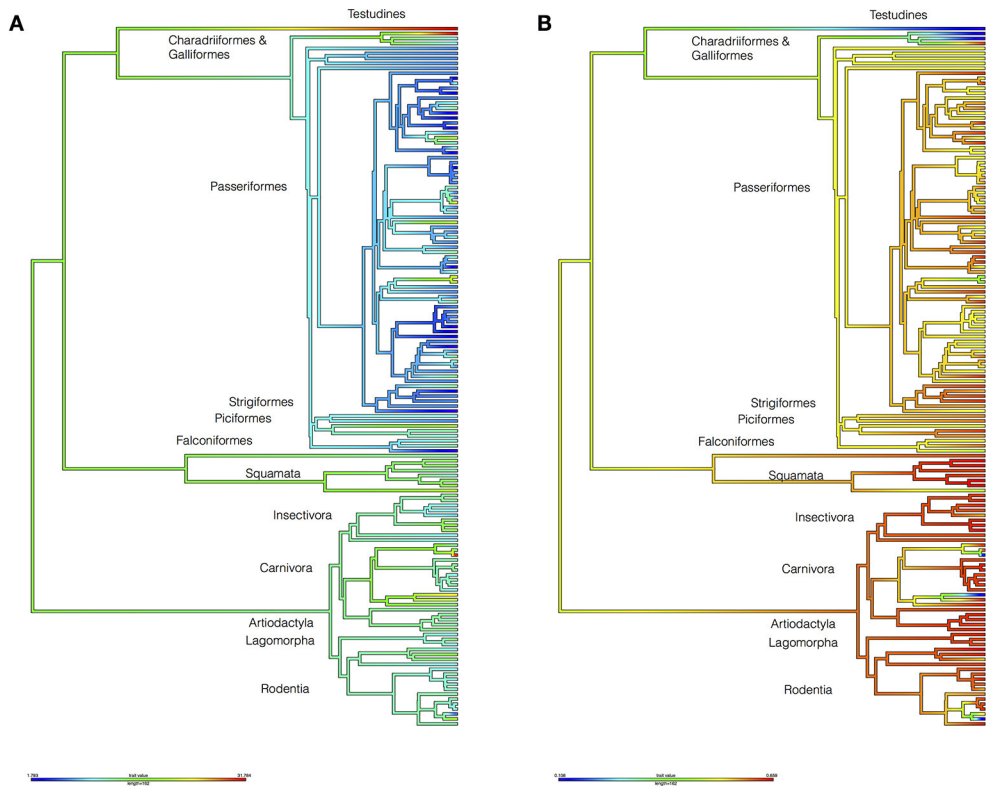

FIGURE 2 | Reconstructions of the environmental niche of the hosts of *I. ricinus*. The figure includes data for Land Surface Temperature (LSTD: **A**) and the Normalized Difference Vegetation Index (NDVI: **B**). The trees were drawn according to the phylogenetic tree in the **Figure 1**. Values in the legend are degree Celsius **(A)** and NDVI units multiplied by 100 **(B)**. Higher taxonomical categories of hosts are included at the tips of the tree. **Supplementary Figure 2** provides a high-resolution version of this figure with the names of species at the tips of the tree.

λ (see **Box 1**) was 0.69 ($p = 3.86E\text{-}8$) for the mean of LSTD and 0.66 ($p = 0.0002$) for the mean of NDVI. Other variables tested, like seasonality of LSTD and NDVI, lack phylogenetic signature ($\lambda < 0.3$). Most non-Aves hosts of *I. ricinus* were linked to warmer portions of the niche, whereas almost all species in Aves colonized a colder portion of the niche. All of the non-Aves vertebrates colonized the greenest portion of the environmental niche, whereas most Aves species were restricted to intermediate values of NDVI. Notably, non-Aves hosts evolved earlier than Aves. The fact that the older hosts occupied the warmer portions of the niche suggests that the ancestral state of the phylogenetic signature of the temperature for the hosts of *I. ricinus* was the warmer portions of the niche. The colonization of a colder niche was inferred to be a derived state according to the dating of the Tetrapoda genetic tree. The ecological explanation of these results is that *I. ricinus* is ecologically tied to two large clusters of hosts. The clear dichotomy in the temperature range (as opposed to a gradient) colonized by both clusters of vertebrates is highly suggestive of a host split in which *I. ricinus* accessed a new environmental niche while simultaneously adapting to a new set of hosts. Interestingly, vegetation (and therefore humidity) seems to play a secondary but still significant role in that split of hosts. The niche overlap between *I. ricinus* and its hosts produced a low phylogenetic signal ($\lambda = 0.188$, $p = 0.337$). The ecological interpretation is that the amount of niche shared between *I. ricinus* and its hosts is unrelated to the genetic relationships of the vertebrates: the tick does not exploit species of vertebrates that are closely related to gain a wider environmental niche.

To test the contribution of different host groups to the total environmental niche available to *I. ricinus*, we modeled the environmental suitability of this tick across different environmental conditions considering that only a specific set of vertebrates was exploited. Calculations were done considering different environmental parameters of temperature (LSTD) and humidity (NDVI) and including only "mammals+reptiles" or "mammals+reptiles+birds." **Figure 3** shows the environmental suitability for *I. ricinus* in the niche described by the mean LSTD and NDVI. The inclusion of Aves in calculations resulted in higher suitability at the range of low temperature. However, the net gain in habitat including birds as hosts was only of 8.2%. These results showed that the adaptation of *I. ricinus* to 85 species of Aves, hypothesized to be a derived state, is not sufficiently explained by environmental factors (i.e., temperature and humidity) crucial for tick physiology. In other words, even if the tick could exploit more hosts (Aves) across a wider environmental gradient (colder temperatures), the net gain in niche is too small to confer an adaptive advantage to the tick. This

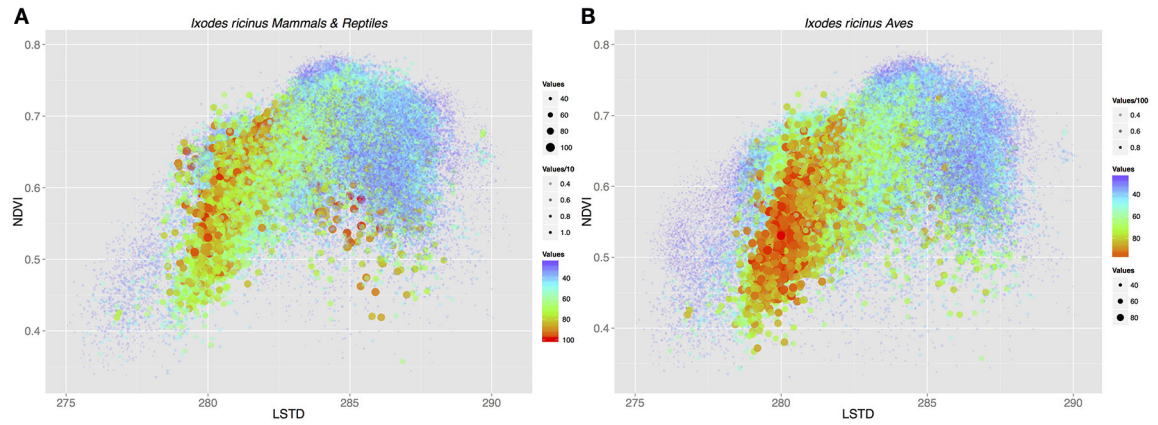

FIGURE 3 | The suitability of the environmental space formed by the intersection of average annual land surface temperature (LSTD) and average annual Normalized Difference Vegetation Index (NDVI) for *I. ricinus*. Dots are the product of the suitability of a specific portion of the abiotic niche for the tick according to environmental traits, the habitat overlap with hosts at that specific point, and the environmental suitability for the host(s) available at that specific point. Because of the large number of points displayed (>6 × 10^6), color, size, and transparency are used together to improve the readability of the charts. **(A)** The niche suitability was calculated with mammals and lizards as the only available hosts. **(B)** Birds were added to calculations of niche suitability for *I. ricinus*.

lead us to the hypothesis that the colonization of new habitats by *I. ricinus* might have played a especial role in pathogen spread and circulation.

A Network of Connections between Ticks, Hosts, and Pathogens Reveals the Structure of the Biotic Relationships of *I. ricinus*

To evaluate the effect of the colonization of Aves and colder regions by *I. ricinus* in pathogen spread and circulation, we developed a network of connections between ticks, hosts, and pathogens. This is a directed network where ticks were recorded on hosts or pathogens detected on vertebrates and/or ticks. The network contains records of vertebrate hosts and transmitted pathogens associated to *I. ricinus*. To gain a complete view of the network, we also included other tick species recorded on the same species of vertebrates. The complete network has 379 nodes (organisms) and 1020 links (connections) among them (**Supplementary Figure S3**). We identified seven communities of organisms that are more associated with each other than with other members of the network. *I. ricinus* is the pivot of a large cluster in which other tick species (*Ixodes redikorzevi, Ixodes acuminatus, Ixodes persulcatus,* and *Haemaphysalis concinna*) are included. In the network, *I. ricinus* links 160 host species and 19 pathogens. *I. ricinus* is linked to 48 families and 86 genera of vertebrates. **Table 1** summarizes the features of centrality regarding the cluster of *I. ricinus*, higher centrality values meaning for a more prominent role of these nodes in supporting the network. Passeriformes have twice the Authority of Rodentia, three times higher PageRank, and twice the Weighted Clustering Coefficient. The ecological translation is that the Passeriformes contribute more to the resilience of the tick and allow better circulation of the transmitted pathogens.

TABLE 1 | Centrality indexes of the hosts forming the network of vertebrates and transmitted pathogens in which *Ixodes ricinus* acts as a carrier.

	Order of hosts	#Species of hosts	Authority	PageRank	Weighted clustering coefficient
Aves	Accipitriformes	3	0.00739	0.00954	0.0000
Aves	Charadriiformes	2	0.00422	0.00632	0.0000
Aves	Coraciiformes	1	0.00211	0.00316	0.0000
Aves	Falconiformes	1	0.00528	0.00451	0.0000
Aves	Galliformes	3	0.01056	0.01228	0.0000
Aves	Gruiformes	1	0.00211	0.00316	0.0000
Aves	Passeriformes	75	**0.32154**	**0.26377**	**5.6237**
Aves	Piciformes	2	0.00422	0.00632	0.0000
Aves	Strigiformes	2	0.00528	0.00680	0.0000
Mammalia	Rodentia	18	**0.15470**	**0.08599**	**2.1718**
Mammalia	Artiodactyla	9	0.09715	0.05254	1.1443
Mammalia	Carnivora	14	0.07497	0.06055	0.5935
Mammalia	Erinaceomorpha	3	0.03273	0.01771	0.1536
Mammalia	Lagomoprha	4	0.02746	0.01931	0.0463
Mammalia	Soricomorpha	9	0.03485	0.03064	0.6470
Reptilia	Testudines	1	0.00528	0.00619	0.0000
Reptilia	Squamata	12	0.05491	0.04545	0.5012
	Total Aves	90	0.36272	0.31584	5.6237
	Total Mammalia	57	0.42186	0.26674	4.7565
	Total Reptilia	13	0.06019	0.05164	0.5012

Numbers in bold refer to the highly prominent hosts in the network of transmitted pathogens.

These observations were further confirmed by comparing the niche overlap of ticks and hosts according to the network-derived properties (**Figures 4**, **5**). *I. ricinus* shares higher fractions of the environmental niche with the hosts that have greater measures

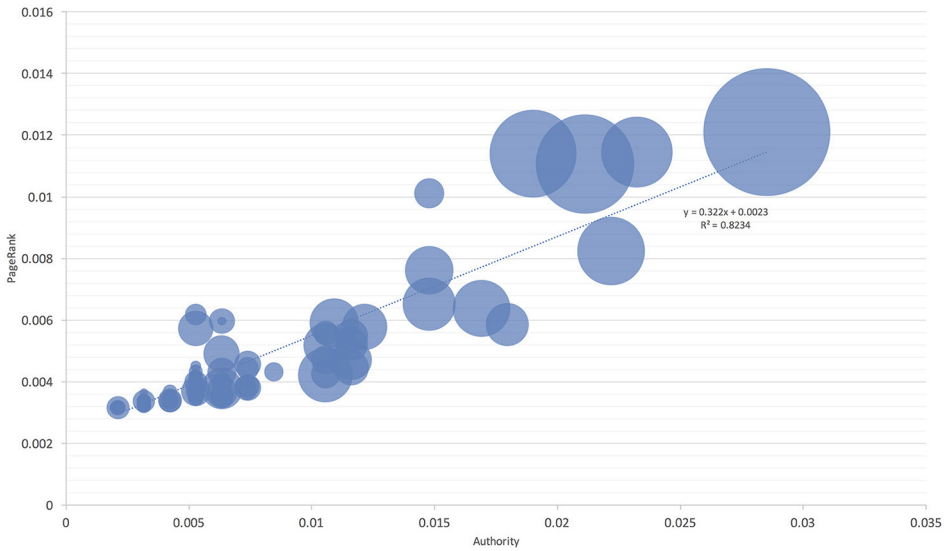

FIGURE 4 | The proportion of niche overlapping between *I. ricinus* and its hosts. The area of each circle is proportional to niche overlap and related to two measures of host centrality, namely the Authority and PageRank. The chart includes all reported hosts for the tick. The line shows the regression between the niche overlap, Authority, and PageRank.

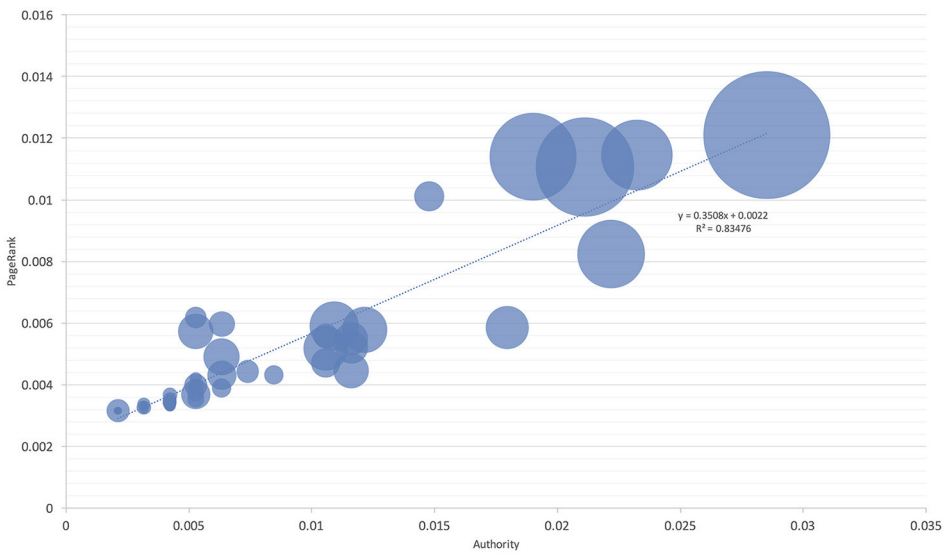

FIGURE 5 | The proportion of niche overlapping between *I. ricinus* and its hosts without Aves. The area of each circle is proportional to niche overlap and related to two measures of host centrality, namely the Authority and PageRank. The chart excludes Aves as hosts for the tick in the calculations and is intended to show the lack of habitat overlap at a large range of the Authority-PageRank gradient. The line shows the regression between the habitat overlap, Authority, and PageRank.

of centrality in the network. Therefore, *I. ricinus* maximizes the niche overlap with the hosts that have a more prominent role in the circulation of the pathogens, independently of their phylogenetic relationships. Since the maximization of the niche sharing improves the circulation of the tick and transmitted pathogens, removing birds from these calculations results in severe reduction of large areas of suitability of the tick and pathogens at intermediate positions of the gradient of centrality measures. The results demonstrated that the use of Aves as hosts by *I. ricinus* (and the gain of a new niche for the tick) shapes the persistence and resilience of the transmitted pathogens.

DISCUSSION

This study captured the structural details of a community of pathogens transmitted by a tick and circulating through different clades of vertebrates. It is the development of a proof-of-concept (Estrada-Peña et al., 2015) intended to accommodate the

structure of a network to the biotic relationships between parasites and hosts, and combines techniques from phylogenetics and niche modeling. The different parts of this study were derived from distinct disciplines with their own intrinsic assumptions, limitations, and requirements. Therefore, integration is critical for the success of this methodological approach. Significant challenges are apparent because these datasets are commonly affected by bias due to the abundance of common hosts or interest in a given pathogen. Therefore, the dataset must be weighted adequately to not reflect bias affecting the structure of relationships. We adhered to reliable protocols to reconstruct the climate niches (Warren et al., 2010), extract the basic environmental envelope of both the tick and its hosts (Phillips et al., 2006; Elith et al., 2011), or weight the records of interactions for the network (Gómez et al., 2013; Estrada-Peña et al., 2015). To examine how niches of hosts evolved, ancestral state reconstruction methods were used on a phylogenetic tree of Tetrapoda (Munkemuller et al., 2012; Roquet et al., 2014).

Results revealed that the tick does not exploit vertebrates because they are phylogenetically related. The reconstruction of the ancestral state of the environmental niche of hosts is strongly suggestive of a split in the abiotic niche of the tick, when it adopted several species of Aves as hosts. The event probably occurred around 120 M years ago and seems to not be related to recent glaciations, which are known to shape the refugia for vertebrates (Jaarola et al., 1999; Deffontaine et al., 2005; Kasapidis et al., 2005; Sommer and Nadachowski, 2006; Venditti et al., 2011). From our results, the ancestral hosts for *I. ricinus* are interpreted to be mammals and reptiles. The colder portions of the niche are assumed to be a derived state because Aves evolved later in geological times. However, it is unclear whether the tick or an ancestor was a parasite of an extinct relative of the current hosts, which further speciated, spreading the tick into new ecological niches to which it adapted. In this hypothesis, other tick lineages would have become extinct because of a lack of adaptation to the new set of environmental conditions. The opposite hypothesis is that the tick was a parasite of mammals and reptiles, and changes in climate pushed the tick to contact new niches finding a new set of hosts to exploit. The former hypothesis is host-driven, allowing the tick to adapt to new environmental traits; the latter is driven by environmental traits, with the tick exploiting a new set of hosts that were available under these new conditions. Environmental filtering has been predicted to generate phylogenetic clustering (Jaarola et al., 1999; Deffontaine et al., 2005; Kasapidis et al., 2005; Brooks et al., 2006; Sommer and Nadachowski, 2006; Venditti et al., 2011; Suzán et al., 2015) because closely related host species share similar niches.

Speciation of the *I. ricinus* group could have occurred after the separation of Laurasia. The time during which Passeriformes evolved was an unusually warm geological interval, followed by a long period of colder temperatures lasting until the recent glaciations. These dates overlap well with the estimated split in the temperature traits based on the phylogenetic signature in the dated host tree. These are key events for the tick's ecological innovation that allows the exploitation of new resources or habitats (Heard and Hauser, 1995). Ecological innovation enhances competitive ability, or permits exploitation of new resources, and has commonly been reported to increase the divergence rates of clades of species (Peterson and Holt, 2003). The adoption of a colder niche implies an ecological cost for the tick, because lower temperatures imply adaptations for a longer life cycle with concurrent higher mortality (Estrada-Peña and de la Fuente, 2014). The split to new hosts could also involve a cost in terms of adaptation to the new "molecular environment" of the hosts, such as the evasion of immune responses (de la Fuente et al., 2015). Thus, it is central to this study to address why the tick persisted to circulate in such a diverse community of new hosts and wide range of thermal conditions.

Networks are pervasive across all levels of biological organization (Watts and Strogatz, 1998). These structures have been commonly used to represent food webs (Cattin et al., 2004) or plant-pollinator relationships (Dormann, 2011), and only recently were applied for describing the epidemiological context of ticks, vertebrates, and pathogens (Estrada-Peña et al., 2015). Our results revealed conceptual aspects of the ecological interactions and showed that the host split by the tick has a considerable impact on the transmitted pathogens. *I. ricinus* pivots around a rich array of relationships in nested sub-networks: phylogenetically distant vertebrates enhance the circulation of pathogens by providing a functional redundancy along the environmental gradient. Pathogens, even if restricted to a set of reservoirs (Kurtenbach et al., 1994) would have greater resilience if the vector (i) maximizes the number of interactions with the most central hosts of the network, boosting the circulation of pathogens across the sub-networks, and (ii) exploits many species of hosts established along the gradient of the niche variables.

We hypothesize that the biotic interactions of the tick with the community of vertebrates improve the circulation of the pathogens which are in turn involved in the adaptation of the tick to a wide environmental niche occupied by genetically unrelated hosts. The mechanisms that support the resilience of the tick vector in such diverse environment are far from being well-understood. Work in progress suggests that the co-evolutionary mechanisms of tick-transmitted micro-organisms could benefit the persistence of the tick. In particular, we recently proposed that tick-pathogen associations evolved to form "*intimate epigenetic relationships*" in which the pathogen induces transcriptional reprogramming in infected ticks (Cabezas-Cruz et al., 2017). This will ultimately favor pathogen propagation, but will also select for the most suitable ecological adaptations in the tick vector. These phenotypic and genetic changes may have the potential to be transmitted to the next generation of ticks. As a result, the ecological associations between tick, vertebrates and pathogens would evolve to maximize pathogen circulation in these communities (Estrada-Peña et al., 2015; Cabezas-Cruz et al., 2017).

An interesting example is the intracellular pathogen *Anaplasma phagocytophilum* that manipulates tick protective responses to facilitate infection and preserve tick feeding and vectorial capacity to guarantee the survival of both pathogens and ticks (Neelakanta et al., 2010; Merino et al., 2011; Busby et al., 2012; Ayllón et al., 2015a,b; de la Fuente et al., 2017). These

mechanisms include the expression of an antifreeze glycoprotein to increase tick survival at cold temperatures (Neelakanta et al., 2010). It was further showed that improved survival of infected ticks correlated with higher *A. phagocytophilum* infection, therefore providing a direct link between pathogen infection and tick fitness in cold environments (Neelakanta et al., 2010). Heat shock proteins (HSP) are also induced by *A. phagocytophilum* infection and protect ticks from stress and pathogen infection (Busby et al., 2012). The HSP responses help increase tick survival by protecting them from stress and preventing desiccation at high temperatures after enhancing questing speed in order to increase the chances of a tick attaching to a host. Similarly, *A. phagocytophilum* subverts the tick RNA interference response to preserve tick feeding (Ayllón et al., 2015b).

Another example of tick-pathogen association is *Borrelia* spp. In the field, *Borrelia*-infected rodents have higher tick burdens than uninfected rodents (Hanincova et al., 2003; Gassner et al., 2013). Studies have also shown that *Borrelia*-infected ticks are more tolerant to desiccating conditions, which commonly stop questing activity and increase tick mortality (Herrmann et al., 2013). Hosts with high nymphal tick burden have a greater chance of becoming infected with *Borrelia,* and rodents infested with nymphs have higher larval tick burdens than rodents without nymphs (Craine et al., 1995; Bown et al., 2008). The molecular mechanisms involved in this regulation are still unknown, but together they contribute to demonstrate how the cargo rewards the circulation of the infected carriers, which are promoted to find a host earlier than non-infected ticks and infect more vertebrate reservoirs. Additional studies with other tick-borne pathogens and deeper analysis of these co-evolutionary adaptations are necessary before we can generalize these findings (de la Fuente et al., 2017).

In this study, we integrated phylogenetics and network analyses to demonstrate that the diversity of hosts increases the niche available for a tick and promotes the circulation of transmitted-pathogens. *I. ricinus* uses a large assemblage of hosts that are phylogenetically unrelated and that split into different values of environmental conditions with an obvious phylogenetic signature. The tick maximizes the niche overlap with the most important hosts in the network, but is further supported by a profusion of secondary hosts that provide the functional redundancy to the network. We interpreted this finding not only as a way for the tick to persist in a variety of conditions, but also as a strategy to enhance the circulation of pathogens. The data are highly suggestive of a dramatic decrease in the circulation of *I. ricinus*-transmitted pathogens if the dozens of species of birds used as hosts by the tick (interpreted as an acquired event in the geological times) are removed. This is a change of paradigm providing evidence that the functional redundancy of the vertebrates enhances the circulation of transmitted pathogens, even if the net gain of environmental niche is low. Though tick larvae would feed mostly on rodents on the local scale, birds are the stepping stones for disseminating the nymphs and enhance the circulation of the community. These results strongly suggest that pathogens may manipulate ticks to occupy sub-optimal environmental niches. Transmission rates between ticks and vertebrates should be incorporated into the network structure to evaluate the contribution of each host to the system and the regulatory mechanisms operating at each level of complexity. These results have important implications for a deeper understanding of the idiosyncratic factors regulating the prevalence of tick-borne diseases.

AUTHOR CONTRIBUTIONS

AE designed the work and prepared the figures. AE and AC obtained the results. All authors wrote the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00234/full#supplementary-material

Supplementary Figure S1 | The phylogenetic tree of 168 species of hosts of *Ixodes ricinus* based on a supertree of Tetrapoda of Western Palearctic. This is a high-resolution version of **Figure 1** with the names of the species of hosts included at the tips of the tree.

Supplementary Figure S2 | Reconstructions of the environmental niche of the hosts of *I. ricinus.* The figure includes data for Lands Surface Temperature (LSTD: **A**) and the Normalized Difference Vegetation Index (NDVI: **B**). The trees were drawn according to the phylogenetic tree in the **Figure 1**. Values in the legend are degree Celsius **(A)** and NDVI units multiplied by 100 **(B)**. Higher taxonomical categories of hosts are included at the tips of the tree. This is a high resolution version of **Figure 2** with the names of the species of hosts included at the tips of the tree.

Supplementary Figure S3 | The network of tick *I. ricinus*, the satellite species of ticks, vertebrate hosts, and transmitted pathogens. Circles are organisms (ticks, hosts, pathogens) and lines are links (interactions) among them. Colors in the figure indicate clusters retrieved by an algorithm that groups together the organisms that are closer than others. Clusters include other species of ticks that are satellite to the focal species because they share hosts. The size of each circle indicates the centrality of the organism. The width of each link is proportional to the strength of the interaction between two given organisms.

Supplementary Table S1 | The tree in newick format used to draw the phylogenetic relationships between the vertebrates recorded as hosts of *Ixodes ricinus*.

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REVIEW
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Bovine Immune Factors Underlying Tick Resistance: Integration and Future Directions

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The mechanisms underlying tick resistance within and between cattle breeds have been studied for decades. Several previous papers on bovine immune parameters contributing to tick resistance discussed findings across DNA, RNA, protein, cellular, and tissue levels. However, the differences between bovine host species, tick species and the experimental layouts were not always taken into account. This review aims to (a) give a comprehensive summary of studies investigating immune marker differences between cattle breeds with varying degrees of tick resistance, and (b) to integrate key findings and suggest hypotheses on likely immune-regulated pathways driving resistance. Experimental issues, which may have skewed conclusions, are highlighted. In future, improved experimental strategies will enable more focused studies to identify and integrate immune markers and/or pathways. Most conclusive thus far is the involvement of histamine, granulocytes and their associated pathways in the tick-resistance mechanism. Interestingly, different immune markers might be involved in the mechanisms within a single host breed in contrast to between breeds. Also, differences are evident at each tick life stage, limiting the level to which datasets can be compared. Future studies to further elucidate immune molecule dynamics across the entire tick life cycle and in-depth investigation of promising markers and pathways on both molecular and cellular level are in dire need to obtain a scientifically sound hypothesis on the drivers of tick resistance.

Keywords: cattle, tick, resistance, tick resistance, immune factors, parasite, host

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INTRODUCTION

The economic importance of ticks and the need to control them was realized alongside the discovery of their potential as vectors of harmful parasites, particularly to livestock (Hunter and Hooker, 1907; Theiler, 1911). The variability in the degree to which cattle display resistance to ixodid ticks was first suggested by Johnston and Bancroft (1918). It is known that tick resistance in cattle varies from more tick-susceptible *Bos taurus taurus* (*B. t. taurus*) to more tick-resistant *B. t. indicus* breeds, between bovine crosses as well as within a single cattle breed (George et al., 1985; Rechav et al., 1991b; Mattioli and Cassama, 1995; Mwangi et al., 1998; Mattioli et al., 2000; Nascimento et al., 2011). However, the biological factors underlying bovine resistance to tick infestation are still poorly understood. Tick resistance is a multi-factorial trait suggested to involve host-related factors such as sex, age, lactation, grooming behavior, skin composition and host

surface area, coat length and environmental factors (Wharton et al., 1970; Seifert, 1971; Doube and Wharton, 1980; Binta and Cunningham, 1984; Ali and de Castro, 1993; Meltzer, 1996; Norval et al., 1996; Mattioli, 1998; Martinez et al., 2006; Kongsuwan et al., 2010). It is also well established that the tick-resistance phenotype is heritable, as is evident from breed-specific resistance patterns. Furthermore, it was proposed that tick attachment sites on resistant cattle rapidly become unsuitable for feeding due to host immune responses (Roberts, 1968b). The majority of studies indicate that resistance is acquired through exposure to ticks (Wagland, 1975, 1980; George et al., 1985; Momin et al., 1991) and that resistance is acquired sooner and to a higher degree in *B. t. indicus* than in *B. t. taurus* breeds (Riek, 1962; Wagland, 1978, 1980; Rechav et al., 1990). This phenotype only becomes apparent after subsequent (and not initial) tick exposure in *B. t. taurus*, *B. t. indicus* and mixed breed cattle (Roberts, 1968a; Wagland, 1975; Hewetson and Lewis, 1976).

To further elucidate potential mechanisms underlying differences in tick resistance, several studies have investigated host immune responses toward ticks on a cellular and molecular level. Gaining an understanding of the molecular mechanisms underlying tick-resistance mechanism will be advantageous in the identification of specific genomic alterations or specific markers that could lead to the ability to screen cattle for their potential resistance status without prolonged tick-infestation trials or counts. This would be helpful in breeding more tick-resistant cattle. In this regard, it was shown that although host-resistance to tick infestation and product yield does not correlated in Holstein-Friesian cattle (Jonsson et al., 2000), but carriers of both *B. t. taurus* and *B. t. indicus* genes may suffer from a trade-off between animal-derived product yield and tick resistance (Wang et al., 2007). A clear understanding of tick-resistance mechanisms would also be beneficial to vaccine trials, where a difference in the resistance status of individual animals could skew results and therefore make accurate data interpretation more difficult. Furthermore, more effective vaccine formulations could be devised as vaccine efficacy is hindered by the modulation of host immune responses through tick saliva (Kazimírová and Štibrániová, 2013). Knowledge regarding molecular mechanisms underlying tick resistance could allow for the optimal selection of appropriate adjuvant/vaccine formulation strategies to provide a cross-breed protective response.

This review therefore provides a summary of studies performed up to date in cattle blood and skin tissue, with critical evaluation of findings followed by hypotheses on key role players, possible immune-regulated pathways as well as improvements for consideration when planning future experiments. Several recent studies were published pertaining to genetic associations with regards to the tick-resistance phenotype (Mota et al., 2016a,b, 2017; Junqueira et al., 2017; Sollero et al., 2017), however, these are outside the scope of this summary. This review should provide readers with the basic knowledge and a critical evaluation of findings to date to make informed decisions for future studies investigating the tick-host-interface with a focus on resistance. Due to differences in experimental layouts, which might skew data interpretation and comparisons such as tick life stage, tick species and type of bovine comparison (between or within breeds), are provided in **Supplementary Table 1**.

BLOOD

Immune cells originate from hematopoietic stem cells in the bone marrow with naïve and mature forms of these cells circulating in the blood and lymphatic systems. Here, they encounter foreign molecules that lead to their proliferation, differentiation and maturation (Janeway et al., 2001). However, due to the constant circulation and changing dynamics of immune response components, experimental designs (especially time points) must be chosen carefully. Various gene expression, translational and cytological studies have investigated blood to elucidate immune responses linked to tick resistance/susceptibility in cattle and these are described in the next section.

Gene Expression Studies in the Blood of Tick-Infested Cattle

Gene expression studies of peripheral blood mononuclear cells identified transcripts for IL-2, IL2Rα, TNFα, and CCR1 to be significantly upregulated in resistant cattle relative to susceptible cattle, while a significantly higher expression of *CXCL10* was detected in susceptible Holstein-Friesian compared to resistant Brahman cattle (Piper et al., 2009). Pathway analysis indicated that genes that are more highly expressed in the resistant breed are associated with the hematopoietic cell lineage and cytokine-cytokine receptor interaction pathways. Another study found a significant upregulation of *CD25, IL10, FoxP3,* and *CXCL10* in samples from cattle infested with larvae compared to samples obtained from uninfested animals. In susceptible animals, *CXCL8* was downregulated in susceptible animals 24 and 48 h after infestation compared to samples from uninfested animals (Domingues *et al.*, 2014). Although *CXCL10* was identified in both studies as differentially regulated, major differences in the study designs hindering any direct comparisons. Piper et al. (2009) obtained blood samples at the peak of tick infestation without reference to a specific time point after infestation and found significantly higher chemokine expression in susceptible compared to resistant cattle breeds. Domingues et al. (2014) on the other hand compared tick-infested versus tick-uninfested cattle of the same breed and identified an increase in *CXCL10* in resistant animals 48 h and an increase in susceptible animals 24 h after tick infestation. Therefore, the role of CXCL10 remains to be confirmed in future studies and its contribution to resistance pathways elucidated.

Translational Studies in the Blood of Tick-Infested Cattle

Immunoglobulins

A link between tick resistance and immunoglobulins was proposed in 1987 by Rechav and colleagues who found a positive correlation between tick numbers and total serum gamma globulin levels in naturally infested *B. t. taurus* and *B. t. indicus* cattle. During the acquisition of tick resistance, a negative correlation between tick weight and total serum gamma

globulin levels was, however, documented in *B. t. taurus* cattle infested with *Rhipicephalus decoloratus* (Rechav et al., 1991a). This discrepancy could be a result of differences in experimental design, as the animals studied by (Rechav et al., 1991a) were most likely in the process of acquiring tick resistance as opposed to the more established resistance of cattle studied by Rechav (1987). In this regard, resistance in the former study was supported by reduced tick weights only.

In general, the number of ticks feeding on cattle were found to positively correlate with salivary gland specific IgG levels in previously infested (Sahibi et al., 1998) and naïve (Cruz et al., 2008) *B. t. taurus* cattle. Cruz et al. (2008) furthermore reported that there was no change in the avidity of antibodies developed by either *Rhipicephalus microplus* resistant or susceptible animals against salivary soluble extracts (Cruz et al., 2008).

Differences in the IgG1 isotype was observed when comparing cattle breeds displaying varying tick-resistance phenotypes. After multiple infestations of tick-naïve cattle, IgG1 levels (against several tick extracts) were found to be significantly higher in susceptible compared to resistant cattle (Piper et al., 2017), with similar results obtained the studies of Garcia et al. (2017) and Piper et al. (2009). Although no differences were observed for tick-naïve animals at the beginning of the study by Piper et al. (2017), higher tick-saliva specific IgG1 levels were seen before and at the beginning of the first infestation in the tick-resistant cattle breed by Garcia et al. (2017). Yet, Kashino et al. (2005) reported a decrease of IgG1 upon heavy tick infestation in naturally infested susceptible animals, compared to resistant animals (Kashino et al., 2005). As such, the question arises whether studies done under controlled housing conditions and those done under field conditions with natural infestation, and possible co-infections/infestations, can be compared.

In contrast to IgG1 levels, no significant differences were identified for the IgG2 isotype between breeds (Piper et al., 2009, 2017), with similar results seen by Garcia et al. (2017) for resistant animals throughout the study. The latter study does however describe an increase of this isotype in the susceptible breed at the third infestation compared to the baseline. At the same time point, IgG2 levels were significantly higher in susceptible compared to resistant animals. Again, in contrast, Kashino et al. (2005) identified decreased levels of IgG2 in naturally infested susceptible compared to resistant animals during heavy infestation.

Only two studies have investigated IgE levels between resistant and susceptible cattle breeds. Garcia et al. (2017) determined levels of total IgE in the sera of cattle infested with *R. microplus*. No difference in these levels were noted between resistant and susceptible breeds. Tick-specific IgE antibody levels, however, were shown to be significantly lower in resistant compared to susceptible animals during heavy infestation as well as during subsequent light infestation (Kashino et al., 2005). This difference in IgE levels between cattle breeds seems to be a result of an increase in this immunoglobulin in susceptible animals instead of a decrease in resistant animals. The same trend was seen in some studies investigating IgG isotypes. IgE with associated receptors and cellular responses are believed to have evolved to counter helminths and other parasites that cannot be phagocytosed (Fitzsimmons et al., 2014). In this paper, we propose a role for IgE-dependent responses as one of the drivers of resistance (see sections Dynamics of Granulocytes and Histamine and their Suggested Involvement in the Tick- Resistance Mechanism Over the Tick Lifecycle and Future Directions: Potential Drivers Involved in Tick Resistance), and as such, daily data on the IgE levels throughout the period of tick attachment and subsequent life stages will be of great importance.

Considering the consensus from the majority of studies, resistant animals seem to display a more constant tick-specific immunoglobulin isotype profile with fewer changes observed throughout infestation cycles. Susceptible animals on the other hand show an increase of tick-specific immunoglobulin levels over multiple infestations. Differences in host immune responses are furthermore evident by the observation that a great variation in tick salivary gland extract profiles are recognized between individual cattle sera (Cruz et al., 2008). Furthermore, sera from a resistant compared to a susceptible cattle breed reacted with more tick salivary proteins, which require further investigation (Garcia et al., 2017). On the other hand, differences in tick numbers and thus the amount of tick antigens in different hosts also requires more in-depth studies to determine immune responses independent of varying tick numbers.

Other

Additional host immune components have also been associated with tick resistance to date, including histamine, complement, acute-phase proteins and bovine lymphocyte antigens. Increased histamine and complement levels were found to be associated with lower tick numbers and resistant animals, respectively (Riek, 1962; Wambura et al., 1998; Zhao et al., 2013). Three proteins of the acute-phase response, which is generally initiated in response to tissue damage (Baumann and Gauldie, 1994), were linked to the tick-resistance mechanism by Carvalho et al. (2008). Briefly, susceptible Holstein-Friesian cattle showed a significant increase in haptoglobulin levels resulting from heavy tick infestation as well as constantly higher alpha-1 acid glycoprotein levels compared to resistant Nelore (*B. t. indicus*) animals. This might be a result of increased tick numbers and the associated increase in tissue damage on susceptible animals. Lastly, only during intense infestation did the more resistant cattle breed show higher levels of serum amyloid A when compared to the more susceptible cattle breed. In two separate studies, a total of 19 bovine lymphocyte antigens were tested in blood collected from a mixed breed cattle population with no overlapping findings. In total, two antigens were significantly associated with tick resistance (W8 and W16) and three with tick-susceptibility (W5, W6, CA31) (Stear et al., 1984, 1989). Additional data is therefore required to resolve knowledge gaps in the pathways associated with the above-mentioned compounds.

Cytological Studies

Identification of Immune Cell Subtypes in Circulating Blood of Tick-Infested Cattle Using Associated Markers

Identification and quantification of immune cell subtypes in circulating blood has been performed in *B. t. indicus*, *B. t.*

taurus and mixed breed cattle with the use of associated markers. Piper et al. (2017) did not find any significant differences while Piper et al. (2009) identified significantly higher levels of CD4+, CD25+ activated and WC1+ γδ T-cell populations in more tick-resistant cattle. Significantly higher levels of CD14+ monocyte and MHC II presenting cells were obtained in more tick-susceptible cattle. As these cell subtypes are known to be associated with a variety of immune responses and pathways, linking them to a putative resistance mechanism will only be possible when analyzing them in combination with additional markers (**Figure 2**).

Identification of Immune Cell Subtypes in Circulating Blood of Tick-Infested Cattle Using Morphological Characteristics

The cellular composition of blood is regarded as an important identifier of the overall health of humans and animals and as such changes in the percentage of different white blood cell populations may be used as an indication of a systemic immune response. Three main studies have relied on the use of different white blood cell population counts (basophils, eosinophils, lymphocytes, total leukocytes, neutrophils, monocytes) in describing the immune response of cattle with varying levels of tick resistance with no differential regulation identified to date (Brown et al., 1984; Rechav, 1987; Rechav et al., 1990). Only blood eosinophil levels were significantly higher in the more susceptible *B. t. taurus* breed (which carried more ticks) in the study by Rechav et al. (1990). The investigation of cell subtypes in blood represents a daunting task as shown by the lack of identified differential regulation of markers obtained. In contrast, research to date has detected significant differences between hosts with varying tick-resistance status in skin tissue (refer to skin section below). This is the case since the dynamics of immune cells in blood only provide a snapshot of what is occurring at a specific time point. Experimental layouts must thus be considered carefully as studies incorporating and comparing several time points and/or tissues might represent a more realistic view of immune drivers of resistance.

SKIN TISSUE

The skin represents the first site of encounter to tick infestation and thus the first line of host immune defense. Upon penetration and successful attachment, ixodid ticks alternate salivation and blood intake every 5–20 min (Francischetti et al., 2009). Numerous salivary components mediate suppression of host responses such as blood coagulation, immunity, inflammation and the ability of the host to develop new blood vessels (Hovius et al., 2008; Kazimírová and Štibrániová, 2013; Kotál et al., 2015). The identification of immunological defense responses at the site of tick infestation have been extensively studied, as evident from the next section.

Gene Expression Studies in the Skin of Tick-Infested Cattle

Based on transcriptional studies in cattle skin, three studies have identified the involvement of the complement cascade in the feeding of ticks in both susceptible and resistant cattle (Wang et al., 2007; Piper et al., 2010; Carvalho et al., 2014). The complement system is composed of a number of molecules and plays a vital part in the immune system for the clearance of foreign cells via a number of mechanisms (Nesargikar et al., 2012). Upregulation of gene expression for complement components in tick-resistant cattle (*C1QA*) (Wang et al., 2007) and tick-susceptible cattle (*complement component 3*) (Piper et al., 2010) have been shown, while the general pathway downregulation of complement has also been described in susceptible animals (Carvalho et al., 2014). More in-depth studies are required regarding these components potentially involved in the tick-resistance mechanism. This is due to the various complement components investigated to date combined with differences amongst results reported. Interestingly, all immunoglobulin associated transcripts were identified to be more abundant in less resistant animals (Wang et al., 2007; Piper et al., 2010) which correlates with findings obtained from blood and could be linked to increased tick numbers on these animals. Furthermore, CD14 (on transcriptional level in skin and translational level in blood) was identified in both tissues to be associated with tick-susceptibility (Piper et al., 2008, 2009). CD14 is known to be a marker for monocytes and macrophages and can therefore be involved in several immune response mechanisms (Ziegler-Heitbrock and Ulevitch, 1993).

Other components that were found to be upregulated in susceptible cattle include transcripts for IL13RA1, CD44, CD63, TNFα, IL-1β, IL-10, NFKBp50, CD1a, CCR-1, CCL2, CCL26, TLR9, MyD88, CD14, FTH1, BDA20, and Traf-6 (Wang et al., 2007; Piper et al., 2008; Nascimento et al., 2011). However, no transcript was reported in more than one of these studies and as such all require validation. Most recently, Franzin et al. (2017) reported on a microarray study of skin from uninfested cattle, larvae (2 days after larvae infestation) and nymph (9 days after larvae infestation) life stages fed on *B. t. taurus* and *B. t. indicus* breeds. Samples were compared within and between breeds. An observed allergic contact-like dermatitis was found to be delayed in susceptible animals detected by the involvement of *IL-6, CXCL-8, CCL-2, HMGB1, ISG15,* and *PKR* which in turn result in the production of chemokines and cytokines involved in the inflammatory response. In another study, downregulation of inflammatory response gene expression was observed within 24 h after tick infestation in susceptible animals, while at the 48-h sampling point genes associated with antigen presentation and oxidative stress were found to be upregulated in resistant cattle (Carvalho et al., 2014). One study identified *CXCL-8* expression as being downregulated in resistant cattle between different genetic crossbred cattle groups from which skin and lymph node samples were obtained 9 days after larvae challenge (Regitano et al., 2008). However, it was unclear to which tissue this finding refers to. Differential gene expression was furthermore identified for genes encoding Blimp-1 (Kongsuwan et al., 2010), cathepsin L2 precursor, MHC class antigen I (Nascimento et al., 2011), various adhesion molecules (Carvalho et al., 2010), TNF receptor-associated factor 6, TATA-binding protein, lumican and beta-2 microglobulin (Marima, 2017).

Due to the variation in experimental designs of different studies, caution should be taken when trying to compare results emanating from transcriptional studies, especially with regards to sampling time points. Since gene expression rarely involves absolute quantification and is based on the relative quantification of transcripts between two populations or between transcripts and reference genes under a specific set of conditions, the results generated may be study specific. RNA sequencing would be an alternative approach not yet utilized in this field of study for the obtainment of large-scale results based on absolute quantification which could allow the identification of novel transcripts. In addition to this, studies on a protein and cellular level, to validate potentially relevant findings from gene expression studies, should be undertaken.

Translational Studies and Metabolites in the Skin of Tick-Infested Cattle

Few studies investigated immune factors underlying the tick-resistance mechanism in skin of cattle on the protein or metabolite level. To date, only one paper has focused on proteins. However, no significant findings regarding proteins directly involved in immune response pathways were identified (Kongsuwan et al., 2010). On a metabolite level, higher histamine levels were linked to tick resistance by Schleger et al. (1981) and Willadsen et al. (1979). These findings also correlate with results obtained from studies done on blood (Riek, 1962; Zhao et al., 2013). Histamine is an immunomodulator produced by a variety of cell types including mast cells, basophils, dendritic cells, and T-cells and can regulate both innate as well as adaptive immune response cells (O'Mahony et al., 2011). The expression of the histidine decarboxylase, which results in the decarboxylation of L-histidine and subsequent production of histamine, is influenced by several immune factors including a variety of cytokines. This secondary metabolite regulates, amongst others, antigen-specific Th1 and Th2 cells in addition to antibody isotype responses (Jutel et al., 2006). Histamine seems to be an effector molecule in tick resistance. This is evident from studies showing that histamine injection at tick attachment sites lead to detachment of some tick larvae, indicating a direct involvement of histamine rather than a general inflammatory reaction being the cause of tick rejection (Kemp and Bourne, 1980).

Furthermore, higher tick numbers were observed in cattle treated with an antihistaminic drug (Tatchell and Bennett, 1969). A response localized to the sites of skin damage is generally the first immunological reaction of a body. Histamine is well documented to be involved in proinflammatory responses and the immediate-type hypersensitivity response, characterized by increased vascular permeability, smooth muscle contractions, activation of certain nerves, wheal-and-flare reactions and itch responses (O'Mahony et al., 2011). Acquired resistance was linked to the occurrence of a hypersensitivity reaction to tick salivary gland components (Riek, 1962). The type of hypersensitivity is, however, not known since contradicting results have been obtained (Kemp et al., 1986; Smith et al., 1989; Latif et al., 1991; Bechara et al., 2000; Piper et al., 2010; Prudencio et al., 2011; Marufu et al., 2013).

Cytological Studies

Identification of Immune Cell Subtypes via Surface Markers at the Site of Tick Attachment

On a cellular level, two potential cell subtypes have been identified across independent studies. Markers used for the identification of γδ T-lymphocytes were found to be present in higher levels in resistant compared to susceptible animals (Constantinoiu et al., 2010; Franzin et al., 2017). Gamma delta T-cells are suggested to function as regulatory T-cells in bovines (Hoek et al., 2009).

The expression of CD3+ T-lymphocytes was found to be increased at different time points in *B. t. indicus* cattle (compared to *B. t. taurus)* in two studies (Constantinoiu et al., 2010; Franzin et al., 2017). This could be explained by different infestation protocols. Constantinoiu et al. (2010) made use of naïve cattle which were infested weekly with *R. microplus* larvae. Samples were taken at 1 day, one, 3 and 7 weeks post-primary infestation. CD3+ T-lymphocytes were found to be more abundant in resistant cattle at 1 day and at 3 weeks. In contrast, Franzin et al. (2017) used naïve cattle, which were challenged only once. Samples were taken at 2 and 9 days post infestation and significantly higher CD3+ T-lymphocyte levels were found in resistant compared to susceptible animals for the later sampling timepoint only. Based on the above studies and the importance of CD3+ T-lymphocytes in innate and adaptive immune responses, these cells are likely involved in the tick-resistance mechanism.

Neutrophils at the Site of Tick Attachment

Although an increase in neutrophil levels at the site of tick attachment is well described and has been related to the number of previous tick exposures (Allen et al., 1977; Binta and Cunningham, 1984; Brown et al., 1984; Gill, 1986; Walker and Fletcher, 1986), no differences have been reported from studies investigating the association of neutrophils to tick resistance. This led to the hypothesis that this cell type is not linked to the tick-resistance mechanism. Latif et al. (1991) found a decrease in the infiltration of neutrophils in less resistant (*B. t. indicus* and *B. t. taurus*) animals when compared to resistant Zebu cattle infested with *Rhipicephalus appendiculatus* nymphs. In contrast, no differences for cattle infested with *Amblyomma variegatum* nymphs (comparing resistant and more susceptible Zebu animals) were found. Furthermore, no differences in the number of neutrophils at the site of adult *R. microplus* attachment could be observed between resistant and susceptible breeds by Carvalho et al. (2010). The same results were obtained by Marufu et al. (2014) for naturally infested cattle. It is evident that there is an increase in the number of neutrophils upon larvae and adult tick infestation, where equal numbers of this cell subtype were found to be present irrespective of the resistance classification of the host (Carvalho et al., 2010; Franzin et al., 2017). Although the reaction of neutrophils upon larval maturation to nymphs presents with conflicting results (Latif et al., 1991; Franzin et al., 2017), the lack of differential levels of this granulocyte seen in the

larvae and adult life stage may support the hypothesis that this cell subtype remains unchanged across the tick life cycle.

Basophils at the Site of Tick Attachment

The fluctuation in the number of basophils between tick-resistant and tick-susceptible cattle breeds infested with either one- or multi-host ticks have been studied. Interestingly, Latif et al. (1991) again identified a potential variation in host immune responses to different multi-host tick species. At *R. appendiculatus* nymph attachment sites, significantly fewer basophils were present in more susceptible compared to resistant cattle within and between breeds. In the same study, no significant differences in the number of basophils were identified between tick-susceptible and tick-resistant cattle within the *B. t. indicus* breed at the sites of *A. variegatum* nymph attachment. Yet, cattle of intermediate resistance showed the highest numbers of basophils. This can be explained by the study layout as the cattle group of lower resistance had previous exposure to much lower *R. appendiculatus* numbers compared to *A. variegatum*. Therefore, animals could be presenting with a higher resistance level against the latter species and thus account for observed discrepancies.

In the one-host tick, *R. microplus*, basophil infiltration levels were also found to alter between cattle of varying resistance in response to tick infestation (**Figure 1**). Overall, basophil numbers at the site of adult tick attachment have been shown to be more abundant at tick attachment sites in resistant cattle than in their susceptible counterpart (Carvalho et al., 2010). Similarly, naturally infested cattle were found to have differing levels of basophils at the site of adult female *R. microplus* attachment, with tick counts negatively correlating with basophil counts (Marufu et al., 2014). The finding that basophil counts increase at the site of tick attachment in cattle was further corroborated by Franzin et al. (2017). This study showed that not only did both tick-resistant and tick-susceptible cattle recruit basophils at the site of *R. microplus* infestation, but also that upon maturation of *R. microplus* larvae to their nymph life stage, significantly more basophils were found to be present in resistant compared to susceptible hosts.

In summary, upon tick attachment basophil levels seem to increase in all cattle. The rate and level of increase is however dependent on the number of previous tick infestations and the level of tick resistance in the respective cattle breed. An increase in the number of infestations of a less susceptible breed to multi-host adult tick species not only showed an association with increased time taken to recruit basophils, but also increased number of basophils compared to previous tick infestations (Allen et al., 1977; Brown et al., 1984; Walker and Fletcher, 1986). Increased levels of this cell subtype in the nymph and adult life stages were found to be higher in resistant animals, with no difference identified between cattle breeds infested with tick larvae (Latif et al., 1991; Carvalho et al., 2010; Marufu et al., 2014; Franzin et al., 2017). These results suggest that it is not necessarily the difference in immune response pathway between cattle breeds that play a part in resistance but rather the level and reaction time of such immune responses.

Eosinophils at the Site of Tick Attachment

Between breed comparisons of eosinophil levels

Altered patterns of eosinophil regulation were identified across different tick life stages, and will hence be discussed accordingly. Three conclusions can be drawn regarding the attachment of larvae on cattle. Firstly, upon tick larvae attachment, there was an overall increase in the number of eosinophils at the site of tick attachment in all cattle breeds. Susceptible cattle did, however, display a higher influx of eosinophils compared to their tick-resistant counterparts (**Figure 1**; Moorhouse and Tatchell, 1969; Piper et al., 2010; Franzin et al., 2017). Secondly, the infestation history of the host does not seem to play an important role regarding the levels of eosinophils at the larval life stage. This is supported by the observation that a higher influx of eosinophils to the attachment site occurs in susceptible breeds in naïve cattle as well as in cattle that have been repeatedly infested (Piper et al., 2010; Franzin et al., 2017). Lastly, differences are observed amongst larval infestation using different tick species. In the case of Moorhouse and Tatchell (1969) it was found that hours after the attachment of *R. microplus* larvae to cattle (with previous tick exposure), susceptible cattle presented with a greater number of eosinophils. In contrast, no difference in the influx of eosinophils was observed between resistant and susceptible cattle breeds in response to infestation with the multi-host tick, *Haemaphysalis longicornis*.

With regards to nymph infestation, Franzin et al. (2017) showed that a reversal of the larval eosinophil response is observed, where upon maturation of tick larvae to nymphs, a greater number of eosinophils occur in resistant breeds (**Figure 1**). The same trend seen during the nymph life stage continues into the adult life stage (**Figure 1**). This has been confirmed in studies using Shorthorn-Zebu vs. Shorthorn (Riek, 1962) and Nelore vs. Holstein-Friesian (Carvalho et al., 2010) cattle infested with adult *R. microplus*. One study did, however, not confirm this observation. Marufu et al. (2014) identified that the more susceptible Bonsmara (*B. t. afrikanus*) cattle displayed higher eosinophil levels compared to that of the resistant Nguni (*B. t. indicus*) cattle breed. To date, it is unknown what the cause of this discrepancy could be.

Within breed comparisons of eosinophil levels

In contrast to studies between cattle breeds, no difference in eosinophil levels at the tick larval life stage was found between animals of the same breed (**Figure 1**). Schleger et al. (1976) showed that in *B. t. taurus* infested with *R. microplus* larvae, similar numbers of eosinophils were present between animals of varying resistance. However, eosinophils were more localized to the site of tick attachment in more resistant cattle (Schleger et al., 1976). This is in contrast to what is seen in blood, where eosinophil levels are significantly higher in susceptible cattle, even under conditions of natural infestation (Rechav et al., 1990).

Regarding nymph infestation, differences in the response were observed with regards to what tick species the cattle were infested with. In a study by Latif et al. (1991) Zebu cattle infested with *A. variegatum* have a greater influx of eosinophils as opposed to cattle infested with *R. appendiculatus*. However, upon conducting intra-breed comparisons, Zebu cattle that

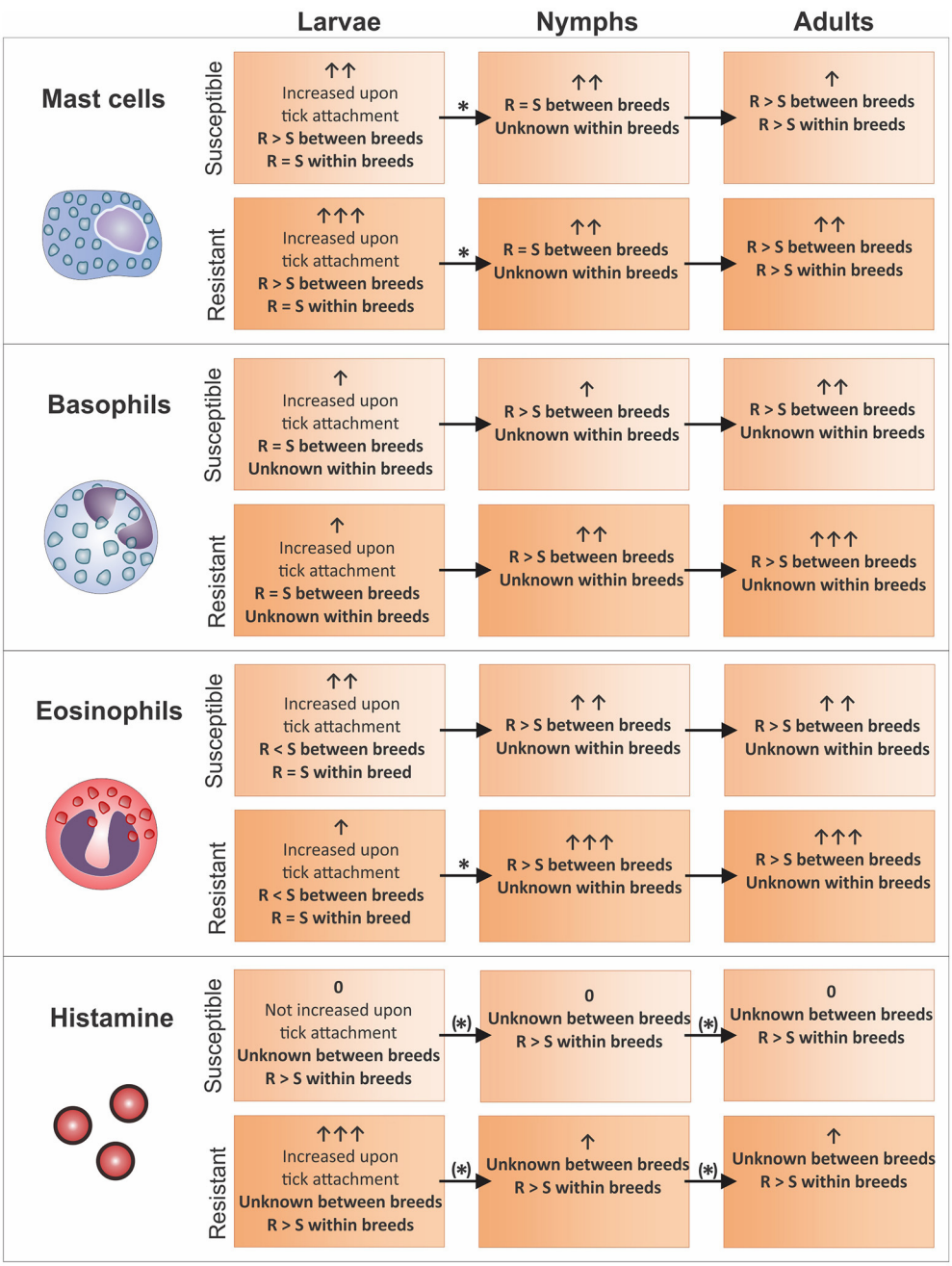

FIGURE 1 | Mast cell, basophil, and eosinophil levels identified in the skin of cattle (sections on Basophils, Eosinophils and Mast Cells at the Site of Tick Attachment) with different tick-resistance status and their correlation with blood (section Translational Studies in the Blood of Tick-Infested Cattle) and skin (section Translational Studies and Metabolites in the Skin of Tick-Infested Cattle) histamine levels (based on literature investigating changes as result of *R. microplus* infestation). Granulocyte and histamine levels are indicated per tick life stage with arrows. An asterix indicates that a specific cell subtype dynamic between life stages was confirmed by literature as per relevant skin and blood section. Where dynamics were obtained from within breed comparisons the asterix was placed in brackets. Resistant and susceptible animals are abbreviated with "R" and "S", respectively. Graphical representations correspond to the key of **Figure 2**.

displayed resistance to *R. appendiculatus* had lower eosinophil levels while no difference in eosinophil levels could be detected in *A. variegatum* susceptible or resistant animals. The latter observation could, however, be due to the presence of higher *A. variegatum* tick numbers compared to *R. appendiculatus* before the commencement of this study. As such, the tick species effect on eosinophil biology remains to be validated.

Studies focusing on the effects of multiple infestations (independent of the host-resistance status), indicated that eosinophil and degranulation levels progressively increase

with the number of infestations with *Hyalomma anatolicum anatolicum* (Gill, 1986) and *R. appendiculatus* (Walker and Fletcher, 1986) adults. In contrast, Allen et al. (1977) showed that all *B. t. taurus* cattle infested with adult *Ixodes holocyclus* had increased eosinophil levels, irrespective of whether the animals were previously infested or not. However, the latter study should be confirmed due to the low numbers of biological repeats per cattle group and low tick numbers used.

Discrepancies observed for within a single breed to between different cattle breeds infested with tick larvae could indicate that eosinophils play different roles in the resistance mechanism in genetically more resistant breeds compared to acquired resistance within breeds. Since studies looking at the changes in eosinophil levels within breeds have mainly focused on *B. t. taurus* cattle, more studies should investigate changes in *B. t. indicus* animals of various resistance.

Mast Cells at the Site of Tick Attachment

Differences in mast cell numbers have been related to the tick life stage and differences have furthermore been found when comparing results from within and between breed studies. Upon *R. microplus* larvae attachment, there is an increase in the number of mast cells at the site of tick attachment in all cattle (**Figure 1**). This increase is intensified in more tick-resistant cattle (*B. t. indicus*) when compared to more susceptible animals (*B. t. indicus*) (Franzin et al., 2017) which was not seen in a study investigating effects within a *B. t. taurus* breed (Schleger et al., 1976; **Figure 1**).

Yet, upon maturation to nymphs, the number of mast cells are similar for both resistant and susceptible animals while a significant decrease in the number of mast cells in more resistant hosts is seen in cattle infested with nymphs compared to larvae (**Figure 1**; Franzin et al., 2017). Similarly, no significant changes in the number of mast cells in the skin of resistant and more susceptible animals infested with *R. appendiculatus* or *A. variegatum* nymphs was found between and within cattle breeds except for a suggested decrease of cells in less resistant animals within the Zebu breed (Latif et al., 1991).

When ticks reached the adult life stage, a greater number of mast cells at the site of tick attachment in more resistant cattle was observed in all studies (**Figure 1**; Engracia Filho et al., 2006; Veríssimo et al., 2008; Marufu et al., 2014). In a study by Engracia Filho *et al.* (2006), Gyr x Holstein cattle were grouped into resistant and susceptible groups based on previous infestations. Upon adult attachment of *R. microplus* it was shown that the number of mast cells in the more resistant group was greater than in the more susceptible group (Engracia Filho et al., 2006). Furthermore, this was confirmed in a similar study using a wider range of cattle breeds and resistance groups including Nelore, Holstein-Friesian, Brown, Gyr and crossbred animals which showed that in the upper dermis of *R. microplus* adult infested cattle skin there was a negative correlation between the number of ticks on the animals and the number of mast cells present (Veríssimo et al., 2008). Both tick-susceptible and more tick-resistant cattle skin naturally infested with *R. microplus* adults also showed a negative relationship between tick counts and mast cell numbers (Marufu et al., 2014).

In addition, investigation of the skin of *B. t. taurus* cattle infested with adult *H. a. anatolicum* suggested that irrespective of previous exposure to ticks, there is a negative correlation between the number of mast cells in the skin of cattle and the number of ticks attached to these animals (Gill, 1986). An increased number of mast cells was found at the tick bite lesion of tertiary as compared to primary infested animals. Additionally, degranulation of mast cells was seen in tertiary infested animals as opposed to naïve cattle. Allen et al. (1977) showed that in the case of adult *I. holocyclus* attachment on European cattle breeds, the number of mast cells in the skin increased upon tick attachment irrespective of previous exposure. It was also shown that mast cell infiltration and mast cell degranulation increased in previously exposed cattle as opposed to naïve cattle (Allen et al., 1977). In contrast, to the above results, *B. t. taurus* cattle infested multiple times with adult *R. appendiculatus* showed a decrease of mast cells at the tick attachment site (Walker and Fletcher, 1986).

In summary, as for the dynamic of the eosinophil cell subtype, differences in results were seen within and between cattle breeds. Mast cells were found to be at similar levels in susceptible and resistant cattle within a cattle breed. While between breeds, resistant cattle showed higher mast cell levels for the larval life stage (Schleger et al., 1976; Franzin et al., 2017). Similar results were obtained for studies investigating tick nymph and adult life stages between breeds.

DYNAMICS OF GRANULOCYTES AND HISTAMINE AND THEIR SUGGESTED INVOLVEMENT IN THE TICK-RESISTANCE MECHANISM OVER THE TICK LIFECYCLE

Changes in histamine and cell infiltration patterns over the life cycle of *R. microplus* stress the importance of taking the dynamics of cellular changes in response to the maturing tick into account when planning a study (**Figure 1**). In the case of histamine, it is increased in the tick larval life stage (in resistant animals) pointing toward it acting as an effector molecule within cattle breeds. In addition, we hypothesize that histamine is increased in resistant animals throughout all tick life stages based on four observations. Firstly, a study comparing susceptible and intermediate-resistant cattle identified higher blood histamine levels throughout the tick life cycle for the latter group within a single cattle breed (Riek, 1962). Secondly, higher histamine levels were found at the site of larval attachment of more resistant cattle within the same breed (Willadsen et al., 1979; Schleger et al., 1981). Thirdly, resistant breeds have equal or higher basophil (Carvalho et al., 2010; Marufu et al., 2014; Franzin et al., 2017); and mast cell (Schleger et al., 1976; Engracia Filho et al., 2006; Marufu et al., 2014; Franzin et al., 2017;) levels throughout all life stages compared to susceptible breeds. Lastly, as histamine can be released from mast cells as well as basophils via an IgE and/or eosinophil-dependent mechanism, the presence of these cells correlate with the increase in histamine observed (Ishizaka et al., 1972; Zheutlin et al., 1984; Janeway et al., 2001; Galli et al., 2005; Stone et al., 2010) (**Figure 2**).

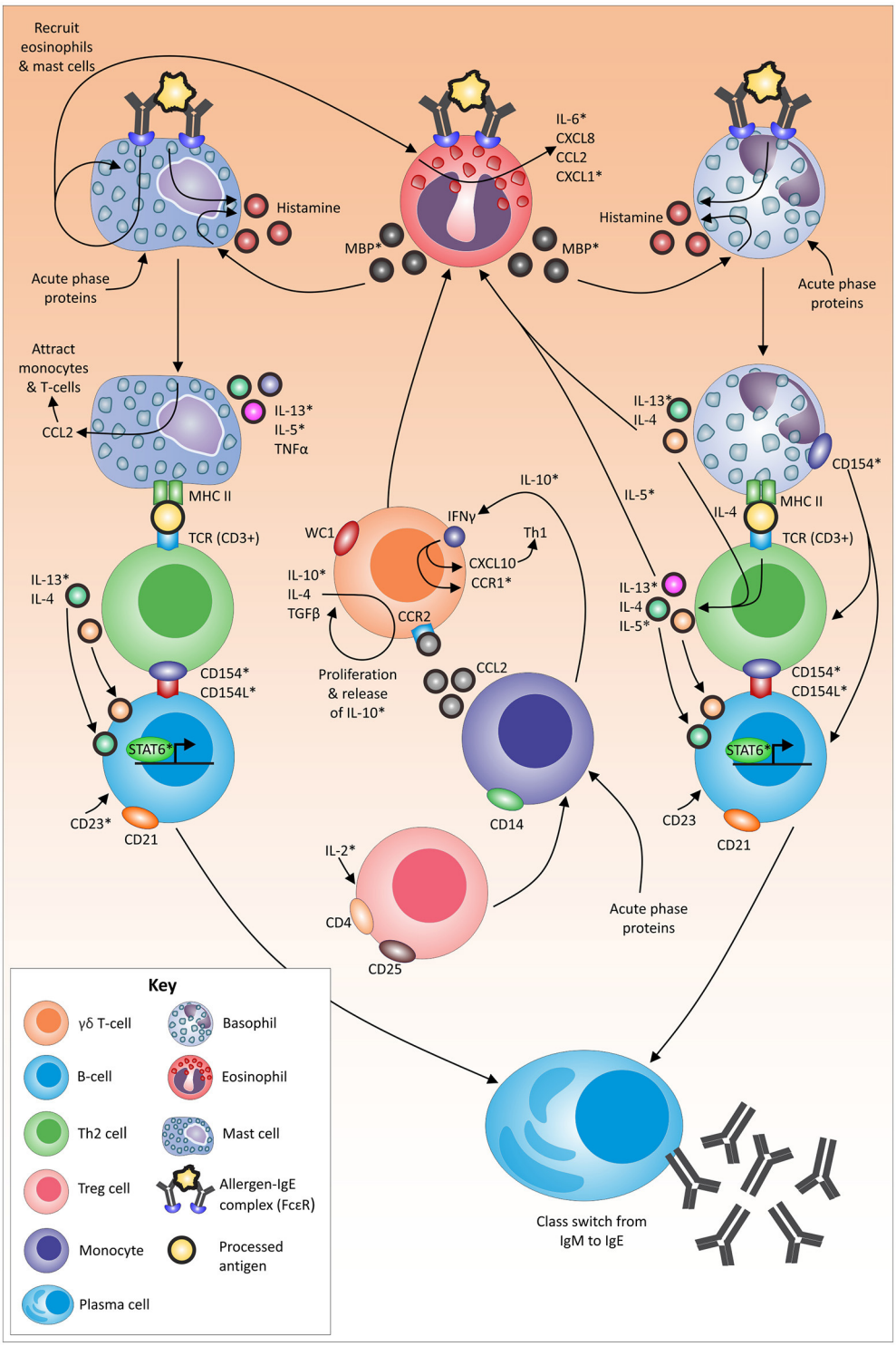

FIGURE 2 | Proposed mechanism, role players and associated pathways in the tick-resistance mechanism. An asterix (*) indicates immune molecules that have not yet been identified to be linked to tick resistance/susceptibility in cattle (based on chosen exclusion criteria indicated in text). Molecules without an asterix have been linked to tick resistance/susceptibility in literature (as per relevant skin and blood section). Arrows indicate a direct or indirect link between molecules.

Comparison of Findings Obtained within a Single Cattle Breed

Although no differences were observed for mast cell and eosinophil levels at the larval life stage, histamine levels were found to be higher in resistant compared to susceptible animals (**Figure 1**) (Riek, 1962; Willadsen et al., 1979; Schleger et al., 1981). Potentially, histamine is thus released via basophils, however, no study up to date has extensively investigated this cell subtype within a single breed. It should be noted that Riek (1962) only investigated the dynamics of this compound during the larval life stage in a limited study using only one highly resistant animal. Higher histamine levels upon nymph and adult tick attachment in blood were also identified when comparing medium resistant to susceptible cattle (**Figure 1**; Riek, 1962). If histamine levels were linked to tick numbers as result of tissue damage, it would be expected that higher histamine levels are present in more susceptible animals which is not the case. This indicates that histamine could be involved in the tick-resistance mechanism within cattle breeds. Since not enough data regarding the number of granulocytes are available to date for the nymph and adult life stages, it cannot be hypothesized by which cell subtype histamine may be released. However, increased mast cell numbers in resistant compared to susceptible animals at the adult life stage could indicate a delay in mast cell dependent release of histamine as seen for inter-breed comparisons at the larval life stage (section Comparison of Findings Obtained between Different Cattle Breeds).

Comparison of Findings Obtained between Different Cattle Breeds

Although mast cells increased upon larvae attachment for all cattle breeds evaluated, this cell subtype was found to be more abundant in resistant breeds (**Figure 1**) (Franzin et al., 2017). This, together with the equal levels of basophils in both resistant and susceptible cattle at the tick larval life stage (**Figure 1**; Franzin et al., 2017), indicates that histamine might be increased as a result of mast cell degranulation and contributes to the first line of tick defense. The higher eosinophil levels in susceptible compared to resistant cattle breeds at the larval life stage (**Figure 1**; Moorhouse and Tatchell, 1969; Piper et al., 2010; Franzin et al., 2017) furthermore suggests that the immune response in susceptible cattle increases histamine levels via an eosinophil-dependent mechanism. This mechanism might be less efficient and slower in susceptible cattle due to its involvement in late-phase reactions (Piliponsky et al., 2001). Mast cell levels in the nymph life stage were found to be present at similar levels in resistant and susceptible breeds (Latif et al., 1991; Franzin et al., 2017), while a relatively higher number of mast cells was seen in more tick-resistant cattle in the adult tick life stage (**Figure 1**). This could be a result of a decrease of this cell subtype in susceptible and not an increase in resistant animals. The apparent decline in the number of mast cells that was observed in the more resistant cattle breed from the larvae to the nymph life stage may thus be delayed and occurring in the more susceptible cattle at the adult life stage. Since the tick-resistance mechanism in *B. t. indicus* animals results in a generally faster response to tick infestation compared to *B. t. taurus* cattle (Riek, 1962; Wagland, 1978, 1980; Rechav et al., 1990), resistance within susceptible cattle breeds might be achieved through a delayed histamine release via basophils. This mechanism is suggested to occur at the nymph life stage in resistant cattle breeds (**Figure 1**). To specifically elucidate this resistance mechanism in depth, especially granulocyte levels for animals within a breed, presenting with varying tick-resistance phenotypes, need to be determined throughout the tick life cycle. Additionally, investigation of histamine within and between cattle breeds at all tick life stages are essential.

FUTURE DIRECTIONS: POTENTIAL DRIVERS INVOLVED IN TICK RESISTANCE

This integrative discussion will give an evaluation of key role players investigated up to date to establish a global view of components potentially involved (directly or indirectly) in the tick-resistance mechanism. It must be kept in mind that some observed immune responses may be a by-product of the effector pathway/molecule or a response to tick infestation without any involvement in the actual resistance mechanism. For example, gene expression results and actual dynamics occurring on protein level often do not correlate due to post-transcriptional, post-translational and degradation regulation (Vogel and Marcotte, 2012). Therefore, results from studies employing gene expression analysis were only included as part of this discussion if their findings have been validated by a second study. Furthermore, since gamma globulin levels, apart from IgE, were generally increased in susceptible cattle, this could be linked to elevated tick numbers. Even though antibody specificity could be a contributing factor, these molecules were thus not included as key role players in this section as evidence remains non-conclusive. Lastly, due to a constant dynamic of immune molecules, identified markers on translational and cellular levels were included if a significant difference (irrespective of the direction) was seen between animals with more and less resistance status. **Figure 2** summarizes potential role players and their possible interactions driving resistance, based on findings up to date with the discussion providing an integrative explanation of identified immune marker interactions of the respective components supported by literature. Indicated with an asterix (*) in the text below and in **Figure 2**, are components that have not yet been identified to be linked to tick resistance/susceptibility in cattle.

During the process of tick attachment, the skin of the host is damaged/pierced, and tick saliva is exposed to sentinel cells, such as granulocytes. Several acute-phase proteins have been shown to be involved at the site of tick attachment (Carvalho et al., 2008). These proteins can be linked to granulocyte (Quaye, 2008; Stone et al., 2010; Eklund et al., 2012) and monocyte (Hochepied et al., 2003) recruitment and/or activation. Furthermore, tick secreted allergens can cross-link to IgE (Galli and Tsai, 2012) and binding of IgE to its high-affinity receptor (FcεRI) on dermal mast cells (and basophils) has been shown to lead to the release

of inflammatory mediators (Stone et al., 2010) such as histamine (Galli et al., 2005).

Following activation, mast cells readily secrete IL-5*, IL-13*, and TNFα (Janeway et al., 2001; Stone et al., 2010). In addition, IgE binding leads to the enhancement of CCL2 (monocyte chemoattractant protein 1) transcription that promotes the migration of monocytes (Oliveira and Lukacs, 2001) and T-cells (Oliveira and Lukacs, 2003) to amplify the local inflammatory reaction. Lastly, due to the antigen presenting nature of mast cells, following the uptake of the IgE-antigen complex, the allergen is presented on mast cells on MHC II, which in turn interacts directly with T-cell receptors (containing CD3) and induces antigen-specific clonal expansion of T-cell populations (Mekori and Metcalfe, 1999; Henz et al., 2001).

Basophils also function as antigen presenting cells in response to certain allergens (Sokol et al., 2008, 2009). The binding of the IgE-allergen complex to FcεRI on basophils activates several pathways in the cell resulting in the release of histamine (Ishizaka et al., 1972) and the expression of IL-4 and IL-13* (Stone et al., 2010). These cytokines are important for the promotion of eosinophil trafficking (Stone et al., 2010) and are also secreted by Th2 cells in response to the presentation of allergen via MHC II and IL-4 production (Perrigoue et al., 2009; Yoshimoto et al., 2009) Activated Th2 cells also secrete cytokines (e.g., IL-5*) which increases eosinophil production (Janeway et al., 2001).

Antigen presentation to Th2 lymphocytes by mast cells and/or basophils, provide two essential signals for isotype switching. The first signal is IL-4 and/or IL-13* which bind to the respective receptors on B-cells and activate transcription at the IgE isotype-specific site via STAT6 (Stone et al., 2010). The second signal involves the binding of CD40L (CD154L*) to the relevant T-cell receptors, which in turn activates DNA

TABLE 1 | Problem identification and potential solutions for studies evaluating the interplay of cattle immune responses to tick infestation.

Problem statement/explanation	Possible solutions and future guidelines
Factors relating to the selection and treatment of host animals	
Extrapolation of findings between different host species (e.g., rodents and bovines).	A rodent model can be used to provide hypotheses, as numerous validated immune markers are available for murine models. However, a significant amount of results cannot be directly extrapolated from murine to bovine hosts and should therefore be confirmed in the appropriate host species to validate an immune response.
Not considering intra-breed differences (range of tick-resistance status between individual hosts within a breed).	Cattle should be sourced from registered breeders to limit genetic differences to a minimum. The resistance status of each animal needs to be taken into account when analyzing data and confirmed prior to the start of a study.
Lack of patient history of experimental animals.	Information on animal source, age and previous exposure(s) to ticks should be provided.
Reporting prophylactic treatments of host animals upon arrival and health status throughout study.	Upon arrival, all treatments should be reported with special emphasis on acaricide treatments and prophylactic treatments (antibiotics for infections, deworming strategies), as all of these influence immunity. General health parameters such as weight, temperature and hematocrits need to be reported as bovine studies are rarely conducted under biosafety level standards.
Factors relating to infestation and sampling protocols	
Comparison of immune responses between animals with different tick attachment efficiencies and thus tick numbers at the respective sampling time points.	Comparing immune responses of susceptible and resistant hosts infested with the same number of ticks (especially at nymph and adult life stages) would allow for a better understanding of which mechanism is at play at which life stage.
Protocol for tick infestation and evaluation of specific tick life stage(s).	Biological question should take into account infestation protocol, as multiple infestations results in numerous life stages being present on a single animal that will bias detection of a life stage specific response.
Focus on between animal comparisons at specific time points instead of immune dynamics.	Focus should be placed on the progression of immune responses within breeds and then determine between breed differences.
Choice of biological sample for analyses.	To date, skin and blood have been studied extensively. Insights from secondary lymphoid organs are in dire need to fully understand tick-mediated immune suppression as well as factors underlying tick resistance.
Selection of immune markers	
Translation of findings to all cellular levels.	Gene expression profiling studies should be validated using protein and/or cellular markers. Genotyping studies should take into account cellular immune markers to link genotypes and phenotypes.
Most immune markers are not confirmed as cross-reactive to specific cell subpopulations in bovines or are lacking.	Immune markers must be confirmed to be cross-reactive to a specific cell subpopulation.
Data analysis and interpretation	
Comparison between studies investigating different tick species (e.g. one-host vs. multi-host ticks).	Transcriptome analysis across ixodid tick species support differences in proteins being present in a specific species. Therefore, cross-species comparisons should be carefully considered.
Comparison between studies investigating different tick life stages.	A unique set of proteins/molecules can be secreted by each life stage of a tick species, with a unique subset of immune cells affected/targeted (e.g., see section on granulocytes).

switch recombination (Stone et al., 2010). Basophils express high levels of CD154* after activation and have been suggested to play a role in polyclonal amplification of IgE production and in the differentiation of Th2 cells (Stone et al., 2010). In addition, the binding of CD23* to CD21+ B-cells may participate in the control of IgE production (Aubry et al., 1992).

Histamine can also be released from mast cells and basophils via an IgE-independent mechanism (Siraganian and Hook, 1976; Piliponsky et al., 2001) utilizing the major basic protein* released from eosinophils (Zheutlin et al., 1984; Janeway et al., 2001). The binding of the allergen-IgE complex to mast cells is suggested to drive the recruitment and activation of additional mast cells and eosinophils (Wong et al., 2009). Mast cells can also induce the release of IL-6*, CXCL8, CCL2 and CXCL1* by eosinophils (Wong et al., 2009).

The development of eosinophilic allergic inflammation and the initiation of Th2-responses is regulated by a T-cell subtype (Zuany-Amorim et al., 1998). Regulatory T-cells are generally known for their ability to suppress putative deleterious activities of Th cells (Corthay, 2009), with IL-2* playing an important role in the survival and proliferation of CD4+CD25+ regulatory T-cells (Létourneau et al., 2009). The exact role of CD4+CD25+ regulatory T-cells in bovines is, however, unknown. It has been proposed that this cell population is neither anergic nor suppressive in cattle, and that their function(s) can to be linked to γδ T-cells (WC1.1+, WC1.2+) (Hoek et al., 2009). Together with the latter cell type, CD14+ monocytes have been linked to immune suppression in ruminants (Hoek et al., 2009). In addition, γδ T-cells bearing the lineage marker WC1 are associated with the production of the proinflammatory cytokine IFNγ (Rogers et al., 2005), that furthers the action of CXCL10 (an INFγ inducible protein, which recruits activated Th1 cells to the site of inflammation) (Dufour et al., 2002). Interferon gamma (Zella et al., 1998) as well as other cytokines such as IL-10* on monocytes (Loetscher et al., 1996; Sozzani et al., 1998) can mediate upregulation of *CCR1** expression. The release of IL-10*, IL-4 and TGFβ can result in the proliferation of subsets of γδ T-cells (Guzman et al., 2014). The accumulation of γδ T-lymphocytes during allergic inflammation in turn is orchestrated by the CCR2/CCL2 pathway (Costa et al., 2009; de Oliveira Henriques and Penido, 2012).

Several immune markers associated with the above pathways, such as histamine, have been identified in section Blood and Skin Tissue (**Figure 2**). To date, histamine levels in the blood and skin were found to be increased in resistant cattle, while little variation was seen in susceptible animals (Riek, 1962; Willadsen et al., 1979; Schleger et al., 1981). Degranulation of both mast cells (Riley, 1953; Mota et al., 1954) and basophils (Pruzansky and Patterson, 1970) is followed by the subsequent release of histamine in an immediate hypersensitivity reaction (Ishizaka et al., 1970, 1972). This reaction is well known to be linked to more frequent and intense grooming (O'Mahony et al., 2011), which was identified to play a role in tick-resistance (Riek, 1956; Snowball, 1956). Future studies are now required to elucidate these predicted pathways in depth, focusing on likely markers not yet investigated (*) and molecular mechanisms/molecules that have resulted in contradicting findings up to date. Since opposing findings could be linked to varying study time points, a dynamic investigation of all potential role players would be valuable and could lead to a better-defined picture of occurrences.

CRITICAL EVALUATION AND CONCLUDING REMARKS

To date, few immune markers have been investigated with sufficient depth to obtain a picture of events involved in the cattle tick-resistance mechanism. Especially large-scale transcriptome studies have identified various components with hardly any overlap of results between studies. One reason for this is the differences in experimental designs which can drastically influence the occurrence of immune events depicted at the chosen time point corresponding to a specific tick life stage(s). In addition, potential markers or pathways should not only be studied on a molecular level but also on a cellular level. The clearest pictures, as obtained for histamine, granulocytes and gamma globulin levels, were identified using markers on both translational and cellular level. In this regard, some immune markers seem to differ for the resistance mechanism within a host breed compared to between breeds as well as between tick life stages (see section Dynamics of Granulocytes and Histamine and their Suggested Involvement in the Tick-Resistance Mechanism Over the Tick Lifecycle and **Figure 1**). It should be noted that even though the final effector molecules might be the same, different pathways are possibly involved in establishing this mechanism (see section Future Directions: Potential Drivers Involved in Tick Resistance). Several experimental factors should thus be considered when comparing experiments as some of these parameters can potentially skew results and lead to contradicting findings if not addressed correctly (**Table 1**). This includes important aspects and potential solutions regarding (1) factors relating to the selection and treatment of host animals, (2) factors relating to infestation and sampling protocols, (3) selection of immune markers and (4) data analysis and interpretation.

To date, the only partially clear picture of immune events involved in the tick-resistance mechanism involves histamine and associated cell subtypes and molecules (**Figures 1**, **2**). This pathway, with its effector molecules and modes of action, still requires additional in-depth investigation before focus is placed on the identification of other contributing mechanisms. The identified cell dynamics between tick life stages furthermore suggest that future studies should concentrate on the dynamics of immune responses at various time points over the complete tick life cycle. This would reduce between study variations in addition to obtaining a temporal overview of events. In general, it is advisable that experiments are standardized as much as possible and more focus should lie on an in-depth investigation of markers/pathways across genetic, translational and cellular levels to successfully validate

a response. Only then can one attempt to consolidate all information into a feasible blue print for the identification of major drivers underlying the bovine immune mechanism driving tick-resistance and subsequent postulation of viable and effective tick control strategies.

AUTHOR CONTRIBUTIONS

LR, SR, CM-O: conceptualized manuscript focus, critically revised draft manuscript, approved final manuscript; LR, SR: accumulated data, designed Figures and Tables, wrote first manuscript draft.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2017.00522/full#supplementary-material

Supplementary Table 1 | Summary of studies investigating tick resistance/susceptibility categorized according to tissue of study.

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REVIEW
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Cattle Tick *Rhipicephalus microplus*-Host Interface: A Review of Resistant and Susceptible Host Responses

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Ticks are able to transmit tick-borne infectious agents to vertebrate hosts which cause major constraints to public and livestock health. The costs associated with mortality, relapse, treatments, and decreased production yields are economically significant. Ticks adapted to a hematophagous existence after the vertebrate hemostatic system evolved into a multi-layered defense system against foreign invasion (pathogens and ectoparasites), blood loss, and immune responses. Subsequently, ticks evolved by developing an ability to suppress the vertebrate host immune system with a devastating impact particularly for exotic and crossbred cattle. Host genetics defines the immune responsiveness against ticks and tick-borne pathogens. To gain an insight into the naturally acquired resistant and susceptible cattle breed against ticks, studies have been conducted comparing the incidence of tick infestation on bovine hosts from divergent genetic backgrounds. It is well-documented that purebred and crossbred *Bos taurus indicus* cattle are more resistant to ticks and tick-borne pathogens compared to purebred European *Bos taurus taurus* cattle. Genetic studies identifying Quantitative Trait Loci markers using microsatellites and SNPs have been inconsistent with very low percentages relating phenotypic variation with tick infestation. Several skin gene expression and immunological studies have been undertaken using different breeds, different samples (peripheral blood, skin with tick feeding), infestation protocols and geographic environments. Susceptible breeds were commonly found to be associated with the increased expression of toll like receptors, MHC Class II, calcium binding proteins, and complement factors with an increased presence of neutrophils in the skin following tick feeding. Resistant breeds had higher levels of T cells present in the skin prior to tick infestation and thus seem to respond to ticks more efficiently. The skin of resistant breeds also contained higher numbers of eosinophils, mast cells and basophils with up-regulated proteases, cathepsins, keratins, collagens and extracellular matrix

proteins in response to feeding ticks. Here we review immunological and molecular determinants that explore the cattle tick *Rhipicephalus microplus*-host resistance phenomenon as well as contemplating new insights and future directions to study tick resistance and susceptibility, in order to facilitate interventions for tick control.

Keywords: ticks, immunity, tick resistance, tick susceptibility, cattle breeds, genetic variation, gene expression profiling, immune responses

INTRODUCTION

Vector-borne pathogens cause diseases with a great impact on public and veterinary health and have accounted for 22% of emerging infections between 1940 and 2004 (Jones et al., 2008). As obligate hematophagous arthropod pests of vertebrates, ticks pose serious threats to beef and dairy cattle producers. It has been estimated that 80% of the world's cattle population is at risk from tick and tick-borne diseases (TBDs) causing estimated annual losses of US$ 22–30 billion (Lew-Tabor and Rodriguez Valle, 2016). The negative impact of ticks on cattle production is due to the direct effects of feeding, such as weight loss and damage of leather, and indirect effects, such as the transmission of tick-borne pathogens. The resulting diseases can potentially cause major production losses in livestock, thereby reducing farm incomes, increasing costs to consumers, and threatening trade between regions and/or world markets.

Since the establishment of extensive vector control programs, a steady decline in vector-borne diseases was observed last century, however recently the emergence and re-emergence of vector-borne diseases has been observed. This re-emergence may be linked to new global trends associated with changes in animal husbandry, urbanization, animal transboundary transportation, and globalization (Ogden and Lindsay, 2016). In this scenario, various approaches for tick control are in practice around the world in accordance with local legislation, environmental conditions, price based selection, and geography. Acaricide (synthetic pesticides) application is the most common component of tick control strategies, however the use of acaricides impose numerous limitations including the selective pressure for the development of more resistant ticks, environmental contamination, drug residues in food products, the expense of developing new acaricides, and the difficulty of producing tick-resistant cattle while maintaining desirable production characteristics (Willadsen, 2004; Abbas et al., 2014). Anti-tick vaccines are a very promising alternative to acaricide usage, however are still insufficient to confer protection against multiple tick species in various geographical regions (de la Fuente and Contreras, 2015; de la Fuente et al., 2016; Schetters et al., 2016).

Anti-tick immunity has been described in guinea pigs, cattle and rabbits, and refers to the capacity of previously exposed hosts to interfere with tick feeding and reproductive fecundity (Nuttall, 1911; Trager, 1939; Hewetson, 1972). A reduction in tick weight, duration of attachment, number of ticks feeding, egg mass, and molting success are some of the parameters measured to determine host anti-tick immunity (Trager, 1939). For the first time, Nuttall (1911) demonstrated host immunity to ticks as a phenomenon of natural immunity in humans. Experimentally, acquired resistance to tick infestation was observed by Trager (1939), who noticed that after repeated infestation of *Dermacentor variabilis* on guinea pigs, the host developed resistance to subsequent tick infestation, shown by the decreasing number of successfully feeding larvae. Furthermore, it was found that as compared with larvae infesting a host with no previous exposure to ticks, larvae infesting resistant hosts weighed less. Several researchers continued to observe host resistance to tick feeding affecting each tick life stage (Gregson, 1941; Feldman-Muhsam, 1964; Wikel, 1996). Various immunological determinants have been examined that influence host resistance to tick infestation including a high level of eosinophils, basophils, T cells, mast cells, specific immunoglobulins, histamine, and changes to gene transcription profiles (Kemp and Bourne, 1980; de Castro and Newson, 1993; Kashino et al., 2005; Veríssimo et al., 2008; Kongsuwan et al., 2010; Piper et al., 2010; Engracia Filho et al., 2017).

Bovines present contrasting, heritable phenotypes for infestation with *Rhipicephalus microplus* and related tick species as a consequence of co-evolution of resistant cattle with ticks and also decades of selective breeding. The *R. microplus* tick has a strong preference for *Bos taurus taurus* cattle over highly resistant *Bos taurus indicus* cattle (Wambura et al., 1998; Porto Neto et al., 2011b; Jonsson et al., 2014; Biegelmeyer et al., 2015). In this article we review the tick:host physical interface, genetic and molecular studies, and immunological determinants of bovine host resistance to ticks.

TICK-HOST PHYSICAL INTERFACE

The cattle tick *R. microplus* co-evolved with Asian bovines (zebu breeds) and due to the global migration of *B. t. taurus* European breeds for dairy production during the eighteenth–nineteenth centuries, this tick spread across tropical and sub-tropical regions of the world (Frisch, 1999; Estrada-Peña et al., 2006; Barré and Uilenberg, 2010). Currently *R. microplus* is considered to be a species complex, in which there are recognized geographic differences between the 5 clades including 3 clades of *R. microplus* (A, B, and C), as well as *R. australis* and *R. annulatus* (Burger et al., 2014; Low et al., 2015). Each taxa transmits both anaplasmosis and babesiosis and each have a parasitic life cycle on cattle for ∼21 days. They will be described collectively as *R. microplus* or simply as cattle ticks in this review. Cattle ticks are attracted to their hosts through stimuli such as carbon dioxide, temperature, vibrations, visual stimuli, and odor (Osterkamp et al., 1999). The susceptible European (*B. t. taurus*) breeds which

were introduced into regions in which *R. microplus* is endemic failed to resist tick infestation to the same extent as tropical *B. t. indicus* breeds, which have developed an effective anti-tick immune response (Frisch, 1999).

The immune response varies among newly introduced European cattle (Taurine breeds, susceptible hosts) whereas Asian bovines (zebuine breeds or resistant hosts) co-evolved with ticks (Utech et al., 1978). Physical barriers that affect tick resistance include density of the fur coat, skin thickness, skin pigmentation (light or dark), skin vibration and/or self-cleaning ability, tongue papillae, and odor (de Castro et al., 1985; Spickett et al., 1989; Veríssimo et al., 2002, 2015; Martinez et al., 2006; Gasparin et al., 2007). In addition to physical differences between resistant and susceptible hosts, their behavior also affects the *R. microplus* parasitic load. Self-grooming is widely used by cattle as an important defense mechanism against ticks (Riek, 1956; Snowball, 1956; Bennett, 1969) and the level of resistance may possibly be associated with tongue morphology. For example the papillae from tick-resistant breeds have smaller spacing, which is more effective in removing *R. microplus* larvae from the skin (Veríssimo et al., 2015). However, there is also conjecture that resistant breeds simply groom more often (Kemp et al., 1976). It has been suggested that innate characteristics such as thinner coats and lower fur density have direct impacts in decreasing tick preferential attachment and infestation (Spickett et al., 1989; Veríssimo et al., 2002; Gasparin et al., 2007; Marufu et al., 2011). However, other studies have shown that skin features have no influence on tick infestation (Wagland, 1978; Doube and Wharton, 1980). Evidence for resistance of cattle to ticks due to physical parameters is scarce and further studies to examine the mechanisms that govern these physical phenomena are still needed.

IMMUNOLOGICAL DETERMINANTS ASSOCIATED WITH HOST RESISTANCE: HOST COUNTER ATTACK?

It is well-established that cattle have three subclasses of IgG (IgG1, IgG2, and IgG3), and during blood meals ticks ingest a substantial amount of IgG (Knight et al., 1988; Symons et al., 1989; Kacskovics and Butler, 1996; Rabbani et al., 1997; Gudderra et al., 2002; Saini et al., 2007). Host IgGs can be found in the tick hemolymph and are potentially biologically active against specific tick proteins (Ben-Yakir et al., 1987). Furthermore, specific host antibodies neutralize the tick salivary pharmacopeia and can damage the tick by binding to tick internal organs such as salivary glands, midgut, or ovaries (Ackerman et al., 1981; Willadsen and Kemp, 1988; Tellam et al., 1992). In other tick-host systems (*Dermacentor andersoni* and guinea pigs), antibodies have been shown to mediate inflammatory reactions by triggering effector-cell recruitment and cellular immune response as a consequence of both Fc receptor activation of leukocytes and complement activation that are harmful to the tick, also an immune mechanism in human auto-immune disease syndromes (Wikel and Whelen, 1986; Hogarth, 2002).

It was documented previously that the passive transfer of plasma from genetically immune resistant animals to naïve hosts, increases resistance to tick challenge and this response was believed to be mediated by antibodies (Roberts and Kerr, 1976; Shapiro et al., 1986). The pattern of antibody responses to immunogens from tick salivary glands and guts have been reviewed by different research groups (Willadsen, 1980; Wikel, 1982; Kaufman, 1989; Kashino et al., 2005; Cruz et al., 2008; Piper et al., 2009, 2010; Garcia et al., 2017).

Some studies have shown that during laboratory animal infestations, such as guinea pigs, rabbits and mice, reactive antibody titers to tick salivary antigens increased (Allen and Humphreys, 1979; Allen, 1989). The densities of *Amblyomma hebraeum, Rhipicephalus appendiculatus,* and *Rhipicephalus evertsi evertsi* ticks was higher on the susceptible breed (*B. t. taurus* Hereford) as compared to resistant cattle (*B. t. indicus* Brahman) with a positive correlation between the level of tick infestation and the level of IgG in susceptible hosts (Rechav, 1987). Piper and colleagues confirmed this correlation noting that susceptible cattle (Holstein-Friesian) have higher levels of tick specific IgGs compared to Brahmans suggesting that these antibodies do not confer immunity to ticks (Piper et al., 2008, 2009, 2010). As with other immune parameters in high and low resistance animals, the interpretation of data can be problematic as a susceptible animal will have more ticks feeding at any time, which would in turn be expected to result in a higher antigenic challenge. However, the negative relationship between IgG levels and host resistance was later confirmed and shown to be independent of the number of feeding ticks, using Santa Gertrudis cattle [a stable composite breed of *B. t. taurus* (5/8) and *B. t. indicus* (3/8)], in which there is wide variation in host resistance to ticks (Piper et al., 2017). Susceptible animals had significantly higher tick-specific IgG1 antibody titres (to several tick antigens including adult female salivary glands and guts, and whole larvae) compared to tick resistant cattle (Piper et al., 2017). In contrast, Kashino et al reported that tick saliva-specific IgG1 and IgG2 antibodies decreased in susceptible (Holstein) compared to resistant (Nelore) cattle where the IgG levels remained the same, however, only IgG levels to tick salivary antigens were examined (Kashino et al., 2005). Previous studies have surmised that there are genetic differences in the bovine host's ability to elicit antibody responses to antigens in *R. microplus* and *D. andersoni* tick saliva (Whelen et al., 1986; Opdebeeck and Daly, 1990). Despite the fact that differences in the IgG levels against tick antigens between heavy or light infestations have been reported, there is individual variation in the same bovine breed with respect to humoral immune responses to tick antigenic molecules (Cruz et al., 2008). In addition, despite most studies reporting increased total IgG production against wide ranging tick antigens in susceptible breeds compared to resistant, IgG responses to salivary proteins were significantly higher in tick naïve resistant hosts (Nelore) at the first larval challenge (Garcia et al., 2017).

Variation in IgG2 allotypes has been associated with variation in immune responses to pathogens. When two allotypes IgG2a and IgG2b were found to differ in sequence at the CH1–CH3 regions it was reported that IgG2b was more able to

initiate the bovine complement cascade while animals with the IgG2a allotype were more susceptible to extracellular pyogenic pathogens (Heyermann and Butler, 1987; Bastida-Corcuera et al., 1999). Other studies have shown that the distribution and presence of IgG2 allotypes differed significantly between taurine and indicine breeds (Butler et al., 1994; Carvalho et al., 2011). Blakeslee et al. (1971) described that ∼80% of susceptible bovines (Holstein) have the IgG2a allotype and that the IgGγ2b was rare in these animals (Blakeslee et al., 1971). Recently, it was shown that the IgGγ2a allotype was significantly more frequent in taurine hosts (tick susceptible) and IgGγ2b was significantly frequent in indicine cattle (tick resistant) (Carvalho et al., 2011). Male tick saliva contains IgG-binding proteins (IGBPs) secreted into the host which assists the female tick to evade the host immune response (Wang and Nuttall, 1999; Santos et al., 2004; Gong et al., 2014). Carvalho et al. (2011) suggested that certain IgG2 allotypes may hinder the function of these tick IGBPs.

Aside from IGBPs, other tick specific proteins have been examined in terms of their immune recognition by tick resistant and susceptible cattle. A *R. microplus* recombinant serine protease inhibitor (Serpin- rRMS-3) was recognized by resistant bovines and not susceptible, suggesting that RMS-3 could be a protective antigen (Rodriguez-Valle et al., 2012). Another study by the same group demonstrated that host responses to six *R. microplus* lipocalins (LRMs which include tick histamine binding proteins) were higher in resistant cattle (Rodriguez-Valle et al., 2013). Both RMS-3 and the LRM proteins were identified based on the *in silico* identification of B cell binding epitopes. In addition, predicted T cell epitopes from 3 LRMs stimulated the generation of a significantly higher number of interferon gamma (IFN-γ) secreting cells (consistent with a Th1 response) in tick-susceptible Holstein–Friesians compared with tick-resistant Brahman cattle. In contrast, expression of the Th2-associated cytokine interleukin-4 (IL4) was lower in Holstein–Friesian (susceptible) cattle when compared with Brahman (resistant) cattle (Rodriguez-Valle et al., 2013). IL4 is known to decrease the production of Th1 cells and IFN-γ, and is thus a key regulator of both the humoral and adaptive immune responses.

The immunological parameters of tick resistance have been shown to differ between tick susceptible and tick resistant breeds as well as within the same breeds. The studies reported differ in the parameters of trials and tick infestations, for example, the use of tick naïve cattle artificially infested or the use of cattle naturally exposed to ticks (Kashino et al., 2005; Piper et al., 2008, 2009). The study undertaken by Kashino et al. (2005) used susceptible cattle that had been treated with acaricides when high tick numbers were observed and the cattle had been vaccinated with GAVAC (Bm86 based tick vaccine), introducing additional variables to the study. Further studies examining specific tick proteins to compare divergent host immune responses are still warranted.

MOLECULAR GENETIC VARIANTS ASSOCIATED WITH HOST RESISTANCE

Numerous studies have attempted to identify genetic markers for host resistance to tick infestation and they are summarized and discussed by Porto Neto et al. (2011b) and Mapholi et al. (2014). Approaches have included immunological methods (Stear et al., 1984, 1989, 1990); protein-based analyses (Ashton et al., 1968; Carvalho et al., 2008); candidate gene sequence or genotype (Acosta-Rodriguez et al., 2005; Martinez et al., 2006; Untalan et al., 2007); genomic detection of quantitative trait loci (QTL) using SNPs or microsatellites, with or without fine mapping (Barendse, 2007; Gasparin et al., 2007; Regitano et al., 2008; Prayaga et al., 2009; Machado et al., 2010; Porto Neto et al., 2010a, 2011a; Turner et al., 2010; Cardoso et al., 2015; Mapholi et al., 2016; Sollero et al., 2017). There is one example of a meta-analysis of genomic association with transcriptome in tick infestation (Porto Neto et al., 2010b). Although this appears to represent a large body of science, it has generated relatively little data which can be used for improved genetic selection. It can be concluded from the studies on the major histocompatibility complex (MHC, also referred to as the bovine lymphocyte antigen (BoLA) system) that the MHC makes a contribution to variation in resistance however there is no single, consistent genotype of any gene in the MHC that is associated with high or low resistance to ticks across breeds and production systems. A number of QTL markers have been identified using microsatellites and SNPs, however these have mostly been inconsistent and the loci have had relatively weak effects. The research of Barendse (2007) and Turner et al. (2010) found several significant loci but most of them had effects in the order of 1% of the phenotypic variation in tick infestation. The lack of consistent and strong findings is not surprising. Counting ticks is difficult and time consuming thus studies resort to scoring systems, which are less precise than counts, and this can have an effect on heritability. Alternatively, the numbers used tend to be relatively small and studies are underpowered. The most robust report is that by Turner et al. (2010), who reported on a study in which ticks were counted and heritability was a respectable 37%, and which used 1,960 cattle. In contrast, Prayaga et al. (2009) used a scoring system, 900 animals and estimated heritability of tick score to be 9%. Furthermore, a study examining the genomic prediction for tick resistance in Braford (Brahman x Hereford/tick resistant x tick susceptible breed, respectively) and Hereford cattle in Brazil showed that genomic selection for tick resistant Braford cattle may be achievable (Cardoso et al., 2015). A recent trait tag-SNP approach by the same group reported 914 SNPs explaining more than 20% of the estimated genetic variance for tick resistance (Sollero et al., 2017).

Despite the challenges of the genomic approach to identifying either mechanisms or markers for host resistance to ticks in cattle, they have enabled the identification of allelic variation in genes that are very likely to influence the trait. The *ELTD1* gene (EGF, latrophilin, and seven transmembrane domain containing 1) was identified from GWAS in dairy and beef cattle (Prayaga et al., 2009; Turner et al., 2010). Its association with the host resistance phenotype was confirmed but its effect was limited to <1% of the total phenotypic variation in the trait (Porto Neto et al., 2011a). Similarly, haplotypes that included the *ITGA11* gene (integrin alpha 11) were significantly associated with tick burden and explained about 1.5% of the variation in the trait. Finally, the potential functional role of allelic variation in a gene identified by the same GWAS studies (Prayaga et al., 2009;

Turner et al., 2010) *RIPK2* (serine-threonine kinase 2) was further examined using knock out mice (Porto Neto et al., 2012). This gene is known to play an essential role in the modulation of innate and adaptive immune responses and it was found that it influenced the recognition of tick salivary antigens by mice.

Limited association of tick burden or phenotype to the genotype is currently available and large bovine genomic meta-analyses may contribute to the identification of within breed markers for tick resistance in the future.

VARIATION IN GENE EXPRESSION AMONG RESISTANT AND SUSCEPTIBLE HOSTS AND RELATIONSHIP WITH IMMUNE RESPONSES

Bioactive molecules secreted by *R. microplus* ticks into the skin of the host during attachment and blood feeding stimulate host effective responses. The variation in the mechanisms by which each host breed responds to each of these bioactive molecules likely results in different levels of resistance. It is well-established that the resistance to tick infestation is due to a complex set of responses, however the specific mechanisms and their relative importance continues to be the subject of debate.

Table 1 summarizes selected up-regulated genes including those that are potentially associated with immune responsiveness, blood coagulation, calcium regulation, and/or wound healing from several studies undertaken to date. The parameters of all of the studies differ from each other including: the number of biological replicates, the number of larvae used in infestations, the breeds and subspecies used, their prior exposure, the methodology and platform used to measure host responses (immunohistochemistry, microarray platforms, qPCR), the timing of sample collection, and the samples analyzed (skin or blood). Without undertaking a formal meta-analysis of the original data, we have attempted to summarize differences and similarities in relation to susceptible vs. resistant animals among reports. The similarities identified are presented diagrammatically in **Figure 1** with text listing commonly up-regulated cells or genes in susceptible (**Figure 1A**) and resistant (**Figure 1B**) cattle.

Gene expression studies have been undertaken using peripheral blood leukocytes (PBL) and skin tick bite sites. These studies have included host qPCR, EST libraries, microarray analyses and cDNA library/next generation sequencing. The findings are summarized in **Table 1**.

Studies on PBL suggest that resistant hosts are more likely to develop a stable T-cell-mediated response against *R. microplus*, while susceptible cattle demonstrated cellular and gene expression profiles consistent with innate and inflammatory responses to tick infestation (Kashino et al., 2005; Piper et al., 2009). The up-regulation of genes in tick susceptible cattle involved in inflammatory and other important immunological responses mediate a greater natural potential to develop higher pro-inflammatory responses in comparison to tick resistant animals.

Gene expression studies on skin taken from larval attachment sites have demonstrated that cytokines, chemokines, and complement factors were differentially expressed between naïve-skin and infested skin in susceptible Holstein–Friesian cattle. It was also found that immunoglobulin transcripts were differentially expressed in infested skin from Holstein-Friesian compared to resistant Brahman cattle. Therefore, the chronic pathology established in *B. t. taurus* cattle might facilitate the tick feeding process (Piper et al., 2010). In addition, extracellular matrix genes such as: keratocan, osteoglycin, collagen, and lumican were up-regulated in infested-skin from *B. t. indicus* resistant Brahman cattle. In a study involving coagulation in skin from resistant and susceptible cattle infested with *R. microplus* (Carvalho et al., 2010b), susceptible hosts had an increased blood clotting time at tick hemorrhagic feeding pools in comparison to normal skin and the skin of resistant hosts. Furthermore, the host resistant phenotype affects the transcript of genes associated with anti-hemostatic proteins in the salivary glands of *R. microplus,* with transcripts coding for anti-coagulant proteins expressed at a higher level in ticks fed on susceptible hosts compared to ticks fed on resistant hosts (Carvalho et al., 2010b).

In the same experiment where PBL gene expression was studied in infested indicine and taurine cattle, the authors examined the response to infestation and larval attachment in bovine skin (Piper et al., 2010). The susceptible cattle displayed an intense cellular inflammatory response at the tick attachment site, i.e., genes involved in inflammatory processes and immune responses including those which encode for matrix proteins were up-regulated in tick-infested susceptible cattle, but not in resistant hosts. Nascimento et al. (2010) constructed cDNA libraries from skin biopsies from resistant and susceptible cattle to evaluate the pattern of gene expression of three calcium-binding-proteins. The results showed that genes coding for translationally controlled tumor protein (*1-TPT1*), calcium channel protein transient receptor potential vanilloid 6 (*TRPV6*) and cysteine proteinase inhibitor (*CST6*) were highly expressed in susceptible cattle compared to resistant cattle (Nascimento et al., 2010). Also, a microarray study using samples from tick infested cattle to evaluate the profile of gene expression during *R. microplus* larvae attachment showed differentially expressed genes involved in lipid metabolism, inflammation control and impairment of tick infestation in resistant cattle (*B. t. indicus* Nelore) (Carvalho et al., 2014). Conversely, in susceptible cattle (*B. t. taurus* Holstein) the acute phase response appeared impaired but this study confirmed the up-regulation of calcium ion control genes which correlates with the calcium binding proteins reported by Nascimento et al. (2010). Franzin et al. (2017) also report the up-regulation of protein S100G, another calcium binding protein, in susceptible cattle. An earlier qPCR study showed higher up-regulation of calcium signaling genes in a tick resistant composite breed in response to ticks most predominantly at 24 h post-infestation with larvae (Belmont Red) (Bagnall et al., 2009). Calcium signaling, calcium binding and/or calcium ion control genes and their functions in tick resistant and tick susceptible cattle warrant further specific examination.

Another host gene expression study was reported recently demonstrating that resistant cattle (*B. t. indicus* Nelore breed)

TABLE 1 | Summary of differentiating characteristics of tick susceptible vs. tick resistant cattle (bold font indicates correlation between studies of certain transcripts and or other markers—genetic, cellular, immunohistochemistry) in response to *Rhipicephalus microplus* ticks.

Methodology (cattle breeds compared)	Susceptible	Resistant	Main conclusion and reference
Cellular IgG isotyping; Cattle naturally infested on pasture; susceptible cattle treated with acaricide when tick counts were >500. Cattle vaccinated with GAVAC. (Holstein and Aberdeen *B. t. taurus* vs. Nelore *B. t. indicus*)	IgG1 and IgG2 decrease (anti-salivary gland antibodies only)	IgG1 and IgG2 no change	IgG suppression by tick infestation on susceptible cattle. Kashino et al., 2005
Genetics study, cattle naturally infested in pasture and at 10–14 months infested with 10,000 tick larvae, PCR to determine allele frequency (F2 population of composites *B. t. indicus* Gyr 1/2 x *B. t. taurus* Holstein 1/2)		**BoLA Class II** DRB3.2	The MHC Class II DRB3.2 allele is associated with tick resistance. Martinez et al., 2006
Microarray gene expression, natural tick infestation for ∼6 months ("naïve" sample taken post field infest), followed by 5 artificial tick challenges over 17 months with 20,000–25,000 larvae (Hereford Shorthorn cattle- tropically adapted *B. t. taurus*)	**B-cell CLL/lymphoma 10** **Collagen**, pro α **Apolipoprotein** **Keratin** **Immunoglobulins** IL13 receptor α 1 precursor	**Cathepsin** B **Collagens** (type I α2; type III pro; type VI, α 3) **Complement** component 1, q subcomponent, α	Susceptible cattle upregulated immune responsive genes and those involved in protein synthesis. **BDA20, Odorant Binding Protein (OBP)** and the dendritic cell protein-B5 over expressed by both groups. Wang Y. H. et al., 2007
Genetic microsatellite and cytokine qPCR of 4 breeds, 17 months natural tick infestation (*B. t. indicus* Nelore, Composite Canchim 5/8 *B.taurus* 3/8 *B. t. indicus* × Nelore, *B. t. taurus* Aberdeen Angus × Nelore, and *B. t. taurus* Simmental x Nelore)	IL4	**IL8** and **IL2** down-regulated	Tick counts were associated with IL4, IL2 is down-regulated in 3 genetically different groups of infested bovines compared to pre-infestation, and IL8 was down-regulated in resistant bovines compared to susceptible animals Regitano et al., 2008
RT-PCR gene expression of peripheral white blood cells, previous natural exposure to ticks, 7 weekly artificial infestations of 10,000 larvae while held in tick infested pasture. (Holstein Friesian *B.taurus* vs. Brahman cattle *B. t. indicus*)	Toll-like receptor pathway (TLR5, TLR7, TLR9, **NFKBp50**, MyD88, Traf-6, CD14, IL-1β) Chemokine ligands and receptors (**CCL2**, CCL26; CCR-1) **IL-10** **bovine dander allergen 20 (BDA20)**		15 transcripts increased significantly in the susceptible breed to suggest innate inflammatory processes Piper et al., 2008
Cellular, RT-PCR and microarray gene expression (microarray) of leukocytes–infestation as described above (Piper et al., 2008) (Holstein Friesian *B. t. taurus* vs. Brahman cattle *B. t. indicus*)	Higher WBC counts Low hemoglobin CD14+ and **MHC Class II** (macrophage cells) High tick specific immunoglobulins (larvae, adult female salivary gland and gut)—high IgG1 **CXCL10**	**CD4+, CD25+** and **γδ T cells** **IL2**, IL2Rα, IL4Rα, TNFα, CCR-1, CCR-7, CXCL4, CD28, CD3E CD40 ligand	Susceptible cattle produce an innate inflammatory type response with high IgG1 titers suggesting also a T cell response. Resistant cattle produce a stable T cell mediated response. Piper et al., 2009
RT-PCR gene expression of calcium signaling genes in skin biopsies. Natural field exposure to ticks, followed by treatment and at 8 months artificial infestation of 10,000 larvae. Skin biopsies at 0, 3, and 24h post infestation (Belmont Red—composite breed ∼ 1/2 *B. t. taurus* and 1/2 *B. t. indicus*, tropically adapted)		**Calcium signaling genes** (significant at 24 h post-exposure): *AHNAK* nucleoprotein (desmoyokin) *CASQ* (Calsequestrin) **IL2** (Interleukin-2) NFAT2CIP (nuclear factor of activated T-cells, calcineurin-dependent) PLCG1 (phospholipase C, γ1)	The significant elevation of some calcium dependent genes following tick exposure suggests that the calcium pathway might be responsive to parasite exposure and could contribute to host immune response. Bagnall et al., 2009

(Continued)

TABLE 1 | Continued

Methodology (cattle breeds compared)	Susceptible	Resistant	Main conclusion and reference
Protein and mRNA expression- infestation as described above (Bagnall et al., 2009) of skin biopsies at 0, 3 and 24 h post-infestation. (Belmont Red – composite breed ~ 1/2 *B. t. taurus* and 1/2 *B. t. indicus*, tropically adapted)		**Keratins** lipocalin 9 epidermal barrier catalyzing enzyme transglutaminase 1 transcriptional regulator B lymphocyte-induced maturation protein 1 lipid processing proteins	Resistant cattle have physically stronger epidermal layers of the skin Kongsuwan et al., 2010
Host gene expression microarrays (skin); infestation as described above Piper et al., 2008 (Holstein Friesian *B. t. taurus* vs. Brahman *B. t. indicus* cattle)	**Chemokine ligands** (C-X-C motif) 2 and 5; (C-C motif) 2 and 8 – CXCL2, CXCL5, **CCL2**, CCL8 **Complement** component 1 and 3; Complement factor D (adipsin) **IL8** **Major histocompatibility complex Class II DRB3** Chemokine Regakine 1 (chemotactic activity for neutrophils) Spleen trypsin inhibitor; Plasminogen activator, tissue; Serpin peptidase inhibitor, clade F; Proapoptotic caspase adapter protein (inflammatory role, recruitment of immune cells to infected tissue); Peptidoglycan recognition protein 1 (inducer of TNFα and IFN γ); **Apolipoprotein D** **Lysozyme** Prostaglandin D2 synthase (inhibitor of platelet aggregation)	Keratocan, osteoglycin, lumican, **Collagen**, (types I and III); Lysyl oxidase-like 4 (formation of collagen) Down-regulated **Cytochrome P450** (**CYP4F3**, CYP11A1) Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin); Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma); Secreted phosphoprotein 2 (cystatin); Procollagen C-endopeptidase enhancer Lipid metabolism Amino acid metabolism Oxidoreductases	Genes involved with inflammatory processes and immune responsiveness upregulated in susceptible cattle. Genes encoding consistuents of the extracellular matrix were up-regulated in resistant cattle. Piper et al., 2010
qPCR analysis after artificial tick infestation, skin biopsies from tick lesions. (Gyr -*B. t. indicus* × Holstein - *B. t. taurus*)	**Calcium-dependent signal transduction** (**S100A7)**, histamine releasing protein (TPT1), epithelial calcium channel 2 (TRPV6) cysteine proteinase inhibitor (CST6)		Susceptible cattle develop hypersensitive reaction which is not protective Nascimento et al., 2010
EST analysis, natural tick infestation followed by acaricide treatment and artificial infestation of 10,000 larvae, biopsies collected at Days 5 and 12 from base of tail (F2 population from Gyr -*B. t. indicus* × Holstein - *B. t. taurus*)	CD44 antigen (lymphocyte activation) CD63 antigen (marker for activated basophils and IgE-mediated allergy) ADAM metallopeptidase **odorant binding protein** poly A binding protein ribosomal proteins	MHC Class antigen 1 **Cathepsin** L2 precursor **Collagen** (type I alpha; type III alpha) **Keratins** Ribosomal proteins	Structural proteins and MHC Class I in resistant cattle; Immune response transcripts in susceptible cattle Nascimento et al., 2011
Immunohistochemistry, infestation of Holstein Friesian and Brahmans as described by (Piper et al., 2008) above. Infestation of tick naïve Santa Gertrudis cattle—10,000 larvae artificially weekly for 13 weeks followed by natural infestation on pasture (Holstein Friesian *B. t. taurus* vs. Brahman *B. t. indicus*) (Composite Santa Gertrudis breed *B. t. taurus* 5/8 × *B. t. indicus* 3/8)	CD45, CD45RO (Holstein-Friesian) Mixed CD45 and CD45RO reactions (Santa Gertrudis)	**CD4+,** CD8+, **CD25+** and **γδ T cells** **MHC Class II cells** Mixed CD45 and CD45RO reactions (Santa Gertrudis)	Higher number of γδ T cells present in the skin of tick naïve resistant cattle. Epitopes recognized by some antibodies might not be present on the cell populations from all breeds of cattle or might be expressed in different levels. Santa Gertrudis resistance associated with lower cellular reaction at the larval attachment sites. Constantinoiu et al., 2010, 2013; Jonsson et al., 2014

(Continued)

TABLE 1 | Continued

Methodology (cattle breeds compared)	Susceptible	Resistant	Main conclusion and reference
Microarray gene expression (skin), natural infestation on pasture (10 months), acaricide treatment, followed by artificial infestation 10,000 larvae, skin biopsies 0, 24 and 48 h post-artificial infestation. (F2 of composite Gyr -*B. t. indicus* × Holstein - *B. t. taurus*)	**Calcium ion control genes** **Cytochrome P450** (CYP4F3)—**leukotriene** (allergy inducing chemical mediator) degradation	**Complement factors** 1, 2 and 3 complexes (C1, C2, C3); complement factor 4 binding protein; complement factor B properdin, galectin-1 Coagulation Factor XIIIa Lipid metabolism	Lipid metabolism in inflammation control in resistant cattle. Acute phase response impaired in susceptible cattle. Carvalho et al., 2014
RT-PCR gene expression of peripheral white blood cells, natural tick infestation followed by acaricide treatment, subsequent artificial infestation with 20,000 larvae, blood samples taken at 0, 24, and 48 h post-infestation. (F2 of composite Gyr -*B. t. indicus* × Holstein - *B. t. taurus*)	**CXCL10** and CXCL8 were down-regulated	**CD4+, CD25+** and **γδ T cells** **IL10**, FoxP3, **CXCL10**, CD25	A correlation between T γδ cell activity and immunological mechanisms in resistant cattle Domingues et al., 2014
Cellular and humoral responses (blood), 10,000 larvae artificially weekly for 13 weeks followed by natural infestation on pasture, blood collected 21 days post 1st infestation and weekly to measure changes in measure parameters (Composite Santa Gertrudis breed *B. t. taurus* 5/8 x *B. t. indicus* 3/8)	High tick-specific (anti-salivary and gut, anti-larval) **IgG1 titers**; variable IgG2 responses Decreased hemoglobin, decreased RBC count Increased **CD25+**, **CD4+**, WC1+	No IgG2 Increased **CD4+ cells** (prior to infestation) Decreased CD3+, **CD4+**, CD8+ Increased **MHCII+**, WC3+ and CD14+ cells	A non-protective high level of IgG1 in tick susceptible cattle. Piper et al., 2017
Microarray gene expression (skin) and histology Acaricide treatment of calves prior to artificial tick infestation with 10,000, skin biopsies with and without feeding ticks were taken prior to infestation (day 0) followed by 2 days (larvae) and 9 days (nymphs). Biopsies without feeding ticks to emulate "stressed" phenotype. Selected immune response genes differentially expressed (>2-fold) by larvae in comparison to baseline and stress responses (no ticks) in susceptible and resistant cattle (Table S5). (Holstein *B. t. taurus* vs. Nelore *B. t. indicus*)	CD209 antigen (leads to **IL10** production) Fibroleukin (fibrinogen type 2) Secretogranin-2 Secretoneurin (chemotaxis of monocytes and eosinophils) CXC6 (downregulated); CCL3, CCL4 high affinity **immunoglobulin** epsilon receptor subunit alpha precursor (**NF-kB**, MAPK activation) **Complement factor** D **Complement** C1q tumor necrosis factor-related protein 7 precursor T-cell surface glycoprotein CD3 delta chain platelet-derived growth factor receptor-like protein precursor (wound healing) Serine/threonine-protein kinase 1 Secreted phosphoprotein 24 (endopeptidase, platelet degranulation) **Keratin** **Protein S100**-G (calcium binding) **Lysozyme**	CD3+ and **γδ T cells** WC1+ Activation of basophils interferon-induced GTP-binding protein Mx2 Interferon alpha-inducible proteins 6 and 27 IL3 **cathepsin** D precursor	Susceptible cattle produce more tick attracting volatiles. Resistant hosts expose ticks to an earlier inflammatory response. Franzin et al., 2017

up-regulated the expression of fewer genes encoding enzymes producing volatile compounds that render them less "attractive" to ticks compared with susceptible cattle (*B. t. taurus* Holstein breed) (Franzin et al., 2017). This finding is consistent with the theory associated with odor (Osterkamp et al., 1999) described above. The study also reported that resistant hosts when exposed to ticks mount an earlier inflammatory response than susceptible cattle (gene expression studies undertaken at 2 days post larval infestation using tick naïve cattle) (Franzin et al., 2017) which appears to disappear later (feeding nymphs at 9 days) but lingers in susceptible cattle.

Franzin et al. (2017) identified numerous novel immune response genes that were up-regulated in susceptible Holstein cattle including *FCER1A* the high affinity immunoglobulin epsilon receptor subunit alpha precursor which is known to be responsible for initiating an allergic response. The up-regulation

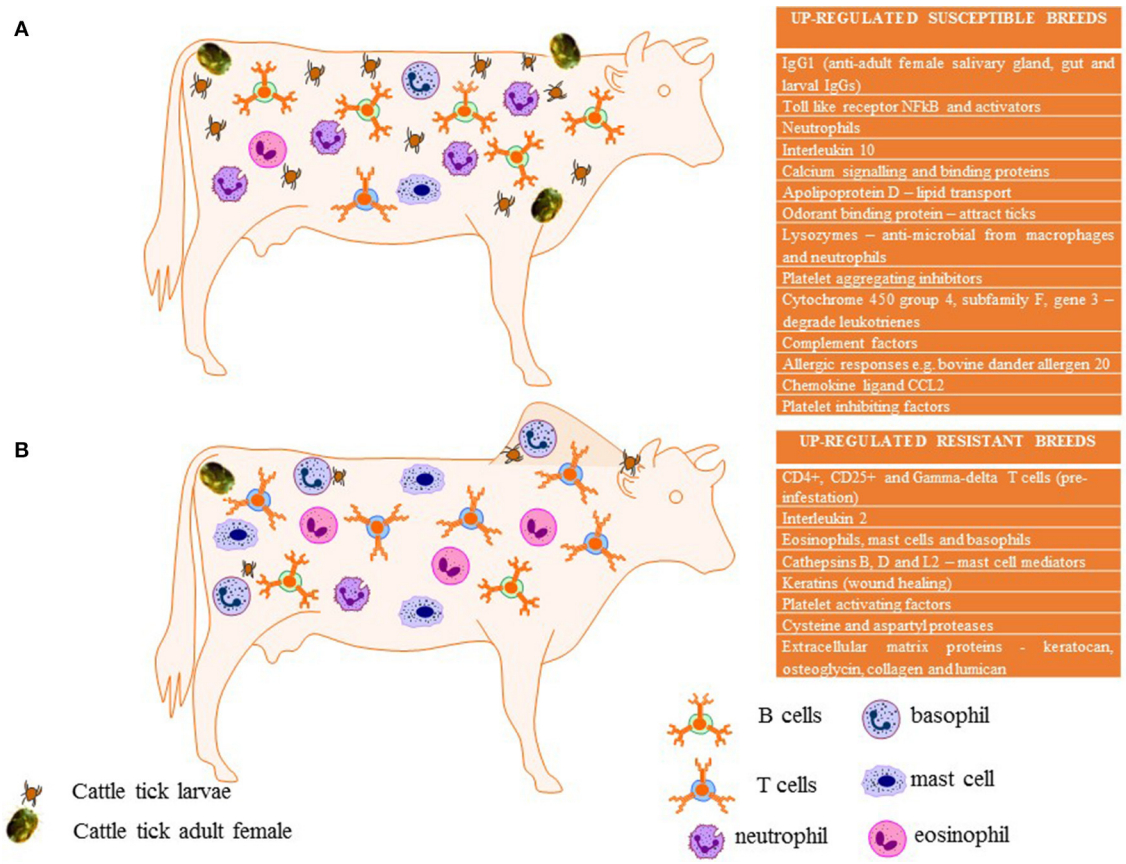

FIGURE 1 | Summary of expression and immunological profiles *commonly* associated in tick susceptible **(A)** compared to tick resistant breeds **(B)** of cattle as identified in **Table 1**. This diagram was created using images from Motifolio Inc.

of complement *C1QTNF7* (C1q tumor necrosis factor-related protein 7 precursor), an inducer of pro-inflammatory activators (Kishore et al., 2004) also concurs with the conclusions of tick susceptible cattle responding in a pro-inflammatory manner (Piper et al., 2010). Piper et al. (2008) had identified the up-regulation of Toll-like receptors (i.e., *NFkB*, nuclear factor kappa-light-chain-enhancer of activated B cells) that correlates with the activity of the complement C1Q identified by Franzin et al. (2017). In addition, *BCL10* (B-cell CLL/lymphoma 10) was up-regulated in susceptible cattle (Franzin et al., 2017) and is also known to activate *NFkB* (Wang M. et al., 2007). In addition fibroleukin is known to have a dual prothrombinase and immunoregulatory activity and was up-regulated in tick naïve (tick susceptible) cattle by larvae (Franzin et al., 2017). Immunoregulatory activity includes the suppression of T cell proliferation and cytokine production, mainly of Th1 and Th17 cells but not Th2 cells (Bézie et al., 2015), which is in contrast to other observations above suggesting that susceptible cattle mount a Th1 response. Functionally, Bézie et al. (2015) found that fibroleukin induced long-term allograft survival in a rat model through regulatory B cells which in turn suppress the proliferation of CD4+T cells. These cells are up-regulated in tick resistant cattle in the majority of studies undertaken. To summarize the results presented by Franzin et al. (2017), early responses to larvae (48 h post-infestation by naïve tick susceptible cattle) appear to show a mixed Th1 and Th2 response with tick susceptibility being associated with a Th1 response.

Several differences are noted among the comparisons of gene expression studies and as noted by Regitano et al. (2008) we generally concur that "*differences in gene expression of resistant cows compared to susceptible cows were breed-specific.*" However, there are some consistencies identified in the above studies. The presence of high densities of CD4+, CD25+, and γδ T cells are seen relatively consistently in resistant indicine cattle (see **Table 1**). The up-regulation of keratins and collagens is also common in resistant indicine cattle, with some divergent upregulation in susceptible breeds in fewer studies. The up-regulation of IgGs in susceptible cattle was reported by most researchers. MHC Class II and calcium binding proteins seem to be mostly up-regulated in susceptible breeds, with the latter commonly associated with susceptible breeds studied in Brazil. The expression of chemokine ligands varied greatly between studies and breeds with no identifiable consistency. Other genes that were up-regulated consistently in susceptible cattle include: apolipoproteins (lipid transport), lysozymes (anti-microbial, also found in macrophages and polymorphonuclear

neutrophils), Toll like receptors, *NFkB* and NFkB activators *C1QTNF7* (complement C1q tumor necrosis factor –related protein 7) and *BCL10* (B-cell CLL/lymphoma 10) and several complement factors. IL10 is considered an anti-inflammatory cytokine perhaps induced early in susceptible cattle but IL10 appears to be associated with long term resistance in *B. t. indicus* Gyr cattle in Brazil (Domingues et al., 2014; Franzin et al., 2017). Cytochrome P450 enzymes are a superfamily of hemoproteins known to be involved in the synthesis or metabolism of various molecules and chemicals within cells. A P450 gene called CYP4F3 (cytochrome 450 group 4, subfamily F, gene 3) is known to degrade leukotrienes which are the chemical mediators of allergic responses (Karasuyama et al., 2011). CYP4F3 was downregulated in the skin of tick exposed resistant cattle (Brahmans, Piper et al., 2010) yet up-regulated in the skin of susceptible cattle (Holsteins, Carvalho et al., 2014) thus showing a correlation for the activity of this enzyme in tick susceptible cattle. A summary of all of these factors is presented in **Figure 1**.

Differential expression of genes coding for other host modifying enzymes were associated with resistant and susceptible phenotypes (**Table 1**). Although not identified in more than one study, the up-regulation of these factors appears to correlate to the relevant phenotype and are thus worthy of further description. For example the following were identified in different studies as up-regulated in tick resistant breeds: Cathepsin B (Wang Y. H. et al., 2007), Cathepsin L2 precursor (cysteine proteases, mast cell mediators) (Nascimento et al., 2011), Cathepsin D (aspartyl protease, mast cell mediator) (Franzin et al., 2017), serine peptidase inhibitor clade A (inhibits neutrophil elastase), phospholipase A2, group VII (platelet activating factor) (Piper et al., 2010), coagulation factors, and procollagen C-endopeptidase enhancer (metalloprotease inhibitor) (Piper et al., 2010); and conversely in tick susceptible breeds: serine peptidase inhibitor clade F (negative regulation of inflammatory response), spleen trypsin inhibitor, plasminogen activator (serine protease which produces plasmin which catalyzes the degradation of fibrin polymers in blood clots), prostaglandin D2 synthase (platelet aggregation inhibitor) (Piper et al., 2010), and phosphoprotein 24 (endopeptidase associated with platelet degranulation) (Franzin et al., 2017) were up-regulated.

Comparative transcriptomic studies of different life stages (larvae and adult females) have shown that ticks respond differentially according to whether they are sensing or feeding on a tick-susceptible or tick-resistant breed of cattle. A microarray study based on a *R. microplus* EST database (Wang M. et al., 2007), using sensing larvae (not attached to the host but contained within a fabric bag and able to sense the host) and feeding, adult females, which were collected from naïve, tick-susceptible Holstein Friesian *B. t. taurus,* and tick-resistant Brahman *B. t. indicus* cattle has been reported (Rodriguez-Valle et al., 2010). Ticks that were feeding on resistant cattle demonstrated the up-regulation of serpin 2, lipocalins and histamine binding proteins. A recent study utilized next generation sequencing to compare tick expression differences from larvae, nymph salivary glands and larval offspring of females - all fed on tick-resistant Nelore *B. t. indicus* and tick-susceptible Holstein *B. t. taurus* (Franzin et al., 2017). That study showed an increased number of transcripts that included evasins, immunosuppressant proteins, lipocalins (including histamine, serotonin, and odorant binding proteins), and reprolysin metalloproteases from ticks associated with susceptible cattle, and an increased number of chitinases and cysteine proteases from ticks associated with resistant cattle. The analysis also included larvae exposed to volatile compounds prepared from the same breeds of cattle and showed that resistant breeds produce less attractive volatiles (Franzin et al., 2017). The latter was thought to be correlated to the fact that ticks on susceptible cattle up-regulated odorant binding proteins. Not enough studies have been undertaken to draw any similarities between the molecular profiles of ticks from susceptible vs. resistant hosts particularly when different stages and breeds are compared. It is clear however that the tick gene expression profiles associated with tick-resistant vs. tick-susceptible cattle appear to be divergent.

Acquired immunity to tick infestation is established after a period of susceptibility to a primary infestation (Wagland, 1978). As confirmed above, gene expression profiles from tick resistant breeds appear to be congruent with a T-cell mediated response, while susceptible cattle exhibit innate and inflammatory responses with higher levels of tick specific IgG1. One quite consistent fact is that resistant cattle appear to be primed to respond to ticks with a higher presence of γδ T cells in the skin of tick naïve resistant cattle in comparison to susceptible. A formal meta-analysis of all gene expression studies where the data are in the public domain is theoretically possible but would likely be compromised by variation in the conditions of each of the studies. Each gene expression comparison study was undertaken using quite different conditions. The variable factors include: environment, season, naïve vs. tick exposed cattle, the use of acaricides post exposure prior to artificial infestations, infestation protocols including frequency and numbers of larvae, and comparative breeds including within breed studies.

CELLULAR PHYSIOLOGY ASSOCIATED WITH HOST RESISTANCE

Granulocytes (or polymorphonuclear leukocytes) are white blood cells characterized by the presence of granules in their cytoplasm and which perform different immune functions. They include neutrophils (most abundant), eosinophils, mast cells, and basophils. The inflammatory profile of the host skin contributes to resistance or susceptibility to tick infestation. Marufu et al. (2013) showed that tick susceptibility (*B. t. taurus* Bonsmara cattle) is associated with an immediate type hypersensitivity reaction. On the other hand, the resistance phenotype was linked to a delayed hypersensitivity reaction in *B. t. indicus* Nguni breed (Marufu et al., 2013) confirming the observations of Constantinoiu et al. (2010) with *B.t.taurus* Holstein Friesian (susceptible) and *B.t.indicus* Brahman (resistant) cattle. **Table 2** summarizes the cellular profiles obtained in response to ticks with differences associated with: the tick stage (larvae, nymph

and adults), use of previously tick-exposed or naïve cattle, timing of sample collection post-infestation, blood or skin samples, and methodologies used.

Neutrophils are usually found in the blood stream and are the most abundant phagocyte. During host infection, neutrophils are quickly recruited to the site of infection i.e., skin in response to tick infestation. Neutrophils may favor infestation by destroying the extracellular matrix around the attached tick and thereby allowing access to blood for feeding (Tatchell and Bennett, 1969; Tatchell and Moorhouse, 1970). Similar numbers of neutrophils were found to be recruited in Holstein (susceptible, *B. t. taurus*) and Nelore cattle (resistant, *B. t. indicus*) (Carvalho et al., 2010a), with slightly higher neutrophils in the skin of susceptible cattle at early stages of infestation using naïve cattle of the same breeds (Franzin et al., 2017). Marufu et al. (2014) showed higher counts of neutrophils at the attachment sites of *R. microplus* in both resistant and susceptible breeds compared to non-infested skin, with higher counts also found in tick susceptible Bonsmara cattle compared to tick resistant Nguni cattle (Marufu et al., 2014). Higher levels of neutrophils do not seem to show a protective role against *R. microplus* infestation and feeding larvae demonstrated a high ingestion of neutrophils in susceptible *B. t. taurus* Holstein Friesian cattle (Constantinoiu et al., 2010). Moreover, activated neutrophils lead to a calcium ion influx which could correlate with the common up-regulation of calcium binding proteins in susceptible cattle gene expression studies (**Table 1**). Overall, higher neutrophil densities in the skin at the site of a tick attachment appear to be associated with the tick susceptible phenotype.

Eosinophils have long been known to be associated with parasite infections and allergy, with several immune functions having been only recently elucidated. For example, recent evidence suggests that eosinophils suppress Th17 and Th1 responses via dendritic cell regulation and also activate basophil degranulation (Wen and Rothenberg, 2016). Eosinophils may influence the tick resistant phenotype due to their role in the translocation of mast cell histamine and lysosomal enzymes to the feeding site lesion, and by impairing tick attachment (Schleger et al., 1981). *B. t. taurus* show higher eosinophil counts during secondary infestations compared with *B. t. indicus* breeds in early studies (Tatchell and Moorhouse, 1968). Marufu et al. (2013) confirmed this observation with higher eosinophil counts in tick susceptible Bonsmara cattle, in contrast to other studies which showed higher eosinophil counts in tick resistant cattle (Nelore *B. t. indicus*) in Brazil and Australian Shorthorn breed (*B. t. taurus* tropically adapted cattle) (Schleger et al., 1976; Carvalho et al., 2010b). Using the same breeds as Carvalho et al. (2010b), Franzin et al. (2017) showed higher eosinophil counts in susceptible cattle during the first infestation of tick naïve cattle, yet higher eosinophil counts at nymph feeding sites in the tick resistant breed. Suppression of Th1 responses by eosinophils may be logically associated with the response of resistant cattle and may also correlate with mast cell activity.

Mast cells (including tissue basophils) are a multifunctional cell population involved in maintaining local homeostasis of connective tissue, control of blood coagulation and defensive functions of innate and adaptive immunity. In addition mast cell dysfunction is associated with several chronic allergic/inflammatory disorders (da Silva et al., 2014). Mast cells contain granules rich in histamine and heparin, and are the main effectors of allergic reactions. Host resistance to ticks appears to concur with mast cell functions such as allergic responses, wound healing and immune tolerance, and a potential mast cell dysfunction in tick-susceptible cattle. Most studies on cattle have shown higher mast cell counts in resistant breeds in response to ticks (**Table 2**). However, one study comparing several breeds found that the Nelore *B. t. indicus* resistant breed had the highest number of mast cells in response to ticks while the Gyr *B. t. indicus* tick resistant breed had similar levels as two tick susceptible *B. t. taurus* breeds, Holstein and Brown Swiss (Veríssimo et al., 2008). In contrast, previously Gyr *B. t. indicus* cattle showed a higher number of mast cells in the dermis compared to susceptible European breeds (Moraes et al., 1992; Veríssimo et al., 2008). A few mast cell activators (da Silva et al., 2014) have been noted in resistant host gene expression studies above including Cathepsins B, D, and L2, platelet activating factors, complement factor C3, IL10, IL2, and TNFα (**Table 1**).

Basophils are known for their allergic effector function and were first described in response to ticks by Trager in 1939. Basophils notably accumulated at tick bite sites causing cutaneous hypersensitivity (Trager, 1939). In the 1950s, it was confirmed that histamines are stored in basophil granules (Graham et al., 1955). Basophils have been associated with immunity against parasites including ticks and helminths, reviewed by Karasuyama et al. (2011). It is thus logical as described above for mast cells, that high levels of circulating basophils would be associated with the tick resistant phenotype, which has been confirmed by two groups (Carvalho et al., 2010a; Marufu et al., 2014; Franzin et al., 2017). The release of histamine has been postulated as a mechanism which dislodges feeding ticks. This was confirmed when mice were injected with cultured mast cells which resulted in tick rejection following infestation of *Haemophysalis longicornis* ticks, with no tick rejection in mast cell deficient mice (Matsuda et al., 1987). A recent review of basophil functions confirms their effector role in allergic reactions, however basophils also share features of innate and adaptive immunity (Steiner et al., 2016) which again associates well with the tick resistant bovine phenotype. Steiner et al. (2016) examined the ever expanding function of basophils including the modulation of several cytokines, Toll-like receptors and chemokines (including CXCL10, CCR1, CCR7 described as up-regulated in certain tick resistant cattle studies, **Table 1**).

The correlation of granulocyte activity (and their immune effector mechanisms) in the skin of tick resistant cattle could further be examined to attempt to correlate immunity with gene expression studies described above. The existence of tick histamine-binding salivary lipocalins have been associated with inhibiting histamines from their receptors (Paesen et al., 1999; Mans et al., 2008) with specific lipocalins up-regulated in resistant vs. susceptible breeds in response to ticks (Rodriguez-Valle et al., 2013). In addition, the central role of histamine in tick resistance was supported by antihistamine administration to cattle which led to increased tick loads on

TABLE 2 | Summary of inflammatory profile between susceptible and resistant breeds of cattle.

Methodology (cattle breeds compared)	Susceptible	Resistant	Main conclusion and reference
Histology of skin using larvae—several different breeds (*B. t. taurus* vs. *B. t. indicus*)	Higher **eosinophils** during secondary infestation		Wide variations in the magnitude of the lesions between different hosts Tatchell and Moorhouse, 1968
Skin histology at larval tick lesions, 3 h feeding on previously tick exposed cattle (tropically adapted Illawarra Shorthorn *B. t. taurus* susceptible vs. resistant)		Higher **mast cells** Higher **eosinophils** Higher **neutrophils**	Degree of mast cell disruption, eosinophil concentration and degranulation and the extent of epidermal vesiculation were all significantly higher in highly resistant hosts. Schleger et al., 1976
Skin biopsies from tick lesions (Gyr -*B. t. indicus* vs Holstein - *B. t. taurus*)	Lower mast cell counts	Higher **mast cell** counts	Mast cells important in host resistance Moraes et al., 1992
Dermal (upper and deep) mast cell counts (Nelore and Gyr -*B. t. indicus* vs. Holstein, Brown-Swiss and Jersey - *B. t. taurus*)	Holsten and Brown Swiss similar to Gyr Lowest mast cell counts – Brown-Swiss	High **mast cells** cell counts– Nelore Gyr - Similar to Holstein and Brown Swiss	Negative correlation between the number of mast cells in the upper dermis and tick counts. Mast cells important in tick resistance. Veríssimo et al., 2008
Immuno staining of skin sections (Brahman *B. t. indicus* vs. Holstein-Friesian *B. t. taurus*)	Higher **neutrophils**	Delayed hypersensitivity	γδ T cells might have a role in limiting the inflammatory process and preservation of the skin homeostasis in *B. t. indicus* cattle. Constantinoiu et al., 2010
In vitro binding of leukocytes and skin histology (Nelore -*B. t. indicus* vs. Holstein - *B. t. taurus*)	Adhesion molecules: leukocyte adhesion glycoprotein 1	Higher **basophils** Higher **eosinophils** Adhesion molecules: E-selectin (promotes adhesion of memory T cells)	Resistant cattle had significantly higher counts of basophils and eosinophils compared to susceptible breeds. Adhesion molecules indicate differences in resistant and susceptible hosts. Carvalho et al., 2010a
Cutaneous hypersensitivity responses to tick larval antigens in previously exposed cattle (Bonsmara *B. t. taurus* vs. Nguni *B. t. indicus*)	Intense cutaneous hypersensitivity response	Delayed hypersensitivity response	Marufu et al., 2013
Cellular responses to adult *R. microplus* in skin biopsies (Bonsmara *B. t. taurus* vs. Nguni *B. t. indicus*)	Higher basophils (lower than resistant cattle) Higher mast cells (lower than resistant cattle) Higher mononuclear cells (lower than resistant cattle) Higher **neutrophils** Higher **eosinophils**	Higher **basophils** Higher **mast cells** Higher mononuclear cells Higher neutrophils (lower than susceptible cattle) Higher eosinophils (lower than susceptible cattle)	Cellular responses showed higher counts of basophils, mast and mononuclear cells and lower neutrophil and eosinophil counts in resistant breeds. Marufu et al., 2014
Histopathology larvae and nymph with naïve cattle. (Holstein *B. t. taurus* vs. Nelore *B. t. indicus*)	Higher **eosinophils** (larvae) Higher **mast** cells (nymph, slightly higher compared to larvae bite site)	Higher **mast** cells (larvae), mast cells degranulated and decreased (nymph) Higher **eosinophils** (nymph) Higher **basophils** (nymphs)	Resistant hosts expose ticks to an earlier inflammatory response which is delayed in susceptible breeds. Franzin et al., 2017

Bold fonts highlight common trends across different publications.

both *B. t. taurus* (Hereford) and *B. t. indicus* (Brahman) breeds (Tatchell and Bennett, 1969).

In summary, these studies confirm that cattle breeds behave differently during *R. microplus* infestation, presenting various intrinsic mechanisms to provide protection against ticks. Overall, resistant cattle appear to be associated with increased mast cells, eosinophils, and basophils in the skin, while the recruitment of neutrophils is potentially associated with tick susceptibility. The release of histamines from these cells appears to be associated with the resistant phenotype. Histamine is thought to inhibit

tick attachment and leads to itching, which subsequently leads to more grooming and tick removal.

MICROBIOTA ROLE IN TICK RESISTANCE

The microbiome contributes to the architecture and function of tissues, host energy metabolism, and also plays an important role in the balance between health and disease as demonstrated recently for intracellular protozoa (Yilmaz et al., 2014; Bär et al., 2015). In vertebrates, semiochemicals can be generated by the activity of the microbiota upon amino acids, short chain fatty acids or hormones secreted in body emissions, such as sweat, tears, sebum, saliva, breath, urine, and feces (Amann et al., 2014; Fischer et al., 2015). This volatile repertoire is of paramount importance with evidence that they can direct host-vector specificity (Smallegange et al., 2011; Davis et al., 2013). The variation in the host chemical production thus causes differential attractiveness to vectors between species and in turn, the bacterial profiles differ according to human genetic background (Benson et al., 2010; Prokop-Prigge et al., 2015). Microbial composition can be affected by diet and other management strategies, such as those used for beef and dairy cattle (Durso et al., 2012; Thoetkiattikul et al., 2013). The differences in the composition of their microbiota (Dowd et al., 2008; Durso et al., 2010, 2012; de Oliveira et al., 2013; Mao et al., 2015), as well as the distinct volatile organic compounds (VOC) produced by *B. t. taurus* and *B. t. indicus* cattle may corroborate the contrasting tick infestation phenotypes observed among these animals (Steullet and Gnerin, 1994; Osterkamp et al., 1999; Borges et al., 2015; Ferreira et al., 2015). Although a few studies have demonstrated that host VOCs play a role in attracting *Rhipicephalus* spp. ticks (Louly et al., 2010; Borges et al., 2015; de Oliveira Filho et al., 2016; Franzin et al., 2017), research is still needed to investigate the interrelationship of host microbiota with VOC production related with tick attraction or repulsion. This may potentially reveal yet other factors contributing to tick susceptibility thereby presenting new opportunities to develop control methods for the livestock ectoparasite, *R. microplus*.

FUTURE RESEARCH AGENDA

Understanding the mechanisms behind genetic resistance to ticks and tick-borne diseases in livestock could improve breeding programs to develop cattle that are more resistant and productive (reviewed by Mapholi et al., 2014). Research to identify host genetic markers associated with tick susceptibility or resistance have been limited and compounded by the comparison of local breeds in different geographic regions as summarized here in this review. In addition, several studies reviewed here applied gene expression analysis of tick resistant breeds such as Nelore or Brahman. Brahman cattle have diverged through "up-breeding" in Australia and are thought to contain ∼7–10% *B. t. taurus* in their genomes (Bolormaa et al., 2011), whereas the Nelore breed is viewed as highly pure *B. t. indicus* in comparison (<1% *B. t. taurus* content). High throughput genomics is increasingly affordable and thus prior to the identification of tick resistance markers, it would be practical to first determine the genomic differences between and within breeds under study.

Further research assessing tick:host preference mechanisms are still needed. Whether these can be manipulated to protect susceptible cattle from ticks is yet to be determined. The volatile organic compounds of susceptible cattle could be influenced by the microbiome which in turn may be controlled by diet. Recently it was demonstrated that butyric acid (also a VOC, commonly found in feces) stimulates bovine neutrophils and potentiates platelet activating factors thus modulating the innate immune response (Carretta et al., 2016). Indeed, to identify links between tick host attraction and bovine immune responses would be interesting. The potential to manipulate volatiles through probiotic treatments and/or diet could be a future option for tick control.

Within the host immune studies compared in this review, it was apparent that the conditions of the experiments preclude direct comparisons of in particular, gene expression data. Nonetheless some similarities were identified. Future research could focus on proteomic analyses of tick lesions between resistant and susceptible breeds with the recommendation to use tick naïve cattle with multiple skin sampling from early infestations (i.e., initial attachment of larvae) until tick resistance is achieved after several infestations. The studies reported most likely are hampered by the costs associated with long cattle experiments. This could be why many studies held cattle in tick infested pastures during artificial tick infestations as this is the most economical option for long term studies.

Tick vaccines can potentially protect the host from tick infestations and tick borne diseases. This review concentrated less on the development of tick vaccines as it was considered that to develop a successful tick vaccine it would be wise to understand the most common immune effectors to emulate this outcome when using a new anti-tick vaccine candidate(s). Perhaps CRISPR/Cas9 gene editing technologies (parasite CRISPR/Cas9 models recently reviewed by (Cui and Yu, 2016) could be manipulated to examine host-tick relationships in order to demonstrate the most effective tick resistance pathway(s) to be exploited for vaccine development. Indeed CRISPR/Cas9 host editing to favor tick resistance could also be exploited in the future. Further insights into the immunomodulatory processes between ticks and tick susceptible/resistant hosts could identify major genes which would facilitate tick control strategies and the development a broad-spectrum anti-tick vaccine.

CONCLUSIONS

Taking advantage of recent advances from new approaches and technologies as applied to the field of vector biology, such as transcriptomics, proteomics, immune-molecular characterization, elucidation of naturally acquired resistance, and the development of innovative arthropod and animal models, may lead to improved investigations of naturally acquired resistant breeds against tick and tick-borne pathogens. Immune-proteomic, sialotranscriptome and

reverse genetics/gene editing (RNAi, CRISPR/Cas9) may help to identify new vaccine candidates that resist ticks and tick-borne pathogens. By understanding the tick:host interface and the most common denominators of immunity to ticks, this acquired immune response could be manipulated to improve the efficacy of novel anti-tick vaccines. Conversely, the knowledge obtained may assist in the selection of tick resistant cattle or the manipulation of susceptible cattle to develop a protective tick immune response. Furthermore, the in depth analysis of host microbiota and volatile organic compounds could lead to probiotic or diet changes or inhibitory chemicals which could render susceptible cattle less attractive to ticks. Future research may lead to a combination of several of these technologies as novel tick and tick-borne disease control options by first identifying viable biological targets and dissecting pathways leading to vaccination or pharmaceutical therapies or cattle management opportunities for tick control.

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AA, GR, and AT conceived the research review. AT prepared the comparative Tables/Figures and with NJ revised all sections written by other co-authors. GRG, AZ, TM and NJ wrote or contributed to specific sections within the review.

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REVIEW
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The Essential Role of Tick Salivary Glands and Saliva in Tick Feeding and Pathogen Transmission

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As long-term pool feeders, ticks have developed myriad strategies to remain discreetly but solidly attached to their hosts for the duration of their blood meal. The critical biological material that dampens host defenses and facilitates the flow of blood—thus assuring adequate feeding—is tick saliva. Saliva exhibits cytolytic, vasodilator, anticoagulant, anti-inflammatory, and immunosuppressive activity. This essential fluid is secreted by the salivary glands, which also mediate several other biological functions, including secretion of cement and hygroscopic components, as well as the watery component of blood as regards hard ticks. When salivary glands are invaded by tick-borne pathogens, pathogens may be transmitted via saliva, which is injected alternately with blood uptake during the tick bite. Both salivary glands and saliva thus play a key role in transmission of pathogenic microorganisms to vertebrate hosts. During their long co-evolution with ticks and vertebrate hosts, microorganisms have indeed developed various strategies to exploit tick salivary molecules to ensure both acquisition by ticks and transmission, local infection and systemic dissemination within the vertebrate host.

Keywords: ticks, tick saliva, tick-borne pathogens, tick salivary glands

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INTRODUCTION

Ticks are obligate hematophagous arthropods and act as vectors of the greatest variety of pathogens including viruses, parasites, and bacteria (de la Fuente et al., 2008; Rizzoli et al., 2014). On a global scale, they represent the most important vectors of pathogens that affect animals, and are second only to mosquitoes where humans are concerned (Dantas-Torres et al., 2012). Their remarkable success as disease vectors is mainly related to their longevity, high reproductive potential and broad host spectrum for several species, as well as to their capacity to imbibe a very large quantity of blood over a relatively long period of time.

For most tick-borne pathogens (TBP), transmission to the vertebrate host occurs via the saliva, underscoring the importance of both salivary glands (SG) and saliva in the transmission process. During feeding, ticks inject saliva and absorb their meal in an alternating pattern through the same canal. They are pool feeders, ingurgitating all of the fluids that are exuded into the haemorrhagic pool generated by the bite. TBP are ingested by ticks during their feeding on infected hosts. From the midgut, TBP cross the digestive epithelium and invade the haemocoel, from which they can penetrate the SG epithelium to invade the SG. From there, TBP can be transmitted to a new host via saliva injected during a new blood meal (**Figure 1**).

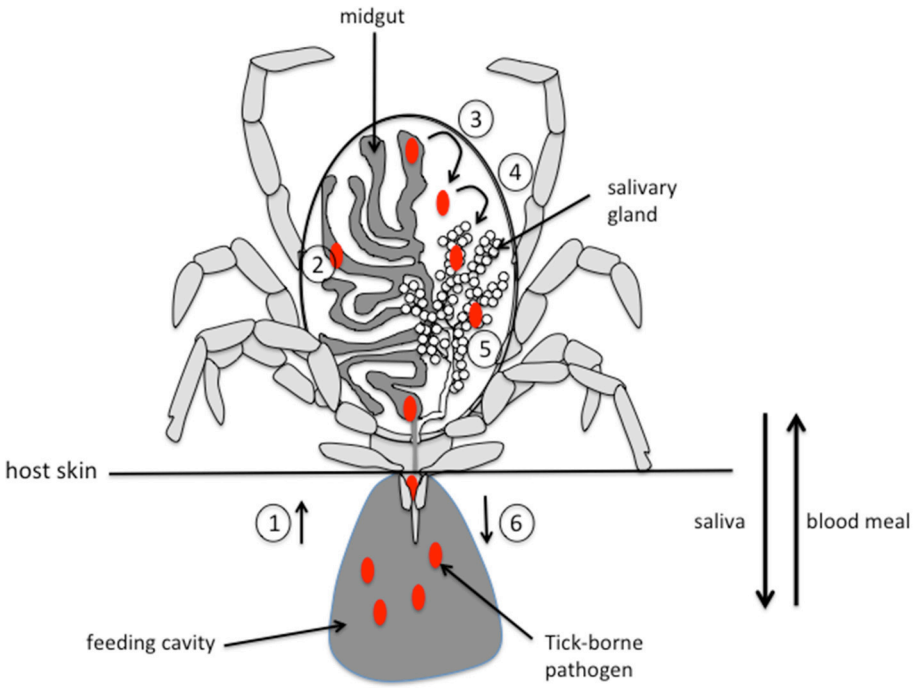

FIGURE 1 | Schematic representation of pathogen acquisition, development and transmission by a tick. (1) pathogens are ingested by the tick along with the blood meal during the bite. (2) Pathogens invade the midgut and, depending on the species, stay in the midgut until the next feeding or immediately cross the epithelium of the digestive tract (3) to invade the tick body. (4) Pathogens move into the salivary glands by crossing the epithelium and invade the acini (5). (6) Pathogens are injected into a new host during feeding, along with saliva that counteracts host hemostasis, inflammation and immune responses, thus facilitating pathogen infection of host. Please note that, for clarity, only half of the digestive tract and a single salivary gland are represented.

Vertebrates react to skin injury inflicted by tick bites by the formation of a haemostatic plug, vasoconstriction, inflammation and tissue remodeling related to wound healing. If unchecked, these processes would cause tick rejection and/or disrupt tick feeding, and arrest their further development. To facilitate the flow of blood and assure feeding, however, ticks have evolved a complex and sophisticated pharmacological armament that blocks pain and itch, inhibits haemostasis, and modulates innate and adaptive immune responses, angiogenesis and wound healing in their hosts (Francischetti et al., 2009; Mans, 2011; Kazimirova and Stibraniova, 2013; Štibrániová et al., 2013; Wikel, 2013; Valdes, 2014; Kotal et al., 2015; Chmelar et al., 2016b). It has been demonstrated that these molecules create a favorable environment for transmission, survival, and propagation of TBP within the vertebrate host (Wikel, 1999; Kazimirova and Stibraniova, 2013). In addition, several studies have reported that tick SG differentially express transcripts and proteins in response to pathogen infection, but only a few SG factors have been identified as directly implicated in pathogen transmission (Liu and Bonnet, 2014). In this review, we will summarize the essential role of both SG and saliva in tick biology as well as in TBP acquisition and transmission.

TICK SALIVARY GLANDS

The tick SG play multiple essential functions during both on- and off-host periods and represent a key route in transmission of TBP. The physiological activity and unique morphology of this tissue are intimately associated with adaptation of the tick to the parasitic lifestyle. Here we briefly describe the structure and function of SG in both ixodid and argasid ticks and discuss the most important findings with respect to the physiology of SG secretion in ixodid ticks.

Structure and Function of Salivary Glands

In both argasid and ixodid ticks the female SG consist of a large number of acini (or otherwise called alveoli) of three different types (type I, II, and III) in ixodid and two different types (type I and II) in argasid ticks. In addition, ixodid males possess a fourth type (type IV) of acini in their SG. Agranular type I acini connect almost exclusively to the anterior part of the main salivary duct, while the granular type II and III acini are associated with more distally located secondary and tertiary ducts, respectively (Coons and Roshdy, 1973; Binnington, 1978; Fawcett et al., 1981a; Walker et al., 1985; Sonenshine, 1991). The agranular acini are morphologicaly similar in both argasid and ixodid ticks, and generally comprise four distinct cell types: a single central lamellate cell, multiple peripheral lamellate cells, peritubular cells, and one circumlumenal cell (Needham et al., 1990; Sonenshine, 1991). In type II and III acini, in addition to various agranular cell types (such as epithelial, adlumenal, ablumenal interstitial, and neck cells), 7–9 various glandular cells (divided into the *a-f* types depending on tick species), enclosing the secretory granules have generally been recognized

(Fawcett et al., 1986; Sonenshine, 1991). The single adlumenal cell, also called the Cap or myoepithelial cell (Meredith and Kaufman, 1973; Krolak et al., 1982), lines the luminal surface of the type II and III acini in web-like fashion, and its contractions facilitate expulsion of the acinar contents into the connecting ducts during tick feeding (Krolak et al., 1982; Coons et al., 1994; Šimo et al., 2014b). During feeding of Ixodid females, the majority of the acinar cells of both type II and III acini undergo marked hypertrophy, resulting in overall increase in the mass of the SG (Binnington, 1978; Fawcett et al., 1986; Šimo et al., 2013). In particular, the lumen of type III acini greatly expands due to fluid uptake from the hemolymph (Meredith and Kaufman, 1973; Fawcett et al., 1981a; Kim et al., 2014), while in type II acini the cell bodies enlarge and the lumen remains proportionally smaller (Binnington, 1978; Walker et al., 1985).

Tick SG mediate diverse functions that ensure the tick's biological success during both on- and off-host periods. Here, we briefly discuss their role in absorption of moisture from unsaturated atmosphere, concentration of the nutrient portion of the blood meal by elimination of excess fluid and, finally, production of the cement that anchors the hypostome in the host skin. The crucial role of tick SG in secretion of biologically active molecules that facilitate acquisition of the blood meal and TBP development is elaborated later in this review.

During the fasting period off the host, the conservation of water is critical for ticks to avoid death due to desiccation. It is generally believed that the type I acini are actively involved in absorption of humidity from the surrounding environment. Under desiccating conditions these structures secrete a highly hygroscopic solution rich in Na^+, K^+, and Cl^- onto the surface of the mouthparts, which is subsequently swallowed back along with absorbed moisture (Knulle and Rudolph, 1982; Needham and Teel, 1986; Needham et al., 1990; Sonenshine, 1991; Gaede and Knülle, 1997). The absorptive property of type I acini was recently confirmed by an elegant experiment in which a fluorescent trace dye (Rhodamine 123) imbibed by desiccated *I. scapularis* females was shown to accumulate exclusively in type I acini (Kim et al., 2016).

Among blood-feeding arthropods, ixodid ticks are unique in the long duration of attachment to the host, which varies from several days up to weeks depending on life stage and tick species. When feeding, female ticks are capable of increasing their weight more than 100-fold due to the blood-meal uptake. At the same time, a large amount of excess fluid (including ions) is excreted back to the feeding site via SG, thus maintaining homeostasis (Balashov, 1972; Sonenshine, 1991; Sauer et al., 1995). In particular, water and ions from the digested blood meal cross the wall of the midgut into the hemocoel where they are taken up by SG and subsequently secreted via the salivary ducts back into the host. In 1973, Kaufman and Philips reported that in *Dermacentor andersoni* females 74% of the water and 96% of the sodium are expelled back to the host via this route. Several studies have suggested that the epithelium of type III acini is the site at which water and electrolytes from surrounding haemolymph gain access into SG (Meredith and Kaufman, 1973; Fawcett et al., 1981b). This hypothesis has been reinforced by a recent study in which the sodium-potassium pump (Na/K-ATPase) involved in formation of the sodium-rich primary saliva was evidenced in the epithelial cells of all three types of SG acini (Kim et al., 2016). In argasid ticks, the mechanism by which excess water is eliminated is different, being accomplished by the coxal glands, which are unique to this tick family (Binnington, 1975).

When attached to a host, the mouthparts of most of the ixodid ticks are encased by a cement cone, allowing the ticks to anchor themselves firmly in the host skin and simultaneously protecting the mouthparts from the host immune system (Kemp et al., 1982). The production of the cement cone has been observed exclusively in the ixodid lineage, although not all of the species from the *Ixodes* genus produce cement (Kemp et al., 1982; Mans, 2014). The origin of this substance, composed of polymerized and hardened glycine-rich proteins, lipids and certain carbohydrates, are the cells of the type II and III SG acini (Chinery, 1973; Jaworski et al., 1992). In addition to glycine-rich proteins, recent proteomic analyses of the cement cone from *Amblyomma americanum* evidenced multiple serine protease inhibitors and metalloproteases (Bullard et al., 2016), some of them considered to be promising anti-tick vaccine candidates due to their antigenic properties (Shapiro et al., 1987; Mulenga et al., 1999; Bishop et al., 2002).

Physiology of Salivary Gland Secretion

The tick SG is controlled by the nerves arising from the synganglion, the central nervous system of ticks (Kaufman and Harris, 1983; Fawcett et al., 1986; Šimo et al., 2009a,b, 2012, 2014a,b). Over the past four decades, pharmacological studies have revealed multiple chemical agents capable of inducing—either directly or indirectly—tick SG secretion (Kaufman, 1978; Sauer et al., 1995, 2000; Bowman and Sauer, 2004; Kim et al., 2014). Among these, catecholamines have been shown to be particularly effective activators of SG fluid secretion in both *in vivo* and *in vitro* assays (Kaufman, 1976; Lindsay and Kaufman, 1986; McSwain et al., 1992; Šimo et al., 2012). The downstream action of dopamine, the most potent activator of SG fluid secretion, has been studied in detail. It has been suggested that dopamine autocrine/paracrine signaling in tick SG (Kaufman, 1977, 1978; Šimo et al., 2011; Koči et al., 2014) activates two independent downstream pathways: cAMP-dependent transduction, which results in fluid secretion, and a calcium-dependent pathway leading to the secretion of prostaglandin E_2 (PGE_2) into the salivary cocktail. PGE_2may subsequently induce the exocytosis of anticoagulant proteins via paracrine signal in tick SG (Qian et al., 1998; Sauer et al., 2000). Dopamine acts via two different cognate receptors expressed in both type II and III SG acini: the D1 dopamine receptor and the invertebrate-specific D1-like dopamine receptor (InvD1L). Pharmacological study of these receptors has revealed that activation of the D1 receptor preferentially triggers the cAMP-dependent downstream pathway, while activation of the InvD1L exclusively causes mobilization of intracellular calcium (Šimo et al., 2011, 2014b). Based on immunohistochemical studies and subsequent physiological experiments performed on isolated SG, distinct structure-function relationships have been proposed for each of these receptors. In particular, the D1 receptor, which has been localized in cell junctions on the luminal surface of type II

and III acini, may regulate the inward transport of fluid into the acini, while the InvD1L receptor, found to be expressed in the axon terminals in proximity to the myoepithelial cell in type II and III acini, is presumed to be involved in the expulsion of the acinar context into the connecting ducts (Šimo et al., 2011, 2014b; Kim et al., 2014).

In addition to dopamine, several other pharmacological agents, such as octopamine, norepinephrine, γ-aminobutyric acid (GABA), ergot alkaloids and pilocarpine, have been shown to exert either a direct or indirect effect on salivary secretion; their precise mode of action, however, remains enigmatic (Needham and Pannabecker, 1983; Pannabecker and Needham, 1985; Lindsay and Kaufman, 1986).

Based on recent studies, it appears that ticks are capable of selectively controlling particular types of acini (and likely individual cells within the acini) via the neuropeptidergic network arising from their synganglion and in which the SG is connected (Šimo et al., 2009a,b, 2012, 2013, 2014a; Roller et al., 2015). In particular, two neuropeptides, myoinhibitory peptide (MIP) and SIFamide, have been found to be co-expressed in the pair of giant neurons in the synganglion whose axonal projections reach the basal regions of type II and III acini (Šimo et al., 2009a,b, 2011, 2012, 2013, 2014a). The SIFamide receptor, as evidenced by immunostaining, was found in proximity to the acinar valve (basal region of acini), suggesting its role in control of this structure (Šimo et al., 2009b, 2013). Using immunohistochemical approaches, two other putative neuropeptides, orcokinin and pigment dispersing factor (PDF), were found in two pairs of neurons innervating exclusively type II SG acini (Šimo et al., 2009a, 2012; Roller et al., 2015). In summary, multiple axonal projections, expressing diverse signaling molecules or their receptors, reach the individual SG acini and regulate a variety of essential processes, presumably in response to the tick's fluctuating physiological requirements.

TICK SALIVA

To understand the complex feeding biology of ticks, as well as the transmission of TBP, the composition of tick saliva must be molecularly resolved. Such resolution would underpin discovery of pharmacologically active compounds of clinical interest, protective antigens for anti-tick vaccines and antigens whose cognate antibody responses represent serological biomarkers of exposure to ticks. The first proteomic studies that addressed tick saliva date back to the first decade of the twenty-first century (Madden et al., 2004; Narasimhan et al., 2007a; Francischetti et al., 2008). Since then, transcript and protein profiling in tick SG have been applied to different developmental stages, sexes, and feeding stages of several species of hard and soft ticks. In addition, comparative analyses of tick SG have revealed that molecular expression varied according to tick life stage, sex, or behavior (Ribeiro et al., 2006; Anatriello et al., 2010; Diaz-Martin et al., 2013b), as well as according to the presence of pathogenic microorganism (Liu and Bonnet, 2014). It should be noted, however, that a minority of the salivary proteins have been functionally annotated, and that of these, the putative function has been verified for fewer than 5% (Francischetti et al., 2009). Nevertheless, these studies have already led to the discovery of multiple factors that contribute to successful tick feeding and evasion of the host immune and haemostatic defenses, which are reviewed below and presented in **Table 1**.

Impact of Tick Saliva on Host Haemostasis

Ticks have developed strategies to block different arms of the haemostatic system of their hosts, and it has been suggested that these antihaemostatic strategies have evolved independently in argasid and ixodid ticks (Mans et al., 2008; Mans, 2011). Haemostasis refers to a set of processes—including vasoconstriction, formation of a platelet plug, blood coagulation, and fibrinolysis—that together control blood loss following vascular injury and ensure normal blood flow (Hoffman et al., 2009). After vascular injury, platelets adhere to exposed subendothelial tissue and are activated, principally owing to the engagement of platelet surface receptors by von Willebrand factor and collagen. Initial activation of platelets leads to release of soluble mediators—such as ADP, serotonin and thromboxane A_2—which activate additional platelets, and to activation of platelet integrins. Integrin binding to multiple ligands in subendothelial tissue or on the surface of other platelets promotes further activation and aggregation of platelets, ultimately leading to the formation of a platelet plug. Moreover, serotonin and thromboxane A_2 trigger vasoconstriction.

To counteract host-derived **vasoconstrictors**, ticks secrete vasodilators into the site of tissue injury, e.g., non-proteinaceous, lipid-derived substances, such as prostacyclin and prostaglandins (see **Table 1**). Certain salivary proteins, such as tick histamine release factor (tHRF) from *Ixodes scapularis* (Dai et al., 2010) and the serine proteinase inhibitor (serpin) IRS-2 from *Ixodes ricinus*, the latter of which inhibits chymase and cathepsin G (Chmelar et al., 2011), may modulate vascular permeability as well (Chmelar et al., 2012). An activity that counters vasoconstriction has also been evidenced in the salivary gland extracts (SGE) of *Dermacentor reticulatus* and *Rhipicephalus appendiculatus*, and while as yet unidentified, the active molecule(s) do not appear to belong to the prostaglandin family (Pekarikova et al., 2015).

Primary haemostasis, that is, **platelet activation and aggregation** at the site of vascular injury, is targeted by ticks in various manners (Francischetti, 2010). The tick adhesion inhibitor (TAI), found in *Ornithodoros moubata*, interferes with adhesion of platelets to soluble collagen and their ensuing activation and aggregation (Waxman and Connolly, 1993; Karczewski et al., 1995). In soft ticks (Ribeiro et al., 1991; Mans et al., 1998a, 2002a), as well as in certain hard tick species (Ribeiro et al., 1985; Liyou et al., 1999), salivary apyrase [an adenosine triphosphate (ATP)-diphosphohydrolase] has been found to degrade ADP. Salivary prostaglandins, such as PGI_2 from *I. scapularis* (Ribeiro et al., 1988) or PGF2á from *A. americanum* (Aljamali et al., 2002) may induce increase in cAMP, an intracellular platelet aggregation inhibitor (Francischetti, 2010). Moubatin, a lipocalin derived from *O. moubata*, inhibits collagen-induced platelet aggregation by scavenging thromboxane A_2 (Waxman and Connolly, 1993;

TABLE 1 | Identified salivary tick molecules involved in modulation of host defense responses.

Target mechanism	Molecule	Tick species	References
Vasodilation/Vasoconstriction	Prostacyclin	*Ixodes scapularis*	Ribeiro and Mather, 1998
	tHRF	*I. scapularis*	Dai et al., 2010
	Prostaglandins	*Amblyomma americanum*	Bowman et al., 1996
Wound healing/Angiogenesis	Metalloproteases	*Ixodes ricinus*	Decrem et al., 2008a
	Metalloproteases	*I. scapularis*	Francischetti et al., 2005a
	ISL 929, ISL 1373	*I. scapularis*	Guo et al., 2009
	Haemangin	*Haemaphysalis longicornis*	Islam et al., 2009
	HLTnI	*H. longicornis*	Fukumoto et al., 2006
	PGE_2	*Dermacentor variabilis*	Poole et al., 2013a,b
Platelet aggregation	Apyrase	*Ornithodoros* spp. *I. scapularis*	Ribeiro et al., 1985; Mans et al., 1998a
	Moubatin	*Ornithodoros moubata*	Waxman and Connolly, 1993
	TAI	*O. moubata*	Karczewski et al., 1995
	Disaggregin	*O. moubata*	Karczewski et al., 1994
	Savignygrin	*Ornithodoros savignyi*	Mans et al., 2002b
	Monogrin	*Argas monolakensis*	Mans and Ribeiro, 2008
	Ixodegrin	*I. scapularis, I. pacificus*	Francischetti et al., 2005b
	SGE, Fraction AV 16/3	*Amblyomma variegatum*	Kazimirova et al., 2002
	Longicomin	*H. longicornis*	Cheng et al., 1999
	Variabilin	*D. variabilis*	Wang et al., 1996
Blood coagulation	Ornithodorin	*O. moubata*	van de Locht et al., 1996
	TAP	*O. moubata*	Waxman et al., 1990
	Enolase	*O. moubata*	Diaz-Martin et al., 2013a
	Savignin	*Ornithodoros savignyi*	Nienaber et al., 1999
	TAP-like protein	*O. savignyi*	Joubert et al., 1998
	Monobin	*Argas monolakensis*	Mans and Ribeiro, 2008
	Ixolaris	*I. scapularis*	Francischetti et al., 2002
	Penthalaris	*I. scapularis*	Francischetti et al., 2004
	Salp 14	*I. scapularis*	Narasimhan et al., 2002
	TIX-5	*I. scapularis*	Schuijt et al., 2013
	Metalloprotease	*I. scapularis*	Francischetti et al., 2003
	IxscS-1E1	*I. scapularis*	Ibelli et al., 2014
	Ir-CPI	*I. ricinus*	Decrem et al., 2009
	IRS-2	*I. ricinus*	Chmelar et al., 2011
	Americanin	*A. americanum*	Zhu et al., 1997
	AamS6	*A. americanum*	Mulenga et al., 2013a
	AamAV422	*A. americanum*	Mulenga et al., 2013b
	Serpin19	*A.americanum*	Kim et al., 2015b
	Calreticulin	*A. americanum*	Jaworski et al., 1995
	Variegin	*Amblyomma variegatum*	Koh et al., 2007
	Amblyomin-X	*Amblyomma cajennense*	Batista et al., 2010; Branco et al., 2016
	Madanin-1; Madanin-2	*H. longicornis*	Cheng et al., 1999
	Chimadanin	*H. longicornis*	Nakajima et al., 2006
	Haemaphysalin	*H. longicornis*	Kato et al., 2005
	Longistatin	*H. longicornis*	Anisuzzaman et al., 2011a
	65 kDa protein	*R. appendiculatus*	Limo et al., 1991
	Rhipilin-1; Rhipilin-2	*Rhipicephalus hemaphysaloides*	Gao et al., 2011; Cao et al., 2013
	BmAP	*Rhipicephalus (Boophilus) microplus*	Horn et al., 2000
	Microphilin	*R. (B.) microplus*	Ciprandi et al., 2006

(Continued)

TABLE 1 | Continued

Target mechanism	Molecule	Tick species	References
	RmS-15	*R. (B.) microplus*	Xu et al., 2016
	Calcaratin	*Boophilus calcaratus*	Motoyashiki et al., 2003
	Hyalomin-1	*Hyalomma marginatum rufipes*	Jablonka et al., 2015
Innate immune responses	MIF homolog	*A. americanum*	Jaworski et al., 2001
	MIF homolog	*H. longicornis*	Umemiya et al., 2007
	Ir-LBP	*I. ricinus*	Beaufays et al., 2008
	Salp16 Iper1, Salp16 Iper2	*Ixodes persulcatus*	Hidano et al., 2014; Liu X. Y. et al., 2014
	IRS-2	*I. ricinus*	Palenikova et al., 2015
	Evasin-1; Evasin-3; Evasin 4	*Rhipicephalus sanguineus*	Frauenschuh et al., 2007; Deruaz et al., 2008
	Hyalomin A, B	*H. asiaticum*	Wu et al., 2010
	Amregulin	*A. variegatum*	Tian et al., 2016
	Ado, PGE_2	*R. sanguineus*	Oliveira et al., 2011
	Metalloproteases	*Amblyomma maculatum*	Jelinski, 2016
	SHBP	*D. reticulatus*	Sangamnatdej et al., 2002
	RaHBP(M), RaHBP(F)	*R. appendiculatus*	Paesen et al., 1999
	TdPI	*R. appendiculatus*	Paesen et al., 2007
Complement	OmCI	*O. moubata*	Nunn et al., 2005
	TSGP2, TSGP3	*O. savignyi*	Mans and Ribeiro, 2008
	Isac	*I. scapularis*	Valenzuela et al., 2000
	Salp 20	*I. scapularis*	Tyson et al., 2007; Hourcade et al., 2016
	Irac I, II, Isac paralogues	*I. ricinus*	Daix et al., 2007
Acquired immune responses	Salp15	*I. scapularis*	Anguita et al., 2002; Ramamoorthi et al., 2005
	IL-2 binding protein	*I. scapularis*	Gillespie et al., 2001
	Sialostatin L, L2	*I. scapularis*	Kotsyfakis et al., 2006
	Iris	*I. ricinus*	Leboulle et al., 2002
	BIP	*I. ricinus*	Hannier et al., 2004
	Salp15-like protein	*I. ricinus*	Liu J. et al., 2014
	Salp15 Iper-1, Salp15 Iper-2	*I. persulcatus*	Mori et al., 2010
	P36	*D. andersoni*	Bergman et al., 2000
	Japanin	*R. appendiculatus*	Preston et al., 2013
	BIF	*Hyalomma asiaticum*	Yu et al., 2006

tHRF, tick histamine release factor; ISL 929 and ISL 1373, I. scapularis salivary proteins 929 and 1373; HLTnI, troponin I-like molecule; PG, prostaglandin; TAI, Tick adhesion inhibitor; TAP, tick anticoagulant peptide; TIX-5, tick inhibitor of factor Xa toward factor V; IxscS-1E1, blood meal-induced I. scapularis (Ixsc) tick saliva serine protease inhibitor (serpin (S); Ir-CPI, coagulation contact phase inhibitor from I. ricinus; IRS, I. ricinus serpin; AamS6, Amblyomma americanum tick serine protease inhibitor 6; AamAV422, Amblyomma americanum AV422 protein; BmAP , Rhipicephalus (Boophilus) microplus anticoagulant protein; RmS-15, Rhipicephalus (Boophilus) microplus serpin 15; MIF, macrophage migration inhibitory factor; Ir-LBP, Ixodes ricinus salivary LTB4-binding lipocalin; Iper, I. persulcatus; Ado, adenosine; SHBP, serotonin- and histamine-binding protein; RaHBP(M), RaHBP(F), Female (F) and male (M) Rhipicephalus appendiculatus histamine-binding protein; TdPI, tick-derived peptidase inhibitor; OmCI, O. moubata complement inhibitor; TSGP2, TSGP3, soft tick lipocalins, Isac, I. scapularis salivary anticomplement; Irac, I. ricinus anticomplement; Salp, salivary protein; Iris, I. ricinus immunosuppressor; BIP, B-cell inhibitory protein; P36, 36-kDa immunosuppressant protein; BIF, B-cell inhibitory factor.

Karczewski et al., 1995; Mans and Ribeiro, 2008). Longicornin, isolated from the SG of *Haemapysalis longicornis*, also inhibits collagen-mediated platelet aggregation, by a mechanism that remains to be defined (Cheng et al., 1999).

While playing an essential role in secondary haemostasis, as discussed below, the serine protease thrombin also activates platelets through cleavage of protein-activated receptors at the platelet surface. Tick salivary antithrombins can inhibit platelet aggregation induced by thrombin (Hoffmann et al., 1991; Nienaber et al., 1999; Kazimirova et al., 2002). The IRS-2 serpin from *I. ricinus* was found to inhibit platelet aggregation induced by both thrombin and cathepsin G (Chmelar et al., 2011), and the serpin IxscS-1E1 from *I. scapularis* to inhibit thrombin- and ADP-induced platelet aggregation (Ibelli et al., 2014).

Post-activation inhibitors of platelet aggregation are known to target the platelet fibrinogen receptor. The disintegrin-like peptides savignygrin from *O. savignyi* (Mans et al., 2002b), monogrin from *Argas monolakensis* (Mans and Ribeiro, 2008), and variabilin from *D. variabilis* (Wang et al., 1996) display the integrin recognition motif RGD and prevent the binding of other ligands to the platelet receptor. By contrast, the fibrinogen receptor antagonist disaggregin, derived from *O. moubata*, lacks the RGD motif and prevents ligand binding and hence platelet aggregation by different means (Karczewski et al., 1994). Ixodegrins identified in *Ixodes pacificus* and *I. scapularis* are integrin antagonists that display sequence similarity to variabilin (Francischetti et al., 2005b; Francischetti, 2010). In addition, ticks have evolved strategies to disaggregate platelet aggregates, either by displacement of fibrinogen from its receptor by competitive binding, e.g., savignygrin (Mans et al., 2002c), or by fibrinolysis (Decrem et al., 2009; Anisuzzaman et al., 2011b; Diaz-Martin et al., 2013a).

Secondary haemostasis, which refers to **blood coagulation**, occurs concomitantly with primary haemostasis. Blood coagulation involves a series of enzymatic reactions during which a coagulation factor (inactive proenzyme) is converted to an active form, which then activates the next proenzyme. Thrombin is involved in the final (common) pathway of the blood coagulation cascade. It converts fibrinogen into fibrin and also regulates the activity of other coagulation factors. Different coagulation factors are countered by multiple tick salivary components, of which Kunitz-type proteinase inhibitors are the most abundant class (Koh and Kini, 2009; Chmelar et al., 2012). Tick-derived thrombin inhibitors target the enzyme at different sites and through various mechanisms (Koh and Kini, 2009). Ornithodorin (van de Locht et al., 1996), savignin (Nienaber et al., 1999), and monobin (Mans et al., 2008) from soft ticks are Kunitz-type antithrombins, while IRS-2 and IRIS from *I. ricinus* (Leboulle et al., 2002; Chmelar et al., 2011), IxscS-1E1 from *I. scapularis* (Ibelli et al., 2014) and RmS-15 from *Rhipicephalus (Boophilus) microplus* (Xu et al., 2016) are serpins. Antithrombins belonging to the hirudin-like/madanin/variegin superfamily include madanin 1 and 2 from *H. longicornis* (Iwanaga et al., 2003), variegin from *Amblyomma variegatum* (Koh et al., 2007) and hyalomin-1 from *Hyalomma marginatum rufipes* (Jablonka et al., 2015). The antithrombins microphilin (Ciprandi et al., 2006) and BmAP (Horn et al., 2000) from *R. (B.) microplus* and calcaratin from *Boophilus calcaratus* (Motoyashiki et al., 2003) cannot be classified in any of the previously mentioned groups.

TAP, the Kunitz-type tick anticoagulant peptide from *O. moubata* (Waxman et al., 1990), the TAP-like protein from *O. savignyi* (Joubert et al., 1998), amblyomin-X from *Amblyomma cajennense* (Batista et al., 2010) and Salp14 (a basic tail-secreted protein) from *I. scapularis* (Narasimhan et al., 2002) are all inhibitors of coagulation factor Xa. Kunitz-type inhibitors that display similarity to the tissue factor (TF) pathway inhibitor have been identified, e.g., in *I. scapularis* (Ixolaris and penthalaris) (Francischetti et al., 2002, 2004), and Rhipicephalus hemaphysaloides (Rhipilin-1 and -2) (Gao et al., 2011; Cao et al., 2013). SGE from *D. andersoni* was observed to inhibit factor V and factor VII (Gordon and Allen, 1991). TIX-5, found in SG of *I. scapularis* nymphs, was shown to inhibit the activation of factor V by factor Xa, and thus delay activation of the coagulation cascade (Schuijt et al., 2013). Haemaphysalin, a plasma kallikrein-kinin system inhibitor, and Ir-CPI, a contact phase inhibitor that impairs the intrinsic coagulation pathway, were identified in *H. longicornis* (Kato et al., 2005) and *I. ricinus* (Decrem et al., 2009), respectively. IRIS, an immunomodulatory serpin from SG of *I. ricinus*, was found to impair the contact phase-activated pathway of coagulation and prolong fibrinolysis (Prevot et al., 2006). Serpins AamS6 (Mulenga et al., 2013a), AamAV422 (Mulenga et al., 2013b), and serpin19 (Kim et al., 2015b), derived from *A. americanum,* and a 65 kDa protein from *R. appendiculatus* (Limo et al., 1991) all delay plasma clotting, although their mode of action remains to be elucidated. Tick-derived calcium-binding proteins with homology to the calreticulin (CRT) sequence may also modulate haemostasis by sequestration of calcium ions, which act as cofactors of the coagulation enzymes (Jaworski et al., 1995).

Finally, tick salivary components may display **fibrinolytic activity**. A metalloprotease mediating such activity was detected in saliva of *I. scapularis* (Francischetti et al., 2003). Longistatin from *H. longicornis*, an activator of plasminogen, was found to cause hydrolysis of fibrinogen and delay formation of the fibrin clot (Anisuzzaman et al., 2011a).

Impact of Tick Saliva on Host Immune Responses

When skin integrity is compromised, processes required to repel microbial invasion and restore the barrier function of the skin are immediately deployed. Pre-positioned sentinel cells, such as mast cells (MC), macrophages and dendritic cells (DC), are activated by particular components released from damaged skin cells or expressed by microbial organisms. Soluble mediators released by MC, such as bradykinin and histamine, cause itch and pain. Sentinel cells release chemoattractants, including chemokines and leukotrienes, that recruit blood-borne innate immune cells, such as neutrophils and monocytes, to the site of damage, as well as pro-inflammatory cytokines, e.g., tumor necrosis factor alpha (TNFα) and interleukin (IL)-1, that enhance activation of local and infiltrating innate immune cells. Monocytes secrete growth hormones that induce proliferation of fibroblasts and deposition of extracellular matrix, thus contributing to wound healing. Activated DC that have acquired foreign antigen migrate via lymphatics to skin-draining lymph nodes, where antigen may be presented to naïve B and T lymphocytes, thereby initiating an adaptive immune response that culminates in the generation of antigen-specific antibodies and T lymphocytes.

Itch and pain—principally triggered by MC- and basophil-derived mediators—cause awareness of injury and, if unchecked, would arouse an alleviative behavioral response. Ticks mitigate itch and pain by means of salivary components that degrade bradykinin and sequester histamine. For example, salivary metalloproteases from *I. scapularis* (Ribeiro and Mather, 1998) and *Amblyomma maculatum* (Jelinski, 2016) hydrolyse bradykinin. Amine-binding proteins of the lipocalin family, e.g.,

the histamine-binding proteins RaHBP(M) and RaHBP(F)-1,2 from *R. appendiculatus* (Paesen et al., 1999) and the serotonin- and histamine-binding protein SHBP from *D. reticulatus* (Sangamnatdej et al., 2002), have been found to interfere with the activity of histamine and serotonin, the latter representing an important inflammatory mediator in rodents (Askenase et al., 1980). Moreover, the activity of human β-tryptases, which are MC-specific serine proteases involved in inflammation, is diminished by the tick-derived protease inhibitor (TdPI) from *R. appendiculatus* (Paesen et al., 2007).

Recruitment of blood-borne innate immune cells, and notably neutrophils, is also strongly suppressed by tick saliva. Salivary inhibitors of CXCL8 and of several CC chemokines (CCL-2, -3, -5, and -11) were evidenced in SGE of several ixodid species (Hajnicka et al., 2001). Three chemokine-binding proteins, called Evasin-1, -2, and -3, were subsequently purified from the SG of *Rhipicephalus sanguineus* (Frauenschuh et al., 2007) and shown to present selectivity for different chemokines (Frauenschuh et al., 2007; Deruaz et al., 2008). Later work has suggested that Evasin-3-like activity may be common among metastriate ixodid tick species (Vancova et al., 2010a). CXCL8-mediated chemotaxis of neutrophils is also inhibited by Salp16 Iper1 and Salp16 Iper2, salivary proteins from *Ixodes persulcatus* (Hidano et al., 2014). Ir-LBP, a lipocalin from *I. ricinus*, was found to bind leukotriene B4, an important inflammatory mediator, with high affinity, thereby interfering with neutrophil chemotaxis and activation (Beaufays et al., 2008). Several tick species express a homolog of the vertebrate macrophage migration inhibitory factor (MIF) (Wasala and Jaworski, 2012). For both *A. americanum* (Jaworski et al., 2001) and *H. longicornis* (Umemiya et al., 2007), MIF has been shown to inhibit migration of macrophages in *in vitro* assays, suggesting that MIF might diminish macrophage recruitment to the bite location *in vivo*.

Tick saliva quells **inflammation** at the bite location by diminishing or enhancing secretion of pro- and anti-inflammatory cytokines, respectively. *D. andersoni* SGE diminished production of IL-1 and TNFα by macrophages and IL-2 and interferon (IFN)-γ production by T lymphocytes (Ramachandra and Wikel, 1992). Hyalomin-A and -B from SG of *Hyalomma asiaticum asiaticum* were found to inhibit secretion of TNFα, CCL2, and IFN-γ, but to increase secretion of the immunosuppressive cytokine IL-10 (Wu et al., 2010). Amregulin from *A. variegatum* saliva was found to suppress the *in vitro* production of TNFα, IL-1, CXCL8, and IFN-γ (Tian et al., 2016). Moreover, non-proteinaceous substances such as PGE_2 and Ado (purine nucleoside adenosine) from saliva of *R. sanguineus* have been found to impair the production of the pro-inflammatory cytokines IL-12p40 and TNFα and stimulate the production of IL-10 by murine DC (Oliveira et al., 2011). Beyond its impact on cytokine production, tick saliva counters multiple effector functions of innate immune cells, including the production of reactive oxygen species (ROS) by neutrophils (Ribeiro et al., 1990; Guo et al., 2009) and macrophages (Kopecký and Kuthejlová, 1998), phagocytosis by neutrophils (Ribeiro et al., 1990) and macrophages (Kramer et al., 2011) and cytotoxicity of NK cells (Kubes et al., 1994). IRS-2, an *I. ricinus* serpin, targets cathepsin G and chymase, enzymes produced by activated neutrophils and MC, respectively (Pekarikova et al., 2015).

Tick saliva also restricts **wound healing and angiogenesis** (Francischetti, 2010; Hajnicka et al., 2011). Salivary molecules from hard ticks are able to bind to the transforming growth factor (TGF)-β1, the platelet-derived growth factor (PDGF), the fibroblast growth factor (FGF)-2 and the hepatocyte growth factor (HGF), depending on the tick species (Hajnicka et al., 2011; Slovak et al., 2014). *Dermacentor variabilis* saliva was found to suppress basal and PDGF-stimulated fibroblast migration and reduce PDGF-stimulated activity of extracellular signal-regulated kinase (ERK) (Kramer et al., 2008). Tick compounds similar to disintegrin metalloproteases and thrombospondin can impair cell-matrix interactions and angiogenesis (Valenzuela et al., 2002; Francischetti et al., 2005a; Fukumoto et al., 2006). The *I. scapularis* proteins ISL 929 and ISL 1373, for example, impair both the expression of β2 integrins and the adherence of polymorphonuclear leukocytes (PMN) (Guo et al., 2009). A troponin I-like molecule (HLTnI) (Fukumoto et al., 2006) and a Kunitz-type protein (haemangin) (Islam et al., 2009) from the SG of *H. longicornis* also impair angiogenesis and wound healing.

The complement system links the host innate and adaptive immune responses and is activated through three pathways (alternative, classical, and lectin). The alternative pathway is the main line of defense against invading pathogens and is also involved in resistance to ticks (Wikel, 1979). Isac, Salp20 and Isac-1 from *I. scapularis* (Valenzuela et al., 2000; Tyson et al., 2007) and IRAC I and II from *I. ricinus* (Daix et al., 2007; Couvreur et al., 2008) inhibit formation of the C3 convertase of the alternative pathway by impeding the binding of complement factor B to complement C3b. The lipocalins OmCI (*O. moubata* complement inhibitor; Nunn et al., 2005) and TSGP2 and TSGP3 (Mans and Ribeiro, 2008) specifically target C5 activation. Inhibition of the classical complement pathway has also been reported for saliva and SGE of *A. cajennense* (Franco et al., 2016).

Tick saliva or SGE is also able to suppress the initiation of **adaptive immunity**, such as by interfering with the capacity of DC to present antigen to T cells and prime appropriate Th responses (Cavassani et al., 2005; Mejri and Brossard, 2007; Oliveira et al., 2008; Skallova et al., 2008; Carvalho-Costa et al., 2015). In some instances these activities have been assigned to molecularly-defined salivary components. Salivary cysteine protease inhibitors (cystatins) of *I. scapularis* were shown to possess inhibitory activity against certain cathepsins. Both Sialostatin L and L2 strongly inhibited cathepsin L, but Sialostatin L also inhibited cathepsin S, which is involved in antigen processing. Inhibition of cathepsin S by Sialostatin L diminished the capacity of DC to induce proliferation in antigen-specific $CD4^+$ T cells (Kotsyfakis et al., 2006; Sa-Nunes et al., 2009), while Sialostatin L2 suppressed the type I IFN response in DC (Lieskovska et al., 2015b). Salp15 from *I. scapularis,* upon stimulation of DC by TLR-2 and -4 ligands, suppressed the production of pro-inflammatory cytokines IL-12p70, IL-6, and TNFα, and the capacity of DC to activate T cells (Hovius et al., 2008a). Japanin, a salivary gland lipocalin from *R. appendiculatus*, modifies the expression of co-stimulatory and co-inhibitory

molecules, the production of diverse cytokines, and inhibits DC differentiation from monocytes (Preston et al., 2013). Not all immunomodulatory salivary mediators are proteins: in *I. scapularis* saliva, PGE_2 was found to be a major inhibitor of DC maturation and TLR-ligand induced secretion of IL-12 and TNFα (Sá-Nunes et al., 2007).

In a number of studies, tick saliva or SGE has been observed to enhance production of T helper 2 (Th2) signature cytokines, such as IL-4, and diminish production of Th1 cytokines, e.g., IFN-γ (Ferreira and Silva, 1999; Mejri et al., 2001). T cell inhibitory salivary molecules include a secreted IL-2 binding protein in *I. scapularis*, which suppresses T cell proliferation and other IL-2 dependent activities (Gillespie et al., 2001), P36 in *D. andersoni* (Bergman et al., 2000), Iris in *I. ricinus* (Leboulle et al., 2002), and Salp15 in *I. scapularis* (Anguita et al., 2002). Iris was found to suppress T cell proliferation, promote a Th2 type response and inhibit the production of pro-inflammatory cytokines IL-6 and TNFα. Salp15 binds to CD4 molecules on the surface of $CD4^+$ T (helper) cells, and consequently inhibits signaling mediated by T cell receptors, resulting in decreased IL-2 production and T cell proliferation (Anguita et al., 2002; Garg et al., 2006). Nevertheless, transcriptional profiling of the cutaneous response in mice to primary and secondary infestation by *I. scapularis* nymphs did not evidence a predominant Th subset in primary infestation and evidenced a mixed Th1/Th2 and possibly regulatory T cell response in secondary infestation (Heinze et al., 2012a,b). For secondary infestation with *D. andersoni* nymphs, the same authors observed an indeterminant Th profile in skin, but a pronounced upregulation of IL-4 expression in draining lymph nodes, suggesting that the systemic response to repeated infestation may display a marked Th2 bias (Heinze et al., 2014).

Tick salivary components also disarm the humoral arm of the adaptive immune response. BIP and BIF derived from *I. ricinus* and *H. asiaticum asiaticum,* respectively, were found to suppress B-cell responses (Hannier et al., 2004; Yu et al., 2006). Salivary immunoglobulin (IgG)-binding proteins bind ingested host IgG and facilitate their excretion in saliva during feeding, thus protecting ticks from ingested host IgG (Wang and Nuttall, 1999).

Owing—at least in part—to active interference by salivary compounds with development of an appropriate immune response, susceptible hosts, such as mice, may fail to develop acquired resistance to ticks despite repeated tick feeding (Schoeler et al., 1999). In tick resistant hosts (e.g. guinea pigs, rabbits), however, the presence of antibodies and effector T lymphocytes with specificity for tick antigens assures a rapid secondary response to infestation that impairs tick feeding. Significant diversity in resistance to tick infestation has been also observed among different breeds of cattle, some of which is related to immunity. In particular, T-cell-mediated responses directed against larval feeding, rather than IgG responses to *R. (B.) microplus* antigens, were shown to be protective (Jonsson et al., 2014). Microarray analysis of differentially expressed genes in bovine skin early after attachment of *R. (B.) microplus* larvae revealed an important role for lipid metabolism in control of inflammation and impairment of tick infestation in tick-resistant cattle, and an impairment of the acute phase response in susceptible animals (Carvalho et al., 2014). Variation among breeds in non-immune characteristics, such as the extracellular matrix of the skin, is also likely to contribute to the variation in resistance to ticks (Jonsson et al., 2014).

TICK SALIVA AND PATHOGEN TRANSMISSION

Host anti-tick immunity may have a major impact on both transmission and acquisition of TBP by ticks. For example, repeated infestation of resistant strains of laboratory animals with pathogen-free *I. scapularis* nymphs afforded protection against tick-transmitted *B. burgdorferi,* suggesting that immunity against tick salivary antigens can impair transmission of *Borrelia* (Wikel et al., 1997; Nazario et al., 1998). Moreover, immunization of guinea pigs with *I. scapularis* SG proteins produced during the first day of tick feeding interfered with *Borrelia* transmission from ticks to hosts, suggesting that at least some of the antigens involved in acquired resistance are secreted in saliva during the first 24 h of tick attachment (Narasimhan et al., 2007a). Indeed, the aforementioned Th2 polarization of the immune response by salivary immunomodulatory molecules has been proposed to enhance transmission of TBP (Gillespie et al., 2000; Schoeler and Wikel, 2001; Wikel and Alarcon-Chaidez, 2001; Brossard and Wikel, 2008).

Enhancement of pathogen transmission by tick saliva—called saliva-assisted transmission or SAT—has been documented for several tick-pathogen associations (Nuttall and Labuda, 2004). However, relatively few tick molecules implicated in pathogen transmission have been identified and characterized.

Saliva Assisted Transmission

In the course of co-evolution with ticks and vertebrate hosts, tick-borne microorganisms have developed myriad strategies to subvert tick salivary molecules so as to ensure their transmission cycle (Brossard and Wikel, 2004; Nuttall and Labuda, 2004; Ramamoorthi et al., 2005; Wikel, 2013). Saliva not only provides the matrix in which TBP are inoculated, but also profoundly modifies the local environment at the bite location, with consequences for not only transmission of TBP from infected ticks to the uninfected vertebrate host but also acquisition of TBP by uninfected ticks.

The first observation of SAT concerned Thogoto virus (THOV). Acquisition of infection by virus-free *R. appendiculatus* nymphs was enhanced when they fed on guinea pigs injected with a mixture of THOV and SGE (Jones et al., 1989). Subsequent studies have demonstrated the SAT phenomenon for tick-borne encephalitis virus (TBEV) (Alekseev and Chunikhin, 1990; Labuda et al., 1993) and *B. burgdorferi* s.l. (Gern et al., 1993; Pechova et al., 2002; Zeidner et al., 2002; Machackova et al., 2006). Tick saliva has also been shown to enhance infection of vertebrate hosts, as documented for Powassan virus (Hermance and Thangamani, 2015), African swine fever virus (ASFV) (Bernard J. et al., 2016), *B. burgdorferi* s.l. (Pechova et al., 2002; Zeidner et al., 2002; Machackova et al., 2006), *Francisella*

tularensis (Krocova et al., 2003), and *Rickettsia conorii* (Milhano et al., 2015).

As a corollary of SAT, saliva is also presumed to enhance so-called non-viraemic transmission (NVT), i.e., transmission of TBP from infected to pathogen-free ticks that feed concomitantly on the same host, in the absence of systemic infection of the host. The pool of saliva produced by ticks that feed in close proximity is thought to enhance pathogen exchange between co-feeding ticks (Randolph, 2011; Voordouw, 2015). Indeed, transmission of THOV to uninfected *R. appendiculatus* ticks was observed to be more efficient while co-feeding with infected ticks on non-viraemic guinea pigs than while feeding on viraemic hamsters (Jones et al., 1987). NVT may assist TBP in circumventing the host immune response, such as by permitting viruses to evade virus-specific neutralizing antibodies (Labuda et al., 1997). Moreover, during NVT of TBEV between *I. ricinus* ticks, infectious virus was evidenced in the cellular infiltrate at the tick bite location, suggesting that migratory cells might transport the virus from infected to uninfected co-feeding ticks (Labuda et al., 1996). While NVT is thought to be most efficient for viruses, it has also been observed for various combinations of tick-borne bacteria and ticks, including *B. burgdorferi* s.l. and *Ixodes* spp (Gern and Rais, 1996; Piesman and Happ, 2001; Richter et al., 2002), *R. conorii* and *R. sanguineus* (Zemtsova et al., 2010). *Rickettsia parkeri* and *A. maculatum* (Banajee et al., 2015) or the *Ehrlichia muris*-like agent and *I. scapularis* (Karpathy et al., 2016).

In most instances SAT has been attributed to the immunomodulatory activity of salivary compounds, a paradigm that has been most extensively addressed for tick-borne bacteria, and more particularly *B. burgdorferi*. Indeed, when mice were infected with *B. burgdorferi* by syringe inoculation they developed a robust humoral response against the protective surface antigens Osp-A and -B, but failed to do so when infected by a tick bite (Gern et al., 1993). Moreover, BALB/c mice have been reported to develop a Th2-biased immune response against *B. burgdorferi* following an infectious tick bite, but a mixed Th1/Th2 response after injection (Christe et al., 2000). Furthermore, when *Borrelia* was injected into mice along with *I. ricinus* saliva, the number of leukocytes and T lymphocytes in the epidermis was diminished at early time-points after inoculation and the total number of cells in draining lymph nodes was reduced (Severinova et al., 2005). For *Borrelia* sp., the mechanisms that underlie SAT have been extensively addressed in relation to DC (for review see (Mason et al., 2014)). *In vitro* treatment of murine DC with *I. ricinus* saliva was found to inhibit DC maturation (Skallova et al., 2008), reduce phagocytosis of *B. afzelii*, reduce cytokine production, and impair proliferation and IL-2 production in *Borrelia*-specific $CD4^+$ T cells (Slamova et al., 2011). Moreover, *I. ricinus* saliva modulated IFN-γ signaling pathways in DC (Lieskovska and Kopecky, 2012b), as well as pathways activated by a TLR2 ligand in *Borrelia*-stimulated DC (Lieskovska and Kopecky, 2012a). SGE of *I. ricinus* and saliva of *I. scapularis* inhibited *in vitro* killing of *Borrelia* by macrophages, through reduced production of ROS (Kuthejlova et al., 2001), and PMN (Montgomery et al., 2004), respectively. In the skin, expression of an anti-microbial peptide (AMP), cathelicidin, was induced by syringe inoculation of *Borrelia* but markedly suppressed when introduced by *I. ricinus* (Kern et al., 2011). Moreover, SGE derived from *I. ricinus* inhibited the *in vitro* inflammatory response of human primary keratinocytes induced by *Borrelia* or by OspC, a major surface antigen. In particular, chemokines (CXCL8 and CCL2) and AMPs (defensins, cathelicidin, psoriasin, and RNase 7) were down-regulated (Marchal et al., 2011). *I. ricinus* saliva also inhibited cytokine production by human primary keratinocytes in response to TLR2/TLR3 ligands during *Borrelia* transmission (Bernard Q. et al., 2016). Nevertheless, the effects of *I. scapularis* saliva on resident skin cells exposed to *Borrelia* were found to depend on the cell type (Scholl et al., 2016): tick saliva suppressed the production of the pro-inflammatory mediators IL-6, CXCL8, and TNFα by monocytes, but enhanced the production of CXCL8 and IL-6 by dermal fibroblasts.

Salivary mediators also appear to influence transmission of Rikettsiales bacteria. Saliva of *I. scapularis* was found to inhibit the pro-inflammatory cytokine response of murine macrophages to infection with the intracellular bacterium, *Anaplasma phagocytophilum* (Chen et al., 2012). For spotted group fever rickettsiae, immunomodulatory factors introduced during feeding of *A. maculatum* seemed to enhance the pathogenicity and dissemination of *R. parkeri* in rhesus macaques (Banajee et al., 2015). Mice experimentally inoculated with *R. conorii* and infested with *R. sanguineus* presented reduced levels of IL-1β and the transcription factor NF-κB and enhanced levels of IL-10 in the lung in comparison with mice inoculated with *R. conori* alone, suggesting that inflammation was inhibited (Milhano et al., 2015).

The mechanisms underlying SAT have less frequently been explored for viruses. Nevertheless, treatment of DC with *I. ricinus* saliva increased the proportion of TBEV-infected cells and decreased the production of TNFα and IL-6 and the induction of apoptosis elicited by the virus (Fialova et al., 2010).

Tick Salivary Molecules Implicated in Pathogen Transmission

The underlying molecular mechanisms implicated in TBP transmission have only begun to be elucidated, and only a few tick molecules directly or indirectly -through interaction with the host- involved in transmission, have been actually identified and functionally characterized (**Table 2**) (Nuttall and Labuda, 2004; Ramamoorthi et al., 2005; Kazimirova and Stibraniova, 2013; Wikel, 2013; Liu and Bonnet, 2014). Among these, some salivary compounds affect the acquisition of TBP by the vector, while others enhance the transmission of TBP to the host.

Expression of members of the 5.3-kDa family of salivary peptides that possess anti-microbial properties (Pichu et al., 2009; Liu et al., 2012) was found to be upregulated in SG of *I. scapularis* during infection with Langat virus (McNally et al., 2012), *B. burgdorferi* (Ribeiro et al., 2006), and *A. phagocytophilum* (Liu et al., 2012). RNAi knockdown of one member of the 5.3-kDa antimicrobial peptide family, encoded by gene-15, increased *A. phagocytophilum* burden in SG of *I. scapularis* and in blood of mice on which gene-15-deficient ticks fed (Liu et al., 2012). Thus, 5.3-kDa antimicrobial peptides are

TABLE 2 | Tick salivary molecules implicated in transmission of tick-borne microorganisms.

Tick species	Salivary molecule		Microorganism	References
I. ricinus	Calreticulin	Calcium-binding protein	*B. burgdorferi* s.l.	Cotté et al., 2014
I. scapularis	5.3-kD protein	Antimicrobial peptide	*A. phagocytophilum*	Liu et al., 2012
I. scapularis	Salp15	Secreted salivary protein	*B. burgdorferi*	Ramamoorthi et al., 2005; Schuijt et al., 2008
I. ricinus	Salp15 Iric-1	Secreted salivary protein, Salp15 homolog	*B. burgdorferi*	Hovius et al., 2008b
I. persulcatus	IperSalp15	Secreted salivary protein, Salp15 homolog	*B. burgdorferi*	Murase et al., 2015
I. scapularis	TSLPI	Tick salivary lectin pathway inhibitor	*B. burgdorferi*	Schuijt et al., 2011
I. scapularis	Salp20	Salivary anti-complement protein	*B. burgdorferi*	Tyson et al., 2007
I. scapularis	Isac	Salivary anti-complement protein	*B. burgdorferi*	Valenzuela et al., 2000
I. ricinus	IRAC I, II	Salivary anti-complement proteins	*B. burgdorferi*	Daix et al., 2007
I. ricinus	BIP	B-cell inhibitor	*B. burgdorferi*	Hannier et al., 2003
I. scapularis	Salp25D	Salivary protein, antioxidant	*B. burgdorferi*	Narasimhan et al., 2007b
I. scapularis	tHRF	Tick histamine release factor	*B. burgdorferi*	Dai et al., 2010
I. ricinus	IrSPI	Serine protease inhibitor	*B. henselae*	Liu X. Y. et al., 2014
I. scapularis	Salp16	Salivary gland protein	*A. phagocytophilum*	Sukumaran et al., 2006
I. scapularis	P11	Salivary gland protein	*A. phagocytophilum*	Liu et al., 2011
I. scapularis	Sialostatin L2	Salivary cysteine protease inhibitor	TBEV	Lieskovska et al., 2015b
I. scapularis	Sialostatins L, L2	Salivary cysteine protease inhibitors	*B. burgdorferi*	Lieskovska et al., 2015a
I. scapularis	Sialostatin L2	Salivary cysteine protease inhibitors	*A. phagocytophilum*	Chen et al., 2014
I. scapularis	subolesin	Tick protective antigen	*B. burgdorferi*	Bensaci et al., 2012
D. variabilis	subolesin	Tick protective antigen	*A. marginale*	Zivkovic et al., 2010

probably able to inhibit both acquisition and transmission of TBP. Moreover, the Janus kinase signaling transducer activator of transcription (JAK-STAT) pathway was implicated in the control of *A. phagocytophilum* infection in ticks by regulating the expression of antimicrobial peptides (Liu et al., 2012).

Distinct tick proteins promote the transmission of *B. burgdorferi* s.l. at different phases of infection, in relation to the phenotypic plasticity of this TBP (Radolf et al., 2012). In the infected tick, *Borrelia* spirochetes express outer surface protein OspA and bind to the midgut wall by means of a tick midgut protein (TROSPA) (Pal et al., 2004). Under the stimulus of a new blood meal and following tick attachment, spirochetes begin to express OspC and move from the midgut through the haemolymph to the SG, where they encounter Salp15. The secreted salivary protein Salp15, considered to be the first tick mediator of SAT to be discovered, was first identified in the SG of *I. scapularis*. Salp15 has been shown to bind to mammalian CD4 (Garg et al., 2006), inhibit activation of $CD4^+$ T lymphocytes (Anguita et al., 2002), impair DC function by inhibiting TLR- and *B. burgdorferi*-induced production of pro-inflammatory cytokines by DCs, as well as DC-induced T cell activation (Hovius et al., 2008a). A Salp15 ortholog from *I. ricinus* inhibited the inflammatory response of human primary keratinocytes during transmission of *Borrelia* (Marchal et al., 2011). The activity of Salp15 has been shown to be critically important in pathogen transmission: RNAi silencing of Salp15 drastically reduced the capacity of ticks to transmit spirochetes to mice (Ramamoorthi et al., 2005), and immunization of mice with Salp15 afforded significant protection from *I. scapularis*-transmitted *B. burgdorferi* (Dai et al., 2009). In the tick SG, spirochetes bind to Salp15 via OspC, which protects them from antibody- and complement-mediated killing (Schuijt et al., 2008) and promotes their transmission and replication in the host skin (Ramamoorthi et al., 2005). Salp15 homologs Salp15 Iric-1 and IperSalp15 have been identified in *I. ricinus* (Hovius et al., 2008b) and *I. persulcatus* (Murase et al., 2015), respectively. These proteins have functions similar to those of Salp15 and appear to protect *B. burgdorferi* s.s., *B. garinii*, and *B. afzelii* from antibody-mediated killing in the host.

To evade complement-mediated killing, *Borrelia* also benefits from tick salivary proteins that inhibit complement activation at the tick bite location (de Taeye et al., 2013). The tick salivary lectin pathway (TSLP) inhibitor from SG of *I. scapularis* was found to interfere with the human lectin complement cascade and impair neutrophil phagocytosis and chemotaxis, thereby protecting *Borrelia* from killing by the lectin complement pathway (Schuijt et al., 2011). Moreover, silencing of TSLPI in *Borrelia*-infected ticks impaired experimental transmission of the spirochetes to mice. Salp20, a member of the *I. scapularis* anti-complement protein-like family of tick salivary proteins, inhibits the alternative complement pathway by binding properdin and causing dissociation of the C3 convertase (Tyson et al., 2007; Hourcade et al., 2016). Salp20 partially protects *B. burgdorferi* from lysis by normal human serum, suggesting that, together with the plasma activation factor H, Salp20 may protect *Borrelia* from components of the host complement system. Anti-complement proteins belonging to the Isac-like family, e.g., Isac from *I. scapularis* (Valenzuela et al., 2000) and its homologs IRAC I and II from *I. ricinus* (Daix et al., 2007) function very similarly to the previously described Salp20, but display different capacities

to inhibit the alternative complement pathway depending upon the host species (Schroeder et al., 2007; Couvreur et al., 2008). Thus, proteins of the Isac-like family may potentially promote transmission of *Borrelia* to the vertebrate host (de Taeye et al., 2013).

BIP from *I. ricinus* SG was found to suppress B lymphocyte proliferation induced by the *B. burgdorferi* OspC, suggesting that BIP may enhance *Borrelia* transmission to the host (Hannier et al., 2003).

Salp25D is an immunodominant antioxidant salivary protein from *I. scapularis.* Silencing of Salp25D expression in SG impaired acquisition of *B. burgdorferi.* The protein is probably involved in acquisition of *B. burgdorferi* by ticks and acts as an antioxidant that promotes pathogen survival in the tick (Das et al., 2001; Narasimhan et al., 2007b).

Salivary tHRF has been shown capable of binding mammalian basophils and triggering release of histamine (Mulenga et al., 2003; Dai et al., 2010). Mulenga et al. (2003) proposed that tHRF was required for vasodilation during the rapid feeding phase of *D. variabilis,* when large volumes of blood are required. tHRF is up-regulated in *I. scapularis* SG during the rapid feeding phase and, by virtue of increasing blood flow to the tick bite location, may facilitate not only tick engorgement but also *B. burgdorferi* infection (Dai et al., 2010). Immunization of mice with the recombinant protein as well as silencing tHRF impaired tick feeding and decreased *Borrelia* burden in the host at 7 days after infection in skin and at 3 weeks in heart and joints (Dai et al., 2010). While reduced bacterial burden in mice may simply have been secondary to reduced burden in ticks, the authors suggested that the vasodilatory activity of histamine might also enhance systemic dissemination of *Borrelia* from the bite site (Dai et al., 2010).

IrSPI, a protein from *I. ricinus* SG belonging to the BPTI/Kunitz family of serine protease inhibitors, probably impairs host haemostasis and facilitates tick feeding and *Bartonella henselae* transmission, as RNAi silencing impaired tick feeding and diminished *B. henselae* load in the tick SG (Liu X. Y. et al., 2014).

Infection with *A. phagocytophilum* was reported to induce expression of the Salp16 gene in *I. scapularis* SG during feeding. Silencing of Salp16 gene expression interfered with trafficking of the bacteria ingested via the blood meal and infection of the tick SG, which demonstrated its role in persistence of this TBP within the tick (Sukumaran et al., 2006).

Silencing of P11, a secreted *I. scapularis* SG protein that was found to be upregulated in *A. phagocytophilum*-infected ticks, demonstrated that the protein enables infection of tick haemocytes and thus facilitates pathogen dissemination in the tick and its migration from the midgut to the SG through the haemolymph (Liu et al., 2011).

Sialostatin L and L2 from *I. scapularis* are potentially implicated in transmission of tick-borne viruses and bacteria. Sialostatin L2 was found to interfere with IFN-γ mediated immune responses in mouse splenic DC, resulting in enhanced replication of TBEV in DC (Lieskovska et al., 2015b) Sialostatin L and L2 modulated responses of murine bone marrow-derived DC exposed to *Borrelia* (Lieskovska et al., 2015a). Moreover, Sialostatin L2 stimulated proliferation of *B. burgdorferi* in murine skin (Kotsyfakis et al., 2010), possibly in relation to inhibition of the type I IFN response in DC (Lieskovska et al., 2015a,b). In addition, Sialostatin L2 inhibited inflammasome formation in mice during *A. phagocytophilum* infection and targeted caspase-1 activity (Chen et al., 2014).

Subolesin is a tick protective antigen with similarity to akirins, an evolutionarily conserved group of proteins in insects and vertebrates, and is suggested to control nuclear factor-kappa B (NF-kB)-dependent and independent gene expression and to play a role in tick immune responses to pathogens (Almazan et al., 2003; Naranjo et al., 2013). Upregulation of subolesin expression was observed in SG of *D. variabilis* ticks infected with *A. marginale* (Zivkovic et al., 2010). The impact of subolesin on transmission encompasses multiple vector-pathogen associations, as both gene silencing or immunization of hosts with recombinant subolesin protein resulted in decreased *A. marginale, A. phagocytophilum*, and *Babesia bigemina* burdens in their respective tick vectors (de la Fuente et al., 2006, 2010a; Merino et al., 2011). Moreover, vaccination of mice with vaccinia virus-expressed subolesin impaired engorgement of *I. scapularis* larvae and reduced acquisition of *B. burgdorferi* by tick larvae from infected mice and *Borrelia* transmission to uninfected mice (Bensaci et al., 2012). These findings suggest that subolesin may be involved in tick innate immunity to microbial agents by reducing their burden in SG while up-regulating factors facilitating pathogen acquisition by ticks.

Finally, a calcium-binding protein (CRT) has been found in the saliva of *A. americanum* and *D. variabilis* (Jaworski et al., 1995), in *R. microplus* (Ferreira et al., 2002) and *I. ricinus* (Cotté et al., 2014). Expression of the gene encoding CRT is up-regulated upon infection of *R. annulatus* with *B. bigemina* (Antunes et al., 2012) and CRT itself is up-regulated in the salivary proteome of *I. ricinus* upon infection with *B. burgdorferi* (Cotté et al., 2014). After immunization of rabbits with a fusion protein comprising the CRT of *A. americanum*, infestation with this tick caused necrotic feeding lesions (Jaworski et al., 1995). While salivary CRT might conceivably facilitate tick feeding and pathogen transmission through inhibition of thrombosis and complement, the precise role of CRT remains enigmatic. Indeed, while the CRT of *A. americanum* is able to bind to C1q, the first component of the classical complement pathway, it was not shown to inhibit activation of the complement cascade (Kim et al., 2015a).

IMPACT OF TBP INFECTION ON GENE EXPRESSION IN TICK SG

Similar to vertebrates, ticks are protected against invading microorganisms by an innate immune system (Hajdusek et al., 2013; Hynes, 2014). During co-evolution, tick-borne microorganisms have developed various strategies to evade or suppress the immune responses of their vectors in order to ensure survival, persistence and transmission. As a countermeasure, ticks have developed means of maintaining pathogen burden at a level that preserves fitness and further development (Smith and Pal, 2014). Indeed, several studies have provided evidence that

acquisition of TBP can exert a profound effect on gene expression in various tick organs, including SG. Multiple families of genes have been shown to be regulated in tick SG following infection, but in most cases, their precise role is unknown (Liu and Bonnet, 2014; Chmelar et al., 2016a).

The impact of infection on the SG transcriptome has been addressed for several species of ticks and TBP, including *D. variabilis* and *Rickettsia montanensis* (Macaluso et al., 2003), *R. appendiculatus* and *Theileria parva* (Nene et al., 2004), *R. microplus* and *A. marginale* (Zivkovic et al., 2010; Mercado-Curiel et al., 2011; Bifano et al., 2014), *I. scapularis* and *A. phagocytophilum* (Ayllon et al., 2015; Cabezas-Cruz et al., 2016, 2017) or Langat virus (McNally et al., 2012) and *I. ricinus* and *B. henselae* (Liu X. Y. et al., 2014) or *B. afzelii* (Valdes et al., 2016). Diverse technologies have been applied to the identification of differentially expressed genes, from seminal studies based on differential-display PCR (Macaluso et al., 2003) and sequencing of expressed sequence tags (Nene et al., 2004), to later studies that applied suppression subtractive hybridization (Zivkovic et al., 2010) and microarrays (Mercado-Curiel et al., 2011; McNally et al., 2012) and to recent studies that have exploited next-generation sequencing (Liu X. Y. et al., 2014; Ayllon et al., 2015). Moreover, the impact of infection on the proteome of the SG, or of saliva itself, has been investigated for infection of *I. scapularis* with *A. phagocytophilum* (Ayllon et al., 2015; Villar et al., 2016) or *B. burgdorferi* (Dai et al., 2009) and *I. ricinus* with *B. burgdorferi* (Cotté et al., 2014). Recently, a novel multidisciplinary approach involving structural analysis of the SG transcriptome and biophysical simulations was used to predict substrate specificity for uncharacterized lipocalins in the SG of *I. ricinus* that might play a role in transmission of *B. afzelii* (Valdes et al., 2016). Depending on the study, relatively modest or extensive modulation of gene expression has been observed following SG infection, possibly reflecting not only the experimental strategy employed, but also the type of relationship established by the tick/pathogen pair in question. It has, for example, been suggested that pathogens that are highly adapted to their tick, such as *A. marginale* for *R. microplus*, and whose presence imposes a minimal fitness cost, have little effect on the SG transcriptome (Zivkovic et al., 2010; Mercado-Curiel et al., 2011), whereas pathogens that have a dramatic effect on tick fitness, such as *B. bovis* for *Rhipicephalus annulatus* (Ouhelli et al., 1987), have a greater impact.

TICK SALIVA ANTIGENS FOR EPIDEMIOLOGY AND CONTROL

Anti-tick immunity was first described in the middle of the twentieth century (Trager, 1939), and many tick salivary proteins have since been shown to be immunogenic in vertebrate hosts (Wikel, 1996). Indeed, during feeding ticks inject multiple salivary proteins that elicit antibody responses, and it has been suggested, from the 1990s, that such responses could be used as biomarkers of host exposure to tick bites (Schwartz et al., 1990). Nevertheless, surveillance of TBD has largely relied on detection of host antibody responses to TBP or of TBP themselves in ticks or host, or on modeling/forecasting approaches (Hai et al., 2014), and evaluation of exposure to tick bites has only rarely been addressed. Biomarkers of exposure to ticks would be useful not only to assess host/vector contact, such as to evaluate the efficacy of anti-vector measures, but also to instruct diagnosis of TBD, by enabling documentation of an antecedent tick bite in patients for whom TBD is suspected. It is, in fact, well-known that self-reported tick exposure is a poor correlate of true tick exposure. The greatest challenge in this endeavor lies in identification of antigenic markers that allow discrimination among the different tick species to which the host has been exposed. Such specificity is particularly critical in areas with a high diversity of hematophagous arthropod species (Fontaine et al., 2011). Indeed, when antibodies against sonicated *Ixodes dammini* SG were first evaluated as markers of tick exposure in humans, cross-reactivity with other arthropods was shown to limit their epidemiologic utility (Schwartz et al., 1990). Since then, the use of a recombinant form of CRT has displayed higher specificity, albeit lower sensitivity, than whole SG for evaluation of exposure to *I. scapularis* bites (Sanders et al., 1999). Antibodies against CRT were thus sought in a longitudinal study that addressed the impact of educational interventions on tick exposure (Malouin et al., 2003), and further studies demonstrated that, in humans, such antibodies were found to persist for as long as a year and a half following tick exposure (Alarcon-Chaidez et al., 2006). Cross-reactivity, however, has been reported between recombinant CRT from *I. scapularis* and *A. americanum* (Sanders et al., 1998b), underscoring the challenge of developing specific ELISA tests. Efforts are thus still in progress to identify discriminant antigenic proteins in tick saliva that could be used to evaluate exposure to different tick species (Sanders et al., 1998a; Vu Hai et al., 2013a,b).

The production of antigen-specific antibodies directed against multiple tick salivary proteins (Vu Hai et al., 2013b), as well as the observation that vertebrates repeatedly exposed to tick bites develop immunity (Brossard and Wikel, 1997; Wikel and Alarcon-Chaidez, 2001) that affords a measure of protection against TBD (Bell et al., 1979; Wikel et al., 1997; Burke et al., 2005; Krause et al., 2009), has sparked interest in development of vaccinal strategies against tick bites. In contrast with conventional control measures, deployment of an anti-tick vaccine would not contaminate the environment and foodstuffs, nor have a deleterious impact on off-target species. Moreover, in light of the transmission of multiple TBP by the same tick species, vaccine strategies that target conserved processes in vector capacity would be expected to afford broad protection against multiple TBD transmitted by the same vector (Willadsen, 2004; Nuttall et al., 2006). Of note, such strategies hold the promise of protecting against uncharacterized TBP currently in circulation or as yet to emerge. The anti-tick vaccine approach is, moreover, compatible with the inclusion of multiple antigens, including those from TBP, so as to reinforce protection, and as demonstrated in the *Borrelia* sp./*I. scapularis* infection model for OsPA and Salp15 (Dai et al., 2009). The only ectoparasite vaccines that are commercially available (in Australia and Cuba) target Bm86, a midgut protein of *R. microplus*—a one-host tick species—and interfere with feeding and subsequent egg

production (Willadsen et al., 1995). Diminution of transmission was thus secondary to reduction in the population of *R. microplus* ticks. Unfortunately, the efficacy of these vaccines is geographically variable due to species and strain specificity and they are no longer commercialized in Australia. Even if orthologs of Bm86 existed in other tick species, however, the strategy was not transposable to tick species that feed on multiple hosts, such as wildlife species, in addition to the host species for which the vaccine is intended. For TBP transmitted by ticks with a broad range of hosts, vaccines that exert a direct effect on tick blood meal acquisition or vector competence must be found. Nevertheless, the anti-Bm86 vaccines provide a powerful proof of principle for the feasibility of creating and deploying anti-tick vaccines.

Salivary proteins represent good vaccine candidates as, owing to their contact with the host immune system, they may allow natural boosting of the host response upon exposure to ticks, limiting the need for repeated administrations (Nuttall et al., 2006). Targeting salivary proteins playing a key role in tick feeding is expected to interfere with completion of the blood meal and subsequently affect their reproductive fitness. Moreover, this strategy may also cause tick rejection and thus abolish or limit pathogen transmission, which typically occurs many hours or even days after tick attachment for hard ticks. Indeed, encouraging results have been obtained for several tick species including *H. longicornis* (Mulenga et al., 1999; Imamura et al., 2005; Zhang et al., 2011; Anisuzzaman et al., 2012), *R. appendiculatus* (Imamura et al., 2006), *O. moubata* and *O. erraticus* (Astigarraga et al., 1995; Garcia-Varas et al., 2010), *A. americanum* (de la Fuente et al., 2010b), *R. (B) microplus* (Andreotti et al., 2002; Merino et al., 2013; Ali et al., 2015), and *I. ricinus* (Prevot et al., 2007; Decrem et al., 2008b). Finally, anti-tick vaccination may also target salivary proteins directly implicated in TBP transmission. Studies carried out to this end concern *I. scapularis* and the salivary antigens Salp15 (Dai et al., 2009), TSLPI (Schuijt et al., 2011), and tHRF (Dai et al., 2010), as regards the transmission of *Borrelia* sp. to mice, and the salivary antigen Salp25D as regards acquisition of *Borrelia* sp. by ticks (Wagemakers et al., 2016). Last, vaccination against the cement protein 64TRP of *I. ricinus* also afforded protection against TBE transmission to mice (Labuda et al., 2006).

CONCLUSION

The rapid evolution of tick distribution and density has created an urgent need for more effective methods for tick control and for surveillance and risk assessment for TBD (Heyman et al., 2010; Leger et al., 2013; Medlock et al., 2013). The deployment of anti-tick vaccines designed to reduce tick populations and/or transmission of TBP and reduce reliance on acaricides and repellents would represent a major improvement over current control measures, being environmentally safe and less likely than acaricides to select resistant strains. Risk assessment for human and animal populations will determine public and veterinary public health priorities, and instruct the implementation of appropriate countermeasures or complementary studies. These goals, however, require a better understanding of tick biology and of the tripartite relationship between ticks, TBP and vertebrate hosts, including the molecular interactions underlying TBP transmission. This includes the subversion of the host response mediated by saliva introduced into the host during tick feeding. Given the central role of tick SG and tick saliva both in tick biology and TBP transmission, their investigation may underpin the discovery of immunological markers for meaningful assessment of exposure to tick bites and as vaccinal candidates to protect against TBD. Ultimately, deciphering the physiology of the essential organ represented by tick SG may lead to the conception of hitherto unimagined strategies for controlling ticks and TBD.

AUTHOR CONTRIBUTIONS

LS, MK, JR, and SB conducted the literature research, wrote the paper and prepared the figures and tables. All authors provided critical review and revisions.

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Analysis of the Salivary Gland Transcriptome of Unfed and Partially Fed *Amblyomma sculptum* Ticks and Descriptive Proteome of the Saliva

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Ticks are obligate blood feeding ectoparasites that transmit a wide variety of pathogenic microorganisms to their vertebrate hosts. *Amblyomma sculptum* is vector of *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF), the most lethal rickettsiosis that affects humans. It is known that the transmission of pathogens by ticks is mainly associated with the physiology of the feeding process. Pathogens that are acquired with the blood meal must first colonize the tick gut and later the salivary glands (SG) in order to be transmitted during a subsequent blood feeding via saliva. Tick saliva contains a complex mixture of bioactive molecules with anticlotting, antiplatelet aggregation, vasodilatory, anti-inflammatory, and immunomodulatory properties to counteract both the hemostasis and defense mechanisms of the host. Besides facilitating tick feeding, the properties of saliva may also benefits survival and establishment of pathogens in the host. In the current study, we compared the sialotranscriptome of unfed *A. sculptum* ticks and those fed for 72 h on rabbits using next generation RNA sequencing (RNA-seq). The total of reads obtained were assembled in 9,560 coding sequences (CDSs) distributed in different functional classes. CDSs encoding secreted proteins, including lipocalins, mucins, protease inhibitors, glycine-rich proteins, metalloproteases, 8.9 kDa superfamily members, and immunity-related proteins were mostly upregulated by blood feeding. Selected CDSs were analyzed by real-time quantitative polymerase chain reaction preceded by reverse transcription (RT-qPCR), corroborating the transcriptional profile obtained by RNA-seq. Finally, high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis revealed 124 proteins in saliva of ticks fed for 96–120 h. The corresponding CDSs of 59 of these proteins were upregulated in SG of fed ticks. To the best of our knowledge, this

is the first report on the proteome of *A. sculptum* saliva. The functional characterization of the identified proteins might reveal potential targets to develop vaccines for tick control and/or blocking of *R. rickettsii* transmission as well as pharmacological bioproducts with antihemostatic, anti-inflammatory and antibacterial activities.

Keywords: *Amblyomma*, tick, salivary glands, blood feeding, spotted fever, RNA-seq, transcriptome, proteome

INTRODUCTION

Ticks are obligate ectoparasites that infest numerous species of vertebrates. As result of their feeding on blood, these arthropods are versatile vectors of a wide variety of pathogenic microorganisms, such as viruses, bacteria, helminths, and protozoa (Jongejan and Uilenberg, 2004; Dantas-Torres et al., 2012; Otranto et al., 2013). Rocky Mountain spotted fever (RMSF), caused by *Rickettsia rickettsii*, is the most lethal tick-borne rickettsiosis that affects humans (Dantas-Torres, 2007). This disease is widely distributed in the Americas (Dantas-Torres, 2007; Walker, 2007; Labruna, 2009), with high case fatality rates (Angerami et al., 2006; Labruna, 2009). Different tick species have been implicated as *R. rickettsii* vectors, being *Dermacentor variabilis* and *Dermacentor andersoni* the main vectors in North America (Dantas-Torres, 2007). *Amblyomma americanum* and *Rhipicephalus sanguineus* are also implicated as vectors in the United States, respectively in the states of North Carolina and Arizona (Demma et al., 2005; Breitschwerdt et al., 2011). In Central and South America, the most important species that transmit *R. rickettsii* belong to the *Amblyomma cajennense* complex (Labruna, 2009; Nava et al., 2014). In the Brazilian territory, *Amblyomma sculptum* (formely named *A. cajennense*; Nava et al., 2014) and *Amblyomma aureolatum*, are incriminated as vectors (Labruna, 2009).

Amblyomma sculptum is widely distributed in Brazil, mainly in the southeast region. This tick species infests many species of both wild and domestic animals, although horses are the preferred hosts (Labruna et al., 2001). Capybaras (*Hydrochoerus hydrochaeris*) are also primary hosts of *A. sculptum*, mostly in RMSF endemic areas, being infested by all tick parasitic stages and acting as amplifier hosts (Labruna, 2009; Szabó et al., 2013). In addition, capybaras are susceptible to *R. rickettsii*, maintaining high bacteremia for several weeks and allowing infection of ticks (Souza et al., 2009). In the last decades, many ecological changes contributed to an increase in the populations of capybaras in the southeast region of Brazil, leading to an augment in *A. sculptum* density and, consequently, the re-emergence of RMSF (Labruna, 2009; Szabó et al., 2013). Importantly, besides transmission of rickettsiae, the bite of *A. sculptum* causes pain, severe inflammatory reaction, fever, and stress, resulting in significant economic losses (Oliveira et al., 2003).

The transmission of pathogens by ticks is mainly associated with the physiology of the feeding process and also with the vector immune system. Generally, the common route of pathogens acquired during the blood meal is the migration from the midgut (MG) to the haemocoel and, subsequently, the colonization of the salivary glands (SG) (Kazimírová and Štibrániová, 2013). Pathogens within the tick SG must then reach the saliva to be transmitted during a subsequent blood feeding. Tick saliva contains a complex mixture of bioactive molecules with anticlotting, antiplatelet aggregation, vasodilatory, anti-inflammatory, and immunomodulatory properties to counteract the host defense mechanisms (Hajdušek et al., 2013; Kazimírová and Štibrániová, 2013; Kotál et al., 2015; Šimo et al., 2017). Besides facilitating tick feeding, the antihemostatic and immunomodulatory properties of tick saliva may also benefit survival and establishment of pathogens in the host (Kazimírová and Štibrániová, 2013; Šimo et al., 2017). Therefore, the identification and characterization of bioactive molecules of tick SG and saliva might help to elucidate the molecular mechanisms of interaction between ticks, pathogens, and vertebrate hosts, revealing new vaccine targets to control ticks and the pathogens they transmit.

In the current study, the gene expression of the SG of unfed and 72 h fed *A. sculptum* was performed by next generation RNA sequencing (RNA-seq). The expression of selected coding sequences (CDSs) in SG of unfed, 24 and 72 h fed ticks was further analyzed by real-time quantitative polymerase chain reaction preceded by reverse transcription (RT-qPCR) in order to determine their temporal transcriptional profile. Finally, we determined the set of proteins contained in saliva of fed *A. sculptum* by high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Data presented in this study amplify the knowledge of proteins possibly involved in tick feeding, which may also play a role on transmission of pathogens. Future functional studies to determine the role of such proteins on *A. sculptum* physiology as well as on the acquisition and transmission of *R. rickettsii* are warranted and might be useful to identify potential vaccine targets.

MATERIALS AND METHODS

Ethics Statement

All procedures involving vertebrate animals were carried out according to the Brazilian National Law number 11794 and approved by the Institutional Animal Care and Use Committees from the Faculty of Veterinary Medicine and Zootecnics (protocol number 1423/2008) and the Institute of Biomedical Sciences (protocol number 128/2011), University of São Paulo, São Paulo, Brazil. Animal purchase and euthanasia procedures were performed as described in Galletti et al. (2013).

Ticks and Sample Collection

Ticks were obtained from a laboratory colony of *A. sculptum* (Pedreira strain, São Paulo, Brazil). Larvae, nymphs, and adults were fed on rabbits (*Oryctolagus cuniculus*) as previously

described (Pinter et al., 2002). Off-host phases were held in an incubator at 25°C and 95% of relative humidity. Adult females were manually removed from the vertebrate hosts after 24 or 72 h of feeding for dissection of SG. Firstly, ticks were washed in 70% ethanol and subsequently in sterile phosphate-buffered saline (PBS) (10 mM NaH_2PO_4, 1.8 mM KH_2PO_4, 140 mM NaCl, and 2.7 mM KCl, pH 7.4) for 10 min each. After dissection, SG were gently washed in sterile PBS and immediately transferred to 50 μL of RNA*later*® Stabilization Solution (Life Technologies, Carlsbad, CA, USA). The SG from unfed *A. sculptum* females (control) were dissected using the same procedure.

Salivation of females fed for 96–120 h on rabbits was induced by injection of approximately 1–3 μL of a solution of 50 mg/mL pilocarpine in 0.7 M NaCl into the tick hemocoel using a 12.7 × 0.33 mm needle BD Ultra-Fine™ (Becton Dickinson and Company, Franklin Lakes, NJ, USA) (Oliveira et al., 2013). The saliva was harvested every 10–15 min using a micropipette and transferred to a polypropylene tube kept in ice. Samples were stored at −80°C until use.

RNA Isolation, RNA-Seq and Bioinformatics Analysis

The total RNA from tick SG was isolated using the NucleoSpin®TriPrep Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's specifications. The RNA extracted from 20 samples (each one composed by SG of three ticks) of each group (ticks unfed or fed for 72 h on rabbits) contributed equally for the composition of the pool RNA samples submitted to high throughput RNA-seq. Samples were tagged with specific barcodes and multiplex sequenced in four lanes using an Illumina HiSeq platform at the North Carolina State University facility (Raleigh, NC, USA).

Approximately 567 million reads of 101 base pairs were obtained using the single read mode [these reads also include the transcriptome of the MG of *A. sculptum* and *A. aureolatum* (Martins et al., 2017) and also of the SG of *A. aureolatum* data not shown]. Reads for each species were assembled using Abyss and Soapdenovo Trans programs with *K*-values varying from 21 to 91 (in 10 interval increments). Resulting assembly of reads were concatenated and clustered following the procedure described by Karim et al. (2011). CDSs were extracted based on matches to public databases or longer open reading frames with a signal peptide as an indicative of secretion (Karim et al., 2011). The comparison of the predicted amino acid sequences translated from the nucleotide sequences to the non-redundant protein database of the NCBI and to the Gene Ontology (GO) database (Ashburner et al., 2000) was performed using the blastx tool (Altschul et al., 1997). In addition, the tool reverse position-specific BLAST (rpsblast) (Altschul et al., 1997) was used to search for conserved protein domains in the Pfam (Bateman et al., 2000), SMART (Schultz et al., 2000), KOG (Tatusov et al., 2003), and conserved domains databases (CDD) (Marchler-Bauer et al., 2002). To identify putative secreted proteins, predicted protein products starting with a methionine residue were submitted to the SignalP server (Nielsen et al., 1997; Petersen et al., 2011). Glycosylation sites on the proteins were predicted by use of the program NetOGlyc (Julenius et al., 2005). The functional annotation of the CDSs was performed based on all the comparisons described above and their *e*-values. Finally, CDSs and their encoded proteins were classified based on function and/or protein families. Reads Per Kilobase Million (RPKM) values for deduced CDSs were calculated as described previously (Kotsyfakis et al., 2015b). Data were organized in a hyperlinked spreadsheet as described by Ribeiro et al. (2004). The complete table (Supplementary Table 1) with links may be downloaded from http://exon.niaid.nih.gov/transcriptome/Amb_sculptum/Supplementary_Table_1-Web.xlsx. The raw data were deposited to the Sequence Read Archives (SRA) of the National Center for Biotechnology Information (NCBI) under Bioproject number PRJNA343654. Only CDSs representing 90% of known proteins or larger than 250 amino acids were deposited to the Transcriptome Shotgun Annotation (TSA) portal of the NCBI [accession number GFAA00000000, version GFAA00000000.1; Biosamples SAMN05792022 (SG of unfed ticks) and SAMN05792023 (SG of ticks fed for 72 h)].

To compare the gene expression between unfed and fed ticks, paired comparisons of the number of reads hitting each contig were calculated by X^2 tests to detect significant differences between samples when the minimum expected value was larger than 2 and $p < 0.05$. Normalized fold-ratios of the sample reads were computed by adjusting the numerator by a factor based on the ratio of the total number of reads in each sample, and adding one to the denominator to avoid division by zero.

The amino acid sequences of proteins encoded by selected CDSs (Acaj-56179, GenBank protein ID: JAU02549.1; Acaj-77950, Genbank protein ID: JAU02547.1; Acaj-65746, GenBank protein ID: JAU03230.1; and AcajSIGP-14784, Genbank protein ID: JAU02578.1) were used as query in blastp searches against Transcriptome Shotgun Assembly (tsa_nr; NCBI) database with the class Arachnida (taxid: 6854) as filter. The protein sequence of a given arachnid species with the best match with *A. sculptum* was selected as representative for that species (accession numbers available in Supplementary Table 2) and used to performing multiple sequence alignment (MSA) with the MUSCLE method (Edgar, 2004) and graphically edited using BioEdit software (Hall, 1999). Phylogenetic analysis of sequences was constructed with the Maximum Likelihood (ML) method with Jones-Taylor-Thornton (JTT) matrix-based substitution model (Jones et al., 1992) using MEGA 5 (Tamura et al., 2011) software. Node support of each clade was evaluated using a bootstrap analysis (1,000 replicates) (Felsenstein, 1985). The distances between the sequences (degree of similarity) are in the units of the number of amino acid substitutions per site (computed by the Poisson correction method) (Zuckerkandl and Pauling, 1965).

RT-qPCR

Ten CDSs detected as differentially expressed by RNA-seq analysis were selected to be evaluated using RT-qPCR. One microgram of the total RNA extracted from the SG of unfed females or females fed for either 24 or 72 h on rabbits were treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA). Resulting RNA was used as template for reverse transcription (RT) in complementary DNA (cDNA) using Platus transcriber

RNase H-cDNA First Strand Kit (Sinapse–Inc, Miami, FL, USA) as described by the manufacturer.

Resulting cDNA was used as template in qPCR. The reactions were performed in a StepOne™ Plus System using SYBR® Green PCR Master Mix (equipment and reagent from Thermo Fisher Scientific, Waltham, MA, USA) and specific primers for selected CDSs (Supplementary Table 3) with cycling parameters of 95°C for 10 min followed by 40 cycles at 95°C for 15 s, 60°C for 60 s, and 72°C for 20 s. A melting curve analysis was carried out to check the specificity of the primers. Primers were designed using Primer3 (Rozen and Skaletsky, 2000). To determine the efficiency of each pair of primers, standard curves were generated using different concentrations of cDNA (400 to 3.12 ng; 2-fold dilution). Only primers presenting efficiency above 90% were used in the analyses. In addition, primer specificity was confirmed using DNA extracted from rabbit blood as control.

The $2^{-\Delta\Delta Ct}$ equation (Livak and Schmittgen, 2001) was utilized to calculate the relative expression (fold-change) of select CDSs in fed vs. unfed ticks. The CDS of the ribosomal protein S3A (Supplementary Table 3), constitutively expressed in SG of fed and unfed ticks (data not shown), was used as reference. Eight biological replicates (each one composed by RNA extracted from SG of three ticks) of each group (ticks fed for either 24 or 72 h and unfed ticks) were analyzed. All samples were analyzed in three technical replicates. The expression of a CDS was considered statistically different by comparing the median of the eight biological replicates of each group using the Wilcoxon test and *p*-values were corrected by the False Discovery Rate (FDR) method (Benjamini and Hochberg, 1995) for multiple tests. Difference in CDS expression was considered significant when $p < 0.05$. Spearman's correlation coefficient was calculated by GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA) using log-transformed fold-change values from qPCR and RNA-seq experiments to verify the replicability between these two techniques.

High-Performance Liquid Chromatography Coupled with Tandem Mass Spectrometry (LC-MS/MS) and Data Analysis

Five samples containing the saliva of five ticks each (females fed for 96–120 h) were independently processed and analyzed. First, protein concentration in each sample was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Proteins were submitted to extraction according to Mudenda et al. (2014), with modifications. After dilution in urea 8 M (1:10; v/v), proteins were reduced with DTT and alkylated with iodoacetamide. Urea was removed and the buffer was changed to 50 mM ammonium bicarbonate with 10% acetonitrile by ultrafiltration with a 10 kDa cutoff Amicon Ultra 0.5 mL filtration unit (Millipore). After digestion with trypsin, resulting peptides were submitted to LC-MS/MS analysis using an Orbitrap Fusion Tribrid (Thermo Fisher Scientific) mass spectrometer coupled with a Proxeon nano-LC through a nanoelectrospray ion source. Peptides were loaded onto an Acclaim™ PepMap™ 100 C_{18} pre-column (5 μm, 100 Å, 75 μm × 2 cm; Thermo Fisher Scientific) and separated on a PepMap™ RSLC C_{18} column (2 μm, 100 Å, 75 μm × 25 cm; Thermo Fisher Scientific) using a linear gradient of acetonitrile (0–40%) in 0.1% formic acid for 80 min at a flow rate of 300 nL/min. This gradient was followed by a 2 min increase to 80% acetonitrile and held at this concentration for 3 min. The nanoelectrospray voltage of the capillary was set to 1.8 kV. The analysis was performed in full scan (m/z 400–1,600) with the resolution of Orbitrap adjusted to 120,000. Using a 3 s cycle time and rapid scan, peptide ions with charge states 2–8 were fragmented in the linear ion trap using low-energy CID (normalized collision energy of 35%) and an isolation width of 1.6 dynamic exclusion was enabled with an exclusion duration of 20 s, and a repeat count of 1.

For protein identification, raw files were imported into PEAKS version 8.5 (Bioinformatics Solutions, Inc.,) and searched against *A. sculptum* transcript fasta database (9,560 CDSs presented in the current study) and the cRAP contaminant database (the common Repository of Adventitious Proteins—http://www.theGPM.org/crap), using a tolerance of 10 ppm for the precursor ion and 0.6 Da for fragment ions. Enzyme specificity was set to trypsin with a maximum of two missed cleavages and one non-specific cleavage. Carbamidomethylation of cysteine (57.021 Da) was set as a fixed modification, and oxidation of methionine (15.994 Da), and deamidation of asparagine and glutamine (0.98 Da) were selected as variable modifications. All peptide identifications were filtered in order to achieve a false discovery rate (FDR) of 0.5% using a decoy database approach. Contaminant proteins were filtered from the final list. The complete dataset was deposited to Pride database (accession number PXD007852).

RESULTS

Gene Expression Profile of *A. sculptum* SG in Response to Blood Feeding

The sialotranscriptomes of unfed and fed ticks were determined using high throughput RNA-seq. Resulting reads were assembled into 9,560 CDSs distributed in different functional classes (**Table 1** and Supplementary Table 1). Over 29 million reads were obtained for unfed ticks, whereas 72 h fed ticks accounted almost 41 million reads (**Table 1**). Reads within transcription, protein synthesis, protein export, proteasome machineries, and transporters/storage functional classes were more represented in sialotranscriptome of unfed than of fed ticks (**Table 1**). On the other hand, reads of proteins predicted to be secreted corresponded to 36.53% of the sialotranscriptome of fed ticks (**Table 1**) and only to 10.95% of sialotranscriptome of unfed ticks (**Table 1**).

The majority of CDSs was shared between unfed and fed ticks (8,802 CDSs). Other CDSs were identified exclusively in sialotranscriptome of unfed or fed ticks. Among the 42 CDSs identified exclusively in the sialotranscriptome of unfed ticks, 18 are predicted to be secreted and all of them code unknown proteins. In addition, 535 CDSs are exclusive of the sialotranscriptome of fed ticks, among which 296 encode putative secreted proteins. Two thirds of the CDSs of putative secreted proteins detected only in sialotranscriptome of fed ticks present

TABLE 1 | Functional classification of CDSs of unfed and fed *A. sculptum* ticks.

Functional class	SG-Unfed			SG-Fed (72 h)		
	Number of CDSs	Number of reads	Relative abundance of reads* (%)	Number of CDSs	Number of reads	Relative abundance of reads* (%)
Cytoskeletal	301	1,108,899	3.79	304	907,896	2.23
Detoxification	85	58,478	0.20	92	126,600	0.31
Extracellular matrix/cell adhesion	291	2,025,509	6.93	302	3,084,348	7.58
Immunity	111	105,175	0.36	121	78,672	0.19
Amino acid metabolism	43	61,283	0.21	45	81,064	0.20
Carbohydrate metabolism	131	258,736	0.88	132	364,731	0.90
Energy metabolism	82	290,754	0.99	85	638,711	1.57
Intermediary metabolism	16	18,384	0.06	16	33,194	0.08
Lipid metabolism	183	258,154	0.88	196	305,605	0.75
Nucleotide metabolism	58	94,988	0.32	62	52,976	0.13
Nuclear export	32	310,812	1.06	32	119,450	0.29
Nuclear regulation	205	545,375	1.86	205	366,541	0.90
Protein export machinery	303	1,069,385	3.66	305	884,552	2.17
Protein modification machinery	192	434,173	1.48	197	1,061,107	2.61
Proteasome	176	819,051	2.80	176	505,338	1.24
Protein synthesis	287	8,053,986	27.54	288	4,133,884	10.16
Signal transduction	807	4,422,649	8.28	814	1,532,993	3.76
Storage	18	99,099	0.34	19	97,785	0.24
Transcription factor	131	406,243	1.39	132	222,886	0.55
Transcription machinery	425	2,686,898	9.19	426	1,426,038	3.50
Transporters and channels	381	1,226,355	4.19	397	793,366	1.95
Unknown	680	1,097,518	3.75	769	1,469,774	3.61
Unknown, conserved	1011	2,097,204	7.17	1024	3,759,621	9.24
Putative secreted	2314	3,202,168	10.95	2592	14,865,642	36.53
Transposable elements	544	314,820	1.08	567	961,273	2.36
Viral products	37	179,294	0.61	39	2,818,989	6.93
Total	8844	29,245,390		9337	40,693,036	

**Relative abundance of reads: number of reads within each functional class in one specific sialotranscriptome (unfed ticks or ticks fed for 72 h) / total number of reads in the same sialotranscriptome.*

unknown function and the remaining belongs to well-known protein families, such as protease inhibitors, metalloproteases, and lipocalins (Supplementary Table 1).

The expression of 10 CDSs identified in the sialotranscriptome as differentially expressed by tick feeding was further assessed by RT-qPCR (**Figure 1** and **Table 2**). The downregulation of sequences encoding glycine-rich proteins (Acaj-81474 and Acaj-81475), eukaryotic translation initiation factor 4 gamma (Acaj-72892), and tick cystatin 1 (AcajSIGP-29822) was confirmed by RT-qPCR (**Figure 1A** and **Table 2**). The transcript levels of all of these CDSs were already significantly lower in SG of ticks fed for 24 h (**Figure 1A** and **Table 2**), except for AcajSIGP-29822, which was not differentially expressed in ticks fed for 24 h, with significant differences in relation to the control (unfed ticks) only in ticks fed for 72 h (**Figure 1A** and **Table 2**). The upregulation of sequences encoding putative secreted cysteine rich protein containing trypsin inhibitor-like (TIL) domain (Acaj-56179), glycine-rich proteins (Acaj-73764 and Acaj-74654), peptidoglycan recognition protein (PGRP) (AcajSIGP-81204), tick Kunitz inhibitor (Acaj-77950), and putative secreted SG antimicrobial peptide (AMP) similar to defensin (Acaj-65746) was also confirmed by RT-qPCR (**Figure 1B** and **Table 2**). The transcript levels of all of these CDSs were higher in SG of ticks fed for either 24 or 72 h than in unfed ticks, although the upregulation of the defensin (Acaj-65746) was significant only after 72 h (**Figure 1B** and **Table 2**). The correlation analysis showed that the gene expression regulation upon blood feeding was highly positively correlated between RNA-seq and RT-qPCR measurements ($r = 0.9756$, $p < 0.0001$), strengthening the transcriptional findings of the current study (**Figure 1C**).

Transcription of Putative Secreted Proteins in SG of *A. sculptum* Ticks during Feeding and Secretion to Saliva

As mentioned above, the majority of sequences encoding proteins predicted to be secreted were upregulated by blood feeding (**Table 3**, **Figure 2** and Supplementary Table 1).

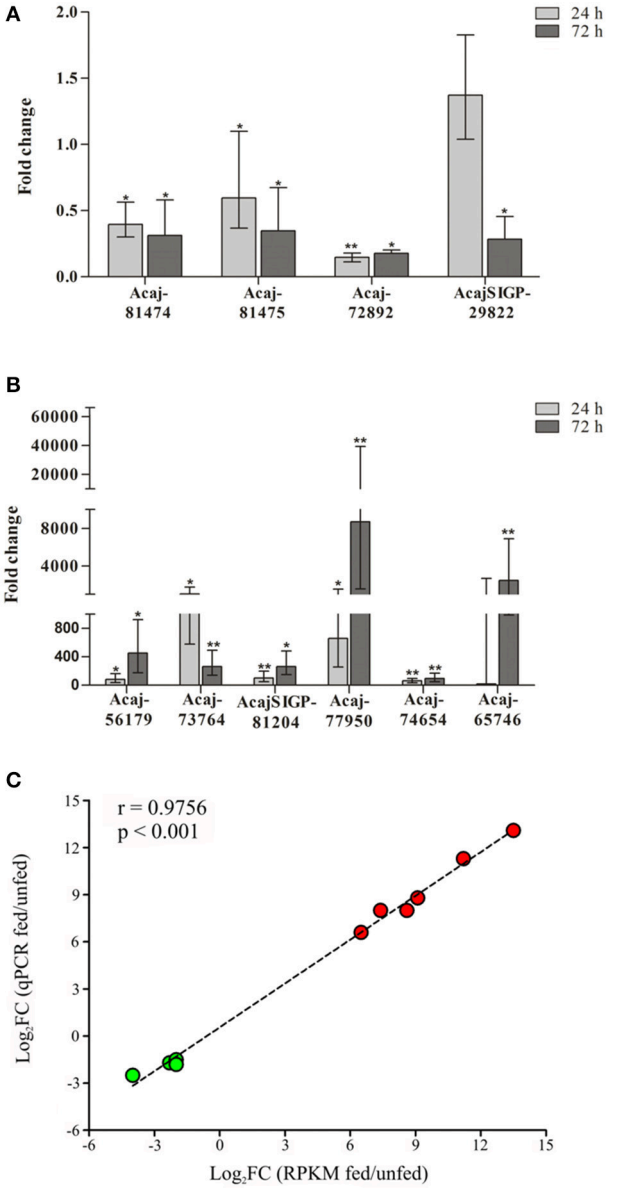

FIGURE 1 | Temporal transcriptional analysis of selected CDSs in SG of unfed and fed ticks by RT-qPCR. **(A)** Downregulated and **(B)** upregulated CDSs identified by RNA-seq were selected for RT-qPCR analysis. The expression levels of CDSs in ticks fed for either 24 or 72 h was compared to expression in SG of unfed ticks (control) using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Error bars represent 95% of confidence interval; $^{*}p < 0.05$ and $^{**}p < 0.001$ are corrected for multiple comparisons by the False Discovery Rate (FDR). **(C)** Spearman correlation between the expression data determined by RT-qPCR and RNA-seq (RPKM values). The fold-changes obtained by either qPCR (y-axis) or RNA-seq (x-axis) was plotted with log-transformed values; therefore negative values means downregulation (CDSs represented by green symbols), while positive values means upregulation (CDSs represented by red symbols). The dashed line represents the goodness of fit of the data calculated by linear regression analysis.

Among them, we highlight members of lipocalin, mucin, metalloprotease, 8.9 kDa, and serine protease inhibitors families as well as immunity-related proteins (such as AMPs)

TABLE 2 | Relative expression of selected CDSs in SG of fed ticks in relation to unfed ticks by RNA-seq and RT-qPCR.

CDSs	Annotation	RNA-seq	RT-qPCR	
		72 h	72 h	24 h
Acaj-81474	Glycine-rich cell wall structural protein	0.20*	0.31*	0.40*
Acaj-81475	Glycine-rich cell wall structural protein	0.24*	0.35*	0.60*
Acaj-72892	Eukaryotic translation initiation factor 4 gamma	0.06*	0.18*	0.15**
AcajSIGP-29822	Tick_cistatins_1	0.25*	0.28*	1.37
Acaj-56179	Secreted cysteine rich protein partial	3,443.20*	454.77*	83.06*
Acaj-73764	Glycine-rich cell wall structural protein 2	164.99*	262.06**	1,032.91*
AcajSIGP-81204	Peptidoglycan recognition protein	374.91*	263.88*	100.78**
Acaj-77950	Tick_Kunitz_135	7,868.61*	8,694.17**	660.38*
Acaj-74654	Glycine-rich cell wall structural protein	89.75*	95.75**	62.79**
Acaj-65746	Secreted salivary gland peptide	1,177.56*	2,460.95**	16.51

$^{*}p < 0.05$; $^{**}p < 0.001$ [Statistically significant differences of expression in the SG of fed ticks in relation to the control (unfed ticks)].

(**Table 3**). Among 72 lipocalin CDSs found in the *A. sculptum* sialotranscriptome, 65 were upregulated by blood feeding (**Figure 2**). Similarly, 12 out of 36 mucin CDSs were upregulated (**Figure 2**). In addition, 24 CDSs of metalloproteases were upregulated and only six were downregulated (**Figure 2**). The sialotranscriptome of *A. sculptum* also revealed the upregulation of several protease inhibitor transcripts by blood feeding, the majority belonging to TIL and Kunitz families. Among the 39 CDSs representing those protease inhibitors, 33 were upregulated by feeding (**Figure 2**). Components of both inhibitor families were previously reported to exhibit antimicrobial properties in ticks (Fogaça et al., 2006; Ceraul et al., 2008). The annotation for the protein encoded by the CDS Acaj-56179 in protein domains databases (Pfam ID 01826 and UniprotKB/Swiss-Prot ID P83516, Supplementary Table 1) shows that it possesses the key features of TIL domain containing proteins. TIL domain is typically composed of five disulfide bonds formed by 10 cysteine residues in a stretch of approximately 54 amino acid residues (Bania et al., 1999), necessary for their biological properties, which include both antimicrobial and serine protease inhibitory activities (Fogaça et al., 2006; Wang et al., 2015). The MSA analysis of the amino acid sequence deduced from the CDS Acaj-56179 with similar sequences of other arachnids (**Figure 3A**) illustrated the highly conserved feature of the 10 cysteine residue positions among the sequences of hard ticks (family Ixodidae). The relationships of these sequences with sequences of soft ticks (family Argasidae) and the mite *Sarcoptes scabiei* showed the expected main clades composed by species of the families Ixodidae and Argasidae separately (**Figure 3B**), and the mite sequence placed into the soft tick branch as outer group.

TABLE 3 | Functional classification of CDSs of putative secreted proteins in unfed and fed *A. sculptum* ticks.

Families of putative secreted proteins	Number of CDSs per protein family	Total reads in SG unfed	Relative abundance of reads (%)	Total reads in SG Fed (72 h)	Relative abundance of reads (%)
ENZYMES					
Metalloproteases	33	130,620	4.08	953,503	6.41
Cysteine proteases	7	22,069	0.69	198,909	1.34
Nucleases	22	21,884	0.68	133,781	0.90
Lipases/Esterases	17	14,441	0.45	24,844	0.17
Other proteases	9	16,156	0.50	21,342	0.14
Serine proteases	6	23,930	0.75	15,524	0.10
Other enzymes	2	2,351	0.07	4,701	0.03
PROTEASE INHIBITORS					
TIL domain	10	9,733	0.30	182,071	1.22
Kunitz domain	29	11,061	0.35	129,613	0.87
Cysteine protease inhibitors	6	2,577	0.08	11,862	0.08
Thyropin	2	868	0.03	3,576	0.02
Kazal domain	2	589	0.02	1,190	0.01
Other serine protease inhibitors	16	74,175	2.32	1,587,200	10.68
Lipocalins	72	8,456	2.64	570,185	3.84
Mucins	36	111,191	3.47	244,246	1.64
IMMUNITY					
Antimicrobial peptides	12	2,462	0.08	43,771	0.29
Evasins	7	2,844	0.09	30,023	0.20
Da-p36 immunosuppressant family	10	130	0.00	29,798	0.20
Lectins	8	16,069	0.50	15,142	0.10
Other immunity-related	6	13,332	0.42	77,167	0.52
TICK-SPECIFIC PROTEINS					
Glycine-rich proteins	17	32,252	10.07	3,325,247	22.37
Basic tail	6	72,556	2.27	469,358	3.16
8.9 kDa superfamily	24	4,095	0.13	118,235	0.80
Toxins	3	968	0.03	47,120	0.32
Ixodegrin	5	2,509	0.08	36,674	0.25
Adhesion	3	2,520	0.08	1,481	0.01
Cuticular	1	59	0.00	556	0.00
Novel putative secreted	82	116,574	3.64	132,096	0.89
Other putative secreted	1060	545,944	17.05	4,029,020	27.10
Unknown putative conserved secreted	103	143,033	4.47	364,287	2.45
Unknown putative secreted	1084	1,430,348	44.67	2,063,120	13.88
Total Secreted		3,202,168		14,865,642	

Importantly, TIL domain containing proteins from *Amblyomma* species were similar enough to constitute a unified subclade (red branch, **Figure 3B**). The annotation for the protein encoded by the CDS Acaj-77950 in protein domains databases (Pfam ID 00014 and UniprotKB/Swiss-Prot ID Q9WU03, Supplementary Table 1) showed that it is member of the Bovine pancreatic trypsin inhibitor (BPTI) family. MSA analysis showed the conserved disposition of the six cysteine residues of Kunitz domain (Ranasinghe and McManus, 2013) of Acaj-77950 and all similar sequences of other arachnids (**Figure 4A**). Regarding sequence relationships, the analysis showed that *Ixodes ricinus* (hard tick) is closer to the spider *Parasteatoda tepidariorum* and to the soft tick *Ornithodorus moubata* than to the other analyzed hard ticks (**Figure 4B**), which reflect the dissimilarity of *I. ricinus* and *O. moubata* sequences in relation to the other tick sequences observed in MSA analysis (**Figure 4A**).

The 8.9 kDa superfamily is composed of proteins exclusively found in hard ticks, but none of its members were functionally characterized so far (Francischetti et al., 2009; Karim et al., 2011). Importantly, 23 CDSs of members of this family were upregulated by blood feeding in *A. sculptum* SG (**Table 3** and **Figure 2**). Glycine-rich proteins correspond to

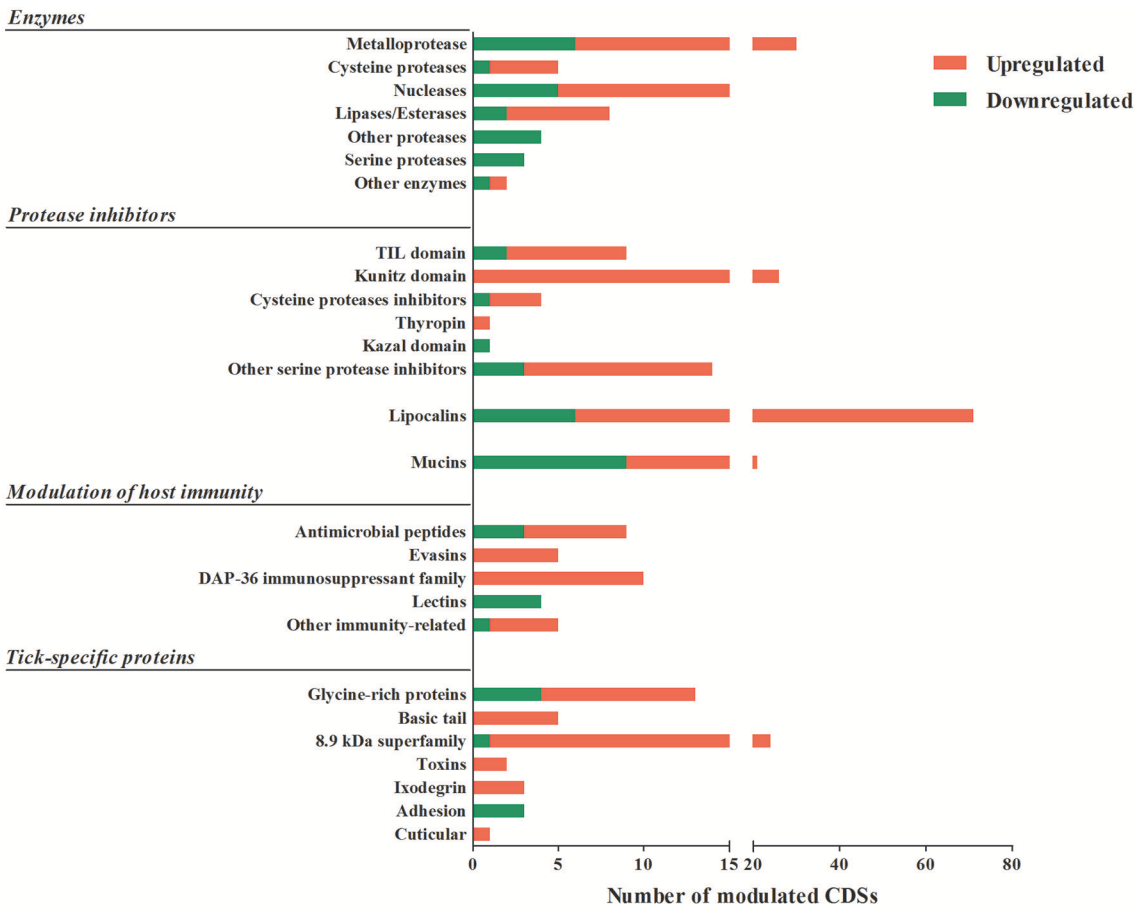

FIGURE 2 | Putative secreted proteins CDSs differentially expressed by feeding in *A. sculptum* SG. The total number of CDSs of proteins predicted to be secreted that were significantly down (green) or upregulated (red) by blood feeding in SG of *A. sculptum* are presented.

another family of proteins that are specifically found in ticks. Seventeen CDSs of glycine-rich proteins were identified in *A. sculptum* sialotranscriptome (**Table 3**), among which nine were upregulated by feeding (**Figure 2**).

The majority of sequences encoding tick immune system components was also upregulated by feeding in SG of *A. sculptum*, except for lectins (**Figure 2**). The CDSs of one defensin (Acaj-65746) was highly upregulated in SG of fed ticks (Supplementary Table 1, **Table 2**, and **Figure 1B**). The annotated information for this sequence in protein domains databases (Pfam ID 01097 and UniprotKB/Swiss-Prot ID Q86QI5, Supplementary Table 1) shows that it is member of the arthropod defensin family, also named as Knottin scorpion toxin-like in InterPro database (Gracy et al., 2008). The mature peptide chain of member of this family ranges from 38 to 51 amino acids in length with six conserved cysteine residues involved in three-disulfide bonds. The conserved cysteine residues can be observed through the alignment of Acaj-65746 with related proteins (**Figure 5A**). The phylogenetic tree resembled the Ixodidae and Argasidae taxonomic clades (**Figure 5B**). Interestingly, the defensin of *Androctonus bicolor* (the only arachnid sequence besides ticks that retrieved from blast analysis) was grouped into hard ticks main clade and not as an outer group, showing that defensins of hard ticks are more similar to scorpion than to soft ticks.

Among the sequences with putative host immunomodulatory activity, 10 CDSs of the Da-p36 immunosuppressant family were identified (**Table 3**) and all of them were upregulated by feeding (**Figure 2**). Five evasin CDSs were also detected as upregulated by feeding in *A. sculptum* SG (**Table 3** and **Figure 2**).

As expected, we also observed a high number of CDSs (2,329) encoding putative secreted proteins classified as (i) novel putative secreted (CDSs of unknown products), (ii) other putative secreted (CDSs of other classes of annotated putative secreted proteins), (iii) unknown putative conserved (CDSs of conserved putative secreted protein precursors) and (iv) unknown putative secreted proteins (CDSs of hypothetical putative secreted protein precursors; **Table 3**).

To identify the proteins encoded by *A. sculptum* SG that are secreted into the saliva, the proteome of the saliva of fed ticks was determined by LC-MS/MS. One hundred twenty-four proteins were identified (**Table 4** and Supplementary Table 4), whose all transcripts were detected in sialotranscriptome. Importantly, 58 of these proteins belong to the putative secreted

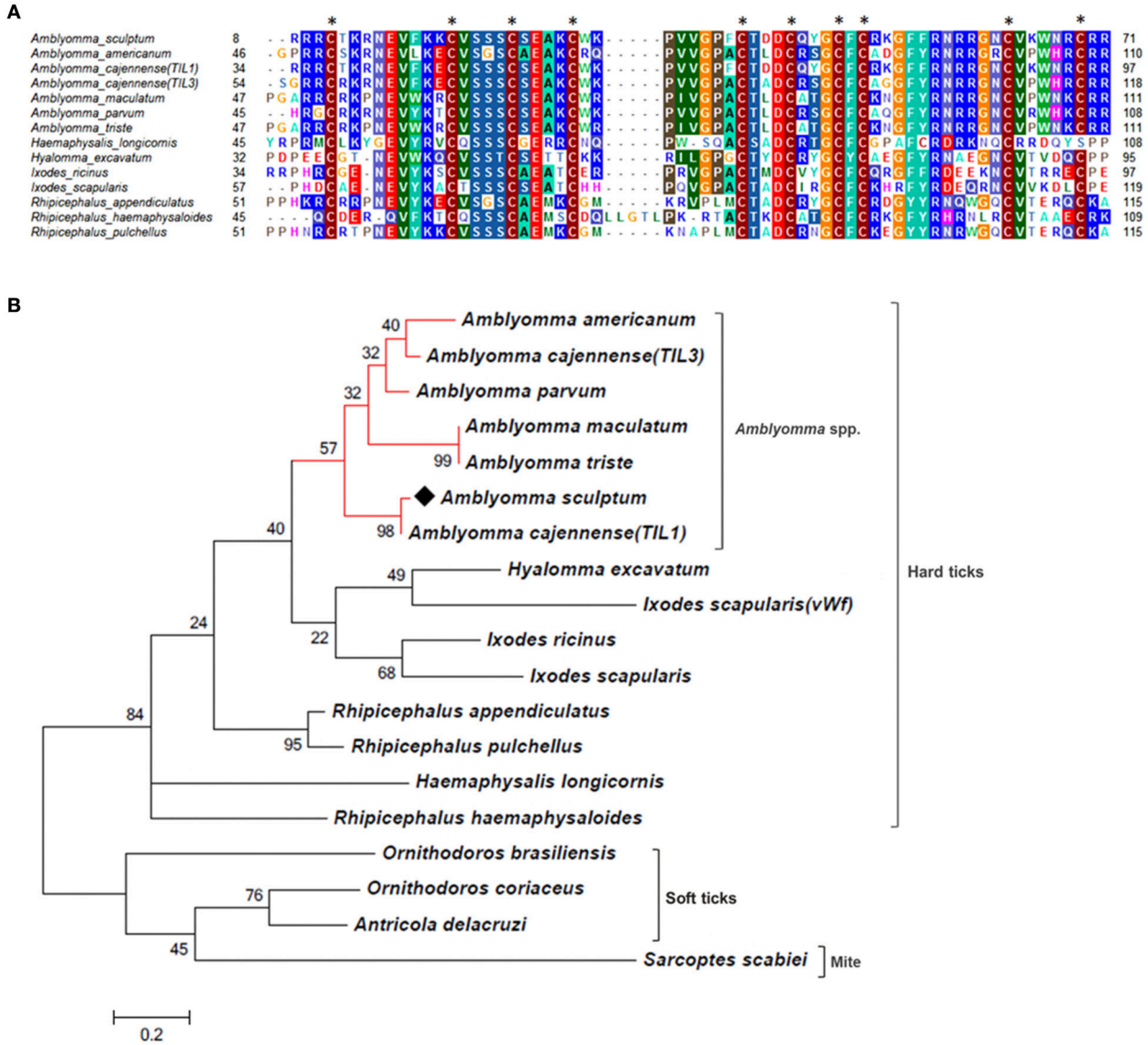

FIGURE 3 | MSA and phylogenetic analysis of TIL domain containing proteins. **(A)** Multiple sequence alignment of protein sequences was performed using MUSCLE method. The numbers flanking the alignment represent the start (left) and end (right) amino acid position of each sequence in the protein domain. Asterisks highlight the conserved cysteine residues. Threshold for shading colors of amino acid similarity was 40%. **(B)** A phylogenetic tree was constructed with protein sequences from ticks and mite using Maximum Likelihood (ML) method. Numbers next to the branches represent the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 19 amino acid sequences (accession numbers available in Supplementary Table 2). All positions containing gaps and missing data were eliminated. There were a total of 51 positions in the final dataset. Bar scale at the bottom indicates 20% amino acid divergence. Diamond symbol refers to the CDS Acaj-56179 from the sialotranscriptome of *A. scultpum* identified in this work.

protein functional class (**Table 4**). Twenty-three of these putative secreted proteins correspond to proteins with non-annotated function (Supplementary Table 4). Regarding putative secreted proteins with annotated function, we highlight six lipocalins, four 8.9 kDa proteins, three glycine-rich proteins, and three AMPs. The corresponding CDSs of five from six lipocalins detected in tick saliva were upregulated in SG of fed ticks (Supplementary Tables 1, 4). Regarding 8.9 kDa proteins, all corresponding CDSs presented high transcriptional levels in SG of fed ticks, while only one CDS of the three glycine-rich proteins detected in tick saliva was upregulated (Supplementary Tables 1, 4). Three histidine-rich AMPs similar to microplusins (Fogaça et al., 2004; Lai et al., 2004) were also detected in tick saliva (Supplementary Table 4). The corresponding CDSs of two of these proteins were upregulated in SG of fed ticks (ACAJ-77500 and ACAJSIGP-14784), while the third CDS (Acaj-57400) was not modulated (Supplementary Tables 1, 4). The protein encoded by the CDS AcajSIGP-14784 was also detected in saliva of ticks fed for 8 days

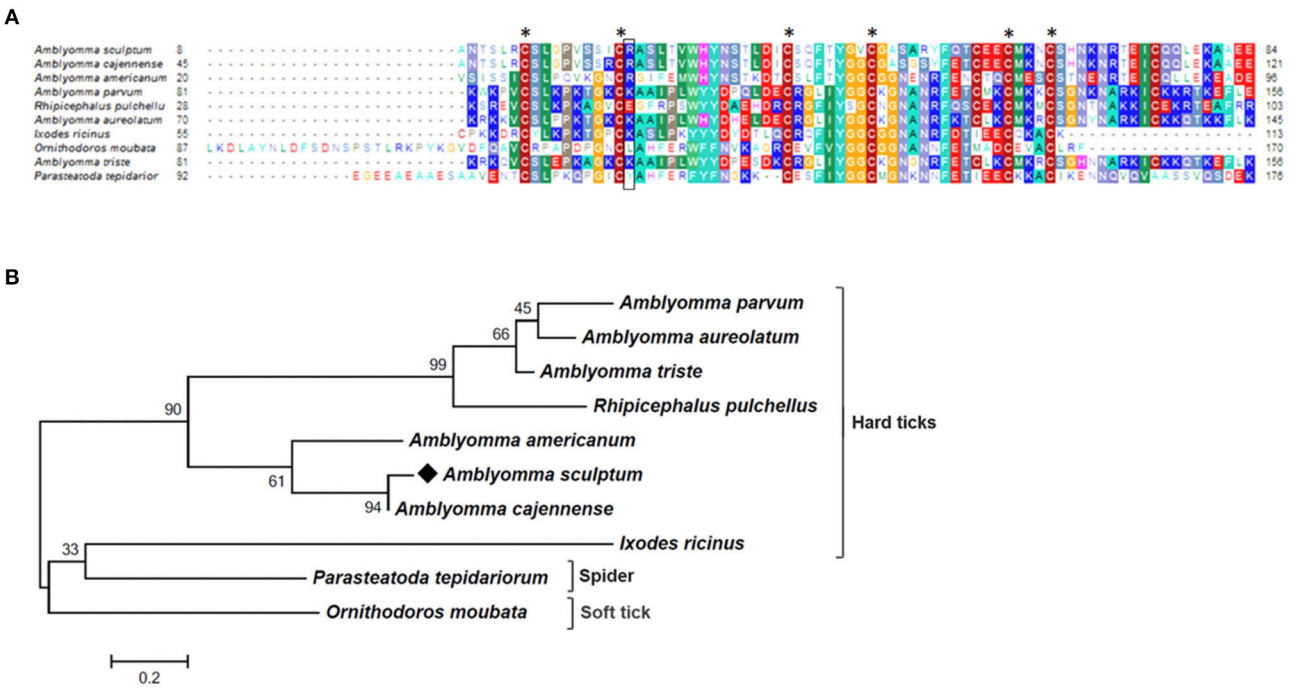

FIGURE 4 | MSA and phylogenetic analysis of Kunitz domain containing proteins. **(A)** Multiple sequence alignment of protein sequences was performed using MUSCLE method. The numbers flanking the alignment represent the start (left) and end (right) amino acid position of each sequence in the protein domain. Asterisks highlight the conserved cysteine residues, while black box correspond to P1 site. Threshold for shading colors of amino acid similarity was 40%. **(B)** A phylogenetic tree was constructed with protein sequences from ticks and spider using Maximum Likelihood (ML) method. Numbers next to the branches represent the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 10 amino acid sequences (accession numbers available in Supplementary Table 2). All positions containing gaps and missing data were eliminated. There were a total of 64 positions in the final dataset. Bar scale at the bottom indicates 20% amino acid divergence. Diamond symbol refers to the Acaj-77950 from the sialotranscriptome of the *A. scultpum* identified in this work.

on rabbits (data not shown). The MSA analysis of this peptide with similar sequences of other ticks showed that they share the six conserved cysteine residues (**Figure 6A**), a characteristic feature of microplusins (Fogaça et al., 2004; Lai et al., 2004). Sequence relationships showed that sequences of all *Amblyomma* species were grouped in one clade, while sequences of other species were grouped in a distinct clade (**Figure 6B**). The most divergent sequence in the later clade is the microplusin identified in the saliva of *Rhipicephalus microplus* (Tirloni et al., 2014; **Figure 6B**).

DISCUSSION

During the tick feeding, SG of hard ticks are able to concentrate blood nutrients by returning the excess of water and also ions to the host via saliva (Bowman and Sauer, 2004; Šimo et al., 2017). The tick saliva also contains a cocktail of antihemostatic, anti-inflammatory and immunomodulatory molecules, guaranteeing the blood meal acquisition (Francischetti et al., 2009; Hajdušek et al., 2013; Kazimírová and Štibrániová, 2013; Kotál et al., 2015; Chmelar et al., 2016b; Šimo et al., 2017). Due to the importance of SG to tick feeding, we compared the sialotranscriptomes of unfed and fed *A. sculptum* ticks. Transcripts of most of the identified CDSs were detected in both sialotranscriptomes, although certain CDSs were found exclusively in only one sialotranscriptome. The sialotranscriptome of fed ticks presented the majority of these exclusive CDSs, suggesting that the proteins encoded by these sequences might play an important role during the feeding process.

In general, transcripts within protein synthesis and transcription machinery classes showed a higher proportion in SG of unfed *A. sculptum* than in fed ticks, suggesting a downregulation effect of blood feeding on protein expression. On the other hand, sequences coding proteins predicted to be secreted were mostly upregulated by blood acquisition. In accordance to our data, putative secreted protein transcripts were also more abundant after feeding in SG of female *Amblyomma maculatum* (Karim et al., 2011) and *A. americanum* (Karim and Ribeiro, 2015). Transcription of selected CDSs were analyzed by RT-qPCR. The high correlation between RNA-seq and qPCR data strengthens the transcriptional findings of the present study.

It is well-known that tick feeding triggers host defense mechanisms, such as hemostasis, inflammation, and immune responses. The SG of ticks, in turn, secrete several molecules into saliva to counteract, modulate and evade host immune responses, ensuring a successful feeding (Chmelar et al., 2012, 2016a,b; Kotál et al., 2015; Šimo et al., 2017). Indeed, sequences encoding putative secreted proteins that present antihemostatic,

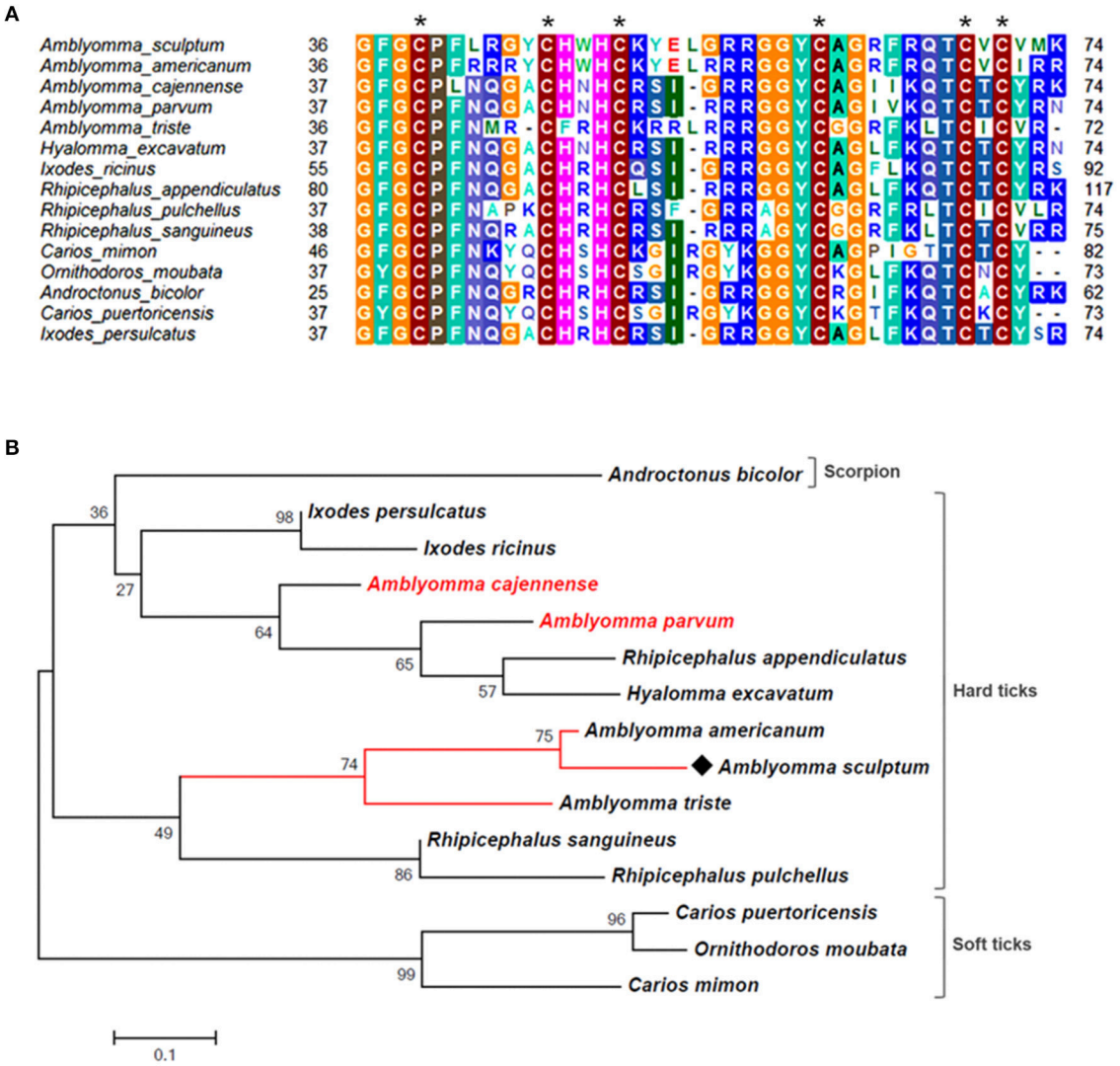

FIGURE 5 | MSA and phylogenetic analysis of defensins. **(A)** Multiple sequence alignment of protein sequences was performed using MUSCLE method. The numbers flanking the alignment represent the start (left) and end (right) amino acid position of each sequence in the protein domain. Asterisks highlight the conserved cysteine residues. Threshold for shading colors of amino acid similarity was 50%. **(B)** A phylogenetic tree was constructed with protein sequences from ticks and scorpion using Maximum Likelihood (ML) method. Numbers next to the branches represent the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 15 amino acid sequences (accession numbers available in Supplementary Table 2). All positions containing gaps and missing data were eliminated. There were a total of 58 positions in the final dataset. Bar scale at the bottom indicates 10% amino acid divergence. Diamond symbol refers to the CDS Acaj-65746 from the sialotranscriptome of *A. scultpum* identified in this work.

anti-inflammatory, and immunomodulatory properties, such as members of lipocalin, metalloprotease, and protease inhibitor families were significantly upregulated in SG of *A. sculptum* by feeding.

Lipocalins are anti-inflammatory proteins that bind both histamine and serotonin (Paesen et al., 1999; Sangamnatdej et al., 2002; Francischetti et al., 2009). It has been previously shown that elevated concentrations of histamine on the feeding site can affect tick attachment, feeding efficiency, and reproductive success, as demonstrated for *D. andersoni* (Paine et al., 1983) and *R. microplus* (Kemp and Bourne, 1980). Accordingly, treatment of infested animals with histamine antagonists was shown to improve tick engorgement and reduce acquired resistance to tick feeding (Tatchell and Bennett, 1969; Wikel, 1982). Therefore, the upregulation of lipocalins seems to be important to prevent excessive plasma exudation, inflammation and grooming behavior associated to vasoactive amines, thus allowing ticks to efficiently acquire the blood meal.

Transcription of metalloproteases was also significantly upregulated in sialotranscriptome of *A. sculptum* by feeding. Some previous studies have reported the expression of metalloproteases in tick SG (Valenzuela et al., 2002; Harnnoi

TABLE 4 | Functional classification of proteins detected in saliva of fed *A. sculptum* ticks.

Functional class	Number of proteins
Cytoskeletal	18
Extracellular matrix/cell adhesion	08
Immunity	02
Carbohydrate metabolism	02
Energy metabolism	01
Intermediary metabolism	01
Lipid metabolism	01
Nuclear regulation	02
Protein export machinery	01
Protein modification machinery	14
Protein synthesis	02
Signal transduction	05
Storage	08
Putative secreted	58
Viral products	01
Total	124

et al., 2007; Decrem et al., 2008). The metalloproteases found in tick sialotranscriptomes belong to the reprolysin family (Francischetti et al., 2005b; Harnnoi et al., 2007; Mans et al., 2008), which present high similarity to the hemorrhagic snake venom metalloproteases (SVMPs) (Francischetti et al., 2003, 2005a). Therefore, it is possible that these proteins promote the fluidity of the blood in the feeding site during the long-extended feeding, by performing antihemostatic activities, such as fibrinogenolysis and fibrinolysis (Francischetti et al., 2003; Barnard et al., 2012). Importantly, the immunization of bovines with the reprolysin BrRm-MP4 of *R. microplus* decreased both feeding and reproductive rates of females (Ali et al., 2015), highlighting the potential of metalloproteinases as vaccine candidates.

The analysis of the sialotranscriptome of *A. sculptum* also revealed the presence of several protease inhibitor transcripts, the majority belonging to TIL and Kunitz families. Protease inhibitors has also been extensively described in SG of ticks (Francischetti et al., 2009; Chmelar et al., 2017). It has been previously shown that these molecules play an important role during tick feeding, preventing host blood clotting and ensuring acquisition of a blood meal (Francischetti et al., 2002, 2004, 2005a; Sasaki et al., 2004; Cao et al., 2013). It is known that ixodidin, a TIL domain containing protein isolated from the hemocytes of *R. microplus*, presents antimicrobial properties besides inhibiting the activity of serine proteases (Fogaça et al., 2006). In addition, it was previously reported that one Kunitz inhibitor of *D. variabilis* exhibits bacteriostatic effect against *Rickettsia montanensis* (Ceraul et al., 2008) and that its knockdown by RNA interference (RNAi) increases the tick susceptibility to infection (Ceraul et al., 2011). Interestingly, MSA and phylogenetic analyses of both TIL and Kunitz domain containing proteins of *A. sculptum* (**Figures 3**, **4**, respectively) showed that they possess the key features required for biological properties of such molecules, suggesting that they may also exhibit antimicrobial and serine proteinase inhibitory activities.

The mucin family is the second class of putative secreted proteins mostly represented in *A. sculptum* sialotranscriptome. The members of this family are serine-and/or threonine-rich secreted proteins that have an O-N-acetylgalactosylation site in common (Karim et al., 2011). Because of dense glycosylation and hydration capacity, mucins can act as protective barriers, providing lubrication of various tick tissues (Hang and Bertozzi, 2005). Therefore, it is plausible to suppose that mucins may play important role in blood acquisition, maintaining the integrity of tick mouthparts (Ribeiro et al., 2006; Anderson et al., 2008; Anatriello et al., 2010).

The transcriptomes of *A. sculptum* evidenced that distinct members of the same protein family present a higher transcriptional level in SG of either fed or unfed ticks. For instance, 65 out of 72 CDSs of lipocalins were upregulated and seven were downregulated by blood feeding. A similar pattern was observed for other protein families, such as mucins, metalloproteinases, and protease inhibitors. In fact, recent studies that evaluated time-dependent expression of proteins by tick SG have found similar results for *I. ricinus* (Kotsyfakis et al., 2015b), *Ixodes scapularis* (Kim et al., 2016), and *A. americanum* (Karim and Ribeiro, 2015; Bullard et al., 2016). Therefore, it is possible that *A. sculptum*, as other tick species, may secrete various isoforms of the same protein and/or different members of the same family (but with similar functions) into saliva during blood feeding as a mechanism of antigenic variation to avoid recognition by the host's immune system.

Notably, almost all CDSs belonging to tick immune system were upregulated by feeding in SG of *A. sculptum*, excepted for lectin encoding sequences. Transcripts of tick immune system components, especially AMPs, were previously identified in tick sialotranscriptomes (Francischetti et al., 2009; Kotsyfakis et al., 2015a). AMPs secreted in tick saliva may prevent the growth of microbes at the feeding site as well as in tick gut (Karim and Ribeiro, 2015). Interestingly, the CDSs Acaj-65746, which encode a defensin, was highly induced in SG of ticks fed for 72 h. MSA and phylogenetic analysis showed that this AMP is member of the arthropod defensin family, also named as Knottin scorpion toxin-like in InterPro database (Gracy et al., 2008). One peptidoglycan recognition protein (PGRP) (AcajSIGP-81204) with amidase catalytic site was also upregulated in SG of *A. sculptum* by feeding. PGRPs are classified into non-catalytic or catalytic depending on the presence of the amidase catalytic site. While non-catalytic PGRPs function as pathogen pattern recognition receptors and activate immune pathways upon infection, catalytic PGRPs cleaves peptidoglycan, acting as effectors and/or negative regulators of the immune response (Palmer and Jiggins, 2015).

Members of the Da-p36 immunosuppressant family, putatively enrolled in host immunomodulatory activity, were also identified in *A. sculptum* SG and all of them were upregulated by feeding. Da-p36 was originally identified in both saliva and SG of *D. andersoni* and it presents an inhibitory

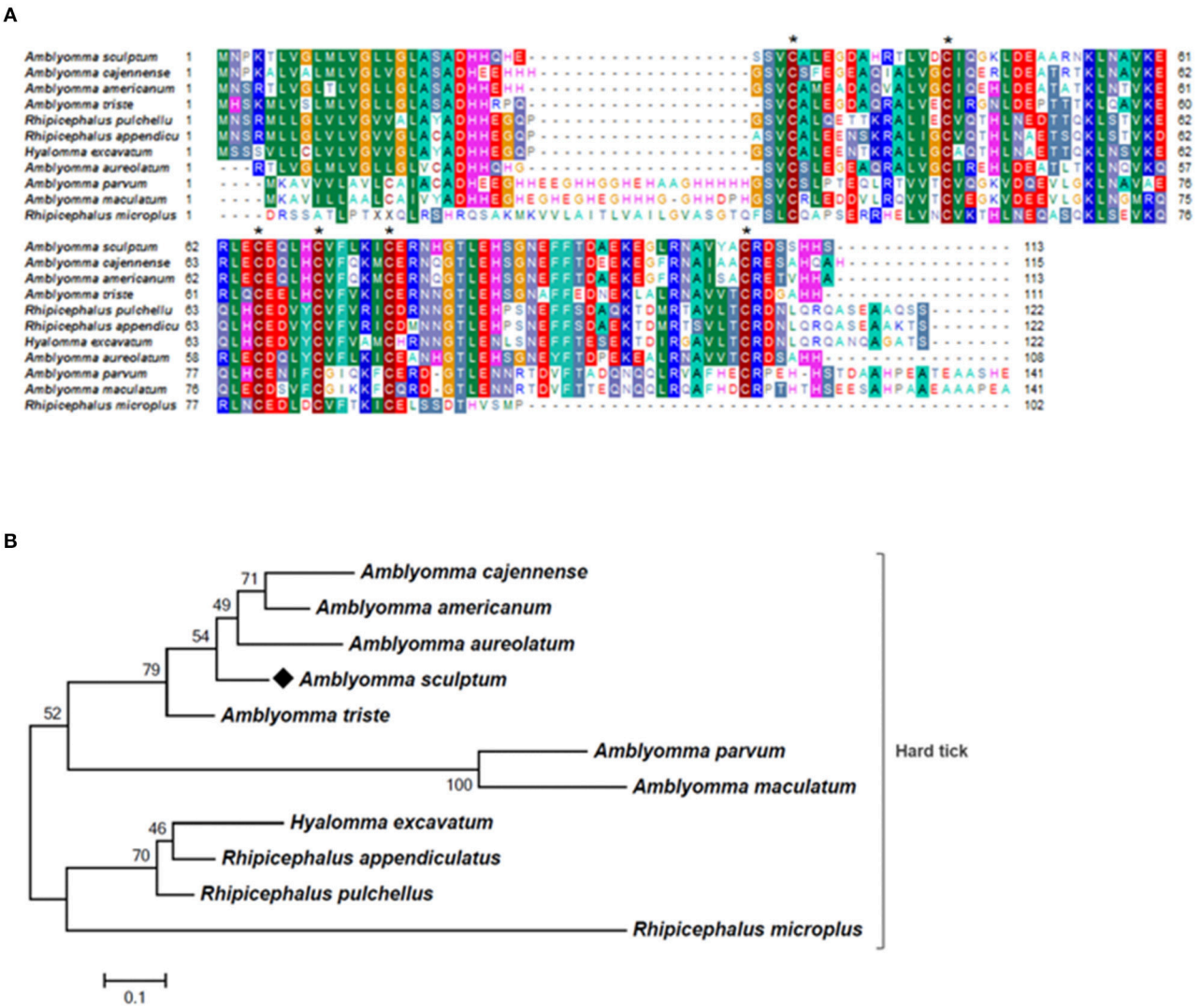

FIGURE 6 | MSA and phylogenetic analysis of microplusin-like AMPs. **(A)** Multiple sequence alignment of protein sequences using MUSCLE method. The numbers flanking the alignment represent the start (left) and end (right) amino acid position of each sequence in the protein domain. Asterisks highlight the conserved cysteine residues. Threshold for shading colors of amino acid similarity was 40%. **(B)** A phylogenetic tree was constructed with protein sequences from ticks using Maximum Likelihood (ML) method. Numbers next to the branches represent the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 11 amino acid sequences (accession numbers available in Supplementary Table 2). All positions containing gaps and missing data were eliminated. There were a total of 75 positions in the final dataset. Bar scale at the bottom indicates 10% amino acid divergence. Diamond symbol refers to the AcajSIGP-14784 from the sialotranscriptome of *A. scultpum* identified in this work.

activity on concanavalin A-induced proliferation of murine splenocytes (Bergman et al., 2000). Homologues of Da-p36 have been reported in others tick species, such as *Amblyomma variegatum* (Nene et al., 2002), *A. maculatum* (Karim et al., 2011), *Haemaphysalis longicornis* (Konnai et al., 2009), and *Rhipicephalus appendiculatus* (Nene et al., 2004). Evasins, small proteins that recognize and bind chemokines, were first described in *R. sanguineus* (Frauenschuh et al., 2007) and were also detected in *A. sculptum* sialotranscriptome. Evasin-1 and Evasin-4 binds CC chemokines, while Evasin-3 binds CXC chemokines, and Evasin-2 has no ligand characterized to date (Frauenschuh et al., 2007; Déruaz et al., 2008). An Evasin-3-like activity was also observed in SG extracts of adult *A. variegatum*, *R. appendiculatus*, and *Dermacentor reticulatus* (Vancová et al., 2010).

The 8.9 kDa superfamily is exclusively found in hard ticks, but none of its members were functionally characterized so far (Francischetti et al., 2009; Karim et al., 2011). Two members of this family are highly expressed in hemocytes of *I. ricinus* (Kotsyfakis et al., 2015a). Therefore, the authors suggested that they might be involved with tick immunity (Kotsyfakis et al., 2015a). Twenty three from 24 CDSs of proteins of this family

were upregulated by blood feeding in tick SG. Glycine-rich proteins are members of another family of proteins specifically find in ticks. CDSs of glycine-rich proteins were also identified in *A. sculptum* sialotranscriptome. Glycine-rich proteins of ticks are associated to salivary cement used to attach mouthparts to host skin (Francischetti et al., 2009; Maruyama et al., 2010).

A blastp search of the sialotranscriptome of *A. sculptum* (formely named *A. cajennense*) against a collection of protein sequences of *A. cajennense* (Garcia et al., 2014; the "cajennense protein database;" please see column BG in Supplementary Table 1) was performed. About 25% of the 9,560 CDSs identified in the current study presented no match against the "cajennense database." As the genome of *A. sculptum* is not available, this study not only provides additional evidences on the transcriptional changes stimulated by blood feeding in ticks, but also extensively contributed with novel transcripts for public sequence databases of this species.

To identify the proteins that are effectively secreted by *A. sculptum* SG, the proteome of the saliva of fed females was determined. A set of 124 proteins was identified, among which 58 are predicted to be secreted, reinforcing the importance of secreted proteins during the feeding process. Among secreted proteins, we highlight lipocalins, 8.9 kDa, glycine-rich proteins and microplusin-like AMPs. It was previously shown that the microplusin of *R. microplus* (Fogaça et al., 2004) exhibits the properties of chelating metallic ions, which seems to be involved in its activity against the Gram-positive bacterium *Micrococcus luteus* (Silva et al., 2009) and the fungus *Cryptococcus neoformans* (Silva et al., 2011). As mentioned above, the presence of proteins with antimicrobial properties in tick saliva might play a role in preventing the growth of microbes ingested with the blood meal (Karim and Ribeiro, 2015). Six vitellogenins were also detected in saliva of fed ticks. Vitellogenin is the major yolk precursor protein, being incorporated in eggs as vitellin (Taylor et al., 1991; Rosell and Coons, 1992; Chinzei and Yano, 1994; James et al., 1999; Thompson et al., 2007). The heme-binding property of both vitellogenin (Thompson et al., 2007) and vitellin (Logullo et al., 2002) has been previously shown. As ticks do not synthesize heme (Braz et al., 1999), these proteins are an important source of this prosthetic group for embryos development (Logullo et al., 2002). It was also demonstrated that vitellins also exhibit an antioxidant property, diminishing the heme-induced lipid peroxidation (Logullo et al., 2002). Importantly, *R. microplus* ticks fed on sheep vaccinated with vitellin showed reduced engorgement and oviposition rates (Tellam et al., 2002).

In conclusion, the current study shows that blood feeding exert a strong effect on the gene expression profile of the SG of *A. sculptum*, upregulating the transcription of putative secreted proteins, which may play pivotal role during the feeding process. In addition, this is the first report on the proteome of *A. sculptum* saliva. Transcriptional and protein data presented in this study amplify the knowledge of proteins possibly involved in tick feeding, which might also play a role on transmission of pathogens. Future functional studies to determine the role of such proteins on *A. sculptum* physiology as well as on transmission of *R. rickettsii* to the vertebrate host are warranted and might have potential as vaccine targets and as pharmacological bioproducts with significant biological activities.

AUTHOR CONTRIBUTIONS

Designed the experiments: EE, AS-N, and ACF. Generated biological samples: EE, LAM, and FBC. Performed the experiments: EE and AAR. Analyzed data: JMCR, SRM, EE, ACF, and RK. Performed statistic data analysis: AF, JMCR, and SRM. Contributed reagents/materials/analysis tools: AS-N, MBL, JMCR, GP, and ACF. Wrote the paper: EE, SRM, AS-N, and ACF. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2017.00476/full#supplementary-material

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Immunomodulatory Effects of *Amblyomma variegatum* Saliva on Bovine Cells: Characterization of Cellular Responses and Identification of Molecular Determinants

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The tropical bont tick, *Amblyomma variegatum*, is a tick species of veterinary importance and is considered as one of major pest of ruminants in Africa and in the Caribbean. It causes direct skin lesions, transmits heartwater, and reactivates bovine dermatophilosis. Tick saliva is reported to affect overall host responses through immunomodulatory and anti-inflammatory molecules, among other bioactive molecules. The general objective of this study was to better understand the role of saliva in interaction between the *Amblyomma* tick and the host using cellular biology approaches and proteomics, and to discuss its impact on disease transmission and/or activation. We first focused on the immuno-modulating effects of semi-fed *A. variegatum* female saliva on bovine peripheral blood mononuclear cells (PBMC) and monocyte-derived macrophages *in vitro*. We analyzed its immuno-suppressive properties by measuring the effect of saliva on PBMC proliferation, and observed a significant decrease in ConA-stimulated PBMC lymphoproliferation. We then studied the effect of saliva on bovine macrophages using flow cytometry to analyze the expression of MHC-II and co-stimulation molecules (CD40, CD80, and CD86) and by measuring the production of nitric oxide (NO) and pro- or anti-inflammatory cytokines. We observed a significant decrease in the expression of MHC-II, CD40, and CD80 molecules, associated with decreased levels of IL-12-p40 and TNF-α and increased level of IL-10, which could explain the saliva-induced modulation of NO. To elucidate these immunomodulatory effects, crude saliva proteins were analyzed using proteomics with an Orbitrap Elite mass spectrometer. Among the 336 proteins identified in *A. variegatum* saliva, we evidenced bioactive molecules exhibiting anti-inflammatory, immuno-modulatory, and anti-oxidant properties (e.g., serpins, phospholipases A2,

heme lipoprotein). We also characterized an intriguing ubiquitination complex that could be involved in saliva-induced immune modulation of the host. We propose a model for the interaction between *A. variegatum* saliva and host immune cells that could have an effect during tick feeding by favoring pathogen dissemination or activation by reducing the efficiency of host immune response to the corresponding tick-borne diseases.

Keywords: *Amblyomma variegatum*, tick saliva, PBMC, immuno-modulation, proteomics

INTRODUCTION

Amblyomma variegatum is among the most important and widely distributed ticks of tropical livestock, and is the subject of veterinary and public health concerns in Africa and islands in the Indian Ocean and the Caribbean (Stachurski et al., 2010; Bournez et al., 2015). The species is the natural vector of *Ehrlichia ruminantium*, the Rickettsia that causes heartwater in ruminants (Allsopp, 2010), and can also transmit human pathogens including several species of Rickettsia and viruses (Jongejan and Uilenberg, 2004). Moreover, *A. variegatum* has been proven to reactivate the development of dermatophilosis, a skin disease with major economic impacts caused by *Dermatophilus congolensis* (Martinez et al., 1992).

Generally, at the bite site, ticks are able to inhibit pain and itch, inflammation, hemostasis, and wound healing, but also to modulate the host innate and adaptive immune responses, which consequently favor transmission of infectious agents (Šimo et al., 2017). The coevolution between ticks, hosts and pathogens has led to a balance of conflicts and cooperations between the different actors, which mainly benefit the ticks and pathogens. Nevertheless, increased production of antibodies against molecular determinants of tick saliva could also increase protection against pathogen infection (Wade, 2007; Wikel, 2013; de la Fuente et al., 2016). In tick-host interactions, tick saliva is the source of biologically active molecules that target a wide spectrum of host physiological mechanisms, mainly inhibiting host defense reactions to the benefit of the feeding ticks (Kazimírová and Štibrániová, 2013; Stibrániová et al., 2013). The immediate inflammatory response to the skin injury induces rapid infiltration of leukocytes at the tick bite site, where both resident and infiltrated cells (keratinocytes, endothelial cells, mast cells, dendritic cells, macrophages, and lymphocytes) are activated by direct contact with tick saliva (Brossard and Wikel, 2004; Francischetti et al., 2009; Wikel, 2013). Omics analyses have enabled the comprehensive characterization of the molecular determinants of tick saliva, with a functional involvement of these bioactive molecules in host immune suppression (Kotál et al., 2015; Chmelar et al., 2016a,b).

The saliva of *Amblyomma* ticks has been the subject of extensive biochemical studies since the early 2000s (Karim et al., 2004, 2011; Madden et al., 2004; Mulenga et al., 2013; Garcia et al., 2014; Araujo et al., 2016), but only a few have been devoted to the sialome of *A. variegatum.* These studies revealed new families of tick-exclusive proteins (Knizetova et al., 2006; Koh et al., 2007; Ribeiro et al., 2011), some related to suppression of inflammation (Tian et al., 2016).

In the present study, using crude saliva from *A. variegatum* semi-fed females (i.e., at the peak of salivation), our aim was to (i) explore the impact of saliva *in vitro* on bovine immune cells and (ii) determine the molecular immunomodulators. The originality of our approach was to combine cellular experiments, demonstrating modulation of lymphocyte proliferation and macrophage activation, with proteomic analysis of saliva to identify and characterize the immunomodulatory proteins potentially responsible for the observed biological effects. Consequences on host response to infestation by *A. variegatum* and transmission of pathogens are discussed.

MATERIALS AND METHODS

A. variegatum Saliva Production

Ten *A. variegatum* female ticks were engorged on naïve creole goats, in accordance with the experimental procedure of the project in animal experimentation approved by the ethics committee Antilles-Guyane (Project Number 69 registered by *Comité National de Réflexion Ethique sur l'Expérimentation Animale*). After 5–7 days, the female ticks were detached at a semi-fed stage and injected in the hemocoele with a Hamilton syringe with 10 µl of a 5% (w/v in PBS) pilocarpine solution to stimulate saliva production. A capillary micropipette placed around the tick hypostome was used to collect the saliva from ticks (duration of salivation about 30 min), and fresh crude saliva was aliquoted and immediately stored at −80°C for further use. For cellular tests, three different pools of saliva were obtained from 10 *A. variegatum* semi-fed female ticks. For proteomic analyses, crude saliva, from either individual ticks' saliva or pools of saliva, was processed directly according the standardized protocol (see below). Protein concentration was measured with a Nanodrop 1000 (ThermoScientific). The concentration of Pilocarpine in the saliva was evaluated using an HPLC-MS/MS method proposed by Ribeiro et al. (2004). Contrary to the pilocarpine concentrations previously reported for *A. americanum* (Ribeiro et al., 2004), we did not detect in our study a concentration of salivary pilocarpine higher than 5 nM, which was set as our pilocarpine control concentration.

Purification of Bovine PBMCs and Proliferation Assay

Peripheral blood was collected in lithium heparin tubes (BD Biosciences, Le Pont de Claix, France) from three healthy female Jersiaise cattle aged between 4 and 6 years (APAFIS#14442015081310300000). PBMC were isolated by

differential centrifugation over Histopaque 1083® (Sigma Aldrich, St. Quentin, France) and washed twice in calcium and magnesium free Hanks balanced salt solution (Life Technologies, Saint Aubin, France). PBMC were suspended at 2.5×10^6 cells/ml in RPMI-1640 culture medium (Life Technologies, Saint Aubin, France) supplemented with 2 mM L-glutamine (Sigma Aldrich, St. Quentin, France), 5×10^{-5} M β2-mercaptoethanol (Sigma Aldrich, St. Quentin, France), 50 μg/ml gentamycin (Sigma Aldrich, St. Quentin, France), and 10% heat-inactivated fetal calf serum (Life Technologies, Saint Aubin, France). One hundred microliters of PBMC (2.5×10^5 cells) were distributed in triplicate in the wells of a 96-well microplate and the cells were stimulated with 2.5 μg/ml of Concanavalin A (ConA, Sigma Aldrich, St Quentin, France) either alone (positive control corresponding to 100% of stimulation) or in the presence of *A. variegatum* saliva at concentrations ranging from 0.3 to 37.5 μg/ml for 72 h at 37°C with 5% CO_2. PBMC were also tested with saliva alone, without ConA stimulation, and with pilocarpine at 5 nM. Cell proliferation was measured using the CyQUANT Direct Cell Proliferation Assay kit (Molecular Probes/ThermoFisher Scientific, Paisley, UK) according to the manufacturer's instructions. Briefly, 100 μl of the 2X detection reagent were added to the cells for a further 60 min of incubation at 37°C, 5% CO_2. The fluorescence of samples was then read at 480/535 nm using an Enspire Multimode Plate Reader (Perkin Elmer, Courtaboeuf, France). Fluorescence in samples treated with ConA and saliva was compared with fluorescence measured with ConA alone to calculate the relative PBMC activation percentage. Similarly, fluorescence in unstimulated PBMC treated with saliva was compared with fluorescence measured in unstimulated cells. Experiments were performed using cells from three different animals (1973, 9567, and 9906), each of the three saliva batches being tested separately. Data are presented as relative PBMC activation, means of values obtained for the three batches of saliva (for each concentration) in triplicate experiments for each of the three animals.

Preparation of Bovine Macrophages and Stimulation Assay

Immuno-magnetic separation of CD14+ monocytes from PBMC was carried out using anti-human CD14 antibody (TUK4) coated beads (Miltenyi Biotec, Bergish Gladbach, Germany), according to the manufacturer's instructions. Flow cytometry analysis was carried out on the positively selected populations and confirmed that the purity of the population was >95%. Bovine monocytes were suspended in complete IMDM (Life Technologies, Saint Aubin, France) supplemented with 2 mM L-glutamine (Sigma, Saint-Quentin, France), 10% heat-inactivated fetal calf serum (Life Technologies, Saint-Aubin, France), 5×10^{-5} M β2-mercaptoethanol (Sigma, Saint-Quentin, France), and 50 μg/ml gentamicin (Life Technologies, Saint-Aubin, France). One hundred microliters of cells at 1.10^6 cells/ml were seeded into 96-well plates (for NO production) or 500 μl of cells were seeded into 48-well plates (for production of cytokines and analysis of co-stimulation surface markers). Half the culture medium was replaced every 3 days by complete IMDM, supplemented with GM-CSF and INF-γ for NO induction in the 96-well plate. After 7 days of culture, more than 95% of cells had the morphological characteristics of macrophages. At day 7, 100 ng/ml of ultrapure LPS from *E. coli* (InVivoGen, Toulouse, France) was added for 1 h prior to saliva stimulation, alone or in the presence of *A. variegatum* saliva at concentrations ranging from 7.8125 to 250 μg/ml for an additional 24 h at 37°C with 5% CO2. Pilocarpine (5 nM) was also tested. Cells were also tested without LPS pre-stimulation, with the different concentrations of saliva or with 5 nM of pilocarpine. Supernatants were collected from the 96-well plate and NO concentration was immediately determined using the Griess method. Supernatants were collected from the 48-well plate for TNF-α, IL-12, and IL-10 quantification by ELISA, according to the protocols of Hope et al. (2002) and Kwong et al. (2002, 2010). Briefly, 5 μg/ml of capture Mabs CC327, CC301, and CC318 for TNF-α, IL-12, and IL-10, respectively (Bio-Rad AbDSerotec, UK) were used as coating antibodies, cultures were left overnight at +4°C. After washing and blocking with 1 mg/ml of casein (Sigma Aldrich, Saint Quentin, France), cell culture supernatants and serial diluted standards were incubated for 1 h at room temperature. Wells were washed and a 8 μg/ml solution of biotinylated detection Mabs (biotinylated-CC328, -CC326, and -CC320, all from Bio-Rad AbD Serotec, UK) were added for 1 h, detected by Streptavidin-HRP (eBiosciences, France) and revealed with TMB liquid substrate system for ELISA (Sigma Aldrich, France). The reaction was stopped with H_2SO_4 0.5 M and the plates were read at 450 nm. All the results were determined with standard curves obtained with serial dilutions of recombinant proteins (ref. RBOTNFA for TNF-α from Pierce, Rockford, USA; ref. 87464 from AbCam, France for IL-12 and ref. RPO379 for IL-10 from Kingfisher, USA).

Cells in the 48-wells plate were collected and labeled with mouse anti bovine monoclonal antibodies for analysis of co-stimulation surface markers: MHC II (clone J11, kindly provided by ILRI, Kenya), CD40 (clone IL-A156, AbD Serotec, UK), CD80 (clone IL-A159, AbD Serotec, UK), and CD86 (clone IL-A190, AbD Seroteck, UK). Isotypic control was used as non-specific labeling (MCA928, AbD Serotec, UK). Antibodies were revealed using goat anti mouse IgG1-RPE (STAR132PE, AbD Serotec, UK), cells were processed for flow cytometry using a FacsCanto II cytometer (BD Biosciences, USA) and data were analyzed using Diva software (BD Biosciences, USA) for FCS/SSC and FL2 fluorescence intensities. Relative expression in MFI (median of fluorescence intensities) was calculated by comparing stimulated cell (without saliva or with different saliva concentrations) MFI with unstimulated cell MFI. Experiments were performed with cells from three different animals (1973, 9567, and 9906), and tested with a pool of saliva from the three batches.

Proteomics Analysis of *A. variegatum* Saliva

A. variegatum saliva proteins (10 μg) were separated on SDS-PAGE gels (12% polyacrylamide, Mini-PROTEAN® TGX™

Precast Gels, Bio-Rad, Hercules USA) and stained with Protein Staining Solution (Euromedex, Souffelweyersheim France). Gel lanes were cut into three continuous gel pieces that were treated independently, in order to isolate majority proteins and to allow identification of low quantity proteins. Gel pieces were first destained with 50 mM triethylammonium bicarbonate (TEABC) and three washes in 100% acetonitrile. After protein reduction (with 10 mM dithiothreitol in 50 mM TEABC at 56°C for 45 min) and alkylation (55 mM iodoacetamide TEABC at room temperature for 30 min) proteins were digested in-gel using trypsin (500 ng/band, Gold, Promega, Madison USA) as previously described (Thouvenot et al., 2008). Digest products were dehydrated in a vacuum centrifuge and reduced to 3 μL. The generated peptides were analyzed by nano-flowHPLC–nanoelectrospray ionization using an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Waltham USA) coupled to an Ultimate 3000 HPLC (Thermo Fisher Scientific). Desalting and pre-concentration of samples were performed online on a Pepmap® pre-column (0.3 × 10 mm, Dionex). A gradient consisting of 0–40% B for 60 min and 80% B for 15 min (A = 0.1% formic acid, 2% acetonitrile in water; B = 0.1% formic acid in acetonitrile) at 300 nL/min was used to elute peptides from the capillary reverse-phase column (0.075 × 150 mm, Acclaim Pepmap 100®C18, Thermo Fisher Scientific). Eluted peptides were electro-sprayed online at a voltage of 1.9 kV into an Oribtrap Elite mass spectrometer. A cycle of one full-scan mass spectrum (400–2,000 m/z) at a resolution of 120,000 (at 400 m/z), followed by 20 data-dependent MS/MS spectra was repeated continuously throughout the nanoLC separation. All MS/MS spectra were recorded using normalized collision energy (33%, activation Q 0.25 and activation time 10 ms) with an isolation window of 2 m/z. Data were acquired using Xcalibur software (v 2.2). For all full scan measurements with the Orbitrap detector a lock-mass ion from ambient air (m/z 445.120024) was used as an internal calibrant as described in Olsen et al. (2005). MS data were analyzed using the MaxQuant software package (v 1.5.0.0) as described by Cox and Mann (2008). Tandem mass spectra (MS/MS) were searched for using the Andromeda search engine (Cox et al., 2011) against the UniProtKB database (release 2015_03) for the *A. variegatum* (AMBVA) taxonomy (753 entries), Amblyomma "all species" taxonomy (22,429 entries), and the UniProtKB proteome UP000001555 database for *Ixodes scapularis* (20,473 entries) using the following parameters: enzyme specificity was set as Trypsin/P, and a maximum of two missed cleavages and a mass tolerance of 0.5 Da for fragment ion were applied. A second database of known contaminants provided with the MaxQuant suite was also used. The "match between runs" option was checked. Oxidation (M) was specified as variable modification and carbamidomethyl (C) as fixed modification. Database searches were performed with a mass tolerance of 20 ppm for precursor ion for mass calibration, and with a 4.5 ppm tolerance after calibration. The maximum false peptide and protein discovery rate was set at 0.01. The MaxQuant software generates several output files that contain information about the peptides and proteins identified. The "proteinGroups.txt" file is dedicated to identified proteins: each single row collapses all proteins that cannot be distinguished based on identified peptides into protein groups. An in-house bioinformatics tool was developed to automatically select a representative protein ID in each protein group. First, proteins with the most identified peptides were isolated in a group called "match group" [proteins from the "Protein IDs" column with the maximum number of "peptides counts (all)"]. For 35% of the remaining match groups in which more than one protein ID existed, the "leading" protein was chosen as the best annotated protein according to the number of gene ontology annotations (retrieval from UniProtKB March 20, 2015) and/or given the following species order preference: *A. variegatum* > *A. maculatum* > *A. cajennense* > *A. parvum* > *A. americanum* > *A brasiliense* > *A. triste* > *A. geayi* > *A. scutatum* > *A. rotundatum*. The mass spectrometry data along with the identification results were deposited in the ProteomeXchange Consortium via the PRIDE (Martens et al., 2005) partner repository with the dataset identifier PXD007821.

Bioinformatics Analysis of Proteomics Data

We used several web servers to investigate the biochemical properties of the identified proteins.

The ProtFun 2.2 server produces *ab initio* predictions of protein function from sequences. The method queries a large number of other feature prediction servers to obtain information on various post-translational and localizational aspects of the protein, which are integrated in final predictions of the cellular role, enzyme class (if any), and selected gene ontology (GO) categories of the submitted sequence (Jensen et al., 2002, 2003).

Propsearch server is designed to find the putative protein family if querying a new sequence using alignment methods has failed. Disregarding the order of amino acid residues in a sequence, Propsearch uses the amino acid composition instead. In addition, other properties like molecular weight, bulky residue content, small residue content, average hydrophobicity, average charge a.s.o. and the content of selected dipeptide-groups are also calculated from the sequence. A total of 144 such properties are weighted individually and used as query vectors. The weights have been trained on a set of protein families with known structures, using a genetic algorithm. Sequences in the database are also transformed into vectors, and the Euclidian distance between the query and database sequences is calculated. Distances are rank ordered, and sequences with lowest distance are reported on top (Hobohm and Sander, 1995). We used Propsearch first to better characterize proteins identified as "Putative Uncharacterized Protein" and second to improve the potential functional information on the identified proteins. In our analytical strategy, we conserved only data for proteins with Euclidian distances between 0.0 and 1.3 (Reliability 99.9%) and between 1.3 and 7.5 (Reliability 99.6%).

Statistical Tests

Graphs and linear modeling were performed with R version 3.4.1 (R Core Team, 2013) and the tidyverse package (v 1.1.1). Datasets

and figures along with the script are available at: https://github.com/loire/amblyoma_paper.

RESULTS

Inhibition of ConA-Induced PBMC Proliferation by *A. variegatum* Saliva

To investigate the effect of *A. variegatum* on PBMC proliferation, cells were stimulated with ConA alone (positive control for PBMC activation) or with various concentrations of saliva for 72 h, and the relative PBMC activation for each saliva concentration vs. no saliva (positive control) was calculated. As shown in **Figure 1**, the relative PBMC activation decreased in a dose-dependent manner when cells were in contact with *A. variegatum* saliva. A concentration of 1.2 µg/ml of saliva reduced the ConA-induced proliferation of PBMC by 9–13%, depending on the animal from which the cells originated (**Figure 1**). The decrease in proliferation was linear depending on the concentration of the saliva, and reached 24–36% for 18.75 µg/ml of saliva (**Figure 1**). Saliva concentrations of <1.2 µg/ml was tested and did not result in statistically significant inhibition of ConA-induced PBMC proliferation, whereas levels of inhibition for higher concentrations (>18.75 µg/ml) were similar to those for 18.75 µg/ml (data not shown). Unstimulated PBMC incubated with saliva did not show any proliferation and pilocarpine control had no effect on ConA-induced PBMC proliferation (data not shown).

Modification of LPS Pre-stimulated Macrophage Activation by *A. variegatum* Saliva

We tested the effects of various concentrations of *A. variegatum* saliva on MHC II and co-stimulation molecules (CD40, CD80, and CD86) on LPS pre-stimulated macrophages. As shown in **Figure 2**, in all conditions tested, *A. variegatum* saliva had a biological effect on the expression of co-stimulation surface markers in LPS pre-stimulated macrophages, compared to unstimulated cells (no LPS). Saliva enhanced the LPS-induced down regulation of macrophages MHC II from 23 to 62%, from 39 to 72%, or from 60 to 75% depending on both the saliva concentration and the animal (overall $p = 0.092$, specific $p < 0.05$), and decreased the LPS-induced up regulation of both CD40 and CD80 in a dose-dependent manner (**Figure 2**). The saliva had no obvious effect on LPS-induced down regulation of CD86, which was already strongly decreased by LPS alone (**Figure 2**). The three independent animals gave similar profiles with individual variability in the level of expression of the co-stimulatory surface markers but, as shown in **Figure 2**, the trend curve shows that the individual variations all followed the same pattern. The effect of *A. variegatum* saliva alone on macrophages, without pre-stimulation with LPS, was less striking, with marked individual variability between animals: MHC II and CD86 were down regulated in a dose-dependent manner, CD40 and CD80 were up regulated in two out of three animals with no dose-dependence, CD86 was down regulated in a dose-dependent manner (**Supplementary Figure 1**). Percent expression of CD40 and CD80 induced by saliva alone was 10 times lower than that with LPS pre-stimulation (**Supplementary Figure 1**). Macrophages derived from animal n° 1973 exhibited a difference MHC II levels depending on the concentration of the saliva compared to the other animals: expression increased for 31.25 µg/ml of saliva, then decreased for 62.5 µg/ml, increased slightly for 125 µg/ml and decreased slightly for 250 µg/ml. Same kind of inverted fluctuations were observed with animal n° 9906 for CD80 and animal n° 9567 for CD86 (**Supplementary Figure 1**).

As shown in **Figure 3**, LPS pre-stimulated macrophages produced NO and production was increased by adding up to 62.5 µg/ml of *A. variegatum* saliva, but decreased at higher saliva concentrations (125 and 250 µg/ml). The production of cytokines was also affected: IL-12 and TNF-α levels decreased dramatically, from a concentration of 31.25 µg/ml of saliva, except for animal n° 1973, in which a small dose-dependent decrease in TNF-α was observed (**Figure 3**). In contrast, the IL-10 level was increased by saliva in a dose-dependent manner (**Figure 3**). The three independent animals exhibited similar NO profiles and cytokine production, with individual variability in the levels reached but, like for co-stimulation surface markers, the trend curve showed that the individual variations all followed the same pattern. The effect of saliva alone on unstimulated cells resulted in very low levels of cytokine production, with increased

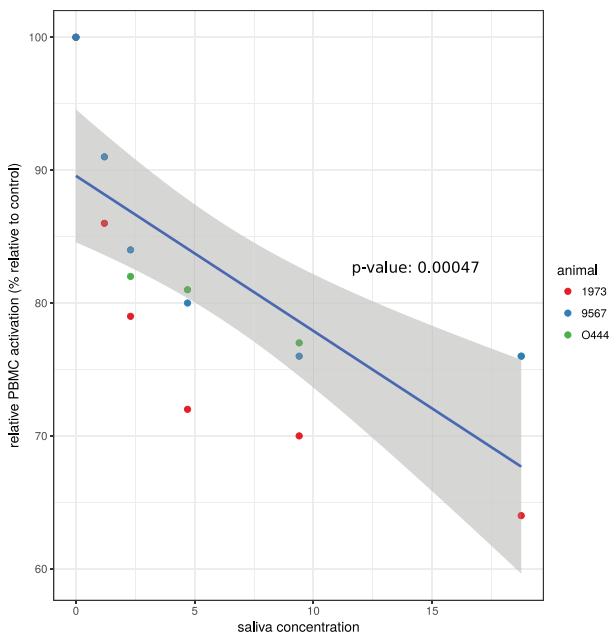

FIGURE 1 | Effect of *A. variegatum* saliva on ConA-induced proliferation of bovine PBMC. Bovine PBMC were stimulated with ConA alone or with different concentrations of *A. variegatum* saliva for 72 h. PBMC responses of cells in contact with ConA and saliva were compared to PBMC in contact with ConA alone (control) to calculate "relative PBMC activation." Data are means of triplicate experiments, for each batch of saliva for the three animals tested. Blue lines and shading show respectively the linear regression fit and the 0.95 confidence interval. The *p*-value of the model is indicated on the panel.

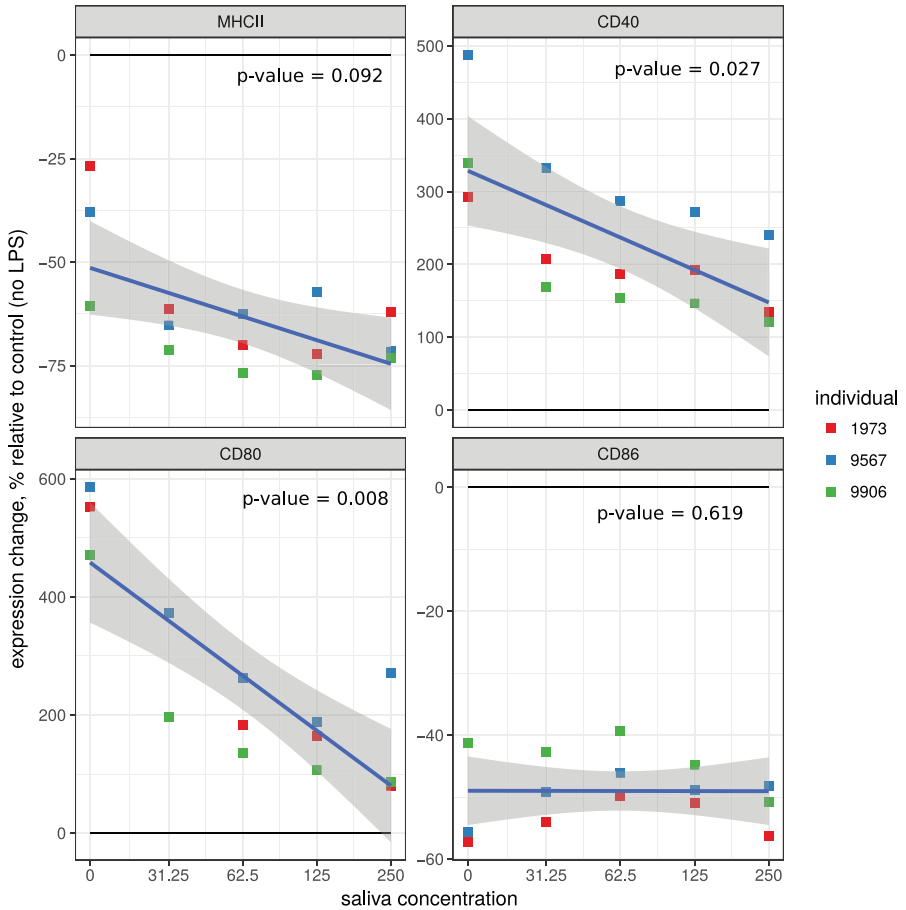

FIGURE 2 | Effect of *A. variegatum* saliva on the expression of surface markers on LPS-stimulated bovine macrophages. Bovine blood monocyte-derived macrophages were pre-stimulated for 1 h with LPS, and then for an additional 24 h with different concentrations of *A. variegatum* saliva. Cells were collected and stained for surface markers. Expression levels (mean of fluorescence intensity, MFI) of MHC II, CD40, CD80, and CD86 markers on macrophages compared with unstimulated control cells were measured by flow cytometry. Data are means of triplicate experiments, for a pool of saliva batches for the three animals tested. Blue lines and shading show the linear regression fit and the 0.95 confidence interval, respectively. The *p*-value of the model is indicated on the panel.

levels of IL-10 up to 125 μg/ml of saliva, increased level of IL-12 up to 31.25 μg/ml then decreased IL-12 in a dose dependent manner, slightly decreased level of TNF-α in a dose-dependent manner (**Supplementary Figure 2**). Unstimulated cells did not produce NO in the presence of saliva (data not shown).

Molecular Characterization of *A. variegatum* Saliva

Proteomics analysis of *A. variegatum* crude saliva led to the identification of 336 proteins, with no significant qualitative and quantitative variation between individuals (PRIDE PXD007821), allowing us to use pools of saliva for both cell biology assays and molecular characterization. The protein sequence data, consisting of the *Amblyomma* taxonomy (22,429 entries) and the *I. scapularis* proteome (UP000001555, 20,473 entries), were retrieved from UniProtKB (release 2015_03) for identification purposes. Among the 336 proteins identified, only 58 (17.3%) *A. variegatum* specific proteins were identified, knowing that the UniProtKB *A. variegatum* specific/particular database contained 753 entries (UniProtKB release 2015_03). Other identified proteins were divided into 236 *Amblyomma* (70.2%) and 42 *I. scapularis* (12.5%), which is the only ixodid tick species with a reference proteome in UniProtKB. It should be noted that 58 out of the 336 (17.3%) proteins identified were described as "Uncharacterized protein."

Analysis of the nature of the identified proteins by the ProtFun 2.2 server indicated that 239 out of 336 (71.1%) of the proteins identified in *A. variegatum* saliva were enzymes. The predicted enzymes were further classified in four out of six different classes (51 Lyases, 25 Ligases, 12 Isomerases, 2 Hydrolases with no Oxidoreductase, and no Transferase), but 149 remained unclassified. Moreover, **Figure 4A** shows the classification of the identified proteins in 12 different functional categories by the ProtFun 2.2 server, the most widely represented for *A. variegatum* saliva being Cell envelope (21.4%), Translation (9.8%), Energy metabolism (9.5%), and Amino acid biosynthesis (8.3%). Nevertheless, 38.4% of the identified proteins were not classified in functional categories (**Figure 4A**). **Figure 4B** shows

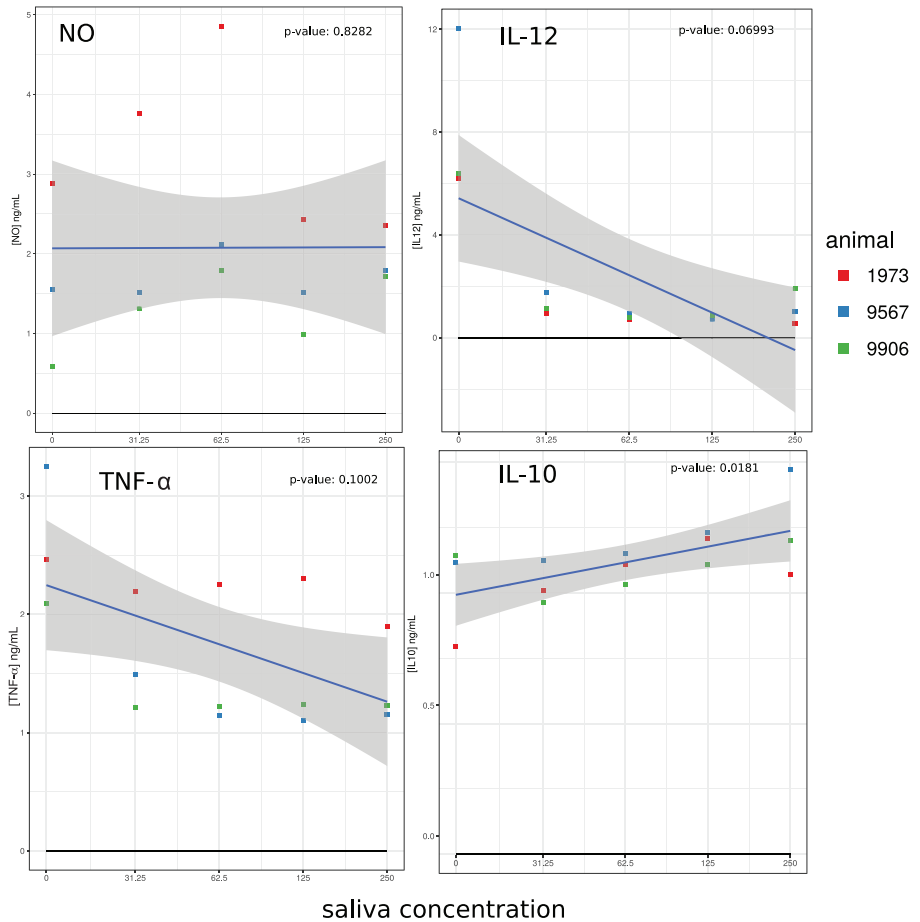

FIGURE 3 | Effect of *A. variegatum* saliva on the production of NO and cytokines by LPS-stimulated bovine macrophages. Bovine blood monocyte-derived macrophages were pre-stimulated for 1 h with LPS, and then for an additional 24 h with different concentrations of *A. variegatum* saliva. Cell culture supernatants were collected and NO production was titrated by the Griess method, IL-10, IL-12, and TNF-α production were titrated by ELISA. Data are means of triplicate experiments, for a pool of saliva batches for the three animals tested. Blue lines and shading show the linear regression fit and the 0.95 confidence interval, respectively. The *p*-value of the model is indicated on the panel.

the prediction scores for 14 gene ontology (GO) categories, the most frequently represented for *A. variegatum* saliva being Growth factor (16.4%) and Immune response (9.8%). Like the functional categories, 55.2% of the identified proteins were not classified in the proposed GO categories.

In order to link the molecular data with the biological data obtained in the cell experiments, we focused on the characterization of *A. variegatum* saliva proteins with immunomodulatory properties already described in the literature in other tick species. We found 89 out of 336 (26.5%) with a potential role in the modulation of the host immune response. Among the immunomodulatory proteins, **Table 1** shows a classification into different groups of salivary proteins with immunomodulatory functions (linked with the observed effect of *A. variegatum* saliva on the host immune cells) as follows: immunogenic proteins including Da-p36 immunosuppressant and Antigen-5 family member (CAP superfamily of proteins), proteases inhibitors including serpins and other serine and systeine protease inhibitors, proteases including serine proteases/serine carboxypeptidases/cathepsin-like cysteine proteases, ferritin and hemelipoproteins, calcium binding proteins, fatty acid, and histamine binding protein, nucleic acid binding proteins, anti-oxidant enzymes, heat shock proteins.

Twelve proteins were identified as ubiquitin with different protein IDs from *Amblyomma* and *Ixodes* taxa, of which 2 (F0J9P3, F0J9K1) specifically in *A. variegatum* (**Table 2**). Using the Propsearch server, we were able to characterize 6 saliva proteins that exhibited properties of ubiquitin-activating enzyme E1 ligase, with respective Euclidian distances, giving a reliability of 99.6% (**Table 2**). By contrast, we found ubiquitin-conjugating enzyme E2 ligase with Euclidian distances giving a percent reliability below the threshold fixed in our analytical approach (from 68 to 94%), and no ubiquitin-ligating enzyme E3 ligase (**Table 2**). However, we found 8 potential de-ubiquitinating enzymes, mainly ubiquitin carboxyl-terminal hydrolases, suggesting a complete and potentially functional ubiquitination system in *A. variegatum* saliva (**Table 2**). We

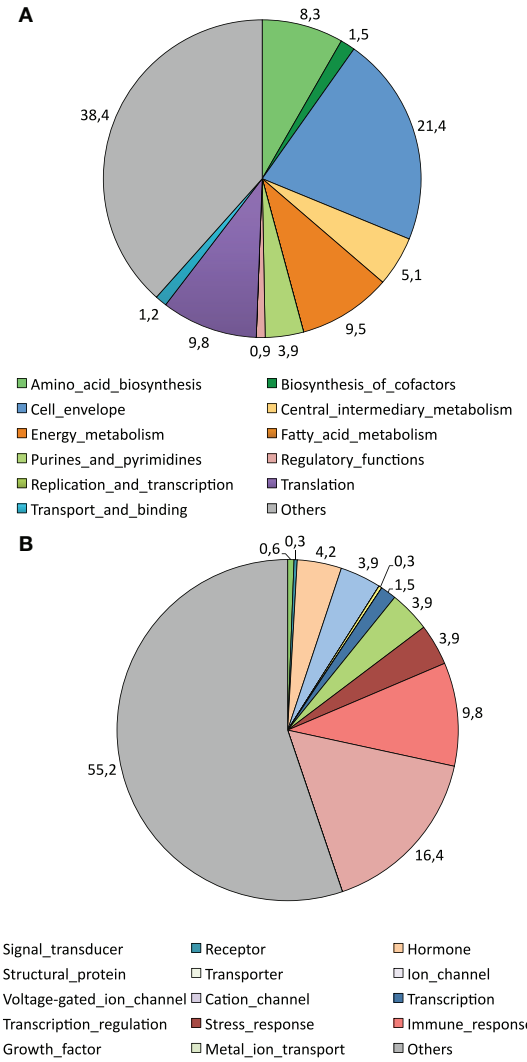

FIGURE 4 | Graphic representation of cellular role **(A)** and selected gene ontology (GO) categories **(B)** attributed to proteins identified in the saliva of *A. variegatum* by the ProtFun 2.2 Server (http://www.cbs.dtu.dk/services/ProtFun/). The function prediction server produces *ab initio* predictions of protein function from sequences by querying a large number of other feature prediction servers to obtain information on different post-translational and localizational aspects of the protein, which are integrated into final predictions of the cellular role, enzyme class (if any), and selected gene ontology categories of the sequence submitted.

found the missing link due to the identification of an alpha spectrin (**Table 2**), which has the particularity of being described as a potential chimeric E2/E3 ubiquitin conjugating/ligating enzyme (Goodman et al., 2015).

Using Propsearch server, we were able to complete the characterization of *A. variegatum* saliva proteins with immunomodulatory properties, by classifying five additional proteins associated with modulation of the host immune response as follows (**Table 3**): a GTP-binding protein (nucleic-acid binding proteins), a superoxide dismutase and a D-dopachrome tautomerase (anti-oxidant enzymes), a 60S ribosomal protein L3 (ribosomal proteins), and another alpha spectrin (Ubiquitin complex).

DISCUSSION

In the present study, we demonstrated high immunomodulation of bovine immune cells, lymphocytes and macrophages by *A. variegatum* saliva. This adds a new tick species to the increasing reports on immunomodulation by tick saliva (Kazimírová and Štibrániová, 2013). Increasing data from the literature on the interactions between tick saliva and the cells of the innate and adaptive immune system of vertebrate hosts tend to demonstrate that excreted-secreted salivary factors are critical both for completing the tick developmental cycle via blood feeding and for the transmission/reactivation of pathogens (Kotál et al., 2015; Šimo et al., 2017). Here, we showed that *A. variegatum* saliva induced inhibition of ConA-induced proliferation of bovine lymphocytes in a dose-dependent manner. This immunosuppression effect has already been observed in different tick models, and with various mitogens, as reviewed by Wikel and Kazimírová (Kazimírová and Štibrániová, 2013; Wikel, 2013). For example, *Ixodes ricinus* salivary glands secreted a protein that suppressed T lymphocyte proliferation and inhibited macrophage pro-inflammatory cytokines (Kovár et al., 2001, 2002). Among *Amblyomma* ticks, *A. cajennense* saliva has also been reported to inhibit lymphocyte proliferation in mouse and horse (Castagnolli et al., 2008). Another study showed that *Dermacentor andersoni* gland extract inhibited the pro-inflammatory cytokine TNF-α and up-regulated the anti-inflammatory cytokine IL-10, with down regulation of the expression of MHC II and of co-stimulation molecules (Wikel, 2013). These effects of saliva from different tick species, associated with our similar findings on *A. variegatum* saliva, strengthen the hypothesis that tick-host interactions generally result in induced lymphocyte anergy and reduced macrophage antigen presenting capacity and the classical activation pathway. In our study, we also showed that *A. variegatum* saliva emphasized the macrophage LPS-induced down regulation of CMH II, decreased the LPS-induced up regulation of CD40 and CD80. While CD40 binds on CD40-L on T cells for activation, inhibition of the expression of this molecule might affect directly the proliferative response of T-cells, as we observed for ConA-stimulated PBMC subjected to saliva. Similarly, while CD80 favors a Th1 response, the inhibition of the expression of LPS-induced CD80 might switch toward a Th2 response of lymphocytes, switching to an anti-inflammatory response. This switch has been demonstrated to be induced by *I. ricinus* salivary gland extracts (Kovár et al., 2001, 2002), and plays a role in countering the Th1-oriented response following tick aggression, as demonstrated for *Rhipichephalus microplus* in bovines (Brake and Pérez de León, 2012). This effect could result in the global inhibition of antigen presentation capacities and pro-inflammatory activation pathway of these immune cells, even if they are activated by the pathogen at the biting site. Furthermore, we evidenced that *A. variegatum* saliva also had an effect on NO production by

TABLE 1 | Potential immunomodulatory proteins identified in *A. variegatum* saliva.

UniProt protein IDs (Leading protein)	Description	Organism	Number of proteins	Peptides	Unique peptides	Sequence coverage [%]	Mol. weight [kDa]
IMMUNOGENIC PROTEINS							
Q75PV7	Protein related to Da-p36	*A. variegatum*	1	6	6	29.7	27.443
F0J8E1	Antigen-5 family member (Fragment)	*A.variegatum*	1	2	2	32.7	12.308
PROTEASE INHIBITORS INCLUDING							
Serpins							
F0J9G9	Serpin (Fragment)	*A. variegatum*	6	11	9	59.3	23.07
F0J8P8	Serpin (Fragment)	*A. variegatum*	1	5	5	57.1	17.759
F0J8R0	Serpin (Fragment)	*A. variegatum*	1	2	1	29.1	17.208
A0A023GPF9	Putative tick serpins 14	*A. triste*	6	6	6	14.7	44.703
A0A023GN51	Putative tick serpins 13	*A. triste*	6	5	1	12.1	42.841
A0A023GP80	Putative tick serpins 27	*A. triste*	2	3	2	7.6	43.538
A0A023FPT3	Putative tick serpins 7 (Fragment)	*A. cajennense*	1	2	2	7.5	35.74
A0A023GGH1	Putative tick serpins 33 (Fragment)	*A. triste*	1	2	2	8.5	26.402
A0A023FUL1	Putative tick serpins 12 (Fragment)	*A. cajennense*	3	2	2	7.8	46.875
A0A023GPF5	Putative tick serpins 13	*A. triste*	2	2	1	9.3	42.797
G3MLB1	Putative uncharacterized protein (tick serpins 27)	*A. maculatum*	15	3	1	9.6	43.103
Other serine protease inhibitors							
F0J9Y3	Kazal-type serine protease inhibitor domain protein	*A. variegatum*	4	3	3	20	26.71
A0A023GE28	Putative serine protease inhibitor *I. scapularis* serine protease inhibitor (Fragment)	*A. triste*	2	6	6	18.5	46.98
A0A023GE28	Putative serine protease inhibitor *I. scapularis* serine protease inhibitor (Fragment)	*A. triste*	2	6	6	18.5	46.98
A0A023FQ10	Putative serine protease inhibitor *I. scapularis* serine protease inhibitor	*A. cajennense*	1	2	2	5.1	44.715
A0A023GPF6	Putative serine proteinase inhibitor	*A. triste*	2	2	1	8.4	43.504
Cysteine protease inhibitors							
A0A023FR27	Putative thyropin	*A. cajennense*	6	2	2	12.4	22.395
F0JA40	Putative thyropin	*A. variegatum*	7	3	3	12.7	22.532
PROTEASES INCLUDING							
Serine proteases							
F0J9J5	Trypsin-like serine protease (Fragment)	*A. variegatum*	1	6	6	41.1	21.867
F0J9E7	Trypsin-like serine protease (Fragment)	*A. variegatum*	3	6	4	48.9	19.618
Serine carboxypeptidases							
A0A023GNB7	Putative serine carboxypeptidase lysosomal cathepsin a	*A. triste*	1	1	1	5.2	53.097
Cathepsin-like cysteine proteases							
F0JA27	Cathepsin L-like cysteine proteinase B	*A. variegatum*	8	10	9	36.8	37.624
F0J903	Cysteine proteinase cathepsin L (Fragment)	*A. variegatum*	2	5	5	38.6	23.017
G3MHB0	Putative uncharacterized protein (Fragment) (peptidase C1 family; cathepsin l-like cysteine proteinase b)	*A. maculatum*	3	2	1	11.5	38.867
FERRITIN AND HEMELIPOPROTEINS							
G3MPJ3	Ferritin	*A. maculatum*	2	3	3	19.5	22.296
F0J8W4	Heme lipoprotein (Fragment)	*A. variegatum*	2	33	22	63.5	50.007
F0J9N1	Heme lipoprotein (Fragment)	*A. variegatum*	1	20	18	67.4	27.005
F0J9N8	Hemelipoglycoprotein (Fragment)	*A. variegatum*	1	14	14	48.2	24.787
F0J9G0	Heme lipoprotein (Fragment)	*A. variegatum*	1	11	8	95.4	14.828
A0MVX0	Heme lipoprotein	*A. americanum*	1	31	5	16.3	177.04

(Continued)

TABLE 1 | Continued

UniProt protein IDs (Leading protein)	Description	Organism	Number of proteins	Peptides	Unique peptides	Sequence coverage [%]	Mol. weight [kDa]
B7Q406	Hemelipoglycoprotein. putative	*I. scapularis*	2	3	1	1.7	177.65
B7QIL0	Hemelipoglycoprotein. putative (Fragment)	*I. scapularis*	1	2	1	1.3	172.44
CALCIUM BINDING PROTEINS							
F0JA25	Calreticulin	*A. variegatum*	1	4	1	16.3	47.318
Q64KA0	Calreticulin	*A. brasiliense*	17	4	1	16.3	47.241
G3MK71	Calponin	*A. maculatum*	6	3	3	24.7	17.614
FATTY ACID AND HISTAMINE BINDING PROTEIN							
A0A023G6C8	Putative lipocal-1 1	*A. triste*	1	1	1	8.5	24.71
A0A023GBM1	Putative phospholipase a2 *I. scapularis* phospholipase a2	*A. triste*	2	4	4	11.4	44.79
NUCLEIC ACID BINDING PROTEINS							
G3MSL8	Putative uncharacterized protein (Putative gtp-binding protein)	*A. maculatum*	10	2	2	12.4	24.689
G3MRA1	Putative uncharacterized protein (gtp-binding protein)	*A. maculatum*	4	1	1	6	22.968
A0A023FGS1	Putative zinc-binding protein of the histidine triad hit family	*A. cajennense*	3	1	1	11.1	13.53
ANTI-OXYDANT ENZYMES							
A0A023FIC2	Putative glutathione s-transferase mu class *Rhipicephalus annulatus* glutathione s-transferase	*A. cajennense*	7	6	6	29.1	25.713
A0A023GJM3	Putative glutathione s-transferase	*A. triste*	2	2	2	14.7	24.394
A0A023FXR2	Putative glutathione s-transferase *I. scapularis* glutathione s-transferase	*A. parvum*	5	2	2	10.4	26.241
A0A023FFS7	Putative glutathione s-transferase *I. scapularis* glutathione s-transferase	*A. cajennense*	1	1	1	5.5	24.303
A0A023FQ81	Putative glutathione s-transferase *I. scapularis* glutathione s-transferase (Fragment)	*A. cajennense*	4	1	1	8.9	23.244
A0A023G5I8	Putative glutathione s-transferase *I. scapularis* glutathione s-transferase (Fragment)	*A. triste*	4	1	1	8.6	27.412
B7PDV0	Glutathione S-transferase. putative	*I. scapularis*	4	1	1	12.2	8.6628
G3MMK0	Putative uncharacterized protein (glutathione s-transferase *I. scapularis* glutathione s-transferase)	*A. maculatum*	2	1	1	5.9	26.159
A0SHR2	Protein disulfide-isomerase	*A. variegatum*	8	3	3	7.2	54.923
A0A023FZW2	Protein disulfide-isomerase	*A. parvum*	5	4	1	9.2	57.252
A0A023FNM6	Putative alkyl hydroperoxide reductase thiol specific antioxidant (Fragment)	*A. cajennense*	2	2	2	10.7	23.542
A0A023FHT1	Putative thioredoxin peroxidase	*A. cajennense*	14	4	4	24	20.494
A0A023FDL5	Putative selenoprotein w 2a	*A. cajennense*	1	1	1	22.1	8.6959
F0J9H3	Peroxidasin (Fragment)	*A. variegatum*	1	1	1	8.1	27.07
A0A023FTX2	Superoxide dismutase [Cu-Zn]	*A. parvum*	1	1	1	9.7	15.744
HEAT SHOCK PROTEINS							
A0A023GNY6	Putative heat shock protein (Fragment)	*A. triste*	11	16	13	35.6	73.124
A0A023GP15	Putative heat shock protein 90	*A. triste*	9	7	2	13.5	84.001
A0A023GLJ3	Putative heat shock protein 70	*A. triste*	7	5	2	10.5	73.014
A0A023FRH0	Putative heat shock protein	*A. cajennense*	1	1	1	5.7	21.73
G3MF27	Putative uncharacterized protein (heat shock 70 kDa protein 5)	*A. maculatum*	7	4	3	8	69.078
G3MF42	Putative uncharacterized protein (heat shock protein)	*A. maculatum*	2	1	1	7.8	21.722

TABLE 2 | Potential ubiquitination complex identified in *A. variegatum* saliva.

UniProt protein IDs (Leading protein)	Description	Organism	Number of proteins	Peptides	Unique peptides	Sequence coverage [%]	Mol. weight [kDa]	Propsearch distance (reliability)	Propsearch putative protein family
UBIQUITIN									
F0J9K1	Ubiquitin (Fragment)	*A. variegatum*	12	2	2	29.8	9.4959	–	–
E1 UBIQUITIN LIGASE									
A0A023GDS5	Putative microtubule associated complex (Fragment)	*A. triste*	2	12	9	14.1	117.06	5.38 (99.6%)	Ubiquitin-activating enzyme E1 (A1S9 protein).
A0A023G500	Putative nidogen (Fragment)	*A. triste*	1	1	1	1.2	124.2	6.21 (99.6%)	Ubiquitin-activating enzyme E1 homolog (D8)
B7QMC8	Alpha-macroglobulin. putative (Fragment)	*I. scapularis*	1	8	1	7	121.21	4.83–5.37 (99.6%)	Ubiquitin-activating enzyme E1
B7PN44	Netrin-4. putative	*I. scapularis*	1	2	2	5.1	41.593	7.21 (99.6%)	Ubiquitin-activating enzyme E1 X (Fragment)
B7PQH0	Hemolectin. putative (Fragment)	*I. scapularis*	1	2	2	2.4	115.88	5.66–5.67 (99.6%)	Ubiquitin-activating enzyme E1
A0A023GPI0	Uncharacterized protein (Fragment)	*A. triste*	3	28	1	32.3	131.52	4.90–5.42 (99.6%)	Ubiquitin-activating enzyme E1
E2 UBIQUITIN LIGASE									
B5M727	Alpha-2-macroglobulin (Fragment)	*A. americanum*	2	5	2	42.6	19.026	8.69–10.05 (94% > x > 80%)	Ubiquitin-conjugating enzyme E2-24 kDa (EC 6.3.2.19)
A0A023G356	Putative secreted protein (Fragment)	*A. triste*	1	3	3	12.2	21.619	11.17 (80%)	Ubiquitin-conjugating enzyme E2-24 kDa (EC 6.3.2.19)
A0A023FDX3	Putative I. scapularis glutathione peroxidase (Fragment)	*A. cajennense*	1	1	1	12.8	10.918	11.13–12.43 (80% > x > 68%)	Ubiquitin-conjugating enzyme variant MMS2 homolog (UEV MMS2)
G3MNL6	Putative uncharacterized protein (alpha crystallins?)	*A. maculatum*	2	1	1	13.1	19.912	9.69 (87%)	Ubiquitin-conjugating enzyme E2-24 kDa (EC 6.3.2.19)
E2/E3 UBIQUITIN LIGASE									
B7P1U8	Spectrin alpha chain. putative	*I. scapularis*	4	4	4	2	272.3	–	–
DE-UBIQUITINATING ENZYME									
A0A023GME3	Putative vitellogenin-1 (Fragment)	*A. triste*	1	38	10	28.4	153.63	6.12–7.44 (99.6%)	Ubiquitin carboxyl-terminal hydrolase 15 (EC 3.1.2.15)

(Continued)

TABLE 2 | Continued

UniProt protein IDs (Leading protein)	Description	Organism	Number of proteins	Peptides	Unique peptides	Sequence coverage [%]	Mol. weight [kDa]	Propsearch distance (reliability)	Propsearch putative protein family
A0A023GMC7	Putative vitellogenin-1 (Fragment)	*A. triste*	2	34	6	30.6	123.4	6.42–6.80 (99.6%)	Ubiquitin carboxyl-terminal hydrolase 15 (EC 3.1.2.15)
A0A023GP74	Putative titin (Fragment)	*A. triste*	2	21	15	10.4	389.34	7.13 (99.6%)	Probable ubiquitin carboxyl-terminal hydrolase FAF (EC 3.1.2.15)
A0A023G0F9	Putative peroxinectin I. scapularis peroxinectin (Fragment)	*A. parvum*	1	16	3	32.1	87.425	5.42 (99.6%)	Ubiquitin carboxyl-terminal hydrolase 20 (EC 3.1.2.15)
A0A023GCH5	Putative neurexin iii-alpha (Fragment)	*A. triste*	1	9	9	8.4	158.55	5.72 (99.6%)	Ubiquitin carboxyl-terminal hydrolase 19 (EC 3.1.2.15)
A0A023GLZ9	Putative extracellular matrix glycoprotein laminin subunit beta (Fragment)	*A. triste*	1	9	4	15.7	92.371	5.25 (99.6%)	Ubiquitin carboxyl-terminal hydrolase 20 (EC 3.1.2.15)
B7P403	Type II transmembrane protein. putative	*I. scapularis*	1	6	6	2.6	319.6	5.79 (99.6%)	Probable ubiquitin carboxyl-terminal hydrolase FAF (EC 3.1.2.15
B7PUM7	Peroxinectin. putative	*I. scapularis*	1	4	1	6.7	90.84	5.18 (99.6%)	Ubiquitin carboxyl-terminal hydrolase 20 (EC 3.1.2.15)

TABLE 3 | Propsearch-defined potential immunomodulatory proteins identified in *A. variegatum* saliva.

UniProt protein IDs (Leading protein)	Description	Organism	Number of proteins	Peptides	Unique peptides	Sequence coverage [%]	Mol. weight [kDa]	Propsearch distance (reliability)	Propsearch putative protein family
NUCLEIC-ACID BINDING PROTEINS									
F0J9Z3	RAS oncogene family member RAB1	*A. variegatum*	5	1	1	11	16.156	5.65–7.34 (99.6%)	GTP-binding protein
ANTI-OXYDANT ENZYMES									
A0A023G0Q2	Putative his-rich 1	*A. parvum*	2	2	2	14.9	15.48	1.08–2.02 (99.9 > x > 99.6%)	Superoxide dismutase [Mn-Fe] (EC 1.15.1.1) (Fragment)
G3MKP9	Carboxypeptidase	*A. maculatum*	2	1	1	2.6	52.321	5.60 (99.6%)	Dopachrome tautomerase precursor (EC 5.3.3.12) (DT) (DCT)
RIBOSOMAL PROTEINS									
G3MMA6	Putative uncharacterized protein (Putative alpha crystallins?)	*A. maculatum*	8	1	1	6	28.586	3.77–7.18 (99.6%)	60S ribosomal protein L3
UBIQUITIN COMPLEX									
A0A023GMF4	Putative beta-spectrin (Fragment)	*A. triste*	2	1	1	0.8	205.72	5.14–5.44 (99.6%)	Spectrin alpha chain

macrophages through complex modulation of this microbicidal and immunosuppressive molecule (Wink et al., 2011): it increased its production at relatively low concentrations but decreased its production at high concentrations. Macrophages use NO together with several other free radicals as a mechanism for killing pathogens and inducing an inflammatory response, but high levels of NO has been shown to have suppressive effects on lymphocyte proliferation (MacMicking et al., 1997). We observed that NO production was increased by small quantities of saliva (31.25–62.5 μg/ml), then strongly decreased by high concentrations of saliva (up to 250 μg/ml). This inhibition correlated with the decrease in TNF-α and the increase in IL-10 we observed when the global *in vitro* immune response became clearly anti-inflammatory. It is possible that high production of NO plays a role in the establishment of lymphocyte anergy, before the deactivation of macrophages by *A. variegatum* saliva avoids tissue damage in the host that would be deleterious to tick feeding. In a previous study on *A. variegatum* saliva performed in the *in vivo* mouse model, we showed that, in skin lesions mimicking tick biting, *A. variegatum* saliva impairs leukocyte infiltration (Vachiery et al., 2015). Experiments in an *in vitro* human model further demonstrated that tick saliva impaired the migration of antigen presenting cells to the draining lymph nodes and affected the recruitment of blood monocytes to the skin lesion and prevented their maturation *in situ* (Vachiery et al., 2015). These results strengthen our findings with bovine cells, and show how the saliva of *A. variegatum*, by modulating chemotaxis, the proliferative capacity of lymphocytes, the regulatory capacities of macrophages, diverts the host's immune system to its own advantage, and the consequences this may have for the transmission or reactivation of pathogens.

Some of the strong and specific effects of tick saliva in modulating cytokine production by macrophage and lymphocytes, and hence the impacts on immune response, can be analyzed at molecular scale to characterize saliva molecules thanks to the advent of proteomics. Among the proteins we evidenced in *A. variegatum* female saliva and shown to have direct immunomodulatory functions, the CAP superfamily of proteins (comprising the CRISP, Antigen-5, and pathogen-related-1 families) has been found in most tick sialomes studied to date, with molecular characteristics similar to wasp-venom proteins and annotated as Antigen-5 (Gibbs et al., 2008). Additionally, recombinant Da-p36 has been demonstrated to suppress T-lymphocyte-mitogen-driven *in vitro* proliferation of splenocytes (Alarcon-Chaidez et al., 2003), and could therefore be part of the molecular mechanism behind our observation of inhibition lymphocyte proliferation in the presence of *A. variegatum* saliva.

The most representative category of proteins with potential immunomodulatory properties we identified were protease inhibitors, mainly serpins. Serpins are abundant in ticks, and one of their functions is to modulate the host immune system (Chmelar et al., 2017). The first tick serpin described for its effect on host defense mechanisms was named *I. ricinus* immunosuppressor (Iris) and was able to inhibit T-cell and splenocyte proliferation and to modify cytokine levels derived from PBMC (Leboulle et al., 2002), as well as to bind

to monocytes/macrophages and to suppress TNF-α secretion (Prevot et al., 2009). These results are very similar to our observations, and we hypothesize the existence of an Iris-like protein among the serpins identified in *A. variegatum* saliva. Other protease inhibitors we identified are cysteine protease inhibitors named thyropins. Recently, an exogenous thyropin was shown to affect IL-12 secretion of dendritic cells (DCs) (Zavašnik-Bergant and Bergant Marušič, 2016), which leads us think that the thyropins in *A. variegatum* saliva could be involved in the decreased production of IL-12 by bovine macrophages, like DCs. In contrast to protease inhibitors, we identified proteases in the saliva of *A. variegatum*, whose carboxypeptidases could play a role in the destruction of inflammatory peptide agonists (Ribeiro et al., 2011) and cathepsin-like cysteine proteases could destroy cellular mediators of inflammation (Radulović et al., 2014).

During tick feeding, the processing of the blood meal is accompanied by a high risk of oxidative stress linked to large amounts of heme, a bi-product of hemoglobin digestion, and free iron in the host's blood (Graca-Souza et al., 2006). We identified ferritin and hemelipoproteins in *A. variegatum* saliva, which could be used to dump heme and free iron in the host to avoid oxidative stress, and at the same time benefit tick-borne pathogens that may need iron to proliferate (Rouault, 2004). We also found numerous anti-oxidant enzymes (including glutathione-S transferase (GST), protein disulfide isomerase, thioredoxin peroxidase, peroxidasin, superoxide dismutase, and selenoproteins) in the saliva of *A. variegatum*, which could play a role in reducing the toxicity of reactive oxygen and nitrogen species (such as NO) produced as part of the wound healing mechanism and anti-microbial defenses (Vider et al., 2001; Rojkind et al., 2002). Preventing damage caused by oxidative stress could benefit both the host and the tick, but given the susceptibility of pathogens to the products of oxidative stress, anti-oxidants in tick saliva could facilitate the transmission of tick-borne pathogens. Interestingly, we found D-dopachrome tautomerase, the functional homolog of macrophage migration inhibitory factor (MIF) (Merk et al., 2011), which is a pro-inflammatory cytokine produced by ticks to regulate host responses to tick feeding (Wasala et al., 2012). D-dopachrome tautomerase/MIF could be involved in the immunological silence induced by *A. variegatum*, with no chemotactic recruitment of host macrophages. Conversly, we evidenced several proteins dedicated to anti-inflammatory response. For instance, extracellular nucleic acids are potent proinflammatory molecules, and nucleic acid binding proteins could be part of the system used by the tick to modulate host inflammation (Radulović et al., 2014). In the same way, ribosomal and heat shock proteins have been described as potent anti-inflammatory molecules (Pockley, 2003; Poddar et al., 2013).

Fatty acid and histamine binding proteins also play roles in preventing inflammation during tick feeding. Histamine is a potent pro-inflammatory molecule that is released by cellular mediators of inflammation such as mast cells and neutrophils (White and Kaliner, 1987). Like *A. americanum*, *A. variegatum* saliva contains histamine-binding protein/lipocalin to sequester histamine and stop the corresponding inflammatory response (Radulović et al., 2014). Although other authors have demonstrated secretion of prostaglandin E2 (PGE2) and prostacyclin (PGI) in tick saliva (Ribeiro et al., 1988; Bowman et al., 1995), we did not identify these proteins among our

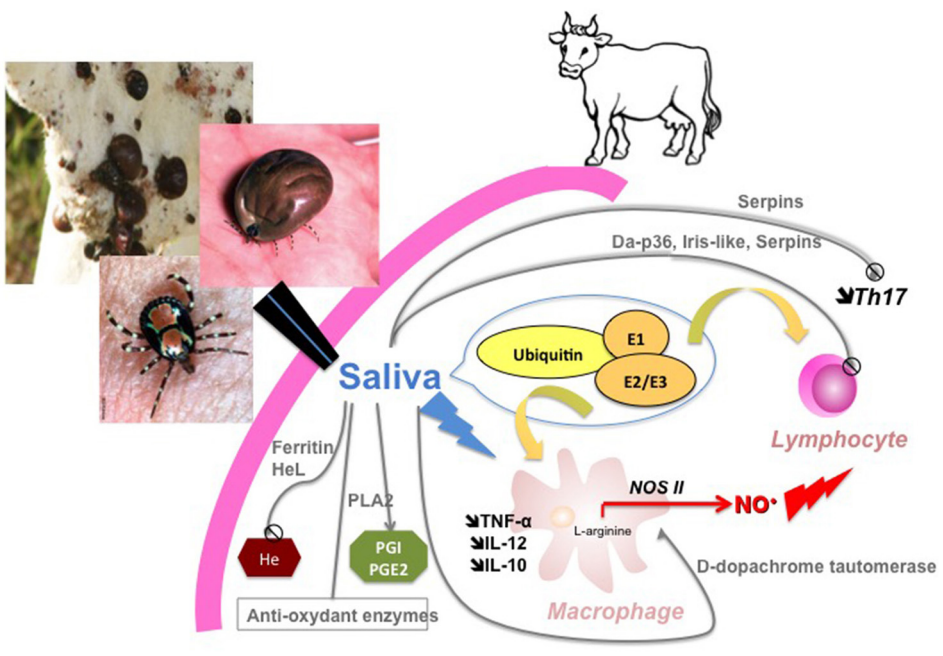

FIGURE 5 | Synthetic view of *A. variegatum* salivary-induced modulation of bovine immune cells Molecular determinants (with immunomodulatory properties identified by proteomics) driving cellular events (inhibition of lymphocyte proliferation, modulation of macrophage activation) evidenced in the study are summarized in the scheme.

proteomics data. However, we did identify a phospholipase A2, which could stimulate prostacyclin production (Bowman et al., 1997) and induce PGE2 production by host cells (Hara et al., 1991; Miyake et al., 1994). Tick saliva PGE2 has been shown to modulate the function of macrophages, particularly cytokine profiles (Poole et al., 2013), as we also evidenced in our study. Indirect PGI and PGE2 regulation could have been developed by *A. variegatum* to manipulate host macrophages, which, as a central immune cell, could pervert the whole immune system of the host.

Multifunctional calcium binding proteins, such as calponin and calreticulin (CRT), are present in the saliva of *A. variegatum* but apart from depleting Ca^{2+} to prevent activation of blood clotting (Astrup, 2009), their function is not yet known. Nevertheless, an interesting hypothesis has emerged from *A. americanum* CRT, which could skew a Th-2 host response to tick feeding (Kim et al., 2015), and which is consistent with our observation of a M2-induced profile of macrophage cytokines/metabolism by *A. variegatum* saliva.

Finally, we also discovered an original ubiquitination complex in the saliva of *A. variegatum*, both on its molecular architecture and on its extracellular localization. In eukaryote cells, ubiquitin is transferred by three enzymes: the ubiquitin-activating enzyme E1 ligase catches ubiquitin and transfers it to the ubiquitin-conjugating enzyme E2 ligase, which associates with ubiquitin-ligating enzyme E3 ligase to transfer ubiquitin to the target substrate (Tanaka et al., 1998). Intriguingly, the ubiquitination we evidenced in *A. variegatum* is simplified with spectrin playing the role of a chemeric E2/E3 ubiquitin-conjugating-ligating enzyme (Goodman et al., 2015). Whereas, ubiquitination has long been well described in connection with proteasome degradation of proteins, in the last decade, an increasing literature has proposed an important role for ubiquitination in immunity (Ben-Neriah, 2002; Zinngrebe et al., 2014), particularly in regulating both innate and adaptive immune responses (Malynn and Ma, 2010; Park et al., 2014; Hu and Sun, 2016). Interestingly, extracellular ubiquitin has been implicated in lymphocyte differentiation, suppression of the immune response, and prevention of inflammation (Sujashvili, 2016), particularly by binding to the CXCR4 receptor (Majetschak, 2011; Scofield et al., 2015). Moreover, extracellular ubiquitin can bind directly to regulatory T cells; enhancing their inhibitory effect on effector T cells proliferation (Cao et al., 2014). Exogenous ubiquitin can also reduce TNF-α production (Majetschak et al., 2004). The last two elements are in complete agreement with our observations on inhibition of lymphocyte proliferation and reduction of TNF-α production by macrophages in the presence of tick saliva, and make us suggest that *A. variegatum* uses its extracellular ubiquitination complex as an effective molecular weapon for render the immune responses of the host ineffective during the blood meal.

To conclude, our data shed new light on the molecular determinants of *A. variegatum* saliva-induced immunomodulation of bovine immunity cells and allows us to propose a new synthesis scheme of the cellular and molecular events that take place at the tick-host interface (**Figure 5**). Further studies on the effect of *A. variegatum* saliva on bovine PBMC and macrophages are needed to better understand the mechanisms underlying our observations, particularly the comparative effect of saliva on different lymphocyte sub-populations such as cytotoxic T cells or NK cells, but also qualitative or quantitative cytokine modulation such as INF-γ, IL-2, IL-4, or IL-17 with impacts on Th1/Th2 or Th17 balance, as shown in *I. ricinus* (Kopecký et al., 1999; Kovár et al., 2001; Leboulle et al., 2002) and in mosquitoes (Wanasen et al., 2004). Effects on macrophages presenting capacities also need to be elucidated in connection with pathogen uptake and presentation in the presence of tick saliva. These cellular investigations will need to be combined with further molecular characterization of saliva proteins to identify the key molecules in immunomodulation mechanisms. Overall, our data provide new insights into tick-ruminant interactions for *A. variegatum*, including their involvement in vectorial competence for pathogen transmission (*E. ruminantium* infection) or on pathogen reactivation (dermatophilosis), and pave the way for integrative strategies to interfere with both the immunosuppressive and infectious processes in the corresponding tick-borne diseases.

AUTHOR CONTRIBUTIONS

VR, BF, FS, and PH: designed the work; VR, AV, LC, RA, KG-G, and PH: generated the biological samples; VR, BF, AV, LC, AA, RA, KG-G, OV, ED, MS, and PH: performed the experiments; VR, BF, AV, LC, AA, OV, ED, MS, FS, EL, NV, and PH: acquired, analyzed, and interpreted the data; VR and PH: wrote the manuscript; VR, BF, AV, LC, AA, OV, ED, MS, RA, KG-G, FS, EL, NV, and PH: revised and approved the final version of the manuscript.

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Mass spectrometry experiments were carried out using facilities of the Functional Proteomics Platform of Montpellier.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2017.00521/full#supplementary-material

Supplementary Figure 1 | Effect of *A. variegatum* saliva on the expression of surface molecules of bovine unstimulated macrophages. Bovine blood monocyte-derived macrophages were cultivated for 24 h with different concentrations of *A. variegatum* saliva. Cells were collected and stained for MHC II, CD40, CD80, and CD86 surface markers. Expression levels (mean of fluorescence intensity, MFI) of markers on macrophages compared to those on

control unstimulated cells were measured by flow cytometry. Blue lines and shading show the linear regression fit and the 0.95 confidence interval, respectively. The *p*-value of the model is indicated on the panel.

Supplementary Figure 2 | Effect of *A. variegatum* saliva on the production of cytokines by bovine unstimulated macrophages. Bovine blood monocyte-derived macrophages were stimulated for 24 h with different concentrations of *A. variegatum* saliva. Cell culture supernatants were collected and IL-10, IL-12, and TNF-α production was titrated by ELISA. (Data are results obtained for one experiment performed on three different animals; note different scales on graphs). Blue lines and shading show the linear regression fit and the 0.95 confidence interval, respectively. The *p*-value of the model is indicated on the panel.

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Salivary Tick Cystatin OmC2 Targets Lysosomal Cathepsins S and C in Human Dendritic Cells

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To ensure successful feeding tick saliva contains a number of inhibitory proteins that interfere with the host immune response and help to create a permissive environment for pathogen transmission. Among the potential targets of the salivary cystatins are two host cysteine proteases, cathepsin S, which is essential for antigen- and invariant chain-processing, and cathepsin C (dipeptidyl peptidase 1, DPP1), which plays a critical role in processing and activation of the granule serine proteases. Here, the effect of salivary cystatin OmC2 from *Ornithodoros moubata* was studied using differentiated MUTZ-3 cells as a model of immature dendritic cells of the host skin. Following internalization, cystatin OmC2 was initially found to inhibit the activity of several cysteine cathepsins, as indicated by the decreased rates of degradation of fluorogenic peptide substrates. To identify targets, affinity chromatography was used to isolate His-tagged cystatin OmC2 together with the bound proteins from MUTZ-3 cells. Cathepsins S and C were identified in these complexes by mass spectrometry and confirmed by immunoblotting. Furthermore, reduced increase in the surface expression of MHC II and CD86, which are associated with the maturation of dendritic cells, was observed. In contrast, human inhibitor cystatin C, which is normally expressed and secreted by dendritic cells, did not affect the expression of CD86. It is proposed that internalization of salivary cystatin OmC2 by the host dendritic cells targets cathepsins S and C, thereby affecting their maturation.

Keywords: cystatin OmC2, tick saliva, cathepsin S, cathepsin C, lysosomal proteases, DPP1, dipeptidyl peptidase 1, dendritic cells

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INTRODUCTION

As external parasites, ticks have adapted to their hosts to successfully feed while warding off the host immune system. The saliva of ticks contains multiple proteins with anti-haemostatic, anti-inflammatory and immunomodulatory properties (Díaz-Martin et al., 2013). Tick saliva influences the production and secretion of numerous cytokines, often simultaneously, from various types of immune cells (Kotál et al., 2015). The tick salivary proteins not only inhibit the components of the host immune system (Francischetti et al., 2009) but also have an impact on the transmission of vector-borne pathogens. Specifically, many species of *Borrelia* causing relapsing fever in infected humans, are transmitted by the soft ticks of *Ornithodoros genus* (Parola and Raoult, 2001). Multiple

species of hard and soft ticks inhibit the production of various cytokines such as TNF-α, IL-6, IL-12, IL-17, and IFN-γ (Sá-Nunes et al., 2009; Fialová et al., 2010; Wu et al., 2010). Further, salivary molecules from *O. moubata* can modulate the host defense system by acting as platelet-aggregation inhibitors [e.g., moubatin (Waxman and Connolly, 1993) and disaggregin (Karczewski et al., 1994)], as complement inhibitors [e.g., OmCI (Nunn et al., 2005)], or by interfering with coagulation and fibrinolysis [e.g., ornithodorin (van de Locht et al., 1996) and tick anticoagulant peptide TAP (Waxman et al., 1990)].

Modulation of the proteolytic activity of targeted host cysteine proteases in antigen-presenting cells (APC) in the host skin may represent an additional mechanism utilized by ticks to modulate the immune response of their host. Cystatin OmC2 is naturally present in the saliva, salivary glands and gut of the soft tick *O. moubata* (Grunclová et al., 2006). Recombinant cystatin OmC2 was screened against a panel of recombinant lysosomal proteases. It was shown to be a broad-specificity inhibitor of mammalian cysteine cathepsins but not of mammalian legumain (asparaginyl endopeptidase), another cysteine protease potentially involved in the processing of antigens in APC. The crystal structure of cystatin OmC2 was determined and used to describe the structure-inhibitory activity relationship (Salát et al., 2010). The biological impact of cystatin OmC2 was demonstrated in mice both using *in vitro* and vaccination experiments. Cystatin OmC2 decreased the levels of TNF-α and IL-12 produced by LPS-activated dendritic cells (DC), as well as it was able to reduce the DC-mediated proliferation of naive $CD4^+$ T cells. In addition, the vaccination of mice with recombinant cystatin OmC2 decreased the success of tick feeding. Ticks that fed on the mice with the highest level of anti-cystatin OmC2 antibodies had the highest mortality (Salát et al., 2010).

This study focused on two lysosomal cathepsins, S and C, which are both members of the papain-like superfamily of cysteine proteases (Turk et al., 2012; Rawlings et al., 2016). The first target, cathepsin S, is involved in the crucial step in the processing of major histocompatibility complex class II (MHC II)-associated chaperone invariant chain (Roche and Furuta, 2015; Lindner, 2017), as well as being involved in the lysosome-mediated response to microbial DNA *via* the TLR9 pathway (Matsumoto et al., 2008). The second target, cathepsin C, is an exoprotease, the function of which in human DC is not yet fully known. The structure of cathepsin C tetramer reveals the tetrahedral arrangement of its active sites. Each monomer consists of three domains: two domains consist of a papain-like structure that contains the catalytic site and an additional exclusion domain that provides features that endow cathepsin C with a dipeptidyl aminopeptidase activity (Dahl et al., 2001; Turk et al., 2001). The general function of dipeptidase cathepsin C is likely to be the degradation of the protein cargo in lysosomes as well as the processing of a diverse set of precursor proteins, which includes proteases. In neutrophils, cathepsin C is essential for the activation of the granule-associated serine proteases. These proteinases require the activity of cathepsin C to remove their propeptides and thus become active. Serine proteases, which are stored in granules in their active forms until they are released after neutrophil exposure to activating stimuli, exhibit broad biological effects including intracellular microbial killing and modulation of the recruitment of inflammatory cells (Pham, 2006). Cathepsin C is involved in the activation of elastase and cathepsin G in neutrophils and chymase and tryptase in mast cells (Pham and Ley, 1999; Pham, 2006; Guarino et al., 2017). Peripheral blood neutrophils upregulate their granzyme B expression and secretion when they are infected with *Mycobacterium tuberculosis* (Mattila et al., 2015). In addition, in natural killer (NK) cells (Maher et al., 2014) and T lymphocytes, cathepsin C is involved in the activation of progranzymes A and B into the proteolytically active enzymes. In patients with neutrophilic lung inflammation, mature cathepsin C is found in large amounts in sputa. It is secreted by activated neutrophils as confirmed through lipopolysaccharide (LPS) administration in a non-human primate model (Hamon et al., 2016). In cathepsin C-deficient mice, the function of the cytotoxic T cells is impaired (Pham and Ley, 1999) whereas in humans, defects in the cathepsin C gene are associated with Papillon-Lefevre disease, Haim-Munk syndrome (Sulák et al., 2016) and aggressive periodontitis (Nagy et al., 2014).

Pathogen invasion together with the proinflammatory signals drives the maturation of skin immature DC which then upregulate the expression of their costimulatory molecules and proinflammmatory cytokines. Distinct subsets of functionally specialized Langerhans cells (LC) and dermal DC have been distinguished among DC in epidermis and dermis (reviewed in Clausen and Stoitzner, 2015). In healthy mouse skin the DC network includes $Langerin^+CD11b^+$ LC, $Langerin^+CD11b^{neg}CD103^+$ dermal DC, $Langerin^+CD11b^{neg}CD103^{neg}$ dermal DC, $Langerin^{neg}CD11b^+$ dermal DC, and $Langerin^{neg}CD11b^{low}XCR1^{neg}$ dermal DC. Human skin DC counterparts include: $Langerin^+CD1a^{high}$ LC, $CD141^{high}XCR1^+$ dermal DC and $CD1c^+CD1a^+$ dermal DC. The human acute myeloid leukemia cell line MUTZ-3 can be differentiated *in vitro* to dermal DC in the presence of GM-CSF, TNF-α and IL-4, and to LC in the presence of GM-CSF, TNF-α and TGF-β (Santegoets et al., 2008). MUTZ-3-derived DC, which have the great advantage of not being dependent on the donor material, express adequate immune-related transcripts (Larsson et al., 2006; Lundberg et al., 2013), supporting their suitability in immune applications (Santegoets et al., 2008). MUTZ-3-derived immature DC differentiated with IL-4 and cultured in the presence of LPS efficiently stimulated the proliferation of $CD4^+CD45RA^+$ T cells (Larsson et al., 2006) but failed to stimulate the proliferation of allogenic NK cells (Kim et al., 2006). In addition, MUTZ-3 cells that are differentiated with TGF-β to MUTZ-3-derived LC (MUTZ-LC) are often applied in the area of risk assessment of chemicals, in allergen and irritant exposure studies etc. (Kosten et al., 2015).

We have further evaluated the possibility of cystatin OmC2 to mimic the function of its host cystatin type 2 homologs, if successfully internalized to the host immune cells. Differentiated MUTZ-3 cells were applied as an *in vitro* cell model of human immature DC and tick cystatin OmC2 was evaluated whether it could interfere with the host proteolytic capacity mediated by the lysosomal cysteine cathepsins. We show

that following its internalization *via* the endocytic pathway, cystatin OmC2 affected the activity of lysosomal cathepsins S and C, two key cysteine proteases of DC proteolytic machinery.

MATERIALS AND METHODS

Tick Cystatin OmC2

The baculovirus expression system was used to prepare the salivary cystatin OmC2 as a recombinant protein with an oligo His-tag added to its C-terminus in insect Sf9 cells (Salát et al., 2010). Salivary tick cystatin OmC2 was also produced in *E. coli* (Grunclová et al., 2006), and this protein was used in the experiments, which are included in Supplementary Figures 1A,B. In addition, the recombinant tick inhibitor OmC2 from insect cells was fluorescently labeled with Alexa Fluor 488 dye (Alexa Fluor 488 Microscale Protein Labeling Kit, Life Technologies-Molecular Probes). The unbound Alexa Fluor 488 dye was removed from reaction mixture using size-exclusion chromatography (as recommended by the producer), additional dialysis and filtration by using Microcon-3 kDa (Millipore). The fluorescence was measured with Tecan microplate reader after each purification step. The labeled cystatin OmC2 was excited at 488 nm, and the fluorescence was followed using a confocal laser scanning microscope Leica SP5 X with a white light laser (Leica MicroSystems) or a Typhoon Variable Mode Imager (GE Healthcare). Prior to the application of the recombinant cystatin OmC2 in the cell studies, its inhibitory activity was determined by active site titration of 10 nM papain (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 6.0). The active concentration of the papain was determined by titration with a synthetic inhibitor E-64 (Sigma-Aldrich). For these assays, 10 μM of the fluorogenic substrate Z-Phe-Arg-AMC (Bachem) was applied, and the fluorescence signal was measured using a Tecan Safire microplate reader. Fluorescently labeled cystatin OmC2 was applied when its internalization was followed by confocal microscopy in viable cells. When cells were assayed for activity measurements or pull-down experiment and mass spectrometry analysis, non-labeled cystatin OmC2 was applied. In addition, the complex formation between cystatin C and cathepsin S was evaluated *in vitro* using IEF. Human recombinant cystatin C was prepared in-house according to published procedures (Cimerman et al., 1999). Software from CBS (CBS Prediction Servers/Post-translational modifications of proteins/NetNGlyc, www.cbs.dtu.dk) was used for the prediction of possible O-glycosylation sites in cystatin OmC2.

Cathepsins

Human recombinant cathepsin S and cathepsin L were expressed in the methylotrophic yeast *Pichia pastoris* and were isolated as previously reported (Mihelič et al., 2008). Human recombinant procathepsin C was expressed in insect Sf9 cells using the baculovirus expression system according to the procedure described elsewhere (Dahl et al., 2001) and was purified by using the modified procedure described by Poreba et al. (2014). Briefly, the cell-free culture medium was collected after 3 days, concentrated by ultrafiltration and dialyzed against 50 mM HEPES with 0.5 M NaCl, pH 7.5. Isolation of procathepsin C (containing an oligo His-tag added to the C-terminus) was performed using immobilized-metal (Ni) affinity chromatography (IMAC) from Roche (cOmplete His-Tag Purification Resin). A 300 mM imidazole solution was used to elute the procathepsin C. The protein was preserved in 30 mM TRIS buffer with 0.4 M NaCl (pH 7.5), or alternatively, 50% glycerol was added to avoid freezing at −20°C. The procathepsin C expression was confirmed by mass spectrometry using an UltrafleXtreme™ III MALDI-TOF/TOF instrument (Bruker). The procathepsin C was then proteolytically processed and activated under acidic condition by the proteases present in the insect cells or by recombinant human cathepsin L, the latter being added at a 1:20 molar ratio. The aminopeptidase activity of the purified cathepsin C was measured as described (Poreba et al., 2014).

Isoelectric Focusing (IEF)

Cathepsins S, C, or L were first activated with 5 mM DTT in 0.1 M phosphate buffer (pH 6.0; cathepsins S and C) or 0.1 M acetate buffer (pH 5.6; cathepsin L) for 5 min. The active cathepsins were next incubated with tick cystatin OmC2 or with human cystatin C at a 1:1 molar ratio for 10 additional min at 37°C. IEF was then performed using the XCell SureLock Mini-Cell system (Invitrogen-Life Technologies). The proteins were separated on vertical pre-cast IEF gels (pH 3–7 and pH 3–10) from Invitrogen. IEF Markers 3–10 (SERVA Liquid Mix) were applied as protein standards. The proteins were fixed in 10% TCA for 30 min, followed by 1% TCA for 10 min. The changes in isoelectric points (pI) of the applied proteins (recombinant proteins and proteins from the cell lysates) were examined. Both recombinant tick cystatins; isolated from insect cells and from *E. coli,* were applied.

SDS-PAGE and Western Blotting

Protein samples (cystatin OmC2, lysates from differentiated MUTZ-3 cells with internalized cystatin OmC2) were separated on 12.5% pre-cast gels from Lonza using the Mini-PROTEAN 3 Cell system (Bio-Rad). Protein standards from Fermentas (PageRuler Prestained Protein Ladder) were applied. The separated proteins were stained with 0.1% PhastGel Blue R (Coomassie R 350 dye) from GE Healthcare or with 0.2% $AgNO_3$ (Fluka). Selected bands were excised and analyzed using mass spectrometry or the proteins were transferred to PVDF membranes. The proteins from the SDS-PAGE or IEF gels were electrotransferred to PVDF membranes and immunolabeled with rabbit anti-cathepsin S pAb (Zavašnik-Bergant et al., 2005), goat anti-cathepsin S pAb (AF1183, R&D Systems), mouse anti-cathepsin C mAb (sc-74590, Santa Cruz Biotechnology), or mouse anti-β-actin antibody (A1978, Sigma-Aldrich). ECL Western blotting detection reagents (Amersham, GE Healthcare) were applied, and the imaging films (BIOMAX Light Film, Carestream) were developed (Konica Minolta SRX-101A). Alternatively, the bands were detected using the DAB chromogenic substrate (Sigma-Aldrich).

Mass Spectrometry (MS) of Recombinant Proteins

Selected bands were excised from the precast SDS-PAGE or IEF gels and prepared for mass spectrometric analysis as described (Sobotič et al., 2015). Briefly, the gel pieces were de-stained, reduced with 10 mM DTT and alkylated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate buffer, pH 7.8. In-gel digestion was performed with sequencing grade modified porcine trypsin (Promega) at 37°C, overnight. The samples were subjected to peptide mass fingerprint analysis using a MALDI-TOF/TOF UltraFlextreme III mass spectrometer (Bruker), and the database was searched using Mascot software. To compare the non-labeled cystatin OmC2 and Alexa Fluor 488-labeled cystatin OmC2, the intact protein molecular weights were determined using MALDI-TOF/TOF UltraFlextreme III. For this purpose, a matrix mixture was prepared by mixing 2 μl of protein sample, 2 μl of 2% TFA in H_2O and 2 μl of a 2,5-dihydroxyacetophenone matrix solution. The mixture was applied to a matrix target plate, allowed to dry at room temperature, and analyzed.

Differentiation and Maturation of Cells

The human MUTZ-3 cell line was purchased from DSMZ (Germany). The cells were grown in α-MEM with 20% heat-inactivated FBS (PAA Laboratories-GE Healthcare Life Sciences), 1% Glutamax (Life Technologies) and 40 ng/ml GM-CSF (CellGro) as previously described (Nelissen et al., 2009; Song et al., 2015). The MUTZ-3 cells were differentiated to immature DC with 62.5 ng/ml GM-CSF, 100 ng/ml IL-4 (CellGro) and 2.5 ng/ml TNF-α (CellGro) for 4 days (Zavašnik-Bergant and Bergant Marušič, 2016). The viability of the cells was evaluated using Trypan Blue (Sigma-Aldrich). The differentiated MUTZ-3 cells were matured with 20 ng/ml LPS (Sigma-Aldrich) for 24 h. Alternatively, the MUTZ-3 cells were stimulated with LPS in the presence of tick cystatin OmC2 (0.4 or 0.8 μM). Cystatin solution was filtered through a 0.22 μM Durapore PVDF membrane/Millex GV filter unit (Millipore). In control experiments, sterile PBS was added instead of cystatin OmC2, and the cells were cultured for 24 h. In addition, immature DC were matured with LPS in the presence of 0.4 or 0.8 μM human cystatin C (Zavašnik-Bergant et al., 2005).

Flow Cytometry

The surface expression of MHC II and CD86 was followed in MUTZ-3 cells (differentiated and stimulated with LPS or cultured in the presence of recombinant tick cystatin OmC2 or human cystatin C). Both cystatins weren't labeled with Alexa Fluor 488. The cells were pre-incubated at 4°C to prevent non-specific internalization of the fluorescently labeled primary antibodies: anti-CD86 (clone FUN-1) conjugated to CY-CHROME and anti-MHC II (anti-HLA-DR, clone G46-6) conjugated to R-phycoerythrin (both from BD Biosciences-Pharmingen). A total of 5000 cells per sample were gated and evaluated for specific labeing using a FACSCalibur instrument (BD Biosciences) and BD CellQuest software (version 3.3). Differentiated MUTZ-3 cells treated with cystatin OmC2 and LPS were compared to non-treated differentiated MUTZ-3 cells.

Immunocytochemistry, Confocal, and Electron Microscopy

MUTZ-3 cells (non-differentiated, differentiated, activated with LPS, or cultured in the presence of 2 or 12 μM tick cystatin OmC2 for 1 or 3 h) were cytocentrifuged to glass slides coated with poly-L-lysine (Sigma Aldrich). The cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The cells were then immunolabeled with rabbit anti-cathepsin S pAb, rabbit anti-human cystatin C pAb, sheep anti-cathepsin L (Zavašnik-Bergant et al., 2005), mouse anti-HLA-DR mAb (clone TÜ-36, BD Pharmingen), goat anti-cathepsin C pAb (clone T-17, Santa Cruz Biotechnology) or rabbit anti-His tag pAb (ab9108, Abcam). Highly cross-adsorbed goat anti-mouse IgG pAb, goat anti-rabbit IgG pAb, donkey-anti sheep IgG pAb, and donkey anti-goat IgG pAb, labeled either with Alexa Fluor 488 or with Alexa Fluor 546, obtained from Life Technologies-Molecular Probes were used as secondary antibodies. Controls in which the primary or secondary antibodies were omitted were included in these runs. Immunofluorescence microscopy of optical sections was performed by using a confocal laser scanning microscope Leica TCS SP5 X (Leica MicroSystems). The fluorophores were excited with selected lines from a tunable white light laser (460–670 nm) or a diode laser (405 nm). Leica Application Suite Advanced Fluorescence software (LAS AF, version 2.7.3.9723, Leica MicroSystems) was used for the image analysis. Transmission electron microscopy and immunogold labeling of cystatin C was performed as described (Zavašnik-Bergant and Bergant Marušič, 2016).

Live Cell Imaging

Differentiated MUTZ-3 cells were cultured in the presence of fluorescently labeled tick cystatin OmC2 (2 μM) for 1 or 3 h. Internalization of cystatin OmC2 to acidic vesicles within the endocytic pathway of treated cells, i.e., to the late endosomes and lysosomes, was followed by using confocal microscopy and fluorescently labeled organelle markers. The LysoTracker Blue DND-22 (a marker for lysosomes), CellMask Orange plasma membrane stain, and CellLight Late Endosomes-RFP/Bac Mam 2.0 system (for the expression of Rab7a-RFP in late endosomes) were from Life Technologies-Molecular Probes. All commercial reagents were used according to the supplier's recommendations. Also, differentiated MUTZ-3 cells were pre-incubated for 30 min at 4°C to decrease their endocytic capacity. Then, cells were cultured with fluorescently labeled cystatin OmC2 at 4°C for 1 and 3 h. Cells cultured at 4°C were compared to the cells cultured at 37°C (and not pre-incubated at 4°C).

Cell Lysates

Differentiated MUTZ-3 cells (1×10^6 cells/ml) were cultured in the presence of 2, 12, or 15 μM cystatin OmC2 for 1 or 3 h. The cells were pelleted, vigorously rinsed and resuspended in 0.1 M phosphate buffer with 1 mM EDTA (pH 6.0). The cells were sonicated on ice using a Branson Digital W-450 Sonifier (Branson, Danbury, CT). The non-soluble fraction was pelleted and removed by centrifugation at 16,000 g. The soluble fraction was aliquoted and stored at −80°C. The residual activity of the

cysteine proteases in the treated cells was compared to the non-treated cells (control). In addition, lysates for analysis by mass spectrometry and Western blotting were prepared from cells that had been cultured in the presence of 12 μM non-labeled cystatin OmC2 (pull-down assay).

Activity of Cysteine Proteases

The residual endoprotease and exoprotease activities were measured using the fluorogenic substrates Z-Phe-Arg-AMC and H-Gly-Phe-AMC (Bachem) as described (Mihelič et al., 2008; Poreba et al., 2014). The increases in the fluorescence signals (ex. 370 nm/em. 460 nm) were measured with Tecan Safire microplate reader. Samples were measured in triplicate and the differences in residual activity after 1 h and after 3 h were analyzed with Student's *t*-test. Prior to the *t*-test an assumption on the homogeneity of variances was tested by the Levene test. Cells cultured in the presence of cystatin OmC2 (two different times of incubation at 37°C, two fluorogenic substrates, and three concentrations of added inhibitor) were all compared to corresponding controls (non-treated cells). *P*-values of 0.05 or less were considered significant. Statistical analysis was performed using IBM SPSS Statistics 20.

Pull-Down Assay

The isolation of the cell proteins bound to internalized cystatin OmC2 (not labeled with Alexa Fluor 488) was performed using immobilized-Ni affinity chromatography. Cell lysates were prepared from differentiated MUTZ-3 that had been cultured in the presence of 12 μM cystatin OmC2 for 1 or 3 h as described above. PBS was added to non-treated cells as a control. The lysates from the treated and non-treated cells were normalized to the same protein content (Bradford assay, Bio-Rad) and incubated with Ni-precharged Sepharose CL-6B (Roche). After 30 min of incubation at 4°C and rigorous shaking (200 rpm), the beads with the bound proteins were rinsed, mixed with SDS-PAGE loading buffer with DTT and boiled. The samples were centrifuged at 16,000 g, and the soluble fractions were subjected to electrophoresis. The separated proteins were stained with Coomassie dye and analyzed using mass spectrometry. Alternatively, the separated proteins were blotted to nitrocellulose membranes and labeled with anti-cathepsin C or anti-cathepsin S antibodies.

Mass Spectrometry of Cell Lysates

Selected comparable bands were cut from the precast SDS-PAGE gels and prepared as described (Sobotič et al., 2015). The extracted peptides were analyzed using an Orbitrap LTQ Velos mass spectrometer (Thermo Scientific, Waltham, MA, USA), coupled to a nanoLC HPLC unit (Proxeon, Thermo Scientific, Waltham, MA, USA). The peptides were loaded on a C_{18} trapping column (Proxeon EASY-Column^{TM}) and separated on a C18 PicoFrit^{TM} AQUASIL analytical column (New Objective, Woburn, MA, USA). The mobile phase A solvent (0.1% formic acid) was used for loading and a 60 min gradient consisting of 5–40% of mobile phase B (100% acetonitrile, 0.1% formic acid) was used for peptide separation. The overall flow rate was 300 nl/min. The MS spectra were acquired in the Orbitrap analyser with a mass range 300–2,000 m/z and a resolution of 30,000. The MS/MS spectra were obtained by CID fragmentation (normalized collision energy at 35) of the nine most intense precursor ions from the full MS scan. Dynamic exclusion was enabled with repeat count of 1 and 60 s exclusion time. The database search and quantification by spectral counting were performed using the MaxQuant proteomics software (version 2.0.18), with the embedded Andromeda search engine (Cox and Mann, 2008; Cox et al., 2011). Searches were performed against the human IPI protein database v.385 (89,952 sequences, 36,291,020 residues), using the trypsin cleavage specificity with a maximum of 2 missed cleavages. Carbamidomethylation of cysteines was set as a static modification, while methionine oxidation and N-terminal acetylation were set as dynamic modifications. A reversed database search was performed and the false discovery rate (FDR) was set at 1% for peptide and protein identifications. Proteins with at least two identified peptides were considered to be positive identifications. Relative quantification was performed by spectral counting as described previously (Old et al., 2005).

RESULTS

Characterization of Cystatin OmC2

Tick cystatin OmC2 was prepared in insect cells as a recombinant protein with oligo His-tag added to its C-terminus (Salát et al., 2010). Recombinant cystatin OmC2 from insect cells, previously checked for endotoxin contamination, was used in the majority of described experiments. Recombinant cystatin OmC2 from insect cells was preferentially applied for two reasons; (1) it was added to the immune cells later on, and (2) it was expressed with His-tag at the C-terminal end of cystatin molecule, i.e., at the part which does not participate in binding to the active site of targeted proteases (lysosomal cathepsins). The isolated protein migrated as a single band at approximately 13 kDa (**Figure 1A**). The inhibitor was 88% active as determined by active site titration with papain (**Figures 1B,C**) whereas the activity of cystatin OmC2 conjugated to the Alexa Fluor 488 fluorophore was 55% (**Figure 1D**). Furthermore, the molecular weights of non-labeled and fluorescently labeled cystatin OmC2 were confirmed by mass spectrometry to verify the fluorophore binding (**Figure 1E**). Three singly charged peaks were observed. The first peak corresponded to the non-labeled cystatin OmC2 with a mass of 13112 Da, which is in agreement with its theoretical Mw of 13115 Da. Two additional species were determined, the first of which corresponded to cystatin OmC2 that was N-terminally conjugated with Alexa Fluor 488 (13629 Da), and the second was a minor species that corresponded to the additional labeling of a lysine side chain (14147 Da). According to the areas of the peaks corresponding to each species, nearly 50% of cystatin OmC2 was N-terminally conjugated and only a minor part was additionally labeled at the lysine side chain (<2%). Additional peaks were observed for doubly charged species at 6557, 6815, and 7073 Da. No other bands or additional peaks that would have indicated the presence of impurities were observed on a silver-stained SDS-PAGE gel (**Figure 1A**) or in the MS spectra (**Figure 1E**). Both

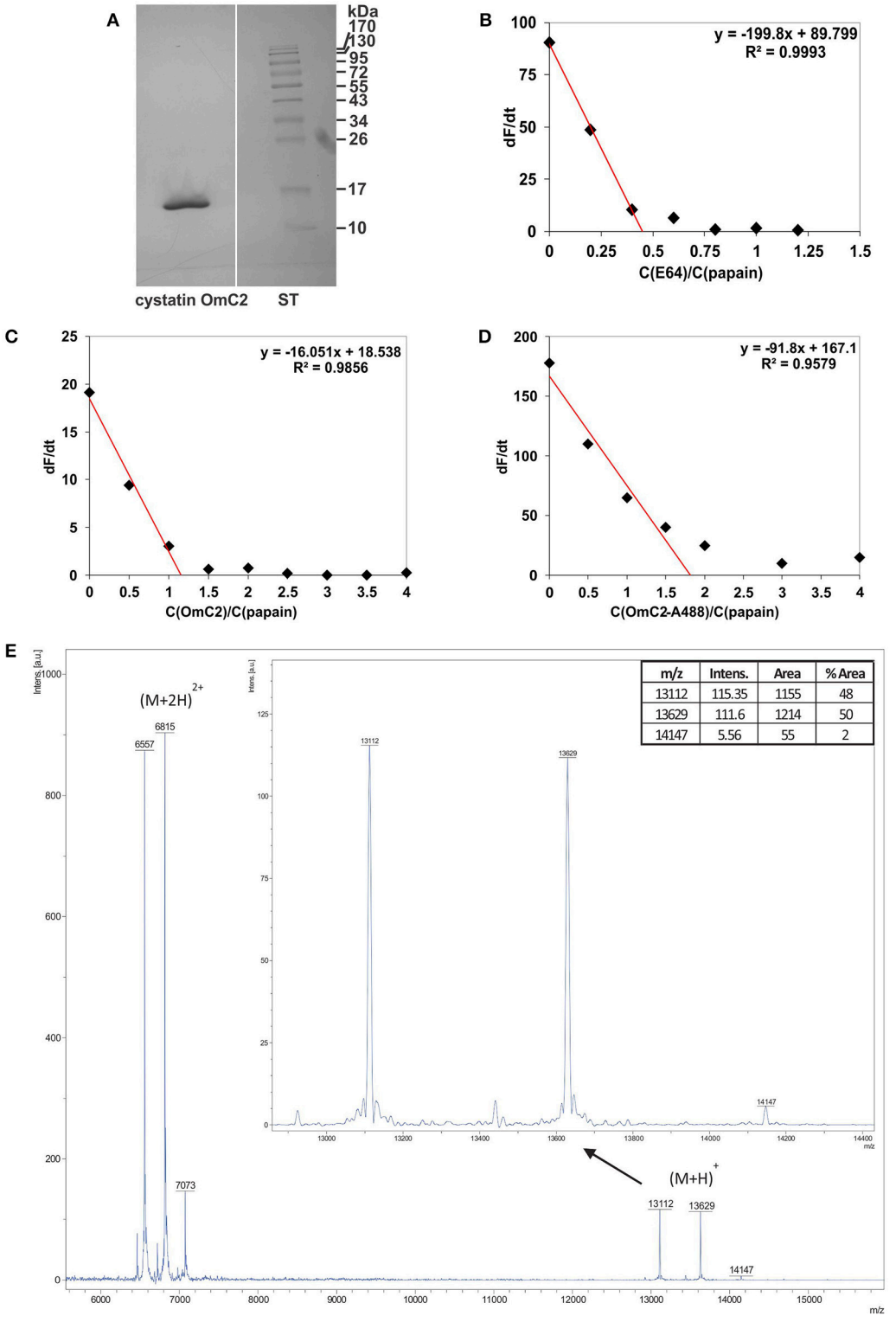


FIGURE 1 | Characterization of cystatin OmC2 by **(A)** SDS-PAGE, **(B–D)** active site titration and **(E)** mass spectrometry. **(A)** The sample was stained with silver, ST—standards. **(B)** Active site titration of papain with inhibitor E64; dF/dt = 0 at a molar ratio of papain/E64 = 0.45. **(C)** Active site titration of papain with cystatin OmC2; dF/dt = 0 at a molar ratio of cystatin OmC2/papain = 1.16. **(D)** Active site titration of papain with cystatin OmC2 conjugated to Alexa Fluor 488; dF/dt = 0 at a molar ratio of cystatin OmC2-A488/papain = 1.82. **(E)** MALDI-TOF spectra of non-labeled cystatin OmC2 and cystatin OmC2 conjugated to Alexa Fluor 488. Singly charged cystatin OmC2 peaks corresponding to different modification levels ($(M+H)^+$): the peak at 13112 Da represents unmodified cystatin OmC2, the peak at 13629 Da represents N-terminally labeled cystatin OmC2, and the peak at 14147 Da represents cystatin OmC2 labeled at its N-terminal and a Lys side chain. The areas of singly charged peaks (enlarged image) were used for relative quantification of labeling efficiency. Doubly charged peaks ($(M+2H)^{2+}$) are also present.

non-labeled and fluorescently labeled cystatin OmC2 were used in the subsequent studies with cells.

Cystatin OmC2 Decreases Surface Expression of MHC II and CD86 in LPS-Stimulated Cells

MUTZ-3 cells (**Figure 2A**) that had been differentiated with IL-4 were used as a model of immature DC. An increase in the surface expression of MHC II (**Figure 2B**) and a high content of endogenous cystatin C in the Golgi apparatus (**Figure 2B**, arrows), which is another characteristic of human immature DC when they are differentiated *in vitro* (Zavašnik-Bergant et al., 2005), were confirmed in the differentiated MUTZ-3 cells (**Figure 2B**). In addition to confocal imaging, an increased immunogold labeling of cystatin C in Golgi cisternae was observed in differentiated MUTZ-3 cells (**Figure 2B**, TEM micrograph, arrows). MHC II was further increased in these cells following a 24-h maturation with LPS (**Figure 2C**). The increases in the expression of MHC II and CD86 that were associated

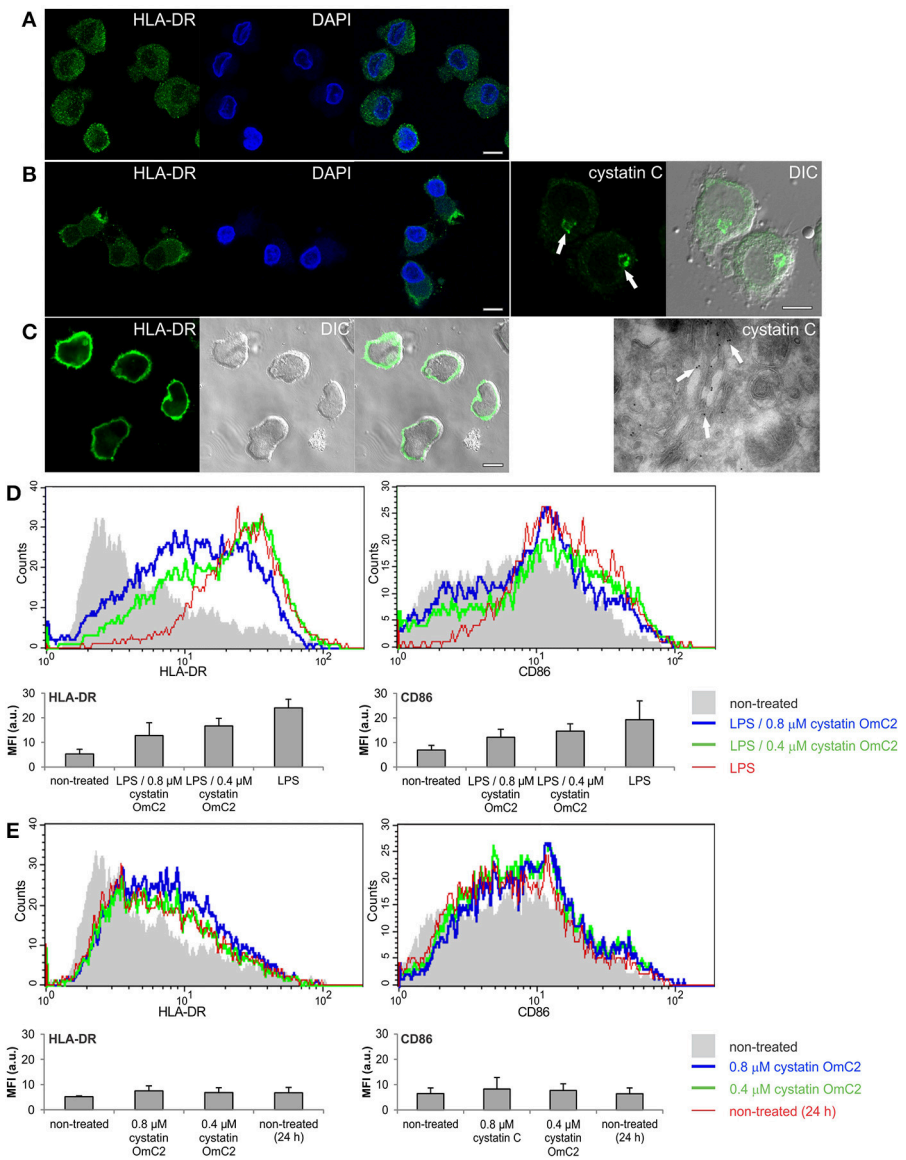


FIGURE 2 | Effect of tick cystatin OmC2 on the expression of MHC II and CD86. The confocal images show MHC II (HLA-DR) in **(A)** non-differentiated MUTZ-3 cells, **(B)** differentiated MUTZ-3 cells, and **(C)** differentiated MUTZ-3 cells stimulated with LPS. The localization of endogenous cystatin C in the differentiated MUTZ-3 cells is shown in **(B)**. Bars: 10 μm. **(D)** LPS-induced maturation of differentiated MUTZ-3 cells in the presence of cystatin OmC2 is compared to non-treated cells (no added cystatin OmC2). **(E)** The histograms show HLA-DR and CD86 in the differentiated MUTZ-3 grown in the presence of cystatin OmC2 (no maturation with LPS). **(D,E)** The shadowed histograms represent the non-treated cells (no added cystatin OmC2 and no maturation with LPS) at the beginning of the experiment. A representative analysis of three independent biological replicates is shown. Mean fluorescence intensities (MFI) of labeled cell populations (geometric means) are shown in bar graphs.

with the maturation induced by LPS (red histograms, bar graphs, **Figure 2D**) were diminished when cystatin OmC2 was added to the culture medium (green and blue histograms, **Figure 2D**). In contrast, cystatin C, which is a human cystatin type 2 homolog that is endogenously expressed (**Figure 2B**) and secreted by human DC (Zavašnik-Bergant et al., 2005), did not notably change the surface expression of CD86 when it was added to the immature DC together with LPS at the same concentrations (0.4 or 0.8 μM) as the tick cystatin OmC2 (Supplementary Figure 2A), or added to the culture medium of the immature DC in the absence of LPS (Supplementary Figure 2B). Furthermore, pre-incubation of the immature DC with cystatin OmC2 in the absence of stimulation with LPS did not noticeably change the surface expression of MHC II or CD86 (**Figure 2E**). If, supposedly, the applied recombinant cystatin OmC2 had been "contaminated" with endotoxins, an increased labeling of surface HLA-DR and CD86 might have been observed also in cells shown in **Figure 2E** (cells not stimulated with LPS).

Cystatin OmC2 Is Internalized via the Endocytic Pathway of Differentiated MUTZ-3 Cells

The Alexa Fluor 488-conjugated cystatin OmC2 was efficiently separated from the unbound Alexa Fluor 488 dye after three steps of purification. After the second dialysis and membrane filtration, the fluorescence of the unreacted dye in the filtrate was low (1125 a.u.) compared to the fluorescence measured in fraction with the labeled cystatin (25900 a.u.) (Supplementary Figure 3A). Compared to the preincubation of cells with the labeled cystatin OmC2 (Supplementary Figure 3C), no pronounced fluorescence was observed when cells were pre-incubated (under the same conditions) with the equivalent volume of the filtrate after the last purification step (Supplementary Figure 3D). The filtrate contained only the remaining unbound dye but not the conjugated protein. We concluded that the conjugated cystatin OmC2 was applicable and it was therefore used for the co-localization study with immunolabeled cathepsin S and cathepsin C.

Differentiated MUTZ-3 cells showed high internalization ability of fluorescently labeled cystatin OmC2 at 37°C (Supplementary Figure 4). Increased fluorescence was observed in Rab7a-positive vesicles after 1 and 3 h (Supplementary Figures 4C,D). On the contrary, the internalization of fluorescently labeled cystatin OmC2 was almost completely abolished in cells that were cultured at 4°C for 1 h and 3 h (Supplementary Figures 4A,B). In addition, a clear distinction between the non-viable cell (being fluorescent due to the loss of its membrane integrity) and other viable cells (without significant intracellular fluorescence) can be seen in Supplementary Figure 4A. The diffused green fluorescence can be observed outside the cells because the fluorescently labeled cystatin OmC2 was not removed from the culture medium before confocal imaging.

The plasma membrane of the cells was stained with CellMask Orange, and the newly formed endocytic vesicles of the cells that were cultured in the presence of cystatin OmC2 were observed under the confocal microscope (**Figures 3A,B**). Following its internalization, cystatin OmC2 was found present in vesicles formed from the labeled plasma membrane (**Figure 3A**). This confirmed that its internalization from the culture medium to the endocytic pathway of the treated cells was successful. After 1 h, cystatin OmC2 was already localized in the Rab7a-positive late endosomes (**Figure 3C**). The uptake of cystatin OmC2 was not homogenous within the population of the treated cells, but the fluorescence associated with cystatin OmC2 clearly increased during the prolonged 3 h incubation (**Figure 3D**). After this time, more than 80% of cells showed an intense perinuclear staining of the internalized cystatin OmC2.

In addition, the predicted binding of internalized fluorescently labeled cystatin OmC2 to target proteins in differentiated MUTZ-3 cells was further indicated by the changes of isoelectric point (pI) of cystatin OmC2 that were observed in the IEF gels under non-denaturating conditions in the range of pH 3–7 (**Figure 3E**). Prior to its internalization, fluorescently labeled cystatin OmC2 was detected at pI 6.3 (**Figure 3E**). In contrast, in the lysates prepared from cells cultured in the presence of cystatin OmC2, additional fluorescent bands at 4.2, 4.5, 4.7, and 6.8 were observed (**Figure 3E**, arrows). The multiple fluorescent bands in the lysate, in addition to the one at pI 6.3, indicated that internalized cystatin OmC2 was bound to various cell constituents. No fluorescent bands were observed in lysates prepared from cells cultured in the absence of the labeled cystatin OmC2.

Cystatin OmC2 Decreases the Activity of Cysteine Proteases in Differentiated MUTZ-3 Cells

The endoprotease activity toward Z-Phe-Arg-AMC (**Figure 3F**) and the exoprotease activity toward H-Gly-Phe-AMC (**Figure 3G**) were both decreased in the lysates from the immature DC that were cultured in the presence of cystatin OmC2 for 1 or 3 h. Compared to the non-treated cells, the endoprotease activity after 3 h was diminished by 18% in the cells treated with 2 μM cystatin OmC2 and by 30% in cells treated with 12 μM cystatin OmC2. The exoprotease activity was diminished more, i.e., by 26% at 2 μM cystatin OmC2 and by 49% at 12 μM cystatin OmC2 when the inhibitor was added to the culture medium of differentiated MUTZ-3 cells. However, even at the highest concentration of cystatin OmC2 applied (15 μM), the endoprotease and exoprotease activities were not completely blocked after 3 h; the first was diminished by 50% and the second by 65%. The addition of cystatin OmC2 did not noticeably change the viability of the treated cells after the 3-h incubation compared to the non-treated cells (Supplementary Table 1). Significant differences in protease activity between the non-treated cells (control) and the cells treated with cystatin OmC2 at different experimental conditions are denoted in **Figures 3F,G**.

Cystatin OmC2 Forms Complexes with Recombinant Cathepsins S and C

The possible formation of complexes of cystatin OmC2 with the selected lysosomal endoprotease cathepsin S, and of cystatin OmC2 with the exoprotease cathepsin C, was verified using IEF

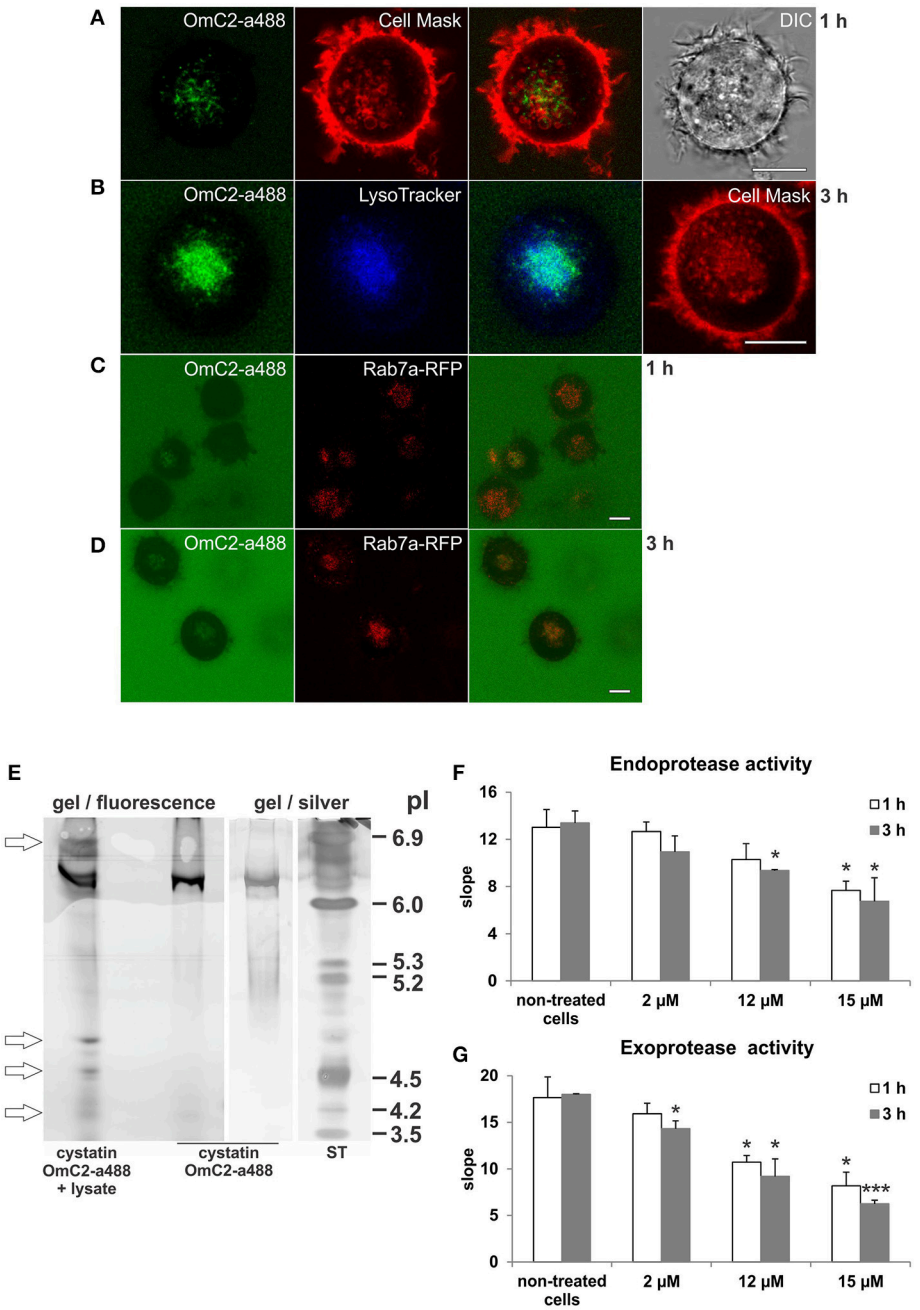


FIGURE 3 | Localization of internalized cystatin OmC2 and the activity of cysteine proteases in treated differentiated MUTZ-3 cells. Cystatin OmC2 labeled with Alexa Fluor 488 **(A–D)**, LysoTracker Blue DND-22 **(B)**, CellMask Orange plasma membrane stain **(A,B)**, and Rab7a-RFP **(C,D)** are shown in viable cells. Bars: 10 μm. **(E)** IEF (pH 3–7) of the proteins from cells that were cultured in the presence of labeled cystatin OmC2 for 3 h. The pI-values of the additional fluorescent bands in the lysate are denoted (arrows) and compared to the pI of the fluorescently labeled cystatin OmC2, which was also stained with silver, at pI 6.3. ST—standards. **(F,G)** The endoprotease (Z-Phe-Arg-AMC) and exoprotease activities (H-Gly-Phe-AMC) in the lysates from cells that were cultured with non-labeled cystatin OmC2 (2, 12 or 15 μM) for 1 or 3 h. The samples were measured in triplicate, and the average values ± *SD* are shown. Cell treated with cystatin OmC2 were compared to non-treated cells after 1 h or after 3 h (*t*-test, $^{*}P < 0.05$, $^{***}P < 0.001$).

(pH 3–7) under native conditions. The observed changes in the pI's of cathepsin S and of cathepsin C, both pre-incubated with cystatin OmC2, were compared to those of the non-treated cathepsins (**Figure 4**). The predicted pI for cystatin OmC2 (Salát et al., 2010) was confirmed to be 6.3 (**Figures 4A,B**). Cystatin OmC2 is a secretory type 2 cystatin. We presume that another band around pI 6.0 (**Figure 4A**, lane 7), in addition to the band at pI 6.3, represents the different glycosylation of recombinant

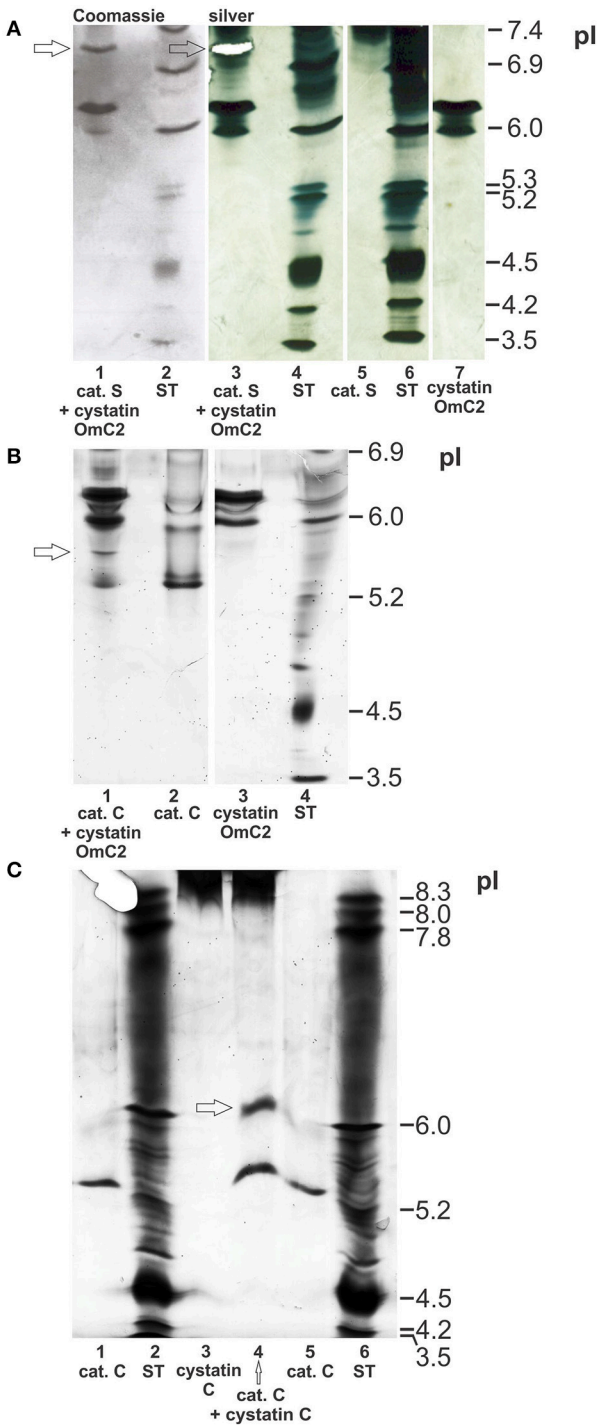


FIGURE 4 | Formation of complexes of cystatin OmC2 with cathepsin S and of cystatin OmC2 with cathepsin C. IEF was performed at pH 3–7 **(A,B)** and pH 3–10 **(C)** under native conditions. The cathepsins were pre-incubated with cystatin OmC2 **(A,B)** or with cystatin C **(C)** prior to IEF. The samples (all recombinant proteins) are: cystatin OmC2 **(A,B)**, cathepsin S **(A)**, cathepsin C **(B,C)** and cystatin C **(C)**. The cystatin OmC2/cathepsin S complex is indicated at pI 7.1, the cystatin OmC2/cathepsin C complex at pI 5.6, and the cystatin C/cathepsin C complex at pI 6.2. The proteins were stained with silver **(A–C)** and Coomassie dye **(A)**. **(A)** The excised bands, analyzed by peptide mass fingerprint (A, lane 3 and B, lane 1) are indicated (white mask, arrows). ST, standards.

cystatin OmC2 molecules expressed in insect cells. According to the cystatin OmC2 sequence, O-glycosylation at four Ser and Thr residues, respectively, has been predicted using NetOGlyc 4.0 software. A pI above 8.0 was determined for human cystatin C, which is an endogenous type 2 cystatin inhibitor of cysteine proteases (**Figure 4C**).

The bands representing cathepsin S (without cystatin OmC2) were observed at pI 7.4 or higher (**Figure 4A**, Supplementary Figure 1). Multiple bands were observed due to the different glycosylation states of the recombinant cathepsin S. The recombinant cathepsin C was observed at pI 5.3 (stronger band) and pI 6.0 (**Figures 4B,C**). Pre-incubation of the cysteine proteases with tick cystatin OmC2 resulted in the appearance of additional bands: an additional band at pI 7.1 was observed in the case of cathepsin S (**Figure 4A**, arrow) and one at pI 5.6 in the case of cathepsin C (**Figure 4B**, arrow).

A band representing a complex between cathepsin S and the tick cystatin OmC2 that was expressed in *E. coli* was also observed at pI 7.1 (Supplementary Figure 1). In addition, a new band indicating the formation of a cathepsin C/human cystatin C complex was observed at pI 6.2 (**Figure 4C**). Cystatin C was observed as a thick diffused band above pI 8.3 (**Figure 4C**, lanes 3 and 4). Applied cystatin C is a basic protein with a very high pI, so it didn't travel far in an electric field toward the other electrode as did travel other proteins with lower pI-values. But, regardless of the high pI of cystatin C, the distinction between the pI-value of cystatin C bound to cathepsin C (at pH 6.2) and the pI-value of cathepsin C alone (at pH 5.3) can be clearly drawn (**Figure 4C**, lane 4).

Credible protease/cystatin complexes of both cathepsin S and cathepsin C with cystatin OmC2 (**Figures 4A,B**) were identified in the excised bands by mass spectrometry (Supplementary Table 2). The identification of peptides that corresponded to the proteins under study in the single band excised from the IEF gels further confirmed the formation of complexes between the two studied proteins. In the case of the complex of cathepsin S with cystatin OmC2, 5 peptides from cathepsin S (15% sequence coverage) and 2 peptides from cystatin OmC2 (28% sequence coverage) were identified. In the case of the complex of cathepsin C with cystatin OmC2, 2 peptides from cathepsin C (4% sequence coverage) and 7 peptides from cystatin OmC2 (64% sequence coverage) were identified.

Cystatin OmC2 Colocalizes with Cathepsins S and C in Differentiated MUTZ-3 Cells

The formation of complexes of cystatin OmC2 with recombinant cathepsin S, and of cystatin OmC2 with recombinant cathepsin C (**Figure 4**), indicated that similar complexes could form between internalized tick cystatin and its target proteases in immune cells if the cystatin OmC2 colocalized with the proteases inside the same cell compartments (late endosomes and lysosomes). We further hypothesized that those complexes would be stable enough to be isolated from the treated cells. Confocal micrographs confirmed that the fluorescently labeled cystatin OmC2 accessed the acidic vesicles (lysosomes) that were

labeled with Lysotracker (**Figure 3B**) and have a high content of lysosomal cysteine proteases (**Figure 5A**). Among these vesicles, the signals for cathepsin C and cathepsin S were found to colocalize with the internalized cystatin OmC2 (**Figure 5C**). The vesicular staining of the internalized non-labeled cystatin OmC2 after 1 and 3 h was confirmed using an anti-His tag antibody (**Figure 5B**). In addition, comparable vesicular patterns of internalized fluorescently labeled cystatin OmC2 (green fluorescence) and cystatin OmC2 labeled with an anti-His tag antibody (red fluorescence) were confirmed in the same cells after a 3 h-incubation (Supplementary Figure 5). Concerning the vesicles with red fluorescence only (Supplementary Figure 5), they represent the internalized cystatin OmC2, not labeled with Alexa Fluor 488 prior to the experiment.

Cystatin OmC2 Binds to Cysteine Cathepsins in Differentiated MUTZ-3 Cells

The cystatin OmC2 that was internalized after 1 and 3 h did not change the processing of cathepsin S (24 kDa) or cathepsin C (21 kDa) in the treated cells (lanes 2 and 4) compared to the cells grown in the culture medium without cystatin OmC2 (controls in lanes 1 and 3; **Figures 6A,B**).

Affinity binding of the oligo His-tag of the recombinant cystatin OmC2 enabled purification of the targeted cellular proteins that were colocalized and successfully associated with the internalized cystatin. Hence, lysosomal proteases bound to the internalized cystatin OmC2 were isolated from the lysates of the differentiated MUTZ-3 cells. As shown using Western blotting, both, cathepsin S (**Figure 6A**) and cathepsin C (**Figure 6B**) were found to be enriched in the pull-down fractions from the treated cells (lanes 2 and 4) compared to the cells grown in the absence of cystatin OmC2 (controls in lanes 1 and 3).

Mass spectrometry was applied to further determine whether cathepsins S and C were among proteins targeted by cystatin OmC2. Several cysteine cathepsins were identified in the pull-down fractions from cells after 1 or 3 h (**Figure 6C**, Supplementary Table 3). Quantification by spectral counting showed that cathepsins B, C, H, and S were enriched in samples from the cells that were cultured in the presence of cystatin OmC2. All four cathepsins showed greater than five-fold increases in the spectral counts compared to the control cells that were not treated with cystatin OmC2, which is significant according to the criteria generally accepted for spectral counting (Old et al., 2005). Further, cathepsins B, H, and S were completely absent from samples of the non-treated cells, which additionally confirmed the relevance of the cystatin OmC2/cathepsin interaction for the enrichment. The same cathepsins (B, C, H, and S) were identified after 1 h and after 3 h of incubation of cells with cystatin OmC2. In contrast, following pre-treatment of cells with cystatin OmC2, cathepsins L and X were not significantly increased (Supplementary Table 3). Cystatin OmC2 was also highly enriched in the samples from the treated cells (**Figure 6C**). A small amount of cystatin OmC2 was identified in a control sample (**Figure 6C**) but was considered to be the result of a carry-over from the previous inhibitor-treated sample.

DISCUSSION

In haematophagous ectoparasites such as ticks, salivary modulators of the host DC have presumably evolved to suppress the host immune response to facilitate blood feeding (reviewed in Kovár, 2004; Schwarz et al., 2012; Kazimírová and Štibrániová, 2013). For those species that are vectors of pathogens, such molecules could also create a permissive environment for pathogen transmission (Preston et al., 2013). DC in the host skin participate in the development of the immune response against various species of bacterial pathogens such as *Borrelia* that are transmitted during the bite of a tick (Slámová et al., 2011; Lieskovská et al., 2015a,b). However, tick saliva inhibits the differentiation and maturation of DC, and subsequently modulates their immune stimulatory functions (Cavassani et al., 2005). Among the proteins that comprise the tick sialome, small secretory protease inhibitors similar to human endogenous cystatins have also been proposed to have a role in modulating the production of cytokines secreted from immune cells due to tick feeding (Sá-Nunes et al., 2009; Schwarz et al., 2012).

The salivary cystatin OmC2 from *Ornithodoros moubata* was evaluated as a model tick type 2 cystatin. This protein is homologous to human cystatin C, which is a member of family I25 of inhibitors of papain-like cysteine proteases [MEROPS database (Rawlings et al., 2016)]. Endogenous cystatin C is increased in monocyte-derived immature DC, but its high content in Golgi, the absence of significant colocalization with lysosomal cathepsins S, L, and H in the late endosomes and lysosomes, as well as an intensive secretion of cystatin C that is dependent on differentiation and maturation status of DC all indicate that the endogenous cystatin C does not directly modulate the activity of the endogenous cysteine proteases in DC (Zavašnik-Bergant et al., 2005; Zavašnik-Bergant, 2008). This conclusion contrasts with the report of Pierre and Mellman (1998) and the evidence that an exogenous cystatin homolog from *Brugia malayi*, Bm-CPI-2, inhibits the MHC II-restricted antigen processing in treated cells (Manoury et al., 2001).

On the basis of its similarity to human cystatin C, salivary tick cystatin OmC2 could be a potent immunomodulatory molecule that could mimic and add to the inhibitory function of its vertebrate homolog during the tick feeding and the transmission of tick-borne pathogens. In the present study, we have investigated the effect of cystatin OmC2 when it was internalized to the endocytic pathway of human immature DC, i.e., to late endosomes and lysosomes. Specifically, we asked whether the exogenous tick cystatin persisted in the acidic vesicles in contrast to endogenous cystatin C, and whether it changed the cell proteolytic potential and possibly also other DC characteristics. Cystatin OmC2 was tested for its abilities to inhibit the lysosomal cysteine proteases in DC prior to their maturation and to alter the surface expression of CD86 during the subsequent DC maturation, which could therefore indicate a possible function independent of its inhibitory activity.

The human acute myeloid leukemia cell line MUTZ-3 has been used as a model of dermal DC in human skin. Immature DC were generated from MUTZ-3 cells, previously differentiated *in vitro* with IL-4 (Song et al., 2015). Immature DC constantly

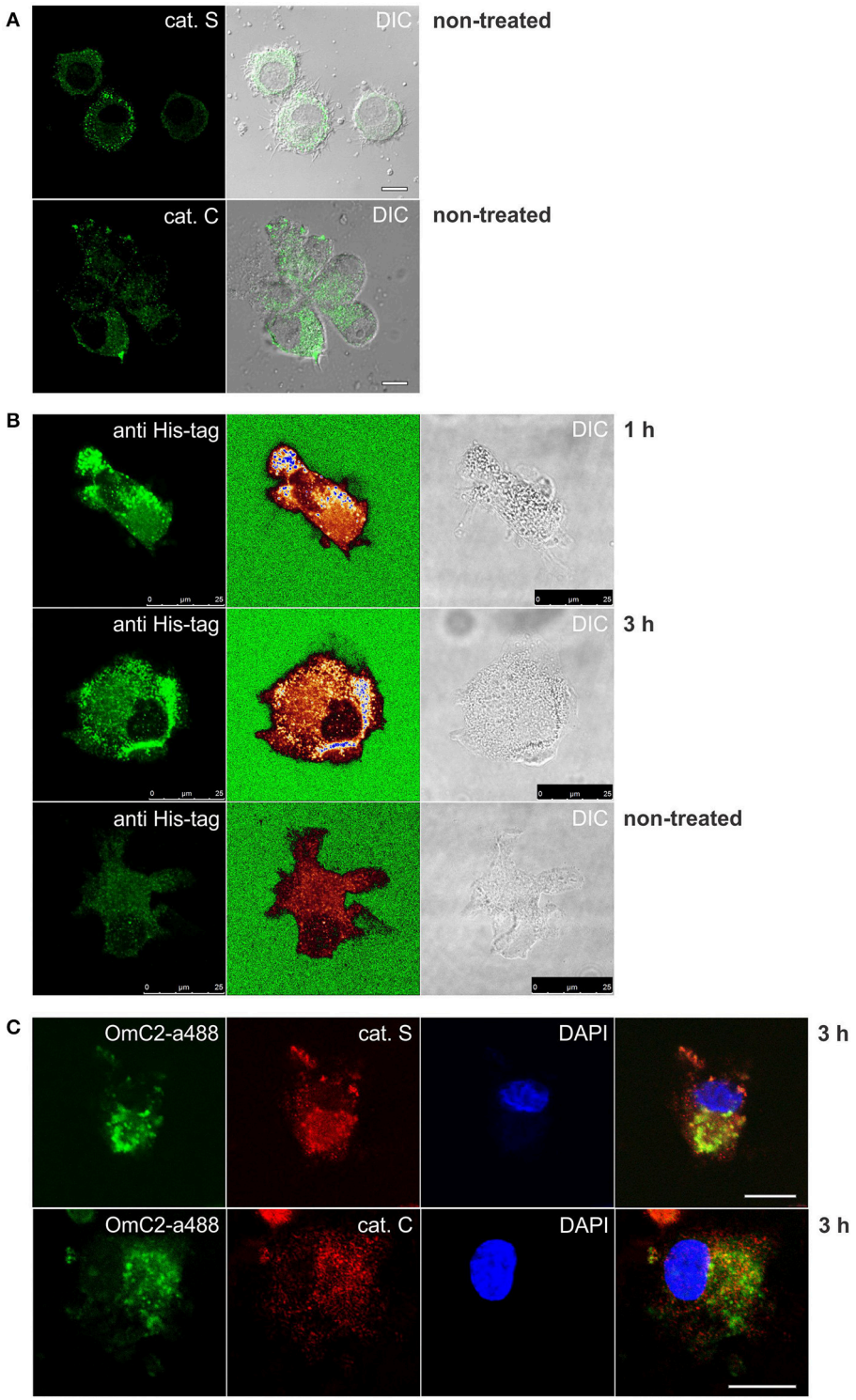
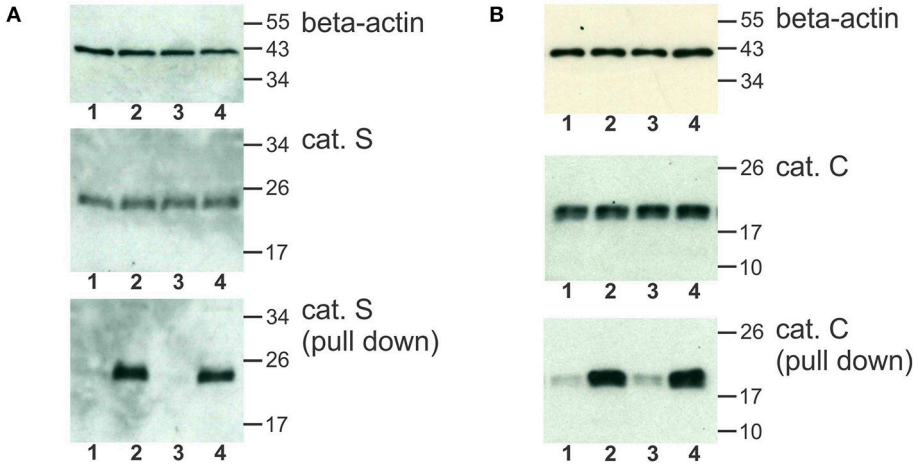


FIGURE 5 | Immunocytochemistry of differentiated MUTZ-3 cells that were cultured in the presence of cystatin OmC2 for 1 and 3 h. The confocal images show **(A)** immunolabeled cathepsins in non-treated cells, and **(C)** their colocalization with Alexa Fluor 488-labeled cystatin OmC2 in treated cells. **(B)** Cells with internalized cystatin OmC2 (without fluorochrome) were labeled with an anti-His tag antibody. The intensity of the fluorescence signal is represented with a rainbow color palette (green–the lowest intensity, blue–the highest intensity). Non-treated cells were used as a control. Bars: 10 μm **(A,C)** and 25 μm **(B)**.



C

	pull down			
	1	2	3	4
	Non-treated cells (1 h)	Treated cells (1 h)	Non-treated cells (3 h)	Treated cells (3 h)

UniProt No.	Protein name	Gene name	No. of peptides	No. of recorded MS/MS spectra			
P25774	cathepsin S	CTSS	8	1	14	0	8
P53634	cathepsin C	CTSC	16	47	252	26	113
P07858	cathepsin B	CTSB	11	0	32	0	25
P09668	cathepsin H	CTSH	4	0	10	0	11
Q6QD55	cystatin OmC2	omc2	14	20*	630	3*	100

FIGURE 6 | Pull-down of internalized cystatin OmC2 and endogenous cathepsins S and C from differentiated MUTZ-3 cells. **(A,B)** Western blotting of cathepsin S and cathepsin C in lysates and pull-down fractions from non-treated (lanes 1 and 3) and treated cells (lane 2 and 4). A total of 40 μg of total protein from lysates was added per well and beta-actin was used as a loading control. For the pull-down fractions, equal volumes (30 μl) per well were used. **(C)** The cathepsins in the pull-down fractions were identified using LC-MS/MS analysis, and the numbers of recorded MS/MS spectra are indicated. (*) A carry-over from an inhibitor-treated sample, analyzed as the preceding sample.

sample the surrounding environment and endocytosis is a functional feature of these cells, which decreases with their maturation. As published by Larsson et al. (2006), the uptake of FITC-dextrane by mature MUTZ-3-derived DC, matured with either LPS or cocktail of proinflammatory cytokines, was reduced compared to immature MUTZ-3-derived DC. We confirmed that following the internalization of cystatin OmC2 by DC, it was extensively translocated to proteolytically active compartments, i.e., to the Rab7a-positive late endosomes (Guerra and Bucci, 2016), in which antigen processing and binding to MHC II take place (Blum et al., 2013), and to lysosomes, which are highly enriched with the active lysosomal cathepsins that are crucial for proteolytic degradation of the lysosome cargo. Further, the regulation of lysosomal degradation pathways is essential to maintain cellular homeostasis (Huber and Teis, 2016).

Cystatin OmC2 inhibits recombinant lysosomal cathepsins with K_i-values in the nanomolar range, whereas the legumain/asparaginyl endopeptidase (AEP) from family C13 (Rawlings et al., 2016) is not inhibited (Grunclová et al., 2006). We demonstrated that both non-labeled and fluorescently labeled cystatin OmC2 were successfully internalized by the immature DC. The reduced but not completely abolished endoprotease and exoprotease activities of the cysteine proteases, and the confirmed colocalization demonstrated that the targeted proteases (including the lysosomal cathepsins S and C) were accessed by cystatin OmC2. The actual concentration of internalized cystatin OmC2 in particular subcellular compartments could not be measured. However, the inhibition of cysteine protease activity in DC was dependent on the concentration of applied cystatin OmC2 and was consistent with the increase of fluorescence signal from internalized inhibitor

after the prolonged 3-h incubation. We did not observe that the added cystatin OmC2 vastly changed the viability of treated cells. To make DC incapable of an appropriate response to the tick antigens, it seems likely that the activity of targeted cysteine proteases in DC would be obstructed but not completely blocked by the incoming exogenous tick inhibitor.

We don't have quantitative data on the concentration of cystatin OmC2 in the tick saliva. In the paper by Grunclová et al. (2006) rough estimation of the amount of cystatin OmC2 in the salivary gland extract was reported (it represented 0.2−0.5% of the total protein content). In the paper by Salát et al. (2010) saliva collected from *O. moubata* adult females was subjected to proteomic analysis to directly confirm the presence of cystatin OmC2. The applied LC-MS/MS strategy was based on enzymatic digestion of a complex protein mixture and MS/MS peptide sequencing. The analysis provided 53% peptide coverage of the cystatin OmC2 sequence and confirmed that OmC2 is secreted in the saliva of *O. moubata*. Quantitative measurement of cystatin OmC2 concentration in secreted saliva is also problematic due to the small amount of obtained material from one adult tick (about 1 μl). Likely, the concentration used in *in vitro* studies is higher than the concentration of cystatin OmC2 in saliva. Then again, the saliva is injected directly into the tissue and the local concentration of its components may be quite high around the cells in that tissue. Though the tick may secrete only small amounts of cystatin OmC2, its inhibitory potency may be adequate to achieve inhibition of the targeted proteases.

Inhibitory activity of cystatin OmC2 has been determined in enzymatic assays with the recombinant mammalian cathepsins (Salát et al., 2010) at their optimal acidic pH, buffer composition and the absence of other proteases. Our first hypothesis was that cystatin OmC2 would preferentially bind to lysosomal cathepsins with endoprotease activity if successfully internalized to MUTZ-3 cells (and therefore affect their proteolytic activity). With respect to the published data, reported IC50 values (determined in assays with recombinant cathepsins) were in favor of that hypothesis. IC50 values of cystatin OmC2 were higher for cathepsin B (8.8 nM), cathepsin H (1.2 nM) or cathepsin C (1.1 nM), all of them exoproteases, whereas IC50 values of cystatin OmC2 were around 0.15 nM for reported endoproteases (papain, cathepsin S, and cathepsin L) (Salát et al., 2010), Thus, we anticipated more vigorous interaction between endoproteases and cystatin OmC2 also in differentiated MUTZ-3 cells.

The formation of complexes of the cystatin OmC2 with the recombinant cathepsin S and of cystatin OmC2 with the recombinant cathepsin C was verified by using IEF under native conditions, thus mimicking the conditions inside the DC endocytic pathway. Further, following the pre-treatment of the immature DC with cystatin OmC2, cathepsins C, B, and H were isolated from cell lysates together with internalized cystatin OmC2. This was also observed for cathepsin S but not for cathepsins L or X, even though cathepsin X is similar to cathepsin B and is also present in human DC (Obermajer et al., 2008). Cystatin OmC2 is a potent inhibitor of recombinant human cathepsins S and L with similar IC_{50} values (Salát et al., 2010). Conversely, a significant increase (over fivefold and more) in spectral counts compared to the control (non-treated cells) was confirmed only for cathepsin S but not cathepsin L (mass spectrometry analysis, **Figure 6C**), although both cathepsins are active in DC (Lennon-Duménil et al., 2002) and are present in differentiated MUTZ-3 cells (**Figure 5**, Supplementary Figure 1C). However, the vesicular staining of immunolabeled cathepsin L (Supplementary Figure 1C) was relatively weak compared to that for cathepsin S or to cathepsin C. The absence of cathepsin L among the proteins isolated in association with cystatin OmC2 indicates that the formation of this complex, although confirmed between cathepsin L and cystatin OmC2 as recombinant proteins (Supplementary Figure 1), might not occur in the treated DC.

Another possibility would be that cathepsin L/cystatin OmC2 complex, while transiently formed inside cells, was not stable enough to be isolated by affinity chromatography. By analogy, the p41 fragment from the p41 invariant chain, which is a chaperone to MHC II and an endogenous thyropin inhibitor of the cysteine proteases in APC, was shown to act as a chaperone of cathepsin L (Lennon-Duménil et al., 2001). By transiently binding to cathepsin L, this thyropin inhibitor helps to maintain a pool of the mature enzyme in the MHC II-loading compartments in mouse bone-marrow derived APC (Lennon-Duménil et al., 2001).

The inhibition of the endoproteases cathepsins S and L by cystatin OmC2 is comparable to that of sialostatin L, another salivary type 2 cystatin from the hard tick *Ixodes scapularis* (Salát et al., 2010). However, the inhibition of the exoprotease activity of cathepsins B, C, H by cystatin OmC2 is much more effective than that of sialostatin L (Salát et al., 2010); cystatin OmC2 inhibits cathepsin C with $K_i = 0.19$ nM (Grunclová et al., 2006). Our results demonstrated that substantial amounts of the analyzed cathepsins C, B, and H were present in the pull-down fractions from treated cells, which indicated that all of them, in addition to cathepsin S, bind to the internalized cystatin OmC2 (**Figure 6C**).

To summarize, we have shown that all three exoproteases (cathepsins C, B, and H) were increased in pull-down fractions from the cells with internalized cystatin OmC2, whereas only cathepsin S (an endoprotease) was increased when compared to non-treated cells. Neither cathepsin L nor other endoproteases were increased. Among the significantly increased proteases in pull-down fractions, we have focused on the two of them; on cathepsin S due to its known involvement in antigen processing and presentation in DC. Second, we have focused on cathepsin C, a dipeptidyl peptidase, often included in proteolytic cascades, but rarely studied in DC (Ishri et al., 2004; Hamilton et al., 2008).

Cysteine cathepsins are optimally active at slightly acidic pH and are mostly unstable at neutral pH. When cathepsins are outside the lysosomes or extracellularly they can be relatively rapidly and irreversibly inactivated. One exception is cathepsin S, which is still stable at neutral or slightly alkaline pH, thus retaining most of its activity (reviewed in Turk et al., 2012). In the acidic lysosomal milieu, cathepsin C is primarily an amino dipeptidase, cleaving two-residue units from the N-terminus of a polypeptide chain. Cathepsin C is also stable at higher pH and can act as a transferase and catalyze the reverse reaction (reviewed in Turk et al., 2001). The common feature of both cathepsins, proposed here as relevant target proteases of internalized tick cystatin OmC2, is that they are also stable and active at higher pH

compared to other lysosomal cathepsins. This may indicate that internalized cystatin OmC2 preferentially bound to cathepsins S and C in vesicles which were not highly acidic but they contained high amount of these two proteases in their active forms.

Cathepsin S is a key protease involved in the processing of invariant chain, a MHC II-associated chaperone, bound to the peptide binding groove instead of antigenic peptides. When cathepsin S is inhibited, invariant chain could not be efficiently processed to CLIP, removed from MHC II and displaced by degraded antigen. By controlling the pace of Ii degradation, cathepsin S is able to influence MHC class II-mediated presentation of antigens to $CD4^+$ T cells (reviewed in Blum et al., 2013; Roche and Furuta, 2015). The effect of cystatin OmC2 on DC-mediated proliferation of $CD4^+$ T cells have already been published (Salát et al., 2010), at least in a mouse system, with the same tick inhibitor as we used here. Enriched mouse $CD11c^+$ cells were pre-treated with cystatin OmC2 for 4 h and then co-cultured with $CD4^+$ T cells from transgenic OT-II mice together with OVA. As shown by Salát and coworkers, cystatin OmC2 reduced DC-mediated proliferation of $CD4^+$ T lymphocytes from transgenic mice that specifically recognize ovalbumin peptide 323–339.

On the other hand, we did not observe that internalized cystatin OmC2 bound to AEP, another lysosomal cysteine protease, although AEP is present in immature DC in which it participates in the MHC II-associated antigen processing and presentation (Sepulveda et al., 2009). Tick cystatins lack the AEP binding site, the SND motif, in their protein sequence. This sequence is present, for example, in human cystatin C (Alvarez-Fernandez et al., 1999) and in the cystatin Bm-CPI-2 from the nematode parasite *B. malayi* (Murray et al., 2005). We propose that the internalized tick cystatin OmC2, when present in addition to endogenous cystatin C, changes the proteolytic degradation and effective processing of antigens. Among others, cathepsin S is inhibited by cystatin OmC2 in late endosomes, i.e., where antigen processing and presentation is taking place. A crucial step in invariant chain processing, the formation of the αβ-CLIP complex (Driessen et al., 1999; Lindner, 2017), may be interrupted.

As for the inhibition of cathepsin C with cystatin OmC2 we hypothesize that the inhibition of dipeptidyl peptidase activity (cathepsin C sequentially removes dipeptides from the N-termini of protein and peptide substrates) could affect the processing of several other proteins (among them also serine proteases). Interestingly, cathepsin C is highly expressed in monocyte-derived DC (Hashimoto et al., 1999; Le Naour et al., 2001), but its specific function in these professional APC has not yet been established (Hamilton et al., 2008). We have confirmed a high content of cathepsin C in MUTZ 3-derived immature DC, although there were no differences in the fluorescence signal of labeled cathepsin C between immature DC and the cells activated with LPS (Supplementary Figure 1C). However, it has previously been reported that the activity of cathepsin C is increased in immature human DC but decreases rapidly as the DC mature (Ishri et al., 2004). Further, a reduced activity of cathepsin C was associated with the stimulation of the maturation of monocyte-derived DC by trimeric CD40L, TNF-α or *Streptococcus pyogenes* (Ishri et al., 2004). Conceivably, the cathepsin C expression is also related (Hamilton et al., 2008) to the demonstration that activated DC have a tumoricidal activity and express perforin and granzyme B (Stary et al., 2007).

In DC that are affected by the components of tick saliva, the diminished exoprotease activity of cathepsin C by cystatin OmC2 would lead not only to the inhibition of one particular cathepsin but would affect all other proteases in proteolytic cascades dependent on the proteolytic cleavage and activation by cathepsin C. Novel, non-cytotoxic role of a serine protease granzyme B, associated to the promotion of antigen uptake and the suppression of premature T cell activation, has been proposed in plasmacytoid DC (Jahrsdörfer et al., 2014; Fabricius et al., 2016).

Neutrophils, similarly as plasmacytoid DC (Gregorio et al., 2010) and NK cells (Carrega and Ferlazzo, 2012; Sojka et al., 2014), are not frequently observed in normal skin, but they are recruited in high numbers after skin injury (Wilgus et al., 2013). We further speculate that the internalization of cystatin OmC2 could take place in neutrophils and in NK cells when they are present in the dermis. A putative interaction of cystatin OmC2 with cathepsin C as a processing enzyme of serine proteases in the secretory granules of neutrophils or NK cells may affect their killing capacity. Besides, the secretion of active cathepsin C from neutrophils has been reported recently by Hamon et al. (2016). Hypothetically, salivary tick cystatin OmC2 could bind to the secreted active cathepsin C outside the immune cells, if both were present in the same tissue.

Cystatin OmC2 diminishes the antigen-specific proliferation of mouse CD4+ T cells (Salát et al., 2010). As reported, cystatin OmC2 reduced the production of TNF-α by 20% and IL-12 by 25% following the LPS-induced maturation of pretreated DC (Salát et al., 2010). Similarly, in mouse DC activated by LPS *in vitro*, sialostatin L interfered with the TLR-mediated release of IL-12 and TNF-α, but not IL-10, and impaired the antigen-specific $CD4^+$ T cell proliferation (Sá-Nunes et al., 2009). Studies of the bone marrow-derived DC from cathepsin $S^{-/-}$ mice further confirmed that the immunomodulatory actions of sialostatin L are mediated by the inhibition of cathepsin S (Sá-Nunes et al., 2009). In addition to the previously reported reduction in the secretion of IL-12 (Salát et al., 2010), we report here that the surface expression of the costimulatory molecule CD86 was diminished when immature DC were stimulated with LPS in the presence of cystatin OmC2. We can't explain how the internalized cystatin OmC2, acting on cysteine cathepsins inside the endocytic pathway of treated cells, might be directly connected to the changes in the surface expression of HLA-DR and CD86 molecules, observed in LPS-stimulated cells, but not in non-stimulated cells (in the absence of LPS). We assume that the maturation of cells was in a way changed by the presence of cystatin OmC2. Currently, the mechanism behind the observed effect of cystatin OmC2 cannot be explained, and whether the reduction of the surface expression of CD86 and the secretion of IL-12 is linked to the reduced proteolytic activity of one or more cysteine proteases remains to be elucidated. Immature but not mature DC secrete large amount of cystatin C (Zavašnik-Bergant et al., 2005). It is tempting to speculate that endogenous

cystatin C could affect the DC from which it was secreted, thus causing a feedback effect on their maturation and the expression of costimulatory molecules such as CD86. However, our existing data do not support the putative autocrine effect of cystatin C.

Another type 2 cystatin, which is present in chicken egg white, induced TLR/MyD88 signaling when combined with IFN-γ. This resulted in the activation of the IκB kinase complex (IKK) and NF-κB pathway in macrophages that were infected with parasitic *Leishmania donovani* promastigotes (Kar et al., 2009, 2011). In our DC model, fluorescently labeled cystatin OmC2 was not retained on the cell surface, which would have indicated its possible binding as a ligand to one of the proteins on plasma membrane. Instead, it was efficiently internalized to late endosomes and lysosomes. At this point, it is only possible to speculate as to whether cystatin OmC2 exhibits intrinsic structural features that would enable it to be engaged in membrane receptor binding.

In summary, exogenous cystatin OmC2 entered the late endocytic compartments and acted effectively on cysteine proteases inside the MUTZ-3-derived immature human DC. Among these proteases, two cathepsins, S and C, which are involved in antigen processing and proteolytic cascades, were accessed. In addition, the expression of CD86, a costimulatory molecule that is associated with the maturation of DC, was diminished. We propose that the diminution of the activity of two key cysteine proteases contributes to the overall ability of tick saliva to affect the immune response of tick's host.

AUTHOR CONTRIBUTIONS

Conceived the study, designed the experiments and wrote the paper: TZ. Performed the experiments: TZ, RV, and AS. Analyzed the data: TZ, RV, and MF. Contributed reagents/materials/analysis tools: TZ, RV, MF, JS, LG, and PK. Contributed insightful suggestions on the study: PK and BT. Supervised the project: TZ and BT.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00288/full#supplementary-material

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REVIEW
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Protease Inhibitors in Tick Saliva: The Role of Serpins and Cystatins in Tick-host-Pathogen Interaction

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The publication of the first tick sialome (salivary gland transcriptome) heralded a new era of research of tick protease inhibitors, which represent important constituents of the proteins secreted via tick saliva into the host. Three major groups of protease inhibitors are secreted into saliva: Kunitz inhibitors, serpins, and cystatins. Kunitz inhibitors are anti-hemostatic agents and tens of proteins with one or more Kunitz domains are known to block host coagulation and/or platelet aggregation. Serpins and cystatins are also anti-hemostatic effectors, but intriguingly, from the translational perspective, also act as pluripotent modulators of the host immune system. Here we focus especially on this latter aspect of protease inhibition by ticks and describe the current knowledge and data on secreted salivary serpins and cystatins and their role in tick-host-pathogen interaction triad. We also discuss the potential therapeutic use of tick protease inhibitors.

Keywords: tick-host interaction, immunomodulation, protease inhibitors, serpins, cystatins

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SERPINS AND CYSTATINS AS HOMEOSTATIC REGULATORS

Proteases (also proteinases or peptidases) are ubiquitous enzymes that cleave proteins to smaller peptides and amino acids. Proteases participate in a range of physiological processes including extracellular digestion, protein degradation, and tissue development (Rawlings and Salvesen, 2013). Relevant to this review, however, is the fact that many proteases, in particular highly substrate-specific endopeptidases, mediate defense and homeostatic processes in both vertebrates and invertebrates. Proteolytic pathways rely on the precise and tightly regulated activation and inhibition of these endopeptidases. As a result of this evolutionary need, many crucial pathophysiological processes are regulated via proteolytic cascades, with notable examples being coagulation of plasma (or haemolymph in arthropods), bacterial wall perforation with complement, or melanization in arthropods (Amara et al., 2008; Tang, 2009; Gulley et al., 2013). Each step involves proteolytic activation of another downstream protease, and all proteases in such cascades usually have their own endogenous inhibitors that balance the system. The role of arthropod protease inhibitors in the defense is supported by the fact that the expression of serpins and cystatins in *Ixodes scapularis* nymphs was attenuated upon infection with *Anaplasma phagocytophilum*, as seen in the transcriptomic data (Ayllon et al., 2015). On the other hand, the expression of protease inhibitors in salivary glands and midguts of adult females differed among individual inhibitors, i.e., some cystatins and serpins were upregulated upon the infection and vice versa (Ayllon et al., 2015). Similar data were collected from *Ixodes ricinus* infected with *Bartonella henselae* (Liu et al., 2014). Therefore, precise involvement of every individual inhibitor in tick infection would have to be evaluated experimentally.

Other intracellular and extracellular processes, such as cytokine activation, phagocytosis, intracellular signaling, and antigen processing, are also dependent on proteolysis (Muller et al., 2012). Serpins and cystatins are the two main superfamilies of endogenous serine and cysteine protease inhibitors involved in the regulation of these processes. It is therefore unsurprising that both groups of inhibitors are well represented in parasites and are important in their interactions with hosts (Schwarz et al., 2012; Meekins et al., 2017). In order to obtain a blood meal, ticks secrete hundreds of different pharmacoactive molecules into the host via their saliva. These molecules have anti-hemostatic, anti-inflammatory, anti-complement and immunomodulatory properties and their function is to overcome or evade host defense mechanisms including immune response (Brossard and Wikel, 2004; Chmelar et al., 2012). Moreover, tick saliva and also several salivary compounds were found to facilitate and enhance the establishment of tick-borne pathogens in the host (Anguita et al., 2002; Pal et al., 2004; Kazimirova and Stibraniova, 2013; Wikel, 2013). Inhibitors of proteases represent the most prominent protein families in tick salivary secretion that are responsible for alteration of many different host defense pathways.

SERINE PROTEASE INHIBITORS IN TICKS

Four groups of serine protease inhibitors have been described in ticks: Kunitz domain inhibitors, Kazal domain inhibitors, trypsin inhibitor-like cysteine rich domain (TIL) inhibitors, and serpins. Inhibitors with 1–7 Kunitz domains mostly act as anti-hemostatic proteins and form a large multigenic family of secreted salivary proteins in ticks that have probably played a crucial role in the development of tick hematophagy (Corral-Rodriguez et al., 2009; Dai et al., 2012; Schwarz et al., 2014). Moreover, single Kunitz-domain inhibitors in other organisms are involved in ion channel blockade and may play a similar role in ticks (Frazao et al., 2012; Valdes and Moal, 2014). Kazal domain inhibitors are described in hematophagous insects such as mosquitoes and triatomine bugs (Rimphanitchayakit and Tassanakajon, 2010), but they are only rarely reported in ticks, in which their function is still unknown (Zhou et al., 2006a; Mulenga et al., 2007a, 2008). TIL-domain inhibitors represent an interesting group of small inhibitors with a conserved 5-disulphide bridge structure that were first reported in *Apis melifera* (Bania et al., 1999) and have also been detected in ticks (Fogaca et al., 2006; Sasaki et al., 2008). The sequences of over 80 TIL-domain inhibitors have been found in arthropod genomes (Zeng et al., 2014), and the unique features of TIL-domain proteins make them an excellent model for designing novel serine protease inhibitors and antimicrobial peptides (Li et al., 2007).

Serpins

Serpins form the largest superfamily of protease inhibitors, and they are ubiquitously distributed in nature including viruses and prokaryotes. With over 1,500 members, serpins are the most studied protease inhibitors (Law et al., 2006), also helped by their unique and highly intriguing mechanism of inhibition (Whisstock et al., 2010) and the evolutionary changes that turned inhibitory serpins into non-inhibitory proteins with completely different functions (Law et al., 2006; Silverman et al., 2010). For example, there are 29 inhibitory and seven non-inhibitory serpins in humans and 60 functional serpin genes in mice (Heit et al., 2013). Angiotensinogen is a non-inhibitory serpin that is proteolytically activated by renin into several oligopeptides (angiotensins) that regulate vasoconstriction and blood pressure (Lu et al., 2016). Cortisol and thyroxine-binding proteins (human *SERPINA6* and *SERPINA7*) are also notable serpins that act as major transport proteins for glucocorticoids and progesterone (Carrell and Read, 2016). Inhibitory serpins have very diverse functions depending on their specificity, but their importance is highlighted by the serpinopathies—diseases caused by serpin dysfunction or deficiency (Belorgey et al., 2007). Emphysema, cirrhosis, angioedema, hypertension, and even familial dementia are caused at least in part by serpin dysfunction (Kim et al., 1995; Davis et al., 1999; Ekeowa et al., 2009; Huntington and Li, 2009; Lomas et al., 2016).

Arthropod serpins have mostly immunological and hemostatic functions. Serpins have been shown to regulate haemolymph coagulation, are involved in phenoloxidase system activation in insects, and regulate an immune toll pathway in haemolymph (Kanost, 1999; Gulley et al., 2013; Meekins et al., 2017). Furthermore, in bloodfeeding arthropods, serpins can act as modulators of host hemostasis and/or immune responses. Indeed, several insect serpins act as anti-coagulants, anti-complement proteins and immunosuppressors (Stark and James, 1995, 1998; Colinet et al., 2009; Calvo et al., 2011; Ooi et al., 2015). Serpins are abundant in ticks, and one of their functions is to modulate host immune system. Recent advances in this area have been facilitated by the publication of *I. scapularis* genome (Gulia-Nuss et al., 2016) and several next-generation sequencing transcriptome studies that added tens of unique sequences from different tick species to already existing and long list of tick serpins. In 2009, Mulenga and colleagues found 45 serpins in the genome of *I. scapularis* (Mulenga et al., 2009). Two years earlier, the same group described 17 serpins (Lospins) in *Amblyomma americanum* (Mulenga et al., 2007b). This number was, however, substantially broadened by the combination of several approaches up to approximately 120 serpins (Karim and Ribeiro, 2015; Porter et al., 2015, 2017). In the work of Porter and colleagues (Porter et al., 2015), the authors compare homologous serpins across tick species, showing both conserved and species-specific inhibitors. The conservation seems to be higher in serpins with basic or polar uncharged amino acid residues at P1 site (Porter et al., 2015). Other 32 serpin transcripts from the *Amblyomma* genus were found in *Amblyomma maculatum* (Karim et al., 2011) and 50 in *Amblyomma sculptum* (Moreira et al., 2017). Two groups described 18 and 22 serpins in *R. microplus,* respectively (Tirloni et al., 2014b; Rodriguez-Valle et al., 2015) and at least 36 serpins were found in several published trancriptomes from *I. ricinus* (our own unpublished data based on the analysis of transcriptomes) (Schwarz et al., 2013; Kotsyfakis et al., 2015a,b; Perner et al., 2016). Another recent publication described 10 different serpin transcripts in the sialotranscriptome of the tick *Hyalomma excavatum* (Ribeiro et al., 2017). Despite

high number of identified transcripts, only small portion was characterized functionally.

Tick Serpins with Known Function

To date, almost 20 tick serpins from different tick species have been functionally validated by *in vitro* assays, *in vivo* experimental models, vaccination and by RNA interference (RNAi) experiments (**Table 1**). These are detailed below.

AamS6 (A. americanum)

Only two serpins (AamS6 and AAS19) have been characterized thus far in *A. americanum*, despite the overall high number of serpins identified in this tick (Porter et al., 2015). *A. americanum* serpin 6 (AamS6) is upregulated during first 3 days of feeding and is likely to be injected into the host during feeding; however, RNAi did not affect tick feeding ability (Chalaire et al., 2011). Recombinant AamS6 inhibited the serine proteases trypsin, chymotrypsin, elastase, and chymase and the cysteine protease papain in a dose-dependent manner (Chalaire et al., 2011). AamS6 also reduced platelet aggregation and delayed plasma clotting time, suggesting that this serpin facilitates blood feeding by ticks (Mulenga et al., 2013). The complement activation pathway, however, was not affected (Mulenga et al., 2013).

AAS19 (A. americanum)

AAS19 is an anti-coagulant that was shown to inhibit five of the eight serine protease blood clotting factors. AAS19 inhibited thrombin—but not ADP—and cathepsin G-activated platelet aggregation and delayed clotting in re-calcification and thrombin time assays (Kim et al., 2015). AAS19 RNAi halved the blood intake and resulted in morphological deformation of ticks (Kim et al., 2016). In rabbits, immunized with AAS19, tick feeding was faster, but smaller blood volumes were ingested, and tick ability to lay eggs was impaired (Kim et al., 2016).

HLS-1 and 2 (Haemaphysalis longicornis)

Sugino and colleagues isolated a serpin from *H. longicornis* in 2003 (HLS1) (Sugino et al., 2003). Recombinant HLS1 displayed anticoagulant activity, and nymph and adult tick feeding on immunized rabbits resulted in 43.9 and 11.2% tick mortality, respectively. Antibodies raised against tick saliva did not react with recombinant HSL1, suggesting that the serpin was not secreted (Sugino et al., 2003). Moreover, HLS1 expression was detected in the midgut rather than the salivary glands, and HLS1 was therefore considered a concealed antigen, similar to the first commercially used anti-tick vaccine based on the Bm86 tick protein (Willadsen et al., 1995). HLS1 does not contain a signal peptide. Therefore, it is likely that HLS1 is not a secreted protein playing an immunomodulatory or anti-hemostatic role in the host during tick feeding.

A second serpin from *H. longicornis* (HLS2) possesses a signal sequence and seems to be secreted by hemocytes into the haemolymph but not by the salivary glands or midgut (Imamura et al., 2005). HLS2 prolonged the coagulation time in a dose-dependent manner (Imamura et al., 2005), and rabbit vaccination with HLS2 resulted in greater immunization than with HLS1 and almost 50% mortality of feeding nymphs and adults (Imamura et al., 2005). This might be explained by better accessibility and inactivation of extracellular HLS2 in the haemolymph by antibodies from the ingested blood of immunized animals.

Ipis-1 (Ixodes persulcatus)

To date, Ipis-1 is the only characterized salivary serpin from tick *I. persulcatus* (Toyomane et al., 2016). Ipis-1 transcripts were detected only in salivary glands of ticks at same level throughout all phases of feeding. It significantly reduced IFN-γ production and the proliferation of bovine PBMC cells after ConA stimulation. Authors suggest that Ipis-1 could inhibit T cells function by direct interaction with this cell population (Toyomane et al., 2016).

Iris (I. ricinus)

The first tick serpin to be described that had an effect on host defense mechanisms was named Iris (*Ixodes ricinus* immunosuppressor) (Leboulle et al., 2002a,b). Iris displayed several notable and important features. First, Iris was noted to inhibit T cell and splenocyte proliferation and altered peripheral blood mononuclear cell (PBMC)-derived cytokine levels (Leboulle et al., 2002a). Second, Iris showed anti-hemostatic properties including suppression of coagulation and fibrinolysis (Prevot et al., 2006). Finally, Iris was shown to bind to monocytes/macrophages and suppress the secretion of TNF (Prevot et al., 2009). Interestingly, these activities were independent on the protease inhibitory function of Iris. Of note, Iris, together with HLS1 and several other proteins, belongs to a group of serpins in *Ixodes* spp. that have methionine and cysteine in their reactive center loop (RCL) and lack a signaling peptide, suggesting intracellular rather than extracellular function. However, Iris has been detected in tick saliva using a polyclonal serum raised against recombinant protein (Leboulle et al., 2002a; Prevot et al., 2007), and vaccination of rabbits with recombinant Iris increased the mortality of feeding ticks and lowered weight after engorgement (Prevot et al., 2007). This contradictory observation might be explained by cross-reactivity with another secreted serpin or by the action of another, non-classical secretory mechanism (Nickel, 2003). Nevertheless, Iris represents a pleiotropic protein that affects multiple processes simultaneously via independent mechanisms.

IRS-2 (I. ricinus)

IRS-2 (*Ixodes ricinus* serpin-2) was the second serpin to be characterized in *I. ricinus*. IRS-2 has tryptophan in its P1 site, confirmed by its resolved crystal structure (Kovarova et al., 2010; Chmelar et al., 2011). IRS-2 displayed inhibitory specificity against mast cell chymase and cathepsin G, two proteases involved in inflammatory responses (Chmelar et al., 2011), with its anti-inflammatory function evidenced by *in vivo* paw edema experiments, in which IRS-2 significantly decreased paw swelling and neutrophil recruitment in treated animals (Chmelar et al., 2011). Moreover, IRS-2 inhibited the production of proinflammatory cytokine IL-6 in dendritic cells (DC) and impaired IL-6-dependent JAK/STAT3 signaling in T-helper (Th) cells, inhibiting the maturation of proinflammatory Th17 lymphocytes (Palenikova et al., 2015). IRS-2 also inhibited

TABLE 1 | Tick serpins with known function.

Serpin	Tick species	Secreted	Effect (where known)	Tissue/stage	Target enzyme	References
AamS6	*A. americanum*	Yes	Reduced platelet aggregation and delayed plasma clotting time	SG, MG, OVA, CA	Plasmin, papain, elastase, chymase	Chalaire et al., 2011; Mulenga et al., 2013
AAS19	*A. americanum*	Yes	Anti-coagulant protein, delayed clotting in recalcification and thrombin time assays		Trypsin, plasmin, fIXa, fXa, fXIa, fXIIa, thrombin, tryptase, chymotrypsin	Kim et al., 2015; Porter et al., 2015
			RNAi led to smaller blood meals and deformed ticks	SG, MG, OVA, SYN, CA, MT		Kim et al., 2016
			Feeding on immunized rabbits led to smaller blood meals and disrupted egg laying			
HLS1	*H. longicornis*	No	Feeding on immunized animals increased tick mortality rate	MG		Sugino et al., 2003
HLS2	*H. longicornis*	No	Prolonged coagulation, Immunization of rabbits increased tick mortality	LY		Imamura et al., 2005
Ipis-1	*I. persulcatus*	Yes	Inhibited proliferation and IFN-γ production of bovine PBMCs	SG		Toyomane et al., 2016
Iris	*I. ricinus*	Yes	Disrupted blood coagulation and fibrinolysis	SG, saliva	Elastase, thrombin, t-PA, fXa, trypsin	Prevot et al., 2006
			Suppressed T cell and splenocyte proliferation			Leboulle et al., 2002a
			Altered cytokine secretion by PBMC			Prevot et al., 2009
			Bound monocytes/macrophages and inhibited TNF secretion			Prevot et al., 2007
			Vaccination resulted in higher mortality and lower engorgement			
IRS-2	*I. ricinus*	Yes	Inflammation inhibitor, bound mast cell protease-4, blocked induced platelet aggregation	SG, OVA, MG	Cathepsin G, chymase, thrombin, trypsin, a-chymotrypsin	Chmelar et al., 2011
			Inhibited Th17 differentiation by reduced production of IL-6 in DC			Palenikova et al., 2015
IxscS-1E1	*I. scapularis*	Yes	Inhibited platelet aggregation and plasma clotting	SG, MG	Thrombin, trypsin, cathepsin G, fXa	Mulenga et al., 2009; Ibelli et al., 2014
RAS-1, 2	*R. appendiculatus*	No	Feeding on immunized animals increased tick mortality rate			Imamura et al., 2006
RAS-3, 4	*R. appendiculatus*	Yes	Feeding on immunized animals increased tick mortality rate and delayed *Theleria* infection			Imamura et al., 2008
RHS-1	*R. haemaphysaloides*	Yes	Anticoagulation activity, RNAi disrupted tick feeding	SG	Chymotrypsin, thrombin, fXa	Yu et al., 2013
RHS-2	*R. haemaphysaloides*	No	RNAi disrupted tick feeding	MG	Chymotrypsin, thrombin, fXa	Yu et al., 2013
RmS-3	*R. microplus*	Yes	Reduced platelet aggregation Feeding of ticks with RMS-3 antibodies impaired reproduction	SG, MG, CA	Chymotrypsin, cathepsin G, elastase, chymase	Tirloni et al., 2014b, 2016; Rodriguez-Valle et al., 2012, 2015
RmS-6	*R. microplus*	Yes		SG, MG, OVA, CA	Trypsin, plasmin, fXa, fXIa, chymotrypsin	Tirloni et al., 2014b, 2016
RmS-15	*R. microplus*	Yes	Delayed plasma clotting	SG, OVA, CA	Thrombin	Tirloni et al., 2014b; Xu et al., 2016
RmS-17	*R. microplus*	Yes	Delayed plasma clotting, reduced platelet aggregation	SG, MG, OVA, CA	Trypsin, plasmin, cythepsin G, chymotrypsin, fXIa	Tirloni et al., 2014b, 2016
rSerpin	*R. microplus*	Yes	Feeding on immunized animals increased feeding time and tick mortality and reduced tick engorgement and egg mass	SG		Kaewhom et al., 2007; Jittapalapong et al., 2010

SG, salivary glands; MG, midgut; OVA, ovaries; FB, fat body; HE, hemocytes; MAL, Malpighian tubules; DC, dendritic cells.

platelet aggregation induced by cathepsin G but not other inducers such as collagen or arachidonic acid derivatives (Chmelar et al., 2011).

IxscS-1E1 (I. scapularis)

A blood meal-induced salivary serpin IxscS-1E1 from *I. scapularis* has been shown to trap thrombin and trypsin in SDS- and heat-stable complexes, reduce their activity and inhibit the activities of cathepsin G and factor Xa, although protease/inhibitor complexes were not detected (Ibelli et al., 2014). Furthermore, IxscS-1E1 inhibited adenosine diphosphate- and thrombin-activated platelet aggregation and delayed plasma clotting time, suggesting an anti-hemostatic role (Ibelli et al., 2014). IxscS-1E1 had no effect on the classical complement activation pathway (Ibelli et al., 2014).

RAS-1, 2, 3, 4 (Rhipicephalus appendiculatus)

Four serpin cDNAs, two putatively secreted (RAS-3 and RAS-4) and two putatively intracellular (RAS-1 and RAS-2), were identified in and isolated from the salivary glands of *R. appendiculatus* (Mulenga et al., 2003). Although RAS-1 and RAS-2 are expressed in the salivary glands, antibodies against them were not found at the bite site as determined by the reactivity of anti-tick saliva sera to recombinant RAS-1 and RAS-2 (Imamura et al., 2006). This finding is, however, consistent with their predicted intracellular location (Imamura et al., 2006). Vaccination of cattle with a RAS-1/RAS-2 cocktail resulted in a 61.4% reduction in nymph engorgement rate and a 28 and 43% increase in mortality rate in female and male adult ticks, respectively (Imamura et al., 2006). Similar results were obtained when cattle were vaccinated with a mixture of two secreted serpins RAS-3 and RAS-4 and a 36-kDa immunodominant cement protein RIM36 (Imamura et al., 2008): immunization resulted in 40% mortality rate for *R. appendiculatus* ticks and almost 50% for *Theileria parva*-infected female ticks (Imamura et al., 2008). However, no significant protective effect against infection with *T. parva* was observed in spite of a 1–2 day delay in the detection of pathogens in the host peripheral blood after immunization (Imamura et al., 2008).

RHS-1 and 2 (Rhipicephalus haemaphysaloides)

Two serpins (RHS-1 and RHS-2) have been identified and characterized from *R. haemaphysaloides* (Yu et al., 2013), both of which were expressed in the salivary glands and midguts of ticks fed for 4 days. Both inhibited chymotrypsin, and RHS-1 also inhibited thrombin (Yu et al., 2013). Consistent with their inhibitory activity, only RHS-1 exhibited anticoagulation activity based on the activated partial thrombin time assay (Yu et al., 2013). Only RHS-1 seems to be secreted into the saliva and the host, as only RHS-1 was detected by serum from rabbits that were exposed to ticks and only RHS-1 possesses a signal peptide sequence (Yu et al., 2013). Nevertheless, RNAi of both serpins negatively affected the attachment rate after 24 h and decreased the engorgement rate (Yu et al., 2013).

RmS-3, 6, 15, 17 (R. microplus)

Serpin RmS-3 from *R. microplus* displayed anti-elastase and anti-chymotrypsin inhibitory activities (Rodriguez-Valle et al., 2015). Tirloni and colleagues subsequently confirmed this specificity (albeit with much lower inhibitory activity), tested more proteases, and found the highest inhibitory activity against chymase and cathepsin G (Tirloni et al., 2016). RmS-3 is likely to be secreted into the saliva and the host as evidenced by differential antibody responses of tick-resistant and tick-susceptible cattle (Rodriguez-Valle et al., 2012). RmS-3 is expressed in nymphs and in the salivary glands of adult ticks, data on RmS-3 transcription in ovaries differ between the two studies (Tirloni et al., 2014b; Rodriguez-Valle et al., 2015). Capillary feeding of ticks with a RmS-3 antibody reduced tick reproductive capacity (Rodriguez-Valle et al., 2012, 2015).

In addition to RmS-3, three other recombinant *R. microplus* serpins were produced for enzymatic and functional characterization (Tirloni et al., 2014a,b; Xu et al., 2016). RmS-6 inhibited factor Xa, factor XIa and plasmin, suggesting an anticoagulant function, while RmS-17 showed weaker inhibitory activity against chymotrypsin, cathepsin G, trypsin, and plasmin (Tirloni et al., 2016). Both RmS-3 and RmS-17 inhibited cathepsin G-induced platelet aggregation. Interestingly, RmS-3, -6, and -17 from *R. microplus* were recognized by antibodies raised by the saliva of *A. americanum*, *I. scapularis*, and *Rhipicephalus sanguineus*, suggesting a potential use for these proteins as an universal tick vaccine (Tirloni et al., 2016) but also highlighting the pitfall of false-positive detection of serpins in tick saliva. RmS-15 was identified as a thrombin inhibitor and, together with RmS-17, delayed plasma clotting in a re-calcification time assay (Tirloni et al., 2016; Xu et al., 2016). Moreover, RmS-15 is an immunogen, as the infestation of cattle with *R. microplus* resulted in increased anti-RmS-15 IgG titers (Xu et al., 2016).

rSerpin (R. microplus)

Rabbits immunized with putatively secreted serpin (rSerpin) from *R. microplus* (Kaewhom et al., 2007) led to extended feeding time, an 83% reduction in adult engorgement, 67% mortality of engorged females and a 34% reduction in egg mass weight (Jittapalapong et al., 2010).

Cystatins

Cystatins form a superfamily of tight-binding reversible inhibitors of papain-like cysteine proteases and legumains and, similar to serpins, they are present in all organisms including prokaryotes (Kordis and Turk, 2009). Cystatins regulate many physiological processes including immunity-related mechanisms such as antigen presentation, phagocytosis, and cytokine expression (Zavasnik-Bergant, 2008). There are four cystatin subgroups: type 1 (stefins), type 2, type 3 (kininogens), and type 4 cystatins (fetuins) (Rawlings and Barrett, 1990). Cystatins' target proteases are usually lysosomal cathepsins involved in protein degradation, but they also target those involved in degradation of antigens presented via MHCII to lymphocytes or in the activation of caspase 1 and thus inflammasome regulation (Jin and Flavell, 2010; Turk et al., 2012).

Cystatins with Known Function

Similarly to serpins, there are around 20 tick cystatins described in the literature and only type 1 and type 2 cystatins have thus

far been reported in ticks. While stefins lack a secretory signal and are most likely involved in the intracellular digestion of hemoglobin or in developmental processes, type 2 cystatins are secreted and expressed in both the midgut and salivary glands (Schwarz et al., 2012). Tick cystatins either regulate hemoglobin digestion, which is driven by cathepsins (Horn et al., 2009), or they can be secreted as immunomodulators into the host with saliva. The majority (84%) of tick cystatin transcripts that are conserved across tick species, belong to the extracellular group, suggesting predominantly immunomodulatory role (Ibelli et al., 2013) Tick cystatins with experimentally validated functions are listed in **Table 2** and detailed below.

Bmcystatin (R. microplus)

Bmcystatin from *R. microplus* is specifically expressed in the salivary glands, ovaries, and fat bodies. Bmcystatin did not inhibit papain but inhibited human cathepsin L and tick vitellin-degrading cysteine endopeptidase (VDTCE), suggesting a role in regulating tick embryogenesis (Lima et al., 2006).

BrBmcys2a, b, c, d, e, (R. microplus)

In addition to Bmcystatin, another five cystatins (BrBmcys2a, b, c, d, e) were identified in the cattle tick *R. microplus*. Their expression differs among various developmental stages and tissues, but since their presence has only been assessed by immunodetection methods, cross reactivity between antibodies is possible and has indeed been reported (Imamura et al., 2013). This study also examined the inhibitory specificity of two cystatins: while BrBmcys2b targeted cathepsins B, C, and L, BrBmcys2c only inhibited cathepsins C and L (Parizi et al., 2015). Antibodies raised against recombinant proteins detected BrBmcys2b in all tick tissues, while anti-BrBmcys2c serum only recognized the protein in the gut from partially engorged females and in the ovaries, salivary glands, and fat bodies from fully engorged females (Parizi et al., 2015). The expression patterns suggest rather homeostatic function of these cystatins in ticks than immunomodulatory activity in the host (Imamura et al., 2013).

Cystatin (A. americanum)

One cystatin was detected in the salivary glands and midguts of unfed and partially fed *A. americanum* ticks (Karim et al., 2005). RNAi of this cystatin led to a 90 and 50% reduction in transcript abundance in the early and late phases of feeding, respectively. RNAi knockdown decreased tick body weight, killed ticks during feeding, and disrupted feeding to full engorgement. Rabbits pre-exposed to dsRNA-injected ticks were re-exposed to naïve ticks, which led to detachment of 34% ticks after 1 day and over 50% mortality of attached ticks (Karim et al., 2005). No such effect was observed in the control group, in which rabbits were pre-exposed to normal ticks. Such a strong immune response indicates an important immunomodulatory function for silenced cystatin that impairs responses to salivary antigens and leads to an overall less intense immune reaction (Karim et al., 2005).

HISC-1 (H. longicornis)

HISC-1 is a type 2 cystatin detected in *H. longicornis* (Yamaji et al., 2009b). It is found mainly in the acinar cells of the tick salivary glands and is therefore likely to be secreted into the host. The number of transcripts was found to be approximately 5-fold higher in the salivary glands than in the midgut, with strong upregulation in early phase of blood feeding and with a pattern suggestive of importance in the feeding process. HISC-1 inhibited cathepsins L and papain but not cathepsin B (Yamaji et al., 2009b).

Hlcyst-1, 2 and 3 (H. longicornis)

While Hlcyst-1 is a type 1 intracellular cystatin with specificity against papain and cathepsin L (Zhou et al., 2009), Hlcyst-2 and Hlcyst-3 are secreted type 2 cystatins (Zhou et al., 2006b, 2010). Hlcyst-2 has been shown to inhibit cathepsin L and cathepsin B, with transcripts found mainly in the midgut and hemocytes of all tick developmental stages. Expression increased with tick development and was induced by blood feeding (Zhou et al., 2006b). Moreover, Hlcyst-2 expression was induced by injecting ticks with LPS or *Babesia gibsoni*, suggesting a role in tick immunity. *In vitro* cultivation of *B. gibsoni* in the presence of Hlcyst-2 significantly inhibited pathogen growth (Zhou et al., 2006b). Hlcyst-1 and Hlcyst-2 also inhibited cysteine protease HlCPL-A with hemoglobinase activity, isolated from *H. longicornis*, which can act as natural target of these cystatins, suggesting an involvement of both the protease and its inhibitors in blood digestion (Yamaji et al., 2009a). Hlcyst-3 inhibited papain and cathepsin L, and its expression was detected preferentially in the midgut (Zhou et al., 2010).

JpIocys2 (Ixodes ovatum)

JpIocys2 was isolated from *I. ovatum* and was shown to modulate the enzymatic activity of cathepsins B, C, and L with cathepsin L as the preferred target (Parizi et al., 2015). Similar to BrBmcys2b and BrBmcys2c, JpIocys2 is considered to be involved in tick homeostasis and egg development.

JpIpcys2a, b, c (I. persulcatus)

Three novel cystatins from *I. persulcatus*, JpIpcys2a, b, and c, have recently been described in terms of sequence and structural analysis and expression profile (Rangel et al., 2017). All three possess a signal peptide and two disulfide bridges in their mature form. Although varying in their tertiary structure, all three *I. persulcatus* cystatins should bind human cathepsin L and papain, based on *in silico* analyses. Transcripts of all three cystatins were detected in almost all tissues (salivary glands, midgut, carcass) and stages (larvae, nymphs, adults) of tick development. The only exception was absence of JpIpcys2c transcripts in unfed larvae. Furthermore, vaccination of hamsters with a structurally similar BrBmcys2c cystatin from *R. microplus* did not show any cross-reactivity and did not lead to impaired *I. persulcatus* feeding or reproduction (Rangel et al., 2017).

Om-cystatin 1 and 2 (Ornithodoros moubata)

Om-cystatin 1 and 2 were described in a soft tick *O. moubata* (Grunclova et al., 2006). While Om-cystatin 1 transcripts were found only in the midguts of unfed ticks, Om-cystatin 2 mRNA was present in all tissues. Transcript levels were rapidly suppressed after tick feeding. Both possessed inhibitory activity against cathepsins B, C, and H and papain (Grunclova et al.,

TABLE 2 | Tick cystatins with known function.

Cystatin	Tick species	Secreted	Effect (where known)	Tissue/stage	Target enzyme	References
Bmcystatin	*R. microplus*	No		SG, OVA, FB	Cathepsin L, VDTCE	Lima et al., 2006
BrBmcys2a	*R. microplus*	Yes		MG, OVA, FB		Imamura et al., 2013
BrBmcys2b	*R. microplus*	Yes		MG	Cathepsin B, C, L	Imamura et al., 2013; Parizi et al., 2015
BrBmcys2c	*R. microplus*	Yes		MG	Cathepsin C, L	Imamura et al., 2013; Parizi et al., 2015
BrBmcys2d, e	*R. microplus*	Yes		larvae		Imamura et al., 2013
Cystatin	*A. americanum*	Yes	RNAi caused decreased tick body weight, dying of ticks during feeding or disrupted feeding to the fully engorged state	MG, SG		Karim et al., 2005
HISC-1	*H. longicornis*	Yes		SG	Cathepsin L, papain	Yamaji et al., 2009b
Hlcyst-1	*H. longicornis*	No	Regulated hemoglobin degradation	MG	Cathepsin B, H, L, papain, HlCPL-A	Zhou et al., 2006b, 2009; Yamaji et al., 2009a, 2010
Hlcyst-2	*H. longicornis*	Yes	Regulated hemoglobin degradation, inhibited *Babesia* growth *in vitro*	MG, SG, OVA, HE, FB	Cathepsin L, papain, HlCPL-A	Zhou et al., 2006b; Yamaji et al., 2009a, 2010
Hlcyst-3	*H. longicornis*	Yes		MG, SG, OVA, HE, FB	Cathepsin L, papain	Zhou et al., 2006b, 2010
Jplocys2	*I. ovatum*	Yes		Assumed MG	Cathepsin B, C, L	Parizi et al., 2015
Jplpcys2a, b, c	*I. persulcatus*	Yes		SG, MG / larvae, nymphs, adult	Cathapsin L, papain	Rangel et al., 2017
Om cystatin 1	*O. moubata*	Yes		MG	Cathepsin B, C, H	Grunclova et al., 2006
Om cystatin 2	*O. moubata*	Yes	Inhibited TNF-α and IL-12 production by DC and proliferation of CD4+ T cells, immunization decreased tick feeding success	SG, OVA, MAL, MG	Cathepsin B, C, H, L, S, papain	Grunclova et al., 2006; Kotsyfakis et al., 2010
RHcyst-1	*R. haemaphysaloides*	No	Inhibitors, RNAi of RHcyst-1 impaired tick attachment rate and decreased hatching rate	Egg, larvae	Cathepsin B, C, H, L, S, papain	Wang et al., 2015b
RHcyst-2	*R. haemaphysaloides*	Yes		Egg, adult MG, SG, OVA, FB	Cathepsin B, C, H, L, S, papain	Wang et al., 2015a
Rmcystatin3	*R. microplus*	Yes		FB, HE	Cathepsin B, L, BmCl1	Lu et al., 2014
Sialostatin L	*I. scapularis*	Yes	Inhibited CTL proliferation, anti-inflammatory effects	SG	Cathepsin C, L, V, X, papain	Valenzuela et al., 2002
			Impaired DC maturation and differentiation and T cells proliferation		Binds cathepsin S	Kotsyfakis et al., 2006
			Prevented experimental asthma, inhibited IL-9 production by Th9 cells and mast cells by targeting IRF-4			Sa-Nunes et al., 2009
			Decreased IFN-β production in DC and DC maturation			Horka et al., 2012; Klein et al., 2015
			Attenuated IFN-β-triggered JAK/STAT signaling pathway in dendritic cells			Lieskovska et al., 2015a
						Lieskovska et al., 2015b
Sialostatin L2	*I. scapularis*	Yes	RNAi caused tick mortality, reduced weight and less eggs	SG, MG	Cathepsin C, L, S, V	Kotsyfakis et al., 2007
			Immunization caused decreased feeding ability of nymphs			Kotsyfakis et al., 2008
			Enhanced establishment of *Borrelia* infection			Kotsyfakis et al., 2010
			Inhibited caspase-1 maturation and diminished IL-1β and IL-18 secretion by macrophages during *Anaplasma phagocytophilum* infection			Chen et al., 2014
			Attenuated IFN-β-triggered JAK/STAT signaling in DC and promotes TBEV replication, decreases MIP-a and IP-10 production by DC			Lieskovska et al., 2015a,b

SG, salivary glands; MG, midgut; OVA, ovaries; FB, fat body; HE, hemocytes; MAL, Malpighian tubules; VDTCE, vitellin-degrading cysteine endopeptidases; DC, dendritic cell; TBEV, tick-borne encephalitis virus.

2006). Om-cystatin 2 was further functionally and structurally characterized under the name OmC2 (Salát et al., 2010). OmC2 inhibited the secretion of pro-inflammatory cytokines TNF and IL-12 by DC after LPS stimulation and reduced antigen-specific $CD4^+$ T cell proliferation induced by DC (Salát et al., 2010). Exposing OmC2 immunized mice to *O. moubata* nymphs reduced feeding ability and increased mortality during nymphal development to the next stage. Interestingly, nymphs mortality was positively correlated with higher titers of anti-OmC2 antibodies in the serum (Salát et al., 2010).

RHcyst-1 and RHcyst-2 (R. haemaphysaloides)

Two cystatins have been described in *R. haemaphysaloides*, RHcyst-1 and RHcyst-2. RHcyst-1 is an intracellular type 1 cystatin that inhibited cathepsins L, B, C, H, and S and papain, with strongest affinity to cathepsin S (Wang et al., 2015b). RHcyst-1 was expressed at all developmental stages but was most abundant in tick eggs, and its expression decreased throughout the development. RNAi of RHcyst-1 reduced the attachment rate of adult ticks and decreased hatching rate (Wang et al., 2015b). RHcyst-2 is a secreted type 2 cystatin that inhibited the same cathepsins as RHcyst-1 (Wang et al., 2015a) and was again present at all developmental stages with highest expression in eggs. However, RHcyst-2 expression increased during blood feeding, and RHcyst-2 was secreted to the host during tick feeding according to immunodetection methods (Wang et al., 2015a).

Rmcystatin3 (R. microplus)

Rmcystatin3 inhibited cathepsins L and B and *Boophilus microplus* cathepsin L-1 (BmCl1) (Lu et al., 2014). Bmcystatin3 transcripts were found in tick hemocytes, fat bodies, and salivary glands, but protein was only detected in hemocytes and the fat bodies by western blotting. Infection of ticks with *E. coli* significantly downregulated Bmcystatin3 expression (Lu et al., 2014) but increased efficacy of pathogen clearance, suggesting that Rmcystatin3 may be a negative regulator of tick immune responses, probably by regulating cysteine proteases responsible for the production of antimicrobial effectors in hemocytes (Lu et al., 2014).

Sialostatin L (I. scapularis)

One of the best studied tick cystatins is sialostatin L, a type 2 cystatin detected in *I. scapularis*. Sialostatin L has preferential specificity for cathepsin L; however, cathepsins V, C, X, S, and papain were also inhibited in enzymatic assays (Kotsyfakis et al., 2006). In the same study, sialostatin L inhibited the proliferation of the cytotoxic T lymphocyte cell line CTLL-2, suggesting its effect on adaptive immunity. Moreover, the anti-inflammatory activity of sialostatin L was confirmed in a mouse model of carrageenan-induced paw edema, in which sialostatin L reduced edema and neutrophil myeloperoxidase activity (Kotsyfakis et al., 2006).

Sialostatin L has been shown to inhibit IL-2 and IL-9 production by Th9 lymphocytes (Horka et al., 2012). IL-9 production by Th cells is IL-2 dependent (Schmitt et al., 1994), but the addition of exogenous IL-2 did not rescue IL-9 synthesis, suggesting that mechanisms other than IL-2 reduction may be involved in IL-9 inhibition (Horka et al., 2012). Nevertheless, the impairment of Th9 cells by sialostatin L abrogated the eosinophilia and airway hyperresponsiveness of mice challenged with OVA antigen (Horka et al., 2012). The inhibition of IL-9 production together with reduced expression of IL-1β and IRF4 (interferon regulating factor 4) was also observed in mast cells, with IL-9 production rescued by the application of exogenous IL-1β (Klein et al., 2015). The inhibition of IL-9 was IRF4 or IL-1β dependent, as proven by the fact that IRF4-deficient or IL-1 receptor-deficient mast cells failed to produce IL-9. The transcription factor IRF4 binds to IL-1β and IL-9 promoters, implying that sialostatin L inhibits IL-9 production via its effect on IRF4 (Klein et al., 2015). Furthermore, mice with IRF4 knockdown in mast cells or mice administered with sialostatin L showed a strong reduction in eosinophilia and airway hyperresponsiveness, important symptoms of asthma. Conversely, sialostatin L did not affect mast cell degranulation or IL-6 expression (Klein et al., 2015).

Sialostatin L inhibits cathepsin S, resulting in reduced antigen-specific $CD4^+$ T cell proliferation *in vitro* and *in vivo*; sialostatin L treatment during OVA immunization impaired early T cell expansion of splenocytes in OT-II mice and late recall immune responses by impairing the proliferation of lymph node cells (Sa-Nunes et al., 2009). Sialostatin L also potently prevented symptoms of experimental autoimmune encephalomyelitis in mice accompanied by impaired IFN-γ and IL-17 production and specific T cell proliferation (Sa-Nunes et al., 2009).

In addition to modulating T cells, sialostatin L inhibited DC maturation and reduced the production of IL-12 and TNF by DC (Sa-Nunes et al., 2009). These effects on DC can also be attributed to anti-cathepsin S activity, as cathepsin S plays a role in an invariant chain processing (Pierre and Mellman, 1998) and its inhibition thus leads to poor antigen presentation by DC (Sa-Nunes et al., 2009). Similar to another *I. scapularis* cystatin Sialostatin L2 (Lieskovska et al., 2015b), sialostatin L attenuated IFN-β-triggered JAK/STAT signaling in DC (Lieskovska et al., 2015a). However, unlike Sialostatin L2, it did not suppress expression of the IP-10 chemokine or IRF-7, suggesting that these two cystatins can produce the same phenotype by impairing different pathways in the same cell (Chmelar et al., 2016). It also decreased IFN-β production in DC activated by either *Borrelia* or TLR-7 ligand (Lieskovska et al., 2015a).

Sialostatin L2 (I. scapularis)

Sialostatin L2 is an *I. scapularis* cystatin similar in sequence to sialostatin L but with different anti-protease potency, antigenicity, and expression pattern. Unlike sialostatin L, sialostatin L2 transcripts accumulate in the salivary glands during tick feeding (Kotsyfakis et al., 2007). Its target proteases are cathepsins L, V, S, and C with preferential affinity for cathepsins L and V (Kotsyfakis et al., 2007). Sialostatin L2 was shown to inhibit inflammasome formation during infection with *A. phagocytophilum* (Chen et al., 2014) via sialostatin L2-driven inhibition of caspase-1 maturation, leading to diminished IL-1β and IL-18 secretion by macrophages after stimulation with *A. phagocytophilum* (Chen et al., 2014). However, the mechanism was not due to direct caspase-1 or cathepsin L inhibition, but was

instead dependent on reactive oxygen species (ROS) production by NADPH oxidase that was affected by the Loop2 domain of the cystatin (Chen et al., 2014). As mentioned above, sialostatin L2 interfered with JAK/STAT signaling in DC (Lieskovska et al., 2015b), attenuating STAT phosphorylation upon IFN-β treatment and thus inhibiting the IFN-β stimulated IP-10 and IRF7 chemokine genes (Lieskovska et al., 2015b). No interference with the IFN-β receptor was observed, so the downstream components of the pathway were most likely affected. Moreover, this activity enhanced the replication of tick borne encephalitis virus in DC (Lieskovska et al., 2015b). Sialostatin L2 decreased the production of specific DC chemokines MIP-1α and IP-10 in response to *Borrelia* (Lieskovska et al., 2015a). Upon LTA/TLR2 stimulation of DC, sialostatin L2 attenuated Erk1/2 phosphorylation, inhibited the PI3K pathway by reducing Akt phosphorylation, and also reduced NF-κB phosphorylation. Impaired Erk1/2 phosphorylation was the only effect observed for sialostatin L2 after stimulation of DC with *Borrelia* spirochetes (Lieskovska et al., 2015a).

The role of sialostatin L2 in *Borrelia* transmission and tick feeding has also been addressed. RNAi of sialostatin L2 led to 40% mortality in tick feeding, reduced tick size, and reduced the number of eggs by about 70% (Kotsyfakis et al., 2007). Similar effects were seen when *I. scapularis* nymphs were exposed to guinea pigs immunized with sialostatin L2 (Kotsyfakis et al., 2008). The rejection rate of nymphs fed on immunized animals was three times higher compared to controls, and the time needed to finish a blood meal was prolonged by approximately 1 day (Kotsyfakis et al., 2008). Moreover, IgG isolated from immunized animals reduced sialostatin L2 inhibitory activity against cathepsin L (Kotsyfakis et al., 2008). Of note, sialostatin L2 has been referred to as a "silent antigen," meaning that corresponding antibodies cannot be found in naïve animals exposed to ticks despite an increased titer of specific antibodies in animals pre-immunized with recombinant protein. This can be explained by the amount of sialostatin L2 injected via the saliva into the host being too small to elicit a response (Kotsyfakis et al., 2008). Sialostatin L2 has also been shown to play an important role in *Borrelia* infection (Kotsyfakis et al., 2010). The skin of mice simultaneously injected with *Borrelia* and sialostatin L2 contained six-times more spirochetes than controls. Sialostatin L2 does not appear to bind spirochetes directly and had no effect on *Borrelia* growth *in vitro*, so the mechanism of *Borrelia* growth boost in skin remains unknown (Kotsyfakis et al., 2010).

PROTEASE INHIBITORS AT THE TICK-HOST INTERFACE

Tick cystatins and serpins can obviously affect many intracellular pathways and thus impair the functions of host immune cells. Moreover, they can also interfere with extracellular proteolysis, thereby inhibiting hemostasis (**Figure 1**). These activities take place at the site of attachment, where they cause local immunosuppression and inhibition of blood clotting. Of note, different inhibitors can cause similar phenotypes by targeting different pathways or even different components of the same pathway. Their actions are therefore redundant. Conversely, more than one effect is usually observed for a single inhibitor. Such concept of redundancy and pluripotency is probably a strategy developed by ticks during long-term co-evolution with their hosts (Chmelar et al., 2016). There is no doubt that salivary secretion at the tick-host interface is beneficial for the tick and deleterious for the host. From this perspective, tick inhibitors represent an important and interesting research field for the development of anti-tick vaccines and tick control strategies.

As shown on vaccination experiments, tick serpins and cystatins can contribute to the establishment of pathogens in the host (Imamura et al., 2008; Kotsyfakis et al., 2010). Such role of serpins is in accordance with observed positive effect of activated plasminogen activation system (PAS) with upregulated serpin PAI-2 on the establishment of *Borrelia burgdorferi* infection. The facilitation of infection resulted from direct enhancement of *Borrelia* dissemination and from the inhibition of inflammatory infiltration to the site of exposure (Haile et al., 2006). *Borrelia recurrentis* was shown to bind host serpin—C1 inhibitor—on its surface and thus inhibit complement activation (Grosskinsky et al., 2010). On contrary, mammalian cystatins were shown as regulators of cysteine proteases like cathepsin S and L, which contribute to the establishment of several viral infections (Kopitar-Jerala, 2012). Thus, the involvement of cystatins in the establishment of microbial and viral infection is not clear and cannot be easily addressed without experimental evidence.

TICK PROTEASE INHIBITORS AS NOVEL DRUGS

Cystatins

The inhibition of target proteases with tick-derived inhibitors can, however, be beneficial in different scenarios. Almost all the mammalian serine and cysteine proteases that are targets of tick inhibitors described in this review play important roles in various human diseases and pathologies. For a long time, the functions of lysosomal cysteine cathepsins (B, C, F, H, K, L, O, S, V, X, and W) were thought to be strictly limited to intracellular protein degradation and cellular metabolism. Recently, many cathepsins have been shown to be involved in multiple pathological processes. For example, increased serum levels of cathepsin L are associated with metastatic stage of different cancer types and poor patient prognosis (Tumminello et al., 1996; Chen et al., 2011). Tumor cells can produce high amounts of cathepsin L, leading to high serum level, which is considered as blood marker of cancer (Denhardt et al., 1987). High concentration of cathepsin L in tumor and its vicinity leads to extracellular matrix degradation, higher tumor invasiveness, and several cancer-related health complications (Sudhan and Siemann, 2015). Other cysteine cathepsins may also participate in tumor invasion and metastasis (Kuester et al., 2008; Tan et al., 2013), so cystatins are considered possible effectors that could block the deleterious activity of cysteine cathepsins in cancer (Cox, 2009; Hap et al., 2011). Cysteine cathepsins also contribute to neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's disease and amyotrophic lateral

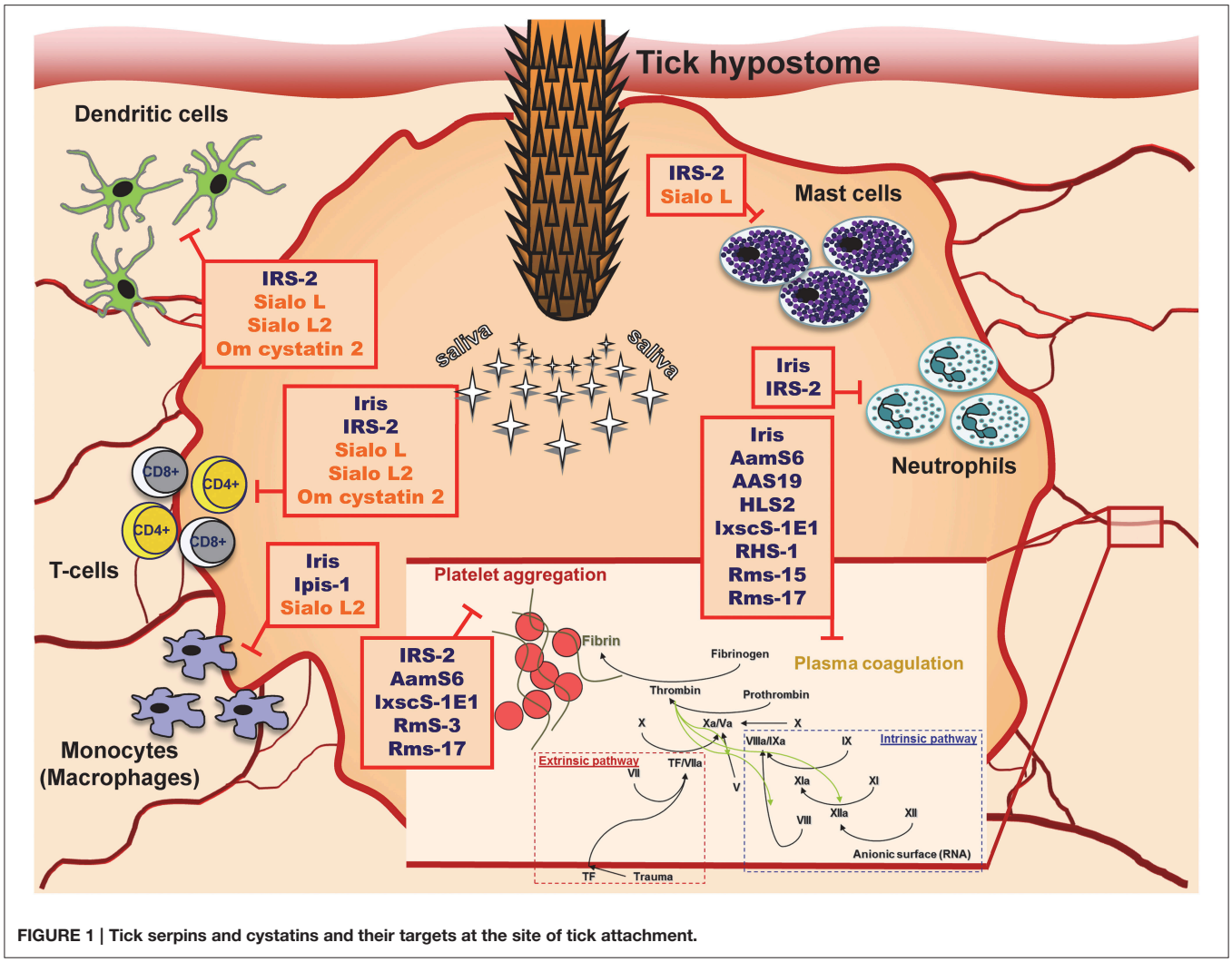

FIGURE 1 | Tick serpins and cystatins and their targets at the site of tick attachment.

sclerosis (**Figure 2A**; Pislar and Kos, 2014). The leakage of lysosomal cathepsins induces neuronal apoptosis and can also increase the inflammatory milieu in the central nervous system (Pislar and Kos, 2014). Cysteine cathepsins are also implicated in the pathogenesis of psoriasis (Kawada et al., 1997), muscular dystrophy (Takeda et al., 1992), abdominal aortic aneurysm and atherosclerosis (Liu et al., 2006), osteoporosis and rheumatoid arthritis (Yasuda et al., 2005), and acute pancreatitis (Halangk et al., 2000). Relatively recent data are accumulating to suggest that cysteine cathepsins are promising therapeutic targets (Kos et al., 2014; Sudhan and Siemann, 2015). The wide spectrum of tick cystatins with varying specificities provides an opportunity to take advantage of this rich source of natural cathepsin inhibitors.

Serpins

Serine proteases are best known as the building blocks of proteolytic cascades in the blood such as coagulation (**Figure 1**) or complement activation. The portfolio of their activities, however, is much wider. Neutrophils, mast cells, natural killer cells, and cytotoxic T cells all produce serine proteases responsible for extracellular matrix remodeling, microbe killing, cytokine activation, signaling via protease-activated receptors (PARs), or chemoattraction of leukocytes. As regulators of many processes, serine proteases often contribute to disease pathologies. Some diseases, in which serine proteases are implicated, are shown in **Figure 2B**. Signaling via PARs and the activation of coagulation in the tumor microenvironment link coagulation proteases with some of the complications seen in cancer (Shi et al., 2004; Han et al., 2011; Lima and Monteiro, 2013). Neutrophil proteases from azurophilic granules, namely cathepsin G, elastase, and protease 3 (PR3), play crucial roles in neutrophil anti-microbial activity and are indispensable for the clearance of some pathogens (Hahn et al., 2011; Steinwede et al., 2012). Many studies have also described neutrophil proteases as important regulators of inflammatory and immune processes (Pham, 2006, 2008), albeit with deleterious effects in some cases. For instance, due to the large amounts of elastin present in the lung connective tissue, lungs are very sensitive to dysregulation and/or increased levels of elastolytic proteases such as neutrophil elastase (Sandhaus and Turino,

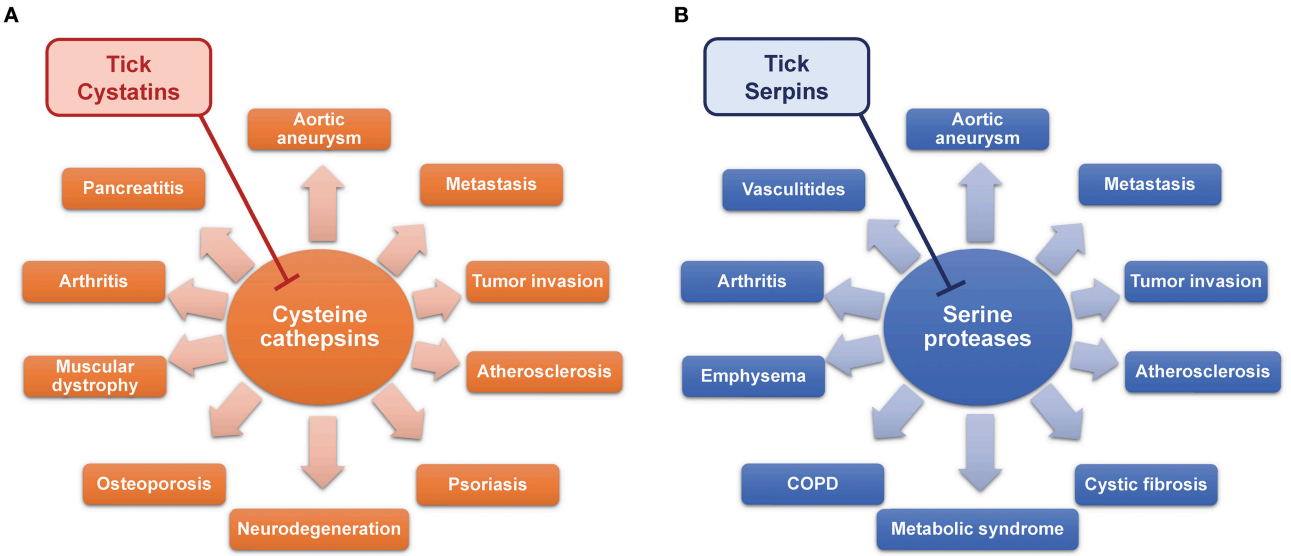

FIGURE 2 | The therapeutic potential of tick cystatins (A) and serpins **(B)** by inhibiting cysteine and serine proteases in various diseases.

2013), which results in several lung diseases. Elastase and cathepsin G facilitate the spreading of metastases to the lungs due to the degradation of antitumorigenic factor thrombospondin-1 (El Rayes et al., 2015). Furthermore, neutrophil proteases have been implicated in the pathogenesis of cystic fibrosis (Twigg et al., 2015; Wagner et al., 2016), chronic obstructive pulmonary disease (COPD) (Shapiro, 2002; Owen, 2008), and emphysema (Ekeowa et al., 2009). In anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides such as Wegener's granulomatosis, neutrophils are activated by auto-antibodies against PR3 (Niles et al., 1989), leading to the production of neutrophil extracellular traps (NETs) containing PR3 and to necrosis (Kessenbrock et al., 2009). Cathepsin G is chemotactic for monocytes in rheumatoid arthritis (Miyata et al., 2007), and the inhibition of neutrophil elastase improved some of the symptoms of this disease (Di Cesare Mannelli et al., 2016). Interestingly, obesity and metabolic syndrome also seem to be affected by neutrophil proteases (Talukdar et al., 2012; Mansuy-Aubert et al., 2013). Mast cells are another significant source of several serine proteases, mainly chymases and tryptases, which are involved in extracellular matrix remodeling, chemoattraction of neutrophils, and protein processing and activation (Pejler et al., 2010). Mast cell chymase and tryptase have been shown to be involved in the pathogenesis of abdominal aortic aneurysm (Sun et al., 2009; Zhang et al., 2011) and atherosclerosis (Sun et al., 2007; Bot et al., 2015).

Due to these diverse and clinically relevant effects of serine proteases, their potential use as therapeutic targets is being thoroughly discussed by scientific community (Guay et al., 2006; Quinn et al., 2010; Caughey, 2016). Tick salivary glands express a large number of serine protease inhibitors with different specificities that could be used as novel drugs against malfunctioning proteases.

CONCLUDING REMARKS

Novel pharmacoactive compounds are being developed either by artificial synthesis or by isolating potential candidates from various organisms including parasites (Cherniack, 2011). For instance, hirudin (a thrombin inhibitor from leeches) and its congener bivalrudin have been useful in the treatment of blood coagulation disorders (Kennedy et al., 2012). Ticks are parasites that have evolved multiple ways to evade or manipulate host immune and hemostatic systems (Chmelar et al., 2012). Tick saliva contains hundreds of proteins not only with anti-hemostatic features (Maritz-Olivier et al., 2007) but also with anti-complement, anti-inflammatory, and immunomodulatory effects on the host (Kazimirova and Stibraniova, 2013).

As discussed in this review, salivary cystatins and serpins display such features and their functions have been studied thoroughly. Moreover, both superfamilies are represented in the vertebrate host and the functions of their members are often known. Therefore, we can predict at least to some degree, which processes or pathways will be targeted by tick proteins. An important advantage of cystatins and serpins is their functional specificity; for example, sialostatins L and L2 cause similar phenotypes (inhibition of IFN-β signaling) either by inhibiting the IFN-β production (sialostatin L) or by inhibiting STAT3 phosphorylation downstream from IFN-β (sialostatin L2) (Lieskovska et al., 2015a,b). The possibility of targeting specific processes is crucial for the development of "patient-tailored" immunotherapeutic strategies (Scherer et al., 2010; Stephenson et al., 2016). Furthermore, tick cystatins and serpins are not the only families in ticks that deserve attention, since there are many tick-specific proteins secreted into the saliva of unknown function. Characterizing ticks using the transcriptomic approach has created a broad field and data

repository, which we can search for novel drugs and potential therapeutics.

AUTHOR CONTRIBUTIONS

JC and JK wrote the manuscript, JK prepared the tables, JC prepared the figures, HL and MK edited and revised the manuscript.

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REVIEW
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Tick-Borne Viruses and Biological Processes at the Tick-Host-Virus Interface

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Ticks are efficient vectors of arboviruses, although less than 10% of tick species are known to be virus vectors. Most tick-borne viruses (TBV) are RNA viruses some of which cause serious diseases in humans and animals world-wide. Several TBV impacting human or domesticated animal health have been found to emerge or re-emerge recently. In order to survive in nature, TBV must infect and replicate in both vertebrate and tick cells, representing very different physiological environments. Information on molecular mechanisms that allow TBV to switch between infecting and replicating in tick and vertebrate cells is scarce. In general, ticks succeed in completing their blood meal thanks to a plethora of biologically active molecules in their saliva that counteract and modulate different arms of the host defense responses (haemostasis, inflammation, innate and acquired immunity, and wound healing). The transmission of TBV occurs primarily during tick feeding and is a complex process, known to be promoted by tick saliva constituents. However, the underlying molecular mechanisms of TBV transmission are poorly understood. Immunomodulatory properties of tick saliva helping overcome the first line of defense to injury and early interactions at the tick-host skin interface appear to be essential in successful TBV transmission and infection of susceptible vertebrate hosts. The local host skin site of tick attachment, modulated by tick saliva, is an important focus of virus replication. Immunomodulation of the tick attachment site also promotes co-feeding transmission of viruses from infected to non-infected ticks in the absence of host viraemia (non-viraemic transmission). Future research should be aimed at identification of the key tick salivary molecules promoting virus transmission, and a molecular description of tick-host-virus interactions and of tick-mediated skin immunomodulation. Such insights will enable the rationale design of anti-tick vaccines that protect against disease caused by tick-borne viruses.

Keywords: tick, arbovirus, transmission, skin, immunomodulation, vaccines

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INTRODUCTION

Ticks are familiar to most people world-wide. They have accompanied humans through their long history, known as blood-sucking creatures that decimate livestock. However, since the pioneering work of Smith and Kilbourne on Texas fever of cattle in 1893, followed by Rickett's discovery of the pathogen causing Rocky Mountain spotted fever transmitted by *Dermacentor andersoni* in 1907, ticks have been identified as vectors of a huge range of viral, bacterial, and protozoan agents of diseases, and have become a major focus of medical and veterinary research (Nicholson et al., 2009; Sonenshine and Roe, 2014). It is now recognized that ticks surpass all other arthropods in the variety of transmitted infectious agents involving nematodes, fungi, protozoa, bacteria, and viruses. Tick-borne viral diseases have significant and increasing medical and veterinary impact due to the geographical spread of the vectors and outbreaks in new regions as a result of changes in global socio-economic and climatic conditions, and lack of efficient control measures (Estrada-Peña et al., 2013; Nuttall, 2014; Vayssier-Taussat et al., 2015; Brackney and Armstrong, 2016). Here, we consider the many viruses transmitted by ticks, the reasons why ticks are such efficient and effective vectors of viruses, and future directions for research.

TICK-BORNE VIRUSES

Tick-borne viruses (TBV) or "tiboviruses" (Hubálek and Rudolf, 2012) comprise a diverse group of viruses circulating between ticks and vertebrate hosts, thriving in two extremely different environments: the homeostatic environment of the vertebrate host and the dramatically changing environment of ticks. TBV and ticks have evolved together, resulting in a complex relationship in which the virus life cycle is perfectly coordinated with the tick's feeding cycle, and the tick can harbor the virus for prolonged periods without affecting its biology. Considering their unique characteristics, ticks are believed to shape the evolution of TBV (Nuttall and Labuda, 2003; Kuno and Chang, 2005).

The initial discoveries of arboviruses such as yellow fever virus (1928) (mosquito-borne), and Nairobi sheep disease virus (1910) and louping ill virus (LIV) (1929) (tick-borne), opened the floodgates for the discovery of over 500 arboviruses during the ensuing years (Bichaud et al., 2014). At least 160 named viruses are tick-borne, of which about 50 are recognized or probable "virus species" (Nuttall, 2014). Taxonomically, TBV comprise a heterogenous group of vertebrate viruses classified into one DNA viral family, *Asfarviridae*, and eight RNA viral families: *Flaviviridae, Orthomyxoviridae, Reoviridae, Rhabdoviridae*, the newly recognized *Nyamiviridae* (order *Mononegavirales*), and the families *Nairoviridae, Phenuiviridae,* and *Peribunyaviridae* in the new order, *Bunyavirales* (**Table 1**).

The occurrence of TBV in different viral families suggests that their tick-borne mode of transmission evolved independently at least seven times (Nuttall, 2014). Almost 25% of TBV are associated with disease and all TBV pathogenic to humans are zoonotic. At present, more than 16 specific tick-borne diseases (TBD) of humans and 19 TBD of livestock and companion animals have been described (Nicholson et al., 2009; Sonenshine and Roe, 2014). Several TBV cause serious human or animal diseases, such as CNS disease (meningitis, meningoencephalitis, or encephalomyelitis), or haemorrhagic disease (**Table 1**). Others are less serious or sporadically reported, and most are without known medical or veterinary significance. Certain viral diseases of feral vertebrates as well as domestic animals and even humans may pass unnoticed or are misdiagnosed, and eventually they may appear as emerging diseases (Dörrbecker et al., 2010; Hubálek and Rudolf, 2012).

In recent decades, a number of recognized TBV, mainly those belonging to the tick-borne encephalitis virus (TBEV) serocomplex, have emerged or re-emerged, and/or spread, posing an increasing threat to human and animal health. For example, a rise in the incidence of human infections caused by Powassan virus (POWV) in the USA (Hermance and Thangamani, 2017), the spread of TBEV into new geographic areas, and the emergence of new viruses such as Alkhurma virus, a subtype of Kyasanur forest disease virus (KFDV) (Charrel et al., 2001), and Deer tick virus, a subtype of POWV (Pugliese et al., 2007; Robertson et al., 2009; Hermance and Thangamani, 2017) have been reported. The latest emerging TBD, caused by Bourbon virus (*Thogoto virus*, *Orthomyxoviridae*), was reported in Kansas in 2014 (Kosoy et al., 2015).

While new TBV are being discovered, unclassified viruses are being allocated to genera or families thanks to improvements in molecular technologies (**Table 1**). The most notable changes have occurred in families *Bunyaviridae* and *Rhabdoviridae*. The *Bunyaviridae* has been revised and elevated to the order *Bunyavirales* comprising 9 families and 13 genera (Briese et al., 2016; Junglen, 2016; Walker et al., 2016b). TBVs are included in three families—*Nairoviridae, Phenuiviridae,* and *Peribunyaviridae*. Except for the most medically important member, Crimean-Congo haemorrhagic fever virus (CCHFV), the genus *Orthonairovirus* of the *Nairoviridae* comprises 11 other species, 9 of which are TBVs (**Table 1**) (Kuhn et al., 2016a,b; Walker et al., 2015, 2016b). The most recent emerging human disease causing significant mortality (up to 30% of cases) is caused by Severe fever with thrombocytopenia syndrome virus (SFTSV), a new member of the *Phlebovirus* genus (*Phenuiviridae)* first reported in China (Xu et al., 2011; Yu et al., 2011; Zhang et al., 2011). Recently, a new virus closely related to SFTSV named Heartland virus was isolated from severely febrile patients in the USA (McMullan et al., 2012) and from field collected ticks (Savage et al., 2013). Moreover, phylogenetic and serological analyses revealed that Bhanja virus and Palma virus (previously unassigned to a genus) are closely related to both SFTVS and Heartland virus (Dilcher et al., 2012; Matsuno et al., 2013).

Within the *Rhabdoviridae* family, a new genus, *Ledantevirus,* comprises 14 new species, four of which are TBVs (Blasdell et al., 2015; Walker et al., 2016a) (**Table 1**). In recent years, further novel rhabdoviruses have been identified from various animal species, but so far transmission by ticks have been confirmed only for a few of them, e.g., Kolente virus (Ghedin et al., 2013) and Yongjia tick virus 2 (YTV-2) (Li et al., 2015). For Long Island

TABLE 1 | Classification of tick-borne RNA viruses including recently described species, with indication of viruses causing major diseases of humans and domesticated animals.

Family	***Flaviridae* (ssRNA +)**			***Reoviridae* (dsRNA)**	
Subfamily/ group	**Mammalian tick-borne flavivirus**	**Seabird tick-borne flavivirus**	**Kadam tick-borne flavivirus**	***Sedoreovirinae***	***Spinareovirinae***
Genus	***Flavivirus***	***Flavivirus***	***Flavivirus***	***Orbivirus***	***Coltivirus***
Species	Tick-borne encephalitis virus (TBEV)††	Tyuleniy virus	Kadam virus	Chenuda virus	Colorado tick fever virus
	Louping ill virus (LIV)†	Meaban virus		Chobar Gorge virus	Eyach virus
	Langat virus	Saumarez Reef virus		Great Island virus	
	Powassan virus (POWV)††			Wad Medani virus	
	Kyasanur Forest disease virus (KFDV)††			St Croix River virus	
	Omsk hemorrhagic fever virus				
	Gadgets Gully virus				
	Royal Farm virus				
Unassigned				Lake Clarendon virus	
				Matucare virus	

Family	***Orthomyxoviridae* (segmented ssRNA-)**		***Rhabdoviridae* (ssRNA-)**		***Nyamiviridae* (ssRNA-)**
Genus	***Thogotovirus***	***Quaranjavirus***	***Vesiculovirus***	***Ledantevirus***	***Nyavirus***
Species	Thogoto virus	Quaranfil virus	Isfahan vesiculovirus	Barur ledantevirus	Nyamanini virus
	Dhori virus	Johnston Atoll virus		Kern Canyon ledantevirus	Midway nyavirus
				Kolente ledantevirus*	Sierra Nevada nyavirus
				Yongjia ledantevirus*	
Unassigned			Connecticut virus		
			New Minto virus		
			Sawgrass virus		

Order	***Bunyavirales* (segmented ssRNA-)**			
Family	***Nairoviridae***		***Peribunyaviridae***	***Phenuiviridae***
Genus	***Orthonairovirus***	**Putative *Nairoviruses***	***Orthobunyavirus***	***Phlebovirus***
Species	Burana orthonairovirus	Artashat virus	Bakau orthobunyavirus	Severe fever with thrombocytopenia syndrome phlebovirus (SFTSV)††*
	Crimean-Congo hemorrhagic fever orthonairovirus (CCHFV)††	Burana virus	Estero Real orthobunyavirus	Uukuniemi phlebovirus
	Dera Ghazi Khan orthonairovirus	Chim virus	Tete orthobunyavirus	
	Dugbe orthonairovirus	Geran virus		
	Hazara orthonairovirus	Nàyǔn tick virus*		
	Hughes orthonairovirus	South Bay virus*		
	Keterah orthonairovirus	Tamdy virus		
	Nairobi sheep disease virus orthonairovirus (NSDV)††			
	Qalyub nairovirus			
	Sakhalin nairovirus			

††Human pathogens and their principal vector ticks: TBEV—Ixodes persulcatus, I. ricinus; KFDV—Haemaphysalis spinigera; POWV—Ixodes scapularis; SFTSV—Haemaphysalis longicornis; CCHFV—Hyalomma spp.

†Non-human pathogens and their principal vector ticks: LIV—Ixodes ricinus; NSDV—Rhipicephalus appendiculatus.

**Recently described species.*

tick rhabdovirus (Tokarz et al., 2014) and Zahedan virus (Dilcher et al., 2015) transmission by ticks has yet to be confirmed.

Recent studies suggest that besides the hitherto only recognized DNA containing arbovirus, African swine fever virus (ASFV) (*Asfarviridae,* genus *Asfivirus*), transmitted by soft ticks, other DNA viruses may be transmitted by ticks. Lumpy skin disease virus (LSDV, *Capripoxvirus, Poxviridae*), the cause of skin disease in cattle, is transmitted by blood-feeding insects such as mosquitoes and stable flies (Carn and Kitching, 1995; Chihota et al., 2001, 2003), but recent studies indicate the potential for biological transmission of LSDV by *Amblyomma* and *Rhipicephalus* ticks (Tuppurainen et al., 2011, 2013a,b; Lubinga et al., 2013, 2014). Another DNA virus potentially transmitted by ticks is Murid herpesvirus 4 (MuHV 4) strain 68 (MHV-68), which has been detected in field collected ixodid ticks (Ficová et al., 2011; Kúdelová et al., 2015; Vrbová et al., 2016). Could this be the cause of the next emerging tick-borne virus disease?

TICKS AS VECTORS OF VIRUSES

Ticks have evolved several unique features - such as their prolonged life-span and complex development, haematophagy in all post-embryonic life stages, long feeding periods, and blood digestion within midgut cells—that contribute to their success as vectors of viruses (Sonenshine et al., 2002; Nuttall and Labuda, 2003). Once ticks acquire a virus, they usually remain infected for the rest of their life. Due to their exceptional longevity, ticks act as excellent reservoirs of TBV, carrying viruses over months or even years, and maintaining them transstadially from one developmental stage to the next prior to transmission to a vertebrate host (Nuttall et al., 1994; Nuttall and Labuda, 2003; Turell, 2015). TBV persistence in a tick population can also be ensured through transmission from infected females via eggs to their progeny, although the rates of transovarial transmission of TBV in nature appear to be low (Nuttall et al., 1994; Kuno and Chang, 2005).

Isolation of a virus (especially from an engorged tick), or detection of the presence of viral RNA or DNA in a tick, do not necessarily prove that a particular tick species is a competent vector of the virus (Nuttall, 2009). To determine vector competence, the following parameters have to be fulfilled: (i) the virus is acquired by a tick during blood-feeding on an infected host; (ii) the virus is transmitted to a host by a tick that takes its blood-meal after it has molted to the next developmental stage. The period between virus acquisition and virus transmission has been termed the "extrinsic incubation period" during which the tick is not able to transmit the virus (Nuttall, 2009).

The association between a tick species and a transmitted virus is very specific. Indeed, fewer than 10% of the known tick species are suggested to be competent vectors of viruses. These belong to the large tick genera, i.e., soft ticks of the genera *Ornithodoros, Carios,* and *Argas,* and hard ticks of the genera *Ixodes, Haemaphysalis, Hyalomma, Amblyomma, Dermacentor,* and *Rhipicephalus* (Labuda and Nuttall, 2004, 2008; Nuttall, 2014). Most TBV are transmitted either by hard ticks or by soft ticks, but rarely by both (Labuda and Nuttall, 2004). Moreover, some tick species (e.g., *I. ricinus, A. variegatum*) are vectors of a few TBV species, whereas others can transmit several different TBV species (e.g., *Ixodes uriae* is suggested to be the vector of at least 7 TBV) (Labuda and Nuttall, 2008).

During co-evolution, molecular interactions have developed between ticks, TBV and vertebrates, and at their interfaces (Nuttall et al., 1994; Labuda and Nuttall, 2004; Robertson et al., 2009; Mlera et al., 2014; Nuttall, 2014). Natural acquisition of the virus takes place when a tick feeds on an infected vertebrate host or co-feeds with infected ticks on a susceptible uninfected host. The virus enters the midgut, passes through the gut wall, disseminates in the tick body, and reaches the salivary glands (SG) so as to be amplified and transmitted to the next host during subsequent feeding. On its route the virus must cross several barriers in the tick body, such as the midgut infection barrier, midgut release barrier, midgut escape barrier, SG infection barrier, and SG release barrier (Nuttall, 2014).

The best understood TBV transmission cycle is probably that of TBEV (*Flaviviridae*) and its principal vectors, *Ixodes persulcatus* and *I. ricinus.* Several experimental studies have been carried out to explain the TBEV—vector—host interactions. For example, tick infestation of viraemic laboratory animals indicated that most of the tested hard tick species (*Ixodes* spp., *Haemaphysalis* spp., *Dermacentor* spp., *R. appendiculatus*) acquired TBEV from the infected blood meal and maintained the virus transstadially (Rajčáni et al., 1976; Nosek et al., 1984; Kožuch and Nosek, 1985; Alekseev et al., 1988, 1991; Alekseev and Chunikhin, 1990a; Labuda et al., 1993a). TBEV was found to infect tick SG prior to attachment and can be transmitted to a vertebrate host by saliva soon after onset of tick feeding (Řeháček, 1965). Similarly, successful transmission of POWV is likely to occur within 15 min of *I. scapularis* attachment (Ebel and Kramer, 2004), and transmission of Thogoto virus (THOV) within 24 h of attachment of *R. appendiculatus* (Kaufman and Nuttall, 2003). Onset of feeding was found to enhance amplification of TBEV in SG of *I. persulcatus* and *I. ricinus* (Alekseev and Chunikhin, 1990b; Khasnatinov et al., 2009; Slovák et al., 2014a) and of THOV in SG of *R. appendiculatus* (Kaufman and Nuttall, 2003). However, knowledge on the critical stages of TBV survival in their vectors is limited (Nuttall, 2014; Slovák et al., 2014a).

TBV must evade tick innate immune responses in order to persist and replicate in their vectors (Hynes, 2014). In general, tick-borne pathogens have developed different strategies to cope with the tick defense system and high-throughput techniques have already provided insights into both the tick immune responses evoked by bacteria and the bacterial evasion strategies (Smith and Pal, 2014). In contrast, information on molecular mechanisms determining interactions of TBV with ticks is scarce (Hajdušek et al., 2013; Hynes, 2014; Gulia-Nuss et al., 2016). RNA interference (RNAi) appears to be the main antiviral mechanism in ticks that, together with Argonaute (Ago) and endoribonuclease Dicer (Dcr) proteins mediates tick antiviral responses (Schnettler et al., 2014; Gulia-Nuss et al.,

2016). However, at present the role of defensin-like peptides displaying *in vitro* virucidal activities against TBV (e.g., longicin or HEdefensin from *Haemaphysalis longicornis*) is unclear in tick antiviral defense (Talactac et al., 2016, 2017).

Tick cell cultures play an important role in many aspects of tick and TBV research (Bell-Sakyi et al., 2012). Since primary tick cell or tissue explant cultures have been introduced, propagation of both arboviruses and non-arthropod-transmitted viruses has been attempted (Řeháček and Kožuch, 1964; Řeháček, 1965; Yunker and Cory, 1967; Cory and Yunker, 1971), and tick cells have been employed to identify and characterize tick genes associated with TBV infection, including those which mediate antiviral activity. For example, by using Langat virus (LGTV)-infected *I. scapularis*-derived cell line, the production of virus-derived small interfering RNAs was revealed (Schnettler et al., 2014). In proteomic studies on *I. scapularis* cells infected with LGTV, 264 differentially expressed tick proteins were identified, out of which the majority were downregulated (Grabowski et al., 2016). The proteins with upregulated expression were associated with cellular metabolic pathways and glutaminolysis. In addition, enzymes that are probably involved in amino acid, carbohydrate, lipid, terpenoid/polykeytide, and vitamin metabolic pathways may also be associated with a decreased replication of LGTV and with a release of infectious virus from *I. scapularis* cells (Grabowski et al., 2017). Analyses of transcriptomes and proteomes of TBEV-infected cell lines derived from *I. ricinus* and *I. scapularis* have identified several molecules that also seem to be involved in the tick innate immune response against flaviviruses and in cell stress responses, such as the heat-shock proteins HSP90, HSP70, and gp96, the complement-associated protein Factor H and trypsin (Weisheit et al., 2015). Furthermore, comparative transcriptome analysis revealed activation of common as well as distinct cellular pathways in *I. ricinus* cells infected with TBEV and LIV and the obligate intracellular bacterium *Anaplasma phagocytophilum*, depending on the infectious agent. Commonly upregulated genes were those that are associated with apoptosis and cellular stress, and genes that affect the tick innate immune responses, whereby only flavivirus infection evoked up- or downregulation of toll genes expression. These data suggest that multiple pathways ensure the control of invading viruses and tick survival (Mansfield et al., 2017).

SALIVA-ASSISTED AND NON-VIRAEMIC TRANSMISSION

According to Nuttall and Labuda (2008) "saliva-assisted transmission (SAT) is the indirect promotion of arthropod-borne pathogen transmission via the actions of arthropod saliva molecules on the vertebrate host."

Ticks succeed in feeding by injecting a cocktail of salivary antihaemostatic and immunomodulatory molecules into the feeding pool (Mans and Neitz, 2004; Mans et al., 2008; Kazimírová and Štibrániová, 2013; Wikel, 2014; Chmelař et al., 2016a). The established route of TBV transmission is via an infectious tick bite in which the virus is carried in tick saliva into the feeding lesion in the host skin that is modified by pharmacologically active compounds present in the saliva. Viraemia (infectious virus detectable in circulating blood) in a vertebrate host was considered an important requirement for biological transmission of viruses (World Health Organization Scientific Group, 1985). However, by mimicking natural conditions of transmission using THOV-infected *R. appendiculatus* adults or nymphs that co-fed with uninfected nymphs or larvae on the same naïve guinea pig, a high percentage of the uninfected ticks became infected, although the host animals did not develop detectable viraemia (Jones et al., 1987). Moreover, non-viraemic guinea pigs supported a higher rate of virus transmission between co-feeding ticks than viraemic hamsters. Based on these findings showing that a vertebrate host free of an apparent viremia can play a role in the epidemiology of an arbovirus, a novel mode of arbovirus transmission, "non-viraemic transmission" (NVT), was proposed (Jones et al., 1987). Subsequently, NVT for which the role of tick salivary compounds is anticipated was considered to be indirect evidence of SAT (Nuttall and Labuda, 2008). Furthermore, NVT of THOV even occurred when co-feeding of ticks took place on virus-immune guinea pigs although levels of virus transmission were reduced compared with NVT involving naïve hosts (Jones and Nuttall, 1989a,b). In addition, *R. appendiculatus* was found to be more efficient in mediating NVT than *A. variegatum,* indicating species-specific differences between the vector ticks (Jones et al., 1990a).

Direct evidence of SAT (originally referred to as "saliva-activated transmission"; Jones et al., 1990b) was demonstrated when increased acquisition of THOV was observed in non-infected *R. appendiculatus* nymphs that fed on naïve guinea pigs inoculated with a mixture of the virus and salivary gland extract (SGE) of partially fed *R. appendiculatus* or *A. variegatum* females in comparison to ticks feeding on guinea pigs inoculated only with the virus (Jones et al., 1989). However, SAT was evidenced only when THOV was inoculated along with SGE into tick attachment sites. Viraemia was not detected in the tested animals, suggesting that THOV transmission was enhanced by factors in tick saliva that are likely to mediate NVT. Furthermore, the effect of SAT factor(s) appears to persist in the host skin for several days. Indeed, the proportion of infected *R. appendiculatus* ticks increased when they fed at the skin site where THOV was inoculated 2–3 days after inoculation of SGE (Jones et al., 1992b). SAT factors are likely to be proteins or peptides (Jones et al., 1990b) and enhance virus transmission through immunomodulation of the host rather than by a direct effect on the virus (Jones et al., 1989, 1990b).

Most of the described SAT and NVT events have been associated with hard ticks (**Table 2**), suggesting that there are differences in the SAT mediators between hard- and soft ticks. Due to their feeding biology (long-lasting blood meal, attachment to hosts in aggregates, and in close proximity), hard ticks appear to be more suitable vectors for NVT than soft ticks (Nuttall and Labuda, 2003). Since the first reports on NVT and SAT, indirect and direct evidence of SAT have been reported for at least 10 different TBV (**Table 2**).

TABLE 2 | Saliva assisted (SAT) and non-viraemic (NVT) transmission of tick-borne viruses.

Virus	Tick species	Described effect	References
SOFT TICKS (ARGASIDAE)			
Asfarviridae			
African Swine Fever virus	*Ornithodoros porcinus*	SAT, host immunomodulation	Bernard et al., 2016
Flaviridae			
West Nile virus	*Ornithodoros moubata*	NVT	Lawrie et al., 2004
HARD TICKS (IXODIDAE)			
Orthomyxoviridae			
Thogoto virus	*Rhipicephalus appendiculatus*	SAT	Jones et al., 1989
		NVT	Jones et al., 1987
	Amblyomma variegatum	NVT	Jones et al., 1990a
	Rhipicephalus evertsi	NVT	Jones et al., 1992a
	Amblyomma hebraeum		
	Amblyomma cajannense		
	Hyalomma dromedarii		
	Hyalomma marginatum rufipes		
	Amblyomma variegatum		
	Boophilus microplus		
Flaviviridae			
TBEV	*Ixodes persulcatus*	NVT	Alekseev and Chunikhin, 1990b
	Ixodes ricinus	SAT	Alekseev et al., 1991
			Labuda et al., 1993c
		NVT	Labuda et al., 1993a,b
		SAT, host immunomodulation	Fialová et al., 2010
	Ixodes scapularis	SAT, host immunomodulation	Lieskovská et al., 2015
	Dermacentor reticulatus	SAT	Labuda et al., 1993c
	Dermacentor marginatus		Labuda et al., 1993a,c
	Rhipicephalus appendiculatus		
Louping ill virus	*Ixodes ricinus*	NVT	Jones et al., 1997
Powassan virus	*Ixodes scapularis*	SAT	Hermance and Thangamani, 2015
Bunyavirales			
CCHFV	*Hyalomma marginatum*	NVT	Gordon et al., 1993
Palma	*Rhipicephalus appendiculatus*	NVT	Labuda et al., 1997a
	Dermacentor marginatus		
Bhanja	*Rhipicephalus appendiculatus*	NVT	Labuda et al., 1997a
	Dermacentor marginatus		
Heartland virus	*Amblyomma americanum*	NVT	Godsey et al., 2016

Studies based on the THOV model demonstrated NVT for TBEV and *I. persulcatus, I. ricinus, Dermacentor marginatus, D. reticulatus,* and *R. appendiculatus* (Alekseev and Chunikhin, 1990b, 1991; Labuda et al., 1993c). To reproduce natural conditions of TBEV transmission, infected and uninfected *I. ricinus* ticks were allowed to co-feed on naïve wild rodents, the main natural hosts of immature stages. Acquisition of the virus was high in ticks feeding on susceptible *Apodemus* mice (*Apodemus flavicollis, A. agrarius*) that had undetectable or very low levels of viraemia. In contrast, co-feeding transmission was about four-times lower to ticks feeding on tick-resistant bank voles (*Clethrionomys glareolus*) that produced significantly higher viraemia and virus loads in lymph nodes and spleen than *Apodemus* mice (Labuda et al., 1993d).

Similar to THOV, transmission of flaviviruses is mediated by SAT factor(s) (Alekseev et al., 1991; Labuda et al., 1993b). For example, SAT was demonstrated when naïve guinea pigs were inoculated with a mixture of TBEV and SGE derived from partially fed uninfected *I. ricinus, D. reticulatus,* or *R. appendiculatus* females, compared with virus alone, and were infested with uninfected *R. appendiculatus* nymphs. Increased acquisition of the virus was observed in ticks feeding on animals inoculated with the mixture of SGE and virus (Labuda et al., 1993b). Enhancement of POWV transmission by SGE derived from *I. scapularis* has been documented recently; the efficiency of SAT was dependent on the inoculated virus dose (Hermance and Thangamani, 2015). Mice inoculated with a mixture of a high virus dose and SGE as well as with virus alone displayed severe

neurological signs of the disease. In contrast, severe clinical signs of the disease were observed in mice inoculated with a low dose of POWV plus SGE, whereas mice inoculated only with a low dose of the virus showed no signs of the disease and displayed low-level viraemia.

SAT has also been documented for bunyaviruses. Transmission of CCHFV from apparently non-viraemic ground-feeding birds to *Hyalomma marginatum rufipes* (Zeller et al., 1994) and between adult *Hyalomma truncatum* co-feeding on naïve rabbits (Gonzalez et al., 1992) was shown. Furthermore, low transmission rates occurred from infected adults of *Hyalomma* spp. to larvae and nymphs that co-fed on non-viraemic guinea pigs (Gordon et al., 1993). NVT appears to be an important amplification mechanism of CCHFV in nature (Bente et al., 2013). Transmission of Palma and Bhanja viruses on non-viraemic laboratory mice was shown by using various donor and recipient tick species (*D. marginatus, D. reticulatus, Rhipicephalus sanguineus, R. appendiculatus,* and *I. ricinus*) (Labuda et al., 1997a). Transmission of the newly described Heartland virus from experimentally infected *A. americanum* nymphs to co-feeding larvae has been documented recently (Godsey et al., 2016).

SAT has rarely been demonstrated in soft ticks. It was reported for the mosquito borne West Nile virus and *Ornithodoros moubata* (Lawrie et al., 2004). In a recent study, modulation of the systemic immune response of domestic pigs and of skin inflammation and cellular responses at the tick bite site by *Ornithodoros porcinus* SGE or feeding was reported. Pigs inoculated with a mixture of AFSV and *O. porcinus* SGE showed greater hyperthermia than pigs inoculated with the virus alone (Bernard et al., 2016).

Although knowledge of the frequency and significance of NVT under natural conditions is still limited, NVT appears to play an important role in the survival of TBV through reducing the pathological impact on the vertebrate host presumably because transmission is more efficient than classical viraemic transmission (Randolph et al., 1996; Labuda and Randolph, 1999; Nuttall and Labuda, 2003; Randolph, 2011).

Several species of wild-living mammals and birds that had not been exposed to TBEV and are known to be natural hosts of *I. ricinus*, were examined for their capacity to support NVT. Infected *I. ricinus* females and uninfected males and nymphs were allowed to co-feed on these animals and were subsequently tested for the presence of TBEV. The examined species differed in their ability to support NVT. While rodents, mainly *Apodemus* mice, were the most efficient amplifying hosts in spite of very low or no detectable viraemia, hedgehogs and pheasants either did not support NVT, or they supported it to a low level. NVT was also observed in bank voles, but their viraemia was higher compared to mice (Labuda et al., 1993d). In order to determine whether virus-immune wild rodents can participate in the transmission of TBEV, yellow necked mice and bank voles were immunized against TBEV and were infested with infected and uninfected *I. ricinus*. In spite of the presence of virus-specific neutralizing antibodies, these animals supported NVT, suggesting that hosts immune to TBEV can participate in the TBEV transmission cycle in nature (Labuda et al., 1997b). Additionally, species-specific differences in the dissemination of TBEV in skin of mice and voles after attachment of infected *I. ricinus* ticks were observed. Indeed, delayed dissemination of the virus from the attachment site of infected ticks to sites where uninfected ticks had fed was confirmed for bank voles but not for mice, partly explaining the difference in the capacity of the two rodent groups to support NVT (Labuda et al., 1996).

NVT plays an important role in the persistence of LIV in nature (Hudson et al., 1995). While red grouse (*Lagopus lagopus scoticus*) are not able to maintain the virus, LIV can persist through NVT in mountain hares (*Lepus timidus*) populations. Mountain hares that did not develop detectable viraemia were shown to support NVT of LIV between co-feeding infected and non-infected *I. ricinus* ticks; the efficiency of NVT was significantly reduced when ticks co-fed on virus-immune hares (Jones et al., 1997).

SAT appears to be correlated with the vector competence of certain tick species for particular viruses (Nuttall and Labuda, 2008). For example, SAT for THOV was demonstrated for *R. appendiculatus* and *A. variegatum* (natural vectors), but not for *I. ricinus* or soft ticks (non-competent vectors) (Jones et al., 1992a). In contrast, SAT for TBEV was observed in natural vectors, *I. persulcatus* and *I. ricinus* (Prostriata), but also in Metastriata species (Alekseev et al., 1991; Labuda et al., 1993b), although *I. persulcatus* and *I. ricinus* appeared to be more efficient donors and recipients of TBEV in NVT than Amblyomminae species (Alekseev and Chunikhin, 1992).

In contrast to the apparent vector specificity of SAT, *D. reticulatus* SGE was found to promote the replication of the insect -borne vesicular stomatitis virus *in vitro* (Hajnická et al., 1998), and the production of the nucleocapsid viral protein (Kocáková et al., 1999; Sláviková et al., 2002) by a yet unexplained mechanism.

Toward Identification of Mediators of SAT

The reported cases of SAT do not provide explanations for the molecular mechanisms involved in TBV transmission. During the last two decades, modern molecular-genetic and high-throughput techniques have been applied in the systemic characterization of tick salivary components, enabling elucidation of the underlying molecular mechanisms of exploitation of tick salivary molecules by tick-borne-pathogens (Liu and Bonnet, 2014; Chmelař et al., 2016a,b). Studies on the sialotranscriptome of *I. scapularis* (Valenzuela et al., 2002; Ribeiro et al., 2006) and the *I. scapularis* genome project (Gulia-Nuss et al., 2016) demonstrated the complexity and the redundancy in saliva protein functions within gene families. A wide range of bioactive proteins have been discovered in tick saliva and different expression profiles for a number of genes, depending on the presence or absence of a microorganism, have been described in various tick tissues, including SG (Chmelař et al., 2016a). However, research in this field is more advanced for the tick - tick-borne bacteria interactions than for TBV (e.g., Kazimírová and Štibrániová, 2013; Liu and Bonnet, 2014; de la Fuente et al., 2016b). One of the reasons may be the high pathogenicity of TBV of medical and veterinary importance that require strict conditions for their handling and usage in

animal experimentation (e.g., laboratories and animal facilities of biosafety levels 3 and 4). Considering these constraints, usage of less pathogenic models as surrogates for highly pathogenic TBV and research on cell lines offer alternative tools to investigate the processes at the tick—TBV interface.

Until recently, the SG transcript expression profile in response to infection with a TBV has only been described for *I. scapularis* nymphs infected with LGTV (McNally et al., 2012). The study demonstrated that in nymphs feeding for 3 days on naïve mice the number of transcripts associated with metabolism increased in comparison to unfed ticks. A total of 578 transcripts were upregulated and 151 transcripts were downregulated in response to feeding. Differences in expression profiles were revealed also between LGTV-infected and uninfected ticks during the 3 days feeding period. The differently regulated transcripts included putative secreted proteins, lipocalins, Kunitz domain-containing proteins, anti-microbial peptides, and transcripts of unknown function (McNally et al., 2012). A transcript upregulated in LGTV-infected nymphs that belonged to the 5.3 kDa family was previously found to be upregulated in *Borrelia burgdorferi*-infected *I. scapularis* nymphs, suggesting that the protein might play a role in tick immunity or host defense (Ribeiro et al., 2006). However, the specific proteins associated with TBV replication and transmission still need to be identified.

The mechanisms of adaptation of TBV to their vectors and hosts are other important aspects that need to be considered to understand TBV transmission. Specific mutations in the viral envelope protein of TBEV have been found to affect NVT between co-feeding *I. ricinus* for Siberian and European TBEV strains (Khasnatinov et al., 2010). Furthermore, it has recently been demonstrated that the structural genes of the European TBEV strain Hypr may determine high NVT rates of the virus between co-feeding *I. ricinus* ticks, whereas the region of the TBEV genome encoding non-structural proteins determines cytotoxicity in cultured mammalian cells (Khasnatinov et al., 2016).

IMMUNOMODULATION OF HOST IMMUNE CELLS AT THE TICK ATTACHMENT SITE—A PREREQUISITE FOR TBV TRANSMISSION

The redundant host defense mechanisms in the skin pose a significant threat to successful tick feeding; however, tick saliva contains an array of pharmacologically active compounds that are vital to overcoming haemostasis, wound healing, and innate and adaptive immune responses of the host. Among the repertoire of bioactive tick salivary molecules are inhibitors of the pain and itch response, anticoagulants, antiplatelet components, vasodilators, and immunomodulators, all of which have been extensively highlighted in several comprehensive reviews (Ribeiro and Francischetti, 2003; Ribeiro et al., 2006; Francischetti et al., 2009; Kazimírová and Štibrániová, 2013; Wikel, 2013). As a tick feeds, salivation is not a continuous process (Kaufman, 1989). Expression of a plethora of tick salivary proteins was found to be differentially up- or downregulated during blood feeding, and differences in saliva composition exist across and within tick genera (McSwain et al., 1982; Ribeiro et al., 2006; Alarcon-Chaidez et al., 2007; Vančová et al., 2007, 2010a; Mans et al., 2008; Peterková et al., 2008; Francischetti et al., 2009; Kazimírová and Štibrániová, 2013; Wikel, 2013). Thus, the composition of tick saliva is dynamic and complex so that it may overcome the many redundancies inherent to the host defenses (Kazimírová and Štibrániová, 2013; Kotál et al., 2015).

Ticks are able to modulate cutaneous as well as systemic immune defenses of their hosts that involve keratinocytes, natural killer (NK) cells, dendritic cells (DCs), T cell subpopulations (Th1, Th2, Th17, T regulatory cells), B cells, neutrophils, mast cells, basophils, endothelial cells, cytokines, chemokines, complement, and extracellular matrix (Kazimírová and Štibrániová, 2013; Štibrániová et al., 2013; Wikel, 2013; Heinze et al., 2014) (**Figure 1**). The general pattern of tick infestation- or tick saliva-induced immunomodulation consists of downregulation of Th1 cytokines and upregulation of Th2 cytokines leading to suppression of host antibody responses. The dynamic balance between host immunity and tick immunomodulation has been found to affect both tick feeding and pathogen transmission (Bowman et al., 1997; Ramamoorthi et al., 2005; Brossard and Wikel, 2008; Nuttall and Labuda, 2008; Wikel, 2013).

Skin is the first host organ that TBV and tick saliva encounter during the tick feeding process. In addition to serving as the host's primary line of defense from the outside environment (Nestle et al., 2009), skin is also the interface for tick-virus-host interactions (Nuttall and Labuda, 2004; Wikel, 2013). Thus, cutaneous immune cells play a crucial role in the initial response of the host to tick feeding and invading pathogenic microorganisms, including viruses (Labuda et al., 1996; Frischknecht, 2007; Wikel, 2013, 2014; Hermance and Thangamani, 2014; Bernard et al., 2015; Hermance et al., 2016). Penetration through the skin brings tick mouthparts into contact with keratinocytes, which possess receptors of innate immune responses, antimicrobial peptides, and pro-inflammatory cytokines (Merad et al., 2008; Martinon et al., 2009; Nestle et al., 2009). TBV delivered into the skin also encounter different cell types, including rich DC networks and neutrophils, which are involved in pathogen elimination during the early stages of infection (Labuda et al., 1996; Wu et al., 2000; Robertson et al., 2009). TBV were found to replicate at the tick bite site within keratinocytes, dermal macrophages, Langerhans' DCs, and neutrophils (Wikel et al., 1994; Labuda et al., 1996; Wu et al., 2000; Ho et al., 2001; Libraty et al., 2001; Marovich et al., 2001).

Modulation of Dendritic Cell Functions

Recognition of TBV by immature DCs occurs via pattern recognition receptor (PRRs) systems such as Toll-like receptors (TLRs) located at the cell surface and within endosomes, or the retinoic acid-inducible gene I (*Rig-1*)-like helicases (RLHs) detecting nucleic acids within the cytosol (Kochs et al., 2010). DCs are known to take up viral antigens which results in DC activation and their migration to local lymphoid tissues. As DCs are the key players in the induction of protective immunity to viral infection, tick salivary molecules that modulate

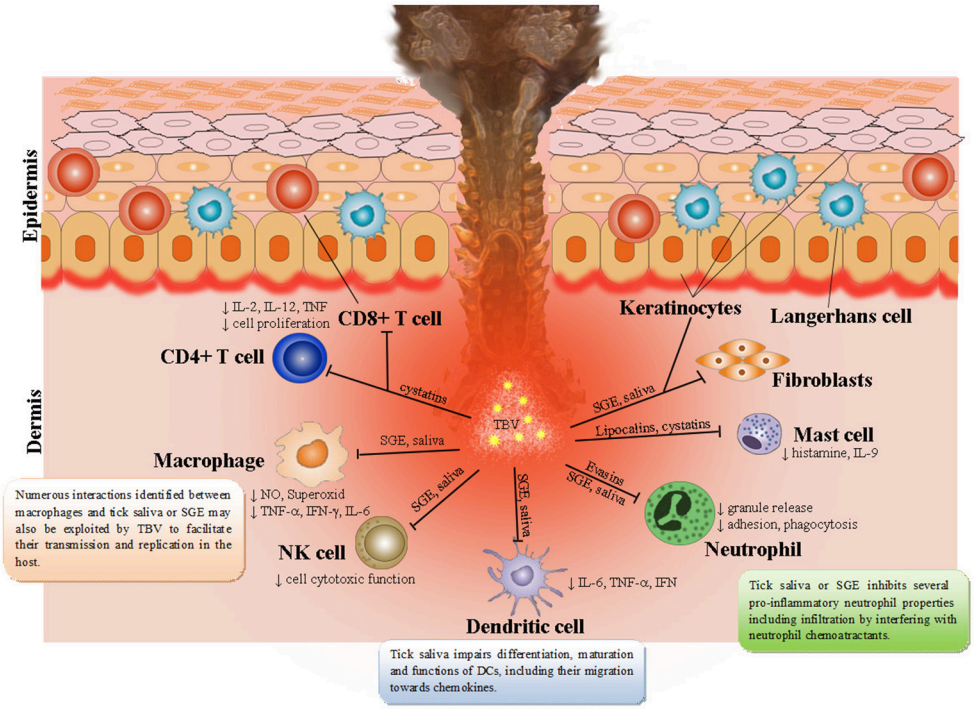

FIGURE 1 | Tick saliva contains a broad spectrum of pharmacologically-active molecules affecting various immune cell populations. Skin is the key interface for tick-virus-host interactions. Resident skin cells—keratinocytes, Langerhans cells (epidermal dendritic cells), dendritic cells, T cells, macrophages, fibroblasts, and endothelial cells are immediately activated after first contact with tick saliva, hypostome, and TBVs. By producing and releasing a wide range of pro-inflammatory chemokines and cytokines they recruit other immune cells, such as neutrophils, T cells, and B cells into the tick attachment site. Tick saliva modulates immune responses to facilitate feeding and consequently facilitate transmission of TBVs, which target and replicate in different skin cells including keratinocytes, dermal macrophages, Langerhans' DCs, and neutrophils.

DC functions are probably exploited by viruses to circumvent host immune responses. During the early phase of infection, a virus replicates within the dermis and subsequently in the skin draining lymph nodes. Activation of DCs confers their ability to activate naïve T cells into T helper type 1 (Th1), Th2, and cytotoxic T lymphocyte (CTL) effector cells. This interaction activates signaling pathways that lead to increased expression of major histocompatibility complex (MHC) class II molecules (required for antigen presentation), T cell co-stimulatory molecules (i.e., CD80 and CD86), and proinflammatory cytokines, such as type I interferon (IFN), interleukin (IL) 6, and IL12, which drive anti-viral Th1 responses (Johnston et al., 2000; Masson et al., 2008).

Tumor necrosis factor (TNF)-alpha is a powerful cytokine secreted by several cell types after viral infection. Together with IL1, TNF-alpha is known to promote DC migration from the skin into regional lymph nodes. TBEV infection was found to induce the release of TNF-alpha and IL6 by DCs, but undetectable levels of IL10 and IL12p70 were measured in DC cultures infected with the virulent Hypr strain in contrast to DCs infected with the less virulent Neudoerfl strain (Fialová et al., 2010). Treatment with *I. ricinus* saliva was found to prolong the survival of TBEV-infected DCs, and suppress the levels of TNF-alpha and IL6, but at the same time, bystander DCs kept the immature phenotype as assessed by low expression of B7-2 and MHC class II molecules (Fialová et al., 2010). Similar results were previously reported for uninfected DCs treated with *I. ricinus* saliva (Sá-Nunes et al., 2007; Hovius et al., 2008). It has been proposed that *I. ricinus* saliva impairs maturation of murine DCs through affecting TLR3, TLR7, TLR9, or CD40 ligation, and reduced TBEV-mediated DCs apoptosis (Skallová et al., 2008). The findings may suggest that in the presence of tick saliva, DCs keep a less mature phenotype and therefore remain permissive for TBEV. On the other hand, tick saliva does not affect virus-induced upregulation of MHC class II and B7-2 molecules.

Differentiation, maturation, and functions of DCs were found to be impaired by *R. sanguineus* saliva (Cavassani et al., 2005) and prostaglandin (PG)E2 from *I. scapularis* saliva (Sá-Nunes et al., 2007). Co-incubation of DCs with *R. sanguineus* saliva promoted attenuation of antigen-specific T cells cytokine production stimulated by DCs (Oliveira et al., 2008). Moreover, saliva of *R. sanguineus* impaired the maturation of DCs stimulated with lipopolysaccharide (LPS), a TLR-4 ligand, by inhibition of the activation of the ERK 1/2 and p38 MAP kinases, leading to increased production of IL10 and reduced synthesis of IL12p70 and TNF-alpha (Oliveira et al., 2010). The above observations suggest that in the presence of tick saliva infected DCs may stay in the skin for a prolonged time and serve as a further source of the

virus, which may, together with the saliva-induced impairment of DC migration, enhance NVT.

Tick saliva was also found to inhibit the chemotactic functions of chemokines and selectively impair chemotaxis of immature DCs by downregulating cell-surface receptors. Saliva of *R. sanguineus* inhibits immature DC migration in response to CCL3 (migration via receptors CCR1 or CCR5), to CCL4 (MIP-1 alpha) (via CCR1), and to CCL5 (RANTES) (migration via CCR1, CCR3, CCR5) (Oliveira et al., 2008). Evasin-1 (derived from *R. sanguineus*) is also able to bind to human CCL3 and mouse CCL3 (Dias et al., 2009). Two salivary cystatins (cysteine protease inhibitors) derived from *I. scapularis* have been shown to inhibit cathepsins L and S, impair inflammation, and suppress DC maturation (Kotsyfakis et al., 2006, 2008; Sá-Nunes et al., 2009). As a result, saliva of ticks can impair early migration of DCs from inflamed skin.

Reduction in the number of DCs was observed around the attachment sites of *D. andersoni* ticks, suggesting that migration of Langerhans cells to lymph nodes occurs after contact with tick salivary components and T cell responses. *In vitro* treatment of DCs from the lymph nodes of tick-bite sensitized tick-resistant guinea pigs with tick saliva induced T cell proliferation (Nithiuthai and Allen, 1985), and co-incubation of DCs with tick saliva lead to attenuation of antigen-specific T cells cytokine production stimulated by DCs (Oliveira et al., 2008). In addition, density and recruitment of Langerhans cells were inhibited by inoculation of SGE or feeding of *O. porcinus* in the skin of domestic pigs infected with ASFV, demonstrating immunomodulatory capacities also for soft tick saliva (Bernard et al., 2016).

A novel mechanism of immunomodulation, potentially facilitating pathogen transmission, has been discovered by Preston et al. (2013). Japanin, a new member of saliva lipocalins from *R. appendiculatus* ticks, was found to specifically reprogram DC responses to a wide variety of *in vitro* stimuli. Japanin was found to alter the expression of co-stimulatory and co-inhibitory transmembrane molecules, modulate secretion of pro-inflammatory, anti-inflammatory, and T cell polarizing cytokines, and also inhibit the differentiation of DCs from monocytes. Based on these findings it was suggested that the failure of DCs to mature in response to viral or tick immunomodulators has important implications for induction of effective antiviral T cell mediated immunity, i.e., it may lead to an aberrant anti-viral immune response and ineffective virus clearance.

Modulation of Interferon Signaling

Although DCs represent an early target of TBV infection, they are major producers of IFN. It has been shown that both early DC and IFN responses are modulated by viruses (Best et al., 2005), but also by tick salivary immunomodulatory compounds. Generally, following virus infection, the host cells deploy the rapid response to limit virus replication in both infected cells and in neighboring cells. Indeed, the IFN-dependent innate immune response is essential for protection against flavivirus infections, whereby type I IFN (including multiple IFN-alpha molecules and IFN-beta) have a central role (Akira et al., 2006; Kawai and Akira, 2006). Although type I IFN signaling is recognized as an important component of antiviral innate immunity, previous studies indicate that its role during vector-borne flavivirus infection is complex and varies, depending on the virus species. Type I and II IFN were found to inhibit flavivirus infection in cell culture and in animals. Type I IFN (alpha or beta) blocks flavivirus infection by preventing translation and replication of infectious viral RNA, which occurs at least partially through an RNAse L, Mx1, and protein kinase (PKR)-independent mechanism. Mx1 and MxA proteins have been determined as the innate resistance factors in mammalian cells against tick-borne orthomyxoviruses (THOV and Batken virus) (Halle et al., 1995; Frese et al., 1997) and bunyaviruses (CCHFV and Dugbe virus) (Andersson et al., 2004; Bridgen et al., 2004). Possible manipulation of IFN signaling by tick SGE was indicated by Dessens and Nuttall (1998) who demonstrated THOV transmission to uninfected ticks feeding on Mx1 A2G mice (a strain resistant to infection) following needle- or tick-borne virus challenge, probably thanks to *Mx1* gene manipulation after injection of virus mixed with tick SGE.

LGTV, a member of the TBEV complex, is sensitive to the antiviral effects of IFN. LGTV-mediated inhibition of JAK-STAT signaling as well as interactions between NS5 and IFNAR2, were demonstrated in infected human monocyte-derived DCs. Non-structural NS5 protein blocked STAT1 phosphorylation in response to either IFN-alpha or IFN-gamma. An association was observed between NS5 and both the IFN-alpha/beta receptor subunit, IFNAR2 (IFNAR2-2 or IFNAR2c), and the IFN-gamma receptor subunit, IFNGR1 (Best et al., 2005). However, arboviruses are generally not recognized as strong inducers of IFN-alpha/beta, with one exception, vesicular stomatitis virus, an insect-borne rhabdovirus. Using this virus, Hajnická et al. (1998, 2000) were the first who provided evidence that SGE from partially fed adult *R. appendiculatus* or *D. reticulatus* increased viral yields by 100- to 1,000- fold in mouse cell cultures. The effect appeared to be due to inhibition of the antiviral effect of IFN by SG factors, possibly acting through the IFN-alpha/beta receptor rather than directly affecting IFN.

The recently observed enhanced replication of TBEV in bone marrow DCs in the presence of *I. scapularis* sialostatin L2 is probably a consequence of impaired IFN-beta signaling (Lieskovská et al., 2015). Both sialostatin L and sialostatin L2 decreased STAT-1 and STAT-2 phosphorylation, and inhibited IFN stimulated genes, *Irf-7* and *Ip-10* in LPS-stimulated DCs. The inhibitory effect of tick cystatin on IFN responses in host DCs appears to be a novel mechanism by which tick saliva assists in the transmission of TBV.

Immune IFN, known as type II IFN or IFN-gamma, is secreted mostly by activated NK cells and macrophages during the early stages of infection (Malmgaard, 2004; Darwich et al., 2009). During later stages of infection, IFN-gamma is produced by activated T lymphocytes (Boehm et al., 1997) in answer to receptor-mediated stimulation (through T cell receptors or NK cell receptors) or in response to early produced cytokines, such as IL12, IL18, and IFN-alpha/beta (Darwich et al., 2009). Type II IFN (IFN-gamma) inhibits flavivirus replication via the generation of pro-inflammatory and antiviral

molecules including nitric oxide (NO). Antiviral activity is not the primary biological function of IFN-gamma. However, through stimulation of the activation of macrophages and increasing the expression of MHC for more effective antigen presentation, IFN-gamma can enhance cell-mediated immune responses that are critical for the development of immunity against intracellular pathogens. It was shown that SGE of *I. ricinus* reduced polyinosinic-polycytidylic acid (poly IC)-induced production of IFN-alpha, IFN-beta, and IFN-gamma (Kopecký and Kuthejlová, 1998) and SGE of female *D. reticulatus* inhibited antiviral effects of IFN-alpha and IFN-beta produced by mouse fibroblasts (Hajnická et al., 2000). SGE from 5-days fed *D. reticulatus* and *I. ricinus* females were shown to inhibit ConA stimulated IFN-gamma production by mouse splenocytes (Vančová, unpublished).

A variety of viruses and different TLR agonists can stimulate Type III IFN (IFN-lambda) gene expression in a similar manner as the expression of type I IFN genes that is induced by transcriptional mechanisms involving IRF's and NF-κB (Onoguchi et al., 2007; Osterlund et al., 2007). Among skin cell populations, keratinocytes and melanocytes, but not fibroblasts, endothelial cells or subcutaneous adipocytes, are targets of IFN-lambda (Witte et al., 2009). Keratinocytes are cells that produce and respond to type III IFN (Odendall et al., 2014). IFN-lambda probably acts primarily as a protection of mucosal entities, e.g., in the lung, skin, or digestive tract (Hermant and Michiels, 2014). According to results provided by Lim et al. (2011), Limon-Flores et al. (2005) and Surasombatpattana et al. (2011), keratinocytes were proposed as key players of early arboviral infection capable of producing high levels of infectious virus in the skin favoring viral dissemination to the entire body.

Modulation of Macrophage Functions

Macrophages are potential targets for TBV. Infection of macrophages with flaviviruses leads to production of NO, which inhibits virus replication (Plekhova et al., 2008). However, the exact roles of NO produced by macrophages in the context of TBV infection remain to be elucidated. It has been demonstrated that mononuclear/macrophage lineages are important sources of local TBEV replication before viraemia occurs (Dörrbecker et al., 2010). Thus, modulation of macrophage functions by tick saliva may also be exploited by TBV to facilitate their transmission and replication in the host. Resident macrophages in the skin act as antigen-presenting cells that elicit a potent proliferative response during secondary tick infestation. Macrophages recruit in increased numbers to the site of injury in response to inflammatory and immune stimulation, and produce cytokines and chemokines that attract inflammatory cells to the tick bite site. The tick macrophage migration inhibitor factor, MIF, identified in SG of *A. americanum*, might impair macrophage functions during virus infection (Jaworski et al., 2001). Moreover, tick saliva was shown to decrease the oxidative activity of mouse macrophages (Kuthejlová et al., 2001).

Modulation of Neutrophil Functions

In addition to DCs and macrophages, neutrophils are recruited to the site of TBV infection (Dörrbecker et al., 2010; Hermance et al., 2016). Neutrophils probably play a role in complementing the cytokine and chemokine responses soon after TBV infection. They may also be involved in the peripheral spread of TBV. However, at the present stage of knowledge we can only speculate about the exploitation of tick saliva neutrophil inhibitors by TBV.

At the tick attachment site, neutrophils are activated by thrombin from the blood-coagulation cascade, by platelet-activating factor, by releasing of proteases modulating platelet function, such as cathepsin G, and/or enzymes that act on tissue matrix, like elastase. Neutrophils are the most abundant cells in the acute inflammatory infiltrate induced by primary tick infestation, but not during subsequent infestations, at least not by all tick species and not in all tick—host associations (Brown, 1982; Brown et al., 1983, 1984; Gill and Walker, 1985). Ticks were found to generate a neutrophil chemotactic factor in their saliva by cleavage of C5 (Berenberg et al., 1972).

Neutrophil infiltration and activation is orchestrated by chemokines such as CCL3, CXCL8/KC. It has been demonstrated that SGE of different hard tick species effectively bind and block in action a broad spectrum of pro-inflammatory cytokines and chemokines. A number of tick species were shown to possess anti CXCL8 activity mediated by one or more molecules (Hajnická et al., 2005, 2001; Vančová et al., 2010b). Inhibition of CXCL8-coordinated neutrophil migration due to inhibition of CXCL8-binding to the cell receptors was demonstrated for *D. reticulatus* SGE (Kocáková et al., 2003). Evasin-1 and Evasin-3 were identified as potent inhibitors of CCL3 and/or CXCL8-induced recruitment of human and murine neutrophils (Déruaz et al., 2008).

Wound Healing

Many tick saliva molecules are involved in modulation of epithelial wound healing and vasculature repair, including cytoskeletal elements (Maxwell et al., 2005; Heinze et al., 2012). Wound-healing events, initiated by haemostasis, are orchestrated by cytokines, chemokines, and growth factors (Behm et al., 2011). Platelets, macrophages, fibroblasts, and keratinocytes release growth factors that initiate a downstream response to promote wound healing. Hypoxic milieu in the wound results in reactive oxygen species (ROS) production and cytokine release. Fibroblast growth factor 7 (FGF-7)-activated peroxiredoxin-6 and Nrf-2 transcription factor protect cells, especially keratinocytes and macrophages, from ROS-induced damage. FGF-2, 7, and 10 are essential in the proliferative phase of wound healing, neoangiogenesis, and re-epithelization. IL1 and IL6 are important in inflammation, angiogenesis, and keratinocyte migration; they affect tissue remodeling by regulation of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs. IL1 molecules are among the first signaling molecules released by keratinocytes and leukocytes in response to disruption of the epidermal barrier. IL1, IL6, and TNF-alpha-activated hepatocyte growth factor (HGF) production in fibroblasts increase tissue granulation, neoangiogenesis, and re-epithelization (Toyoda et al., 2001). IL6, produced by fibroblasts, macrophages, endothelial cells, and keratinocytes play important roles in all steps of wound healing. Through downstream mechanisms, IL6 induces neutrophil and macrophages infiltration, collagen

deposition, angiogenesis, and epidermal cell proliferation. Re-epithelization in wound healing is enhanced by the epidermal growth factor family (EGF). Activated macrophages release platelet-derived growth factor (PDGF) and TGF-beta1 which attract leukocytes, fibroblasts and smooth muscle cells to the wound site in the skin. Infiltration of leukocytes in inflammatory tissues is mediated by the intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule -1 (VCAM- 1). Significant down-regulation of ICAM-1 expression by SGE of *D. andersoni* ticks, and significant reduction of VCAM-1 expression by *I. scapularis* SGE were described (Maxwell et al., 2005). Leukocytes and monocytes actively produce growth factors that prepare the wound for the proliferative phase, when fibroblasts and endothelial cells are recruited. TGF-beta1 that controls signals of fibroblast functions is produced by activated platelets, macrophages and T lymphocytes and affects extracellular matrix deposition, and increases collagen, proteoglycans and fibronectin gene transcription. Furthermore, TGF-beta1 stimulates the tissue metalloprotease inhibitor, and other cytokines (interleukins, fibroblast growth factor FGF, TNF-beta3). TGF-beta1 binding activity, as well as other growth factor binding activities (PDGF, HGF, FGF2) have been detected in saliva of *D. reticulatus, R. appendiculatus, I. ricinus, I. scapularis, A. variegatum,* and *H. excavatum* ticks (Hajnická et al., 2011; Slovák et al., 2014b). Kramer et al. (2011) identified a stimulating effect of *D. variabilis* saliva on basal-and PDGF-stimulated migration of macrophage derived cell line IC-21. In the inflammatory phase of wound healing and angiogenesis, macrophages may transform to produce proliferative mediators in response to IL4 released by mast cells and leukocytes. This switch stimulates collagen synthesis and fibroblast proliferation. Feeding of *D. andersoni* was found to regulate cell signaling, phagocytosis and gene expression, and skewed the immune response toward a Th2 profile, which is characterized by production of anti-inflammatory cytokines IL4 and IL10 (Kramer et al., 2011). Interleukins and TGF-beta are crucial regulators of MMPs that are important for matrix remodeling and angiogenesis (Boniface et al., 2005; Lamar et al., 2008). Chemokines produced by keratinocytes (CXCL11) and by neovascular endothelium (CXCL10) (IP10) are crucial in signaling of the regenerative wound healing phase. Both interact with CXCR3; activation of CXCR3 signaling converts fibroblast from migratory to a contractile state following maturation of collagen fibers (Satish et al., 2003). Modulation of the wound healing processes by tick bioactive compounds may also be exploited by TBVs.

EARLY HOST CUTANEOUS CHANGES AT THE TICK ATTACHMENT SITE

Several studies have used cutaneous feeding site lesions from uninfected ticks to examine the tick-induced changes in cutaneous gene expression and histopathology during the early stages of uninfected tick feeding. Early transcriptional and histopathological changes at the feeding site of uninfected *I. scapularis* nymphs are initially characterized by modulation of host responses in resident cells, followed by progression to a neutrophil-dominated immune response (Heinze et al., 2012). When the cutaneous immune responses and histopathology were analyzed during uninfected *D. andersoni* nymph feeding, chemotaxis of neutrophils and monocytes into the feeding site and keratin-based wound healing responses were prominent (Heinze et al., 2014). During the early phase of primary infestation by *D. andersoni,* significant upregulation of the genes for chemokines (*Ccr1, Ccl2, Ccl6, Ccl7, Ccl12, Cxcl1, Cxcl2,* and *Cxcl4/Pfx4*), cytokines (*Il1b*) and anti-microbial molecules, and downregulation of genes related to DNA repair, transcription, chromatin remodeling, transcription factor binding, RNA splicing, and mRNA metabolism was demonstrated (Heinze et al., 2014). In addition, upregulation was found for the genes for *Nfkbia* and *Tsc22d3* which inhibit NF-κB and AP-1 pro-inflammatory pathways (Heinze et al., 2014). NF-κB and NFAT were previously identified as two of the most important factors coordinating mechanisms of viral evasion by regulation of pro-inflammatory molecules and cytokines which evoke inflammatory responses and recruitment of immune cells (Kopp and Ghosh, 1995). Moreover, during early and late primary *D. andersoni* infestation, murine host genes (*Cyr61*, *SMAD5*, *TNFrsf 12*, *Junb, Epgnc*) that may be related to TNF-alpha, AP-1, and growth factor responses at the tick bite site, were upregulated while genes encoding cytoskeletal elements (*collagen type 1 gene, laminin beta2*), signaling molecules, growth factor receptor (*Pdgfrb*), or growth factor (*Tgfb3*) were downregulated (Heinze et al., 2014). The experiments with cutaneous feeding site lesions from uninfected ticks set the stage for studying the role of localized skin infection and the cutaneous immune response during virus-infected tick feeding.

Cutaneous Immune Response to Tick-Borne Flavivirus-Infected Tick Feeding

Due to the fact that flaviviruses can be transmitted within 15 min of tick attachment (Ebel and Kramer, 2004), attention has been focused on the early stages of tick feeding and TBV transmission. It has long been suspected that localized immunomodulation induced by tick saliva and the cellular infiltrates recruited to the tick feeding site can facilitate TBV replication and transmission, however, there are a limited number of studies that have directly investigated this phenomenon *in vivo*. Prior to gene expression analysis conducted at the POWV-infected *I. scapularis* tick feeding site, no study had used an *in vivo* model to characterize the host's cutaneous immune response during the early stages of TBV transmission. Comparative gene expression analysis between POWV-infected and uninfected *I. scapularis* tick feeding sites was performed at 3 and 6 h after tick attachment (hours post-infection, hpi). After 3 h of POWV-infected tick feeding, cutaneous gene expression analysis revealed a complex proinflammatory environment, which included significant upregulation of proinflammatory cytokines related to neutrophil and phagocyte recruitment, migration, and accumulation (Hermance and Thangamani, 2014). In contrast to the 3 hpi time point, the majority of significantly modulated genes at 6 hpi were down-regulated,

including several proinflammatory cytokines associated with the inflammatory response reaction, suggesting decreased recruitment of granulocytes at the later time point (Hermance and Thangamani, 2014). These data suggest that POWV-infected tick feeding recruits immune cells earlier than uninfected tick feeding.

The murine cutaneous immune response during the early stages of POWV-infected tick feeding was further examined by immunophenotyping infiltrating immune cells and identifying cell targets of POWV infection at the *I. scapularis* feeding site (time points ≤24 hpi). The most distinct histopathological difference between the POWV-infected vs. uninfected tick feeding sites was observed at 3 hpi, when higher levels of cellular infiltrates (mostly neutrophils and some mononuclear cells) were detected at the POWV-infected tick feeding sites compared to the uninfected feeding sites (Hermance et al., 2016). These histopathological findings correlate with gene expression analysis, and together the results demonstrate that neutrophil and mononuclear cell infiltrates are recruited earlier to the feeding site of a POWV-infected tick vs. an uninfected tick (Hermance et al., 2016). Furthermore, POWV antigen was detected in macrophages and fibroblasts located at the tick feeding site, which suggests that these cells are early targets of infection (Hermance et al., 2016). No prior studies used an *in vivo* tick feeding model to report on immune cell targets of infection at the skin interface and the cutaneous immune response during the early hours of tick-borne virus transmission. These findings highlight the complexity of the initial interactions between the host immune response and early tick-mediated immunomodulation, all of which initially occur at the skin interface.

Localized Skin Infection during the Early Transmission of Tick-Borne Flavivirus

The tick attachment and feeding site plays a crucial role in establishing a focus of viral replication during early virus transmission and establishment in the host. This phenomenon was first demonstrated with TBEV where conditions mimicking natural TBEV transmission were incorporated into the experimental design by allowing infected and uninfected *I. ricinus* ticks to co-feed on the same murine host (Labuda et al., 1996). These experiments demonstrated that TBEV is preferentially recruited to tick-infested skin sites compared to uninfested skin sites, and co-feeding TBEV transmission is dependent on localized skin infection at tick feeding sites as opposed to an overt viremia (Labuda et al., 1996). Furthermore, *ex vivo* data from this study suggests that immune cells infiltrating the skin site during tick feeding, and subsequently migrating from such sites, serve as vehicles for TBEV transmission between co-feeding ticks, a process independent of a systemic viremia (Labuda et al., 1996).

Certain immune cells are likely involved in virus dissemination as they emigrate from the skin site of tick feeding. Langerhans cells are the main DC subpopulation in the epidermis, and their major function is to capture antigens in the epidermis and migrate to skin-draining lymphoid tissues where the appropriate immune response is initiated. Langerhans cell migration to draining lymph nodes has been demonstrated in response to cutaneous infections with arboviruses such as West Nile virus and Semliki Forest virus (Johnston et al., 2000). Consequently, in the *ex vivo* experiments conducted by Labuda et al. (1996), the presence of TBE viral antigen in emigrating Langerhans cells suggests that these cells serve as vehicles for TBEV transportation to the lymphatic system, a phenomenon that contributes to overall viral dissemination. The importance of virus-infected cells at the tick feeding site and their contribution to initial viral replication and dissemination was further supported by *in vitro* experiments where *I. ricinus* tick saliva was shown to modulate TBEV infection of dendritic cells. Specifically, when DCs were cultured with TBEV in the presence of *I. ricinus* saliva, the infection rate of the cells was enhanced and there was a decrease in virus-induced TNF- alpha and IL6 production (Fialová et al., 2010). Together these studies illustrate the important role of localized skin infection during the early stages of tick-borne flavivirus transmission.

VACCINES

Globally, the epidemiological impact of TBV infections is small in the context of infectious diseases. This is one reason why there are comparatively few vaccines available for controlling tick-borne viral diseases. There is also the challenge of developing vaccines effective against topological variants of the diverse strains of given viral species. One approach to overcoming this challenge is to develop vaccines that target important tick vector species in such a way that they interfere with the transmission of all tick-borne viruses. Here we review briefly the current state of anti-tick vaccines, and consider future prospects.

An anti-tick vaccine has been marketed since 1994 under the trade name TickGARD; a Cuban version is marketed as Gavac (Willadsen, 2004). The vaccines derive from Bm86/Bm95, midgut antigens of the cattle tick, *R.* (*B.*) *microplus*. They work by eliciting antibodies in immunized animals. When taken up in the bloodmeal of feeding ticks, the antibodies bind to the antigen resulting in damage to the midgut. The consequent impact on feeding success and reproductive output causes a gradual reduction in tick numbers and tick-borne infections (de la Fuente et al., 2007). Despite many attempts to develop a more efficacious vaccine, none has been commercialized.

Development of anti-tick vaccines is driven by the need to control tick infestations of livestock and the diseases caused by tick-borne pathogens, and the increasing resistance of cattle tick populations to commonly used acaricides (Schetters et al., 2016). Considerable effort is directed against the cattle tick [*R.* (*B.*) *microplus*] although other species (e.g., *H. longicornis*, *H. anatolicum*, *I. ricinus*) are being investigated. Most strategies favor the "hidden" or "concealed" antigen approach, as illustrated by the Bm86/Bm95-derived anti-tick vaccines. These are antigens not normally exposed to the host immune system during blood feeding. The most promising candidates include subolesin, ferritins, and aquaporins. Tick subolesin is functionally related to mammalian akirin-2, a downstream effector of the Toll-like receptor required in the innate immune response. A combination

of subolesin and Bm86 has been patented as a more effective formulation for controlling cattle tick infestations (Schetters and Jansen, 2014). Ferritins help ticks cope with the potentially toxic heme from the bloodmeal. Ferritin 2 (Fer2) is a target for vaccine development because it is expressed in the midgut where it mediates transportation of nonheme iron to peripheral tissues, and it is not found in mammals (Hajdusek et al., 2009). Vaccination of cattle with recombinant cattle tick Fer2 elicited protection at least comparable to the Bm86 control antigen (Hajdusek et al., 2010). Aquaporins are integral membrane proteins that serve as channels for the transfer of water across cell membranes. Ticks use aquaporins to remove water from the bloodmeal, a critical process for osmoregulation (Campbell et al., 2010). A recombinant cattle tick aquaporin provided >65% efficacy in two cattle pen trials of a vaccine formulation (Guerrero et al., 2014). However, aquaporins are ubiquitous hence extensive safety testing is needed before they can be licensed in vaccines. Moreover, induced immunity to concealed antigens takes time to act and is usually short-lived.

The alternative "exposed" antigen approach targets proteins or peptides secreted by ticks when they feed and which therefore are exposed to the immune response of the tick-infested host. Several such exposed antigens have been evaluated as recombinant protein anti-tick vaccines with disappointing results (Nuttall et al., 2006; Olds et al., 2016). The concern about this approach is the remarkable diversity (so-called "redundancy") of tick saliva proteins and the likelihood that immune selection pressure on the targeted secreted antigen results in ticks overcoming the vaccine effect.

A third strategy for anti-tick vaccine development is the "dual action" approach involving a secreted ("exposed") antigen that cross-reacts with a midgut ("concealed") antigen (Nuttall et al., 2006). This strategy benefits from the boosting effect of a conserved secreted antigen (the feeding tick elicits an anamnestic response in a vaccinated host), hence inducing long-lasting immunity, while damaging the tick midgut. At least two antigens have been shown to have this dual action: 64TRP cement protein antigen from *R. appendiculatus* and OmC2 cysteine peptidase inhibitor from *O. moubata* (Trimnell et al., 2002; Salát et al., 2010). Trials in cattle of a 64TRP-based vaccine were reported by Merial to show promising results although the data were not published and vaccine development ceased.

More recently, new technologies have made tractable approaches based on a deeper understanding of complex tick-pathogen-host interactions (de la Fuente et al., 2016a; Kuleš et al., 2016). For example, SILK (a salivary gland-expressed flagelliform protein of unknown function) and TROSPA (tick receptor for OspA localized in the gut) facilitate transmission of cattle tick-borne pathogens, *Anaplasma marginale* and *Babesia bigemina*, respectively. While vaccination with SILK reduced tick infestations, oviposition, and levels of *A. marginale* and *B. bigemina* DNA, vaccination with TROSPA did not have a significant effect on any of the tick parameters analyzed and *B. bigemina* (but not *A. marginale*) DNA levels were reduced (Merino et al., 2013). Neither SILK nor TROSPA were significantly more effective than subolesin in reducing tick infestation/productivity or pathogen DNA levels although subolesin is not a recognized facilitator of pathogen transmission and infection. These results illustrate the need for a better understanding of the interface between feeding tick and immunized host/bloodmeal (at the skin site of attachment and within the tick midgut) and how these tick-host interactions affect tick-borne pathogens.

The prospects of developing a single anti-virus vaccine against all TBVs are unrealistic at this point in time as no common target has yet been identified against which vaccines can be developed. However, the idea of a single anti-tick vaccine that provides universal protection against TBV infections is not quite so far-fetched given that TBVs have a common target: they are reliant on a tick vector to survive. Thus, if a tick antigen is found that is common to tick vector species, and immunization with the antigen induces a host response that interferes with virus transmission, the possibility of developing a universal TBV vaccine becomes real. The "wish list" for an ideal universal TBV vaccine looks something like this:

1. Effective against a wide variety of tick species and against different tick developmental stages;
2. Inhibits or suppresses transmission of all viruses vectored by the tick species against which it is effective;
3. Provides long-lasting immunity;
4. Does not induce vaccine resistance (or evasion);
5. Does not induce adverse host responses (e.g., autoimmunity)
6. Cost-effective and practical

Interestingly, although subolesin fulfills many of these criteria, vaccination with subolesin reduced infection with several different bacterial and protozoan tick-borne pathogens but failed to protect against TBEV (Havlíková et al., 2013). Conversely, when 64TRP was used in a cattle tick trial to protect against *Theileria parva*, the protozoan tick-borne agent of East Coast fever in cattle, it was ineffective although 64TRP was effective against TBEV (Labuda et al., 2006; Olds et al., 2016). These contrasting results raise the question of whether a universal vaccine to protect against TBV, if achievable, may not provide similar protection against non-viral tick-borne pathogens. They point to a systems biology approach: we need to understand better the immunological environment at the site of tick feeding that prevents rather than ameliorates infection with TBVs and other tick-borne infectious agents. In the case of 64TRP, mice immunized with various constructs of the *R. appendiculatus*-derived saliva antigen showed significant levels of protection against lethal challenge by TBEV-infected *I. ricinus*, the natural virus vector that feeds on rodents at the immature stage (Labuda et al., 2006). When the surviving mice were inoculated with a lethal dose of TBEV, remarkably, they survived. Hence immunization with 64TRP created conditions at the tick feeding site that controlled the infection by tick bite in such a way that the tick-borne virus transmission effectively acted as a live attenuated anti-TBEV vaccine! This interpretation was supported by the results obtained when, in the same study, mice were immunized with either a licensed anti-TBEV vaccine or anti-tick (TickGARD) vaccine. Although the anti-TBEV vaccine gave slightly better protection against TBEV than 64TRP, it did not reduce significantly the number of mice supporting

co-feeding TBEV transmission whereas 64TRP and TickGARD did. This result indicates that 64TRP and TickGARD induced a host response that interfered with virus uptake from the bloodmeal, possibly though antibody-mediated damage to the midgut, which of course would not occur in mice immunized against the virus. Significantly, although TickGARD reduced virus transmission (measured by the number of uninfected co-feeding nymphs that became infected) and number of mice supporting virus transmission, it did not protect mice against lethal infection with TBEV, in contrast to 64TRP. This strongly supports the hypothesis that inflammatory/immune response to antigenically cross-reactive secreted cement protein at the site of tick feeding on 64TRP-immunized mice (which did not occur in the immune response to the antigenically cross-reactive Bm86 midgut antigen) was responsible for the remarkable protective effect of the 64TRP vaccine. The nature of this host response was not determined although it appeared to be a predominantly CD8+ T lymphocyte response.

The surprising results obtained with 64TRP immunization highlight how little we understand the host responses that control tick infestations and TBV infections. By placing greater emphasis on tick-host-virus interactions as one "interactome" (rather than three separate interactions), we should move closer to specifying the ingredients required to generate an anti-tick vaccine that controls TBVs. Defining an environment at the tick-host interface that is "hostile" to TBVs opens up the possibility of creating a universal vaccine.

CONCLUSIONS AND FUTURE DIRECTIONS

Ticks succeeded in their role as blood-feeders and vectors of TBV thanks to their complex life history and feeding biology. Components in tick saliva have been found to play a crucial role in tick feeding and mediating transmission of TBV. Nowadays, an increasing body of evidence exists on (i) manipulation of host defenses by ticks to enhance feeding and promote pathogen transmission and (ii) strategies used by tick-borne pathogens to evade host immunity and ensure survival in different biological systems. However, much of this knowledge comes from tick and tick-borne bacteria interaction studies. Information on tick and TBV interaction is still limited and so far no tick molecules enhancing virus transmission have been identified. The systems biology approach employing transcriptomics and proteomics has started to reveal molecular mechanisms constituting the survival strategy and persistence of TBV in their vectors and vertebrate hosts as well as the interactions at the tick-virus-host interface determining virus transmission. Identification of SAT factors enhancing TBV transmission during the early phases of tick attachment in the host skin, but mainly the understanding of the complexity of the relationships between ticks, TBV, and their vertebrate hosts, will enable novel strategies for controlling ticks and viral tick-borne diseases.

AUTHOR CONTRIBUTIONS

MK, PB, IS, ST, PN, MH, and VH conducted the literature search and wrote the paper. PB, PN, and MK prepared the figures and tables. All authors critically read and revised the manuscript.

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Tick-Host Range Adaptation: Changes in Protein Profiles in Unfed Adult *Ixodes scapularis* and *Amblyomma americanum* Saliva Stimulated to Feed on Different Hosts

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Understanding the molecular basis of how ticks adapt to feed on different animal hosts is central to understanding tick and tick-borne disease (TBD) epidemiology. There is evidence that ticks differentially express specific sets of genes when stimulated to start feeding. This study was initiated to investigate if ticks such as *Ixodes scapularis* and *Amblyomma americanum* that are adapted to feed on multiple hosts utilized the same sets of proteins to prepare for feeding. We exposed *I. scapularis* and *A. americanum* to feeding stimuli of different hosts (rabbit, human, and dog) by keeping unfed adult ticks enclosed in a perforated microfuge in close contact with host skin, but not allowing ticks to attach on host. Our data suggest that ticks of the same species differentially express tick saliva proteins (TSPs) when stimulated to start feeding on different hosts. SDS-PAGE and silver staining analysis revealed unique electrophoretic profiles in saliva of *I. scapularis* and *A. americanum* that were stimulated to feed on different hosts: rabbit, human, and dog. LC-MS/MS sequencing and pairwise analysis demonstrated that *I. scapularis* and *A. americanum* ticks expressed unique protein profiles in their saliva when stimulated to start feeding on different hosts: rabbit, dog, or human. Specifically, our data revealed TSPs that were unique to each treatment and those that were shared between treatments. Overall, we identified a total of 276 and 340 non-redundant *I. scapularis* and *A. americanum* TSPs, which we have classified into 28 functional classes including: secreted conserved proteins (unknown functions), proteinase inhibitors, lipocalins, extracellular matrix/cell adhesion, heme/iron metabolism, signal transduction and immunity-related proteins being the most predominant in saliva of unfed ticks. With exception of research on vaccines against *Rhipicephalus microplus,* which its natural host, cattle, research on vaccine against other ticks relies feeding ticks on laboratory animals. Data here suggest that relying on lab animal tick feeding data to

select target antigens could result in prioritizing irrelevant anti-tick vaccine targets that are expressed when ticks feed on laboratory animals. This study provides the platform that could be utilized to identify relevant target anti-tick vaccine antigens, and will facilitate early stage tick feeding research.

Keywords: tick, saliva, proteomic, tick-host relationship, host adaptation

INTRODUCTION

Ticks and tick-borne diseases (TBD) cause significant problems to global and veterinary health, impacting huge losses in the livestock industry (Jongejan and Uilenberg, 2004; Grisi et al., 2014). Their impact on public health has been on a steady climb since the 1980s (Dantas-Torres et al., 2012). In absence of vaccines against TBD agents, controlling of ticks using acaricides is the only reliable method to prevent human and animal TBD infections. Limitations of acaricide-based tick control have necessitated the search for alternative tick control methods (Domingos et al., 2013; Abbas et al., 2014) in which immunization of animals against tick feeding has been advocated as a sustainable alternative (de la Fuente and Kocan, 2006; de la Fuente et al., 2007; Parizi et al., 2012). Without the ability to attach and feed on its host, ticks cannot cause skin damage nor transmit TBD agents. Thus, a deeper understanding of tick feeding is needed as a mean to find molecular targets that can be useful for development of novel tick control methods. From this perspective, tick-feeding physiology continues to receive significant research attention.

Fundamental organismal level research has documented a series of tick behavioral and physiological changes through which the tick proceeds to successfully feed and transmit disease agents. Other lines of research have attempted to identify molecular mechanisms underlying tick feeding behavioral and physiological changes leading to successful tick feeding (Nuttall and Labuda, 2004; Mulenga et al., 2007). In this way, molecular targets for innovative tick control could be discovered. We are interested in understanding the molecular basis of how the tick adapts to feed on different animal hosts. The tick's adaptation to feed on different animal species is central to TBD epidemiology. Medically important tick species such as *Amblyomma americanum* and *Ixodes scapularis* that transmit a combined 11 of the 16 human TBD agents in the USA are effective vectors (US Centers for Disease Control and Prevention—CDC, https://www.cdc.gov/ticks/diseases/index.html) because they can feed on multiple hosts including humans (Dantas-Torres et al., 2012). Ticks acquire TBD agents from wild animal reservoirs and transmit to the human population. Likewise, the causative agents of economically important animal diseases such as *Ehrlichia ruminantium* and *Theileria parva* are transferred from wildlife reservoirs to domestic animal population due to the ability of the tick vector to feed on different animal species (van Vuuren and Penzhorn, 2015). The southern cattle fever tick, *Rhipicephalus microplus,* is specialized to feed on cattle, however it may also feed on white tailed deer and other deer species which maintains the tick population in the environment in the absence of cattle (Duarte Cancado et al., 2009), although ticks that feed on deer have a lower fitness (Popara et al., 2013). Likewise, *Rhipicephalus sanguineus,* specialized to feed on dogs can also feed on humans (Dantas-Torres et al., 2006; Dantas-Torres, 2010), in which this tick is capable of transmitting *Rickettsia rickettssii* from dogs to humans in areas where the principal vector ticks *Dermacentor variabilis* and *Dermacentor andersoni* are absent (Piranda et al., 2011; Drexler et al., 2014). Despite its importance, the molecular basis of how the tick adapts to feed on different hosts remains poorly understood.

Ticks are pool feeders, and accomplish feeding by disrupting host tissue and sucking up blood that bleeds into the feeding site (Ribeiro, 1995; Francischetti et al., 2009). This feeding style activates host defense pathways that are aimed at stopping further blood loss. Ticks successfully feed by injecting hundreds of saliva proteins into the host to block host defense to tick feeding (Mudenda et al., 2014; Radulović et al., 2014; Tirloni et al., 2014, 2015; Kim et al., 2016b). Among the molecules present in tick saliva, those that modulate pain/itching, hemostasis, inflammation, wound healing, and host immunity are considered the most important in tick-host-pathogen interaction as these proteins allow blood meal acquisition and facilitate TBD pathogen transmission (Ribeiro, 1995; Nuttall and Labuda, 2004; Francischetti et al., 2009).

The profiles of proteins in tick saliva during blood feeding are different depending on the tick species and the stage of the tick (Mudenda et al., 2014; Radulović et al., 2014; Tirloni et al., 2014, 2015; Kim et al., 2016b). Whether or not ticks of the same species inject the same or different profiles of proteins when feeding on different animal hosts remain unknown. Resolving this question will be particularly interesting for ticks such as *A. americanum* and *I. scapularis* that feed on immunologically diverse animal species, from birds to large mammals (Keirans et al., 1996; Kollars et al., 2000), as the hemostatic and immune responses of their different hosts vary considerably (Gentry, 2004; Boehm, 2012). Furthermore, there is evidence that due repetitive infestations, ticks are able to induce a very strong resistance in some hosts species but not in others, suggesting that resistance is centered on host's particular immune characteristics and/or in the evolution of highly specific evasion mechanisms in ticks due saliva composition (Szabó and Bechara, 1999). In the same way, recently a study demonstrated that *I. scapularis* saliva displays variable fibrinogenolytic activities upon feeding on hosts with different immune backgrounds (Vora et al., 2017). Thus, it is reasonable to hypothesize that ticks could switch their salivary composition in order to modulate different host defense responses.

There is evidence that when ticks engage the host they express certain genes that are thought to represent the tick's

molecular preparation to start feeding. Mulenga et al. (2007) described 40 transcripts that were differentially up regulated in *A. americanum* ticks that were stimulated to start feeding on cattle. Likewise, Lew-Tabor et al. (2010) and Rodriguez-Valle et al. (2010) identified differentially up-regulated genes in *R. microplus* that were stimulated to start feeding on cattle. In a related study, Popara et al. (2013) demonstrated differential protein expression in *R. microplus* that fed on cattle and white-tailed deer. Studies reviewed here (Mulenga et al., 2007; Lew-Tabor et al., 2010; Rodriguez-Valle et al., 2010; Popara et al., 2013) suggested that ticks may express specific genes to prepare for feeding on different host species. In this study, we provide evidence that protein profiles in saliva of ticks that are stimulated to start feeding on different change, as suggested by differential protein profiles in saliva of both *A. americanum* and *I. scapularis* ticks, which were stimulated to start feeding on different hosts.

MATERIALS AND METHODS

Ethics Statement

Ticks used in this study were unfed adult females. As ticks were not fed, modifications of the host to feed ticks were not required, with exception of rabbits. For rabbits, we attached a cotton stockinet on top of the rabbit ear as outlined in the animal use protocol 2011-187 that was approved by Texas A&M University IACUC. The cotton stockinet attachment was used to contain the tick stimulation chamber as detailed below.

Stimulating Unfed Adult *Ixodes scapularis* and *Amblyomma americanum* Females to Feed on Different Hosts

Adult *Ixodes scapularis* and *Amblyomma americanum* ticks that were used in this study were purchased from the tick rearing facilities at Oklahoma State University (Stillwater, OK, USA). Stimulation of unfed adult *A. americanum* and *I. scapularis* ticks to start feeding on different hosts: rabbits, dog, or human was done by exposing ticks to semio-chemicals and temperature as described (Mulenga et al., 2007) with modifications. The modification was that instead of a tick stimulation chamber being made out of a nylon mesh sachet, a tightly capped 2 mL vial that was perforated with a 27-gauge needle was used. The 27-gauge needle perforations were to allow semio-chemicals and body temperature to percolate ticks. Unfed *A. americanum* (n = 40 in each vial) and *I. scapularis* (n = 50–80 in each vial) females ticks were enclosed in a stimuli chamber and placed in close proximity with host skin. To ensure the cap did not open for ticks to escape during the stimulation step, the cap was secured with VWR® general-purpose laboratory labeling tape (VWR International, Radnor, PA, USA) and wrapped with Parafilm M® (Bemis Company Inc., Neenah, WI, USA). To expose ticks to semio-chemicals, the stimulation chamber containing ticks was placed in close proximity with host's skin for ∼12 h. Three individual hosts: (i) human (*Homo sapiens*); (ii) New Zealand white rabbit (*Oryctolagus cuniculus*); and (iii) Dachshund dog (*Canis familiaris*) were used. To expose the ticks to human semio-chemicals and temperature, the stimuli chamber containing *A. americanum* or *I. scapularis* ticks was placed in the front shirt pocket of the volunteer. For exposure to rabbit, stimuli chambers were placed inside cotton stockinet that was attached onto the top of the rabbit ear (Kim et al., 2014). For dogs, stimuli chambers were taped on to the collar/harness. Following exposure to semio-chemicals, ticks were processed for saliva collection as describe below.

I. scapularis ticks mate off the host before interacting with the host (Sonenshine and Roe, 2014). From this perspective, *I. scapularis* female ticks were pre-mated before being stimulated to start feeding. This was done by putting female and male *I. scapularis* ticks in a container, and then visually identifying male and female pairs. Male and female *I. scapularis* pairs were placed in separate container to complete mating. Please note that, since *A. americanum* ticks mate after taking an initial blood meal (Sonenshine and Roe, 2014), we did not pre-mate females prior to stimulating them to start feeding.

Tick Saliva Collection

Tick saliva was collected from unfed *I. scapularis* and *A. americanum* as previously described (Tirloni et al., 2014; Kim et al., 2016b). For *I. scapularis*, we collected saliva from unfed non-stimulated (n = 130 ticks) and those that were stimulated to feed on human (n = 80 ticks), rabbit (n = 130 ticks), and dog (n = 80 ticks) hosts. Likewise, for *A. americanum*, we collected saliva from non-stimulated (n = 40 ticks), and those that were stimulated to feed on human (n = 40 ticks), rabbit (n = 40 ticks), and dog (n = 40 ticks) hosts. Please note that non-stimulated ticks were taken from the incubator (22°C with 90% relative humidity) and were acclimated to room temperature during the saliva collection step. Ticks were rinsed in Milli-Q water, dried on a paper towel and placed dorsal-side down on a glass slide containing tape. Salivation was induced by injecting 0.5–1 μL of 2% pilocarpine hydrochloride (in PBS, pH 7.4) on the ventral side of the lower right coxa using a 34 gauge/0.5 inches/45° angle beveled needle on a model 701 Hamilton syringe (Hamilton Company, Reno, NV, USA). Tick saliva, which in some instances crystalized, was harvested in 2 μL of phosphate buffered saline (PBS) placed on tick mouthparts using a Hamilton syringe every 15 min over ∼4 h at room temperature. Saliva protein concentrations were determined using BCA enhanced protocol (BCA Protein Assay, Pierce, Rockford, IL, USA). Tick saliva was lyophilized and stored at −80°C upon use.

SDS-PAGE and Silver Staining

Approximately 1 μg of *I. scapularis* and 1.5 μg of *A. americanum* total saliva proteins were electrophoresed on 4–20% gradient SDS-PAGE. Gels were silver stained using the Pierce Silver Stain for Mass Spectrometry kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions.

Protein Digestion and Sample Preparation

Total tick saliva proteins (2 μg, in triplicate) of *I. scapularis* or *A. americanum* ticks that were non-stimulated and those that were stimulated to starting feed on different hosts (human, dog, and rabbit) were diluted in 8 M urea/0.1 M Tris, pH 8.5, reduced with 5 mM Tris (2-carboxyethyl) phosphine hydrochloride

(TCEP, Sigma-Aldrich, St Louis, MO, USA) and alkylated with 25 mM iodoacetamide (Sigma-Aldrich). Proteins were digested overnight at 37 °C in 2 M urea/0.1 M Tris pH 8.5, 1 mM $CaCl_2$ with trypsin (Promega, Madison, WI, EUA) with a final ratio of 1:20 (enzyme:substrate). Digestion reactions, in a final concentration of 0.2 μg/mL, were quenched with formic acid (5% final concentration) and centrifuged for debris removal.

Pre-columns and Analytical Columns

Reversed phase pre-columns were prepared in deactivated 250 μm ID/360 μm OD silica capillary (Agilent Technologies, Santa Clara, CA, USA) with a 2 mm Kasil frit at one end. Kasil frits were prepared by dipping 20 cm capillary in 300 μL Kasil 1624 (PQ Corporation, Malvern, PA, USA) and 100 μL formamide solution, curing at 100°C for 3 h and adjusting the length. Pre-columns were packed in-house with 2 cm of 5 μm ODS-AQ C18 (YMC America, INC., Allentown, PA, USA) particles from particle slurries in methanol. Analytical reversed phase columns were fabricated by pulling a 100 μm ID/360 μm OD silica capillary (Molex Polymicro Technologies™, Austin, TX, USA) to a 5 μm ID tip. The same packing material was packed until 20 cm directly behind the pulled tip. Reversed phase precolumns and analytical columns were connected using a zero-dead volume union (IDEX Corp., Upchurch Scientific, Oak Harbor, WA, USA).

LC-MS/MS

Peptide mixtures were analyzed by nanoflow liquid chromatography mass spectrometry using an Easy NanoLC II and a Q Exactive mass spectrometer (Thermo Scientific, Waltham, MA, USA). Peptides eluted from the analytical column were electrosprayed directly into the mass spectrometer. Solutions A and B consisted of 5% acetonitrile/0.1% formic acid and 80% acetonitrile/0.1% formic acid, respectively. The flow rate was set to 400 nL/min. Saliva samples (2 μg per injection) were separated in 155-min chromatographic runs, as follows: 1–10% gradient of solution B in 10 min , 10–40% of solution B in 100 min, 40–50% of solution B in 10 min and 50–90% of solution B in 10 min. Column was held at 90% of solution B for 10 min, reduced to 1% of solution B and re-equilibrated prior to next injection.

The mass spectrometer was operated in a data dependent mode, collecting a full MS scan from 400 to 1,200 m/z at 70,000 resolution and an AGC target of 1×10^6. The 10 most abundant ions per scan were selected for MS/MS at 17,500 resolution and AGC target of 2×10^5 and an underfill ratio of 0.1%. Maximum fill times were 20 and 120 ms for MS and MS/MS scans, respectively, with dynamic exclusion of 15 s. Normalized collision energy was set to 25.

Data Analysis

Tandem mass spectra were extracted from Thermo RAW files using RawExtract 1.9.9.2 (McDonald et al., 2004) and searched with ProLuCID (Xu et al., 2015) against a non-redundant tick databases. *I. scapularis* peptides were searched against the database containing an Ixodidae sequences from NCBI (62,246 sequences and reverse sequences). For *A. americanum*, we searched peptides against an in house database of translated whole tick and dissected organ transcriptome (BioProject accession number PRJNA226980). Searches were done using Integrated Proteomics Pipeline—IP2 (Integrated Proteomics Applications, Inc.). The search space included all fully-tryptic and half-tryptic peptide candidates. Carbamidomethylation on cysteine was used as static modification. Data was searched with 50 ppm precursor ion tolerance and 20 ppm fragment ion tolerance.

The validity of the peptide spectrum matches (PSMs) generated by ProLuCID was assessed using Search Engine Processor (SEPro) module from PatternLab for Proteomics platform (Carvalho et al., 2015). Identifications were grouped by charge state and tryptic status, resulting in four distinct subgroups. For each group, ProLuCID XCorr, DeltaCN, DeltaMass, ZScore, number of peaks matched and secondary rank values were used to generate a Bayesian discriminating function. A cut-off score was established to accept a false discovery rate (FDR) of 1% based on the number of decoys. This procedure was independently performed on each data subset, resulting in a false-positive rate that was independent of tryptic status or charge state. Additionally, a minimum sequence length of six residues per peptide was required. Results were post-processed to only accept PSMs with <10 ppm precursor mass error. A Principal Component Analysis (PCA) plot, performed using PatternLab's Buzios module (Carvalho et al., 2015), was employed to aid in interpreting similarities among samples. Venn's four-set diagrams were generated from the output of PatternLab's Birds Eye view report. Proteins were grouped by maximum parsimony and the presence of proteins in at least two out of three replicates was required for each condition.

Volcano plots were generated by a pairwise comparison between non-stimulated and stimulated tick saliva using PatternLab's TFold module, which uses a theoretical FDR estimator to maximize identifications satisfying both a fold-change cut-off that varies with the *t*-test *p*-value as a power law and a stringency criterion that aims to fish out proteins of low abundance that are likely to have had their quantitation compromised (Carvalho et al., 2015). The following parameters were used to select differentially expressed proteins: proteins were grouped by maximum parsimony, spectral count data was normalized using normalized spectral abundance factor (NSAF) (Zybailov et al., 2006), two (out of the three runs) non-zero replicate values were required for each condition, and a BH *q*-value was set at 0.02 (2% FDR). Low abundant proteins were removed using an L-stringency value of 0.2.

Functional Annotation and Classification

To get insight on the nature of the identified protein sequences, BLASTp searches against several databases were performed. To functionally classify the protein sequences, a program written and provided by Dr. José M. C. Ribeiro in Visual Basic 6.0 (Microsoft, Redmond, WA, USA) was used (Karim et al., 2011). The functionally annotated catalog for each dataset was manually curated and plotted in a hyperlinked Excel spreadsheet designed as **Table S1** (for *I. scapularis*) and **Table S2** (for *A. americanum*).

We have recently identified proteins in saliva of *I. scapularis* ticks that were fed every 24 h on rabbits (Kim et al., 2016b). To determine if some proteins reported here were injected into the host during tick feeding, unfed *I. scapularis* tick saliva proteome from this study were scanned against published *I. scapularis* proteome.

Relative Abundance and Graphical Visualization

Proteomic profiles were compared across samples as functional classes or individual proteins. To determine the relative abundance of proteins, normalized spectral abundance factor (NSAF) was used in a label-free relative quantification approach (Paoletti et al., 2006). Mean NSAF values from the two or three replicates were determined and combined according to functional class, and then divided by the total NSAF for the respective sample. NSAF as an index for relative protein abundance was input in Microsoft Excel (Microsoft, Redmond, WA, USA) as percentage of the total NSAF for respective samples, and visualized on pie charts according to protein classes. To visualize relative expression patterns on a heat map, NSAF values were normalized using Z-score. Normalized NSAF values were used to generate heat maps using the heatmap.2 function from the gplots package in R.

Data Availability

The mass spectrometry proteomics raw data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD00712.

RESULTS AND DISCUSSION

Ticks Stimulated to Feed on Different Hosts Have Unique Protein Profiles in Their Saliva

The hard tick's adaptation to feed on different animal hosts is central to TBD epidemiology as it facilitates the tick's movement of TBD agents from their wild animal reservoir hosts to humans, farm and companion animals. Despite its centrality, the molecular basis of how the tick adapts to feed on different hosts has not been fully evaluated. The tick feeding style of lacerating host tissue and then sucking up host blood that bleeds into the wounded area (commonly known as the tick-feeding site) is thought to stimulate host tissue repair responses that are aimed at stopping further blood loss. However, ticks ensure a full blood meal by secreting a cocktail of proteins that disarm the host's tissue repair response (Francischetti et al., 2009). There is also evidence the tick may express specific sets of genes in preparation to start feeding (Mulenga et al., 2007; Lew-Tabor et al., 2010; Rodriguez-Valle et al., 2010). This study was initiated to answer the question of whether or not the molecular preparation to start feeding by ticks such as *I. scapularis* and *A. americanum* that are adapted to feed on a wide range of hosts utilized was the same regardless of the host. Findings in this study suggest that the tick's molecular preparation to start feeding could be host-specific as indicated by SDS-PAGE and silver staining analysis that revealed unique protein profiles in saliva of *I. scapularis* and *A. americanum* ticks that were stimulated to start feeding on rabbits (SR), dog (SD), human (SH), and those that were not-stimulated (NS) (**Figure 1**). Solid (SB) and broken (BB) line boxes respectively highlight similarity and differences of detectable protein band patterns in saliva of ticks that were stimulated to feed on different hosts (**Figure 1**). Whereas we observed similarities (solid boxes) and differences (broken boxes) in protein banding patterns in saliva of *I. scapularis* ticks that were stimulated to feed on dogs and humans (**Figure 1A**), there are no apparent similarities among protein banding patterns in saliva of the four *A. americanum* treatments (**Figure 1B**). A notable observation in **Figure 1B** is that protein banding patterns in saliva of dog and rabbit-stimulated *A. americanum* ticks were closely similar, while those exposed to humans show more differences. Due to insufficient sample amounts, non-stimulated and rabbit-exposed *I. scapularis* tick saliva proteins were not analyzed by SDS-PAGE (**Figure 1A**). From this perspective, our SDS-PAGE analysis in **Figure 1A** was limited, and thus its unclear if observations in *A. americanum* were consistent with those in *I. scapularis*. The observation that the protein profile in saliva of non-stimulated *A. americanum* ticks is different from those that were stimulated to start feeding (**Figure 1B**) further demonstrates that stimulating ticks to start feeding impacted proteins that were secreted into tick saliva.

There is evidence that exposing the animal to high temperature stress can affect expression levels of different proteins (Villar et al., 2010). However, given that the respective axillary temperatures of dogs (∼38°C), human (∼37°C), and rabbits (∼36°C) are not dramatically different (Vadlejch et al., 2010; Goic et al., 2014), it is unlikely that the observed differences in protein profiles were temperature dependent. For our non-stimulated tick cohorts, saliva was collected from ticks that were obtained straight from the incubator at 22°C and acclimated to room temperature, which is normally around 25°C. Thus, observed shift in protein profiles between NS and those that were stimulated to start feeding on different hosts (which have higher respective axillary temperatures) cannot be ruled out. On the other hand, studies have been shown that ticks respond to mechano-, chemo-, and thermo-sensation and are able to induce different electrophysiological responses (Soares and Borges, 2012; Sonenshine and Roe, 2014). Upon contact with host, release of host-derived semio-chemicals and their interaction with tick sensory organs may result in different electrophysiological responses leading secretion of different proteins during host stimulation process. Since ticks used in this study did not come into contact with the host's skin, tick saliva proteins described reported may not include those are responsive to mechano-sensory stimulation.

To get further insights into differences observed among saliva proteomes from ticks that were exposed to different hosts, we proceeded to identify proteins by LC-MS/MS using an *in-solution* digestion approach as described (Tirloni et al., 2014; Kim et al., 2016b). We respectively identified a total of 276 and 340 non-redundant *I. scapularis* (**Figure 2A**) and *A. americanum* (**Figure 2B**) tick saliva proteins that were determined authentic as they were detected in two or all of

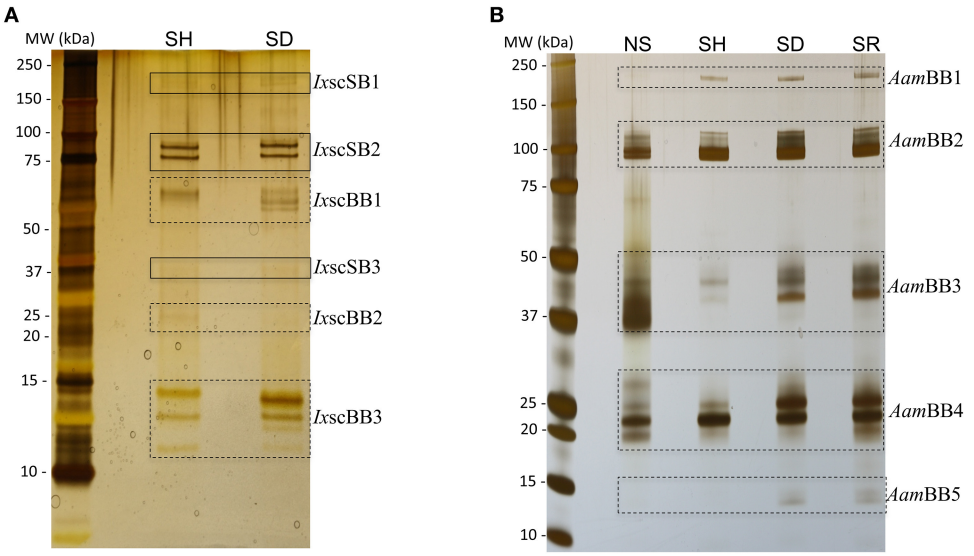

FIGURE 1 | Stimulating ticks to start feeding on different hosts induces expression of differential tick-saliva proteins. To induce salivation, adult female unfed **(A)** *Ixodes scapularis* and **(B)** *Amblyomma americanum* ticks that were stimulated to feed on human (SH), dog (SD), rabbit (SR) for ∼12 h and those not stimulated to start feeding (NS, were taken straight from the incubator) were microinjected with 0.5–1 μL of 2% pilocarpine hydrochloride. Approximately 1 μg of *I. scapularis* and 1.5 μg of *A. americanum* total saliva proteins was electrophoresed on 4–20% SDS gradient gels and silver stained. Solid (SB) and broken (BB) line boxes respectively highlight apparently identical and different protein banding patterns in saliva of *I. scapularis* (*Ixsc*) and *A. americanum* (*Aam*) exposed to different hosts. Please note that due to insufficient samples, we did not run *I. scapularis* NS and SR samples.

the three LC-MS/MS runs that were done for each sample (**Figure 2**, **Tables S1**, **S2**). The remaining 69 (*I. scapularis*) and 57 (*A. americanum*) proteins that were detected in only one of the three runs were considered low confidence hits and not further discussed (**Tables S1**, **S2**). **Figure 2** summarizes the overall total proteins that were identified in saliva of ticks not stimulated to start feeding (NS) and those stimulated to start feeding on human (SH), dog (SD), and rabbit (SR). Of the 276 unique *I. scapularis* tick saliva proteins (**Table S1**), 66, 189, 186, and 165 were respectively identified in saliva of NS, SH, SD, and SR ticks (**Figure 2A**). Of these, 55 were common to all treatments, while 2, 35, 35, and 34 proteins were respectively unique to NS, SH, SD, and SR ticks (**Figure 2A**). Likewise, in *A. americanum* we respectively found 245, 192, 288, and 93 proteins in saliva of NS, SH, SD, and SR ticks (**Figure 2B**). Of these, 67 were common to all treatments, while 19, 12, 59, and 9 proteins were respectively unique to NS, SH, SD, and SR ticks (**Figure 2B**). Functional annotations classified both *I. scapularis* and *A. americanum* tick saliva proteins into 28 protein classes (**Figure 3**, **Tables S1**, **S2**). Based on the total sum of normalized spectral abundance factor (NSAF) (**Figure 3**), the predominant classes of proteins in this study include secreted conserved proteins (unknown functions), proteinase inhibitors, lipocalins, extracellular matrix/cell adhesion, heme/iron metabolism, signal transduction and immunity-related proteins (**Figure 3**).

Graphic visualization data in **Figure 4** summarizes the Z-score of normalized NSAF values for each of the 28 functional classes identified in *I. scapularis* (**Figure 4A**) and *A. americanum* (**Figure 4B**). These data reveal two general trends: (i) the tick might inject the same protein at different levels into different hosts, and that (ii) protein composition in saliva of different tick species that feed on the same host is likely different (**Figure 4**). For instance, saliva of SR *I. scapularis* ticks had high abundance of heme/iron metabolism (22.4%) followed by extracellular matrix/cell adhesion (7%), oxidant metabolism/detoxification (6.2%), cytoskeletal (4.6%), metabolism (amino acid, carbohydrate and energy) (2%), proteasome machinery (1.1%), nuclear regulation (0.8%), conserved protein with unknown functions (0.6%), protein modification (0.4%), protein synthesis machinery proteins (0.4%), and transport/storage (0.02%) (**Figure 4A** and **Table S1**). In contrast, saliva of SR *A. americanum* ticks had high abundance of extracellular matrix/cell adhesion (18.6%) and proteinase inhibitors (18.2%), followed by heme/iron metabolism (11.1%), immunity-related (4.8%), metabolism of energy proteins (1.4%), cytoskeletal (0.7%), protein synthesis machinery (0.5%), and proteasome machinery (0.1%) (**Figure 4B** and **Table S2**).

I. scapularis and *A. americanum* Ticks Stimulated to Feed on Different Hosts Secrete a Core Set of Functionally Similar Proteins in Their Saliva

An interesting finding in our data is that the 55 and 67 proteins that were respectively found in all *I. scapularis* and *A. americanum* treatments (**Figures 2A,B**) belonged to the same functional classes (**Figure S1**, **Tables S1**, **S2**). These data suggest that *A. americanum* and *I. scapularis* utilized a core set of functionally similar proteins that regulated key host defense pathways to successfully feed. Although functional role(s) of

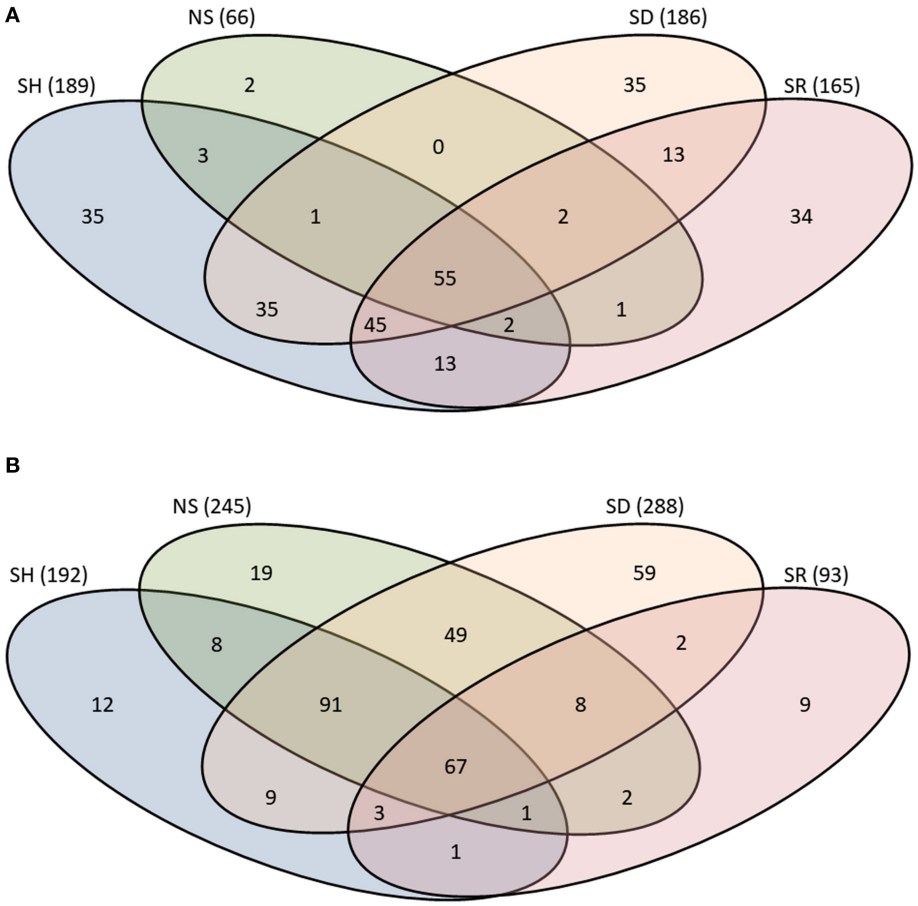

FIGURE 2 | Protein counts in saliva of *Ixodes scapularis* **(A)** and *Amblyomma americanum* **(B)** ticks that were not-stimulated (NS) to start feeding, and those that were stimulated to feed on rabbits (SR), dog (SD), and human (SH). Total number of proteins for each treatment is indicated in parenthesis. The overlap region between Venn diagrams shows proteins present in two or more treatments.

proteins in **Figure S1** remain to be determined, available evidence indicate that some of these proteins regulated important tick feeding pathways. For instance, a cross-tick species conserved AV422 protein that was originally identified among genes that were up regulated in *A. americanum* ticks that were stimulated to feed on cattle (Mulenga et al., 2007) and was injected into animals during *A. americanum* (Mulenga et al., 2013), *I. scapularis* (Kim et al., 2016b), *R. microplus* (Tirloni et al., 2014), and *Haemaphysalis longicornis* (Tirloni et al., 2015) feeding is an inhibitor of blood clotting and platelet aggregation (Mulenga et al., 2013). Likewise, EEC19556.1, which was found in all *I. scapularis* treatments (**Table S1**) is 99% identical to a serine protease inhibitor (serpin, AID54718.1) anti-coagulant and inhibitor of thrombin (Ibelli et al., 2014) that is injected into rabbits during *I. scapularis* feeding (Kim et al., 2016b).

Proteins in Saliva of Ticks Stimulated to Feed on Different Hosts Are Differentially Abundant

In order to investigate if shared proteins were differentially secreted *I. scapularis* and *A. americanum* when stimulated to start feeding on different hosts, pairwise comparison analyses using the PatternLab's TFold module (Carvalho et al., 2015) were conducted (**Figure 5**, **Tables S4**, **S5**). This analysis demonstrated that some of the shared proteins were secreted at equivalent levels (red dots), not significantly different (green and yellow dots), and significantly at different levels (blue dots) (**Figure 5**). Based on fold change (FC), differences in abundance ranged between 18.0 and 1.2 for *I. scapularis* (**Table S4**) and between 40 and 1.2 for *A. americanum* (**Table S5**). Consistent with our analysis in **Figure 4**, majority of SR *I. scapularis* tick saliva proteins were secreted at high concentrations when compared to either SD or SH. When compared to SD or SH, some of the most abundant proteins in saliva of SR *I. scapularis* ticks, included superoxide dismutase (EEC10196.1, FC: 17.7, and FC: 9.3), a glyceraldehyde-3-phosphate dehydrogenase—GAPDH (JAA68969.1, FC: 10.7), a tropomyosin (JAB83342.1, FC: 9.9), a thymosin (JAA70823.1, FC: 7.3), a fructose 1,6-biphosphate aldolase (EEC14101.1, FC: 8.3), and a creatine kinase (JAB78095.1, FC: 6.9). When paired with either rabbit or human, proteins with higher FC in saliva of SD *I. scapularis* ticks, include oxidase/peroxidase enzyme (EEC07462.1, FC: 18.0), a serine protease (EEC02857.1, FC:

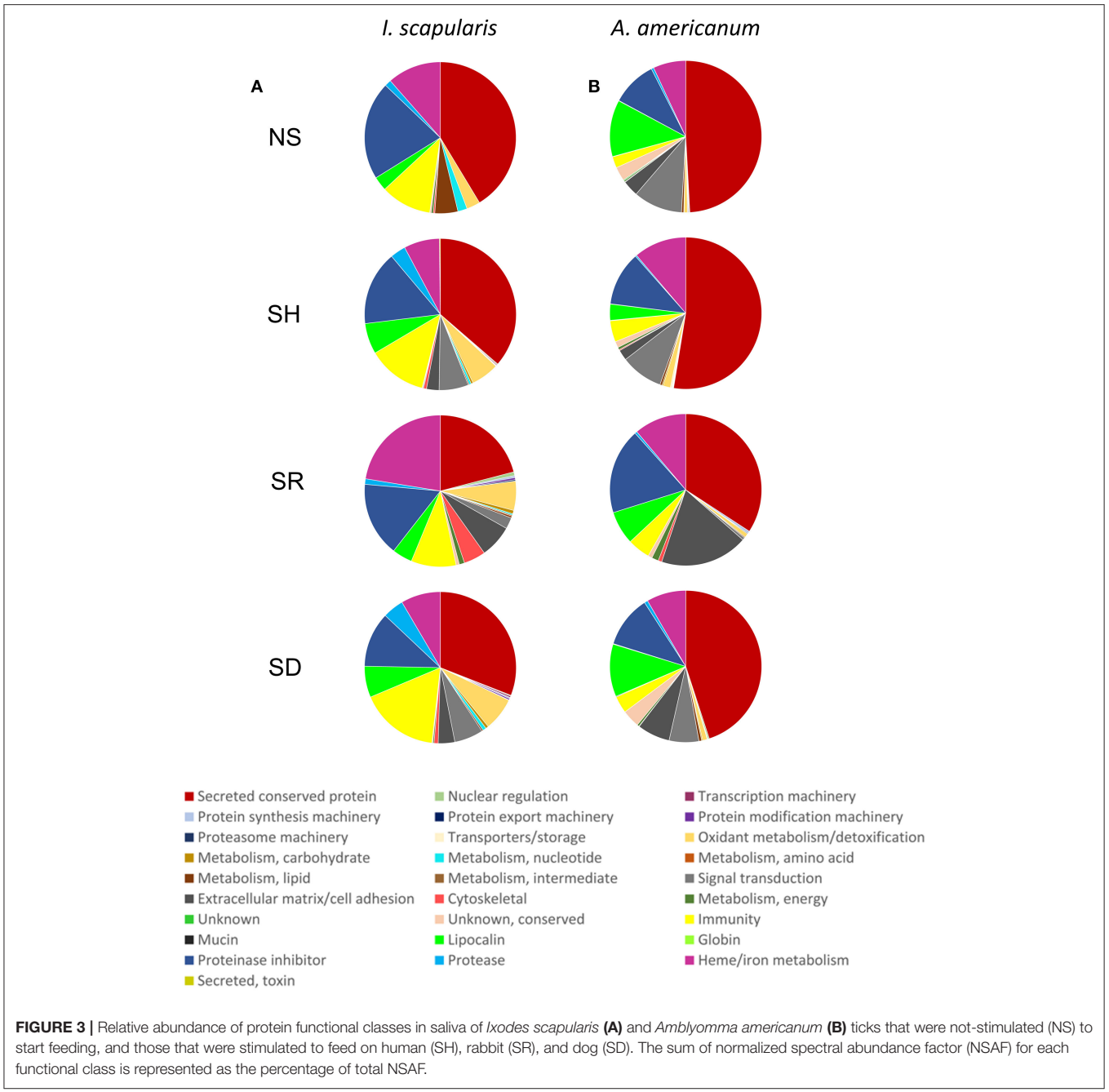

FIGURE 3 | Relative abundance of protein functional classes in saliva of *Ixodes scapularis* **(A)** and *Amblyomma americanum* **(B)** ticks that were not-stimulated (NS) to start feeding, and those that were stimulated to feed on human (SH), rabbit (SR), and dog (SD). The sum of normalized spectral abundance factor (NSAF) for each functional class is represented as the percentage of total NSAF.

6.7), a secreted salivary gland peptide (EEC14213.1, FC: 6.7), a peroxinectin (EEC08358.1, FC: 6.5), a microplusin (AAY66495.1, FC: 4.8), a GAPDH (JAA68969.1, FC: 4.7), and a 14-3-3 zeta protein (JAB76832.1, FC: 4.3). Likewise, for human-exposed ticks an oxidase/peroxidase (EEC07462.1, FC: 9.6), a secreted salivary gland peptide (EEC14213.1, FC: 7.5), an insulin growth factor-binding protein (EEC07853.1, FC: 5.0), a metalloprotease (AAM93652.1, FC: 7.0), and a secreted protein (EEC14470.1, FC: 4.0) were identified with higher FC (**Table S4**).

Although similar to *I. scapularis* (**Table S4**), proteins in saliva of *A. americanum* were likely secreted at high concentration when stimulated to feed on rabbits than either dog or human (**Table S5**). It is notable that fewer proteins were differentially abundant in SH ($n = 2$) or SD ($n = 4$) *A. americanum* tick saliva when compared with SR saliva (**Figure 5B** and **Table S5**). Please note that four of the six proteins that were differentially abundant in SH compared to SR *A. americanum* saliva (**Figure 5B**) are potentially contaminating keratins (**Table S5**), and hence the six blue dots in **Figure 5**. Whether or not the observation here is a consistent biological phenomenon remains to be determined. We also think that this observation could be explained by the limitation of our study: we searched *A. americanum* peptides against a database of a translated *A. americanum* transcriptome that was generated from ticks that were fed on rabbits. We

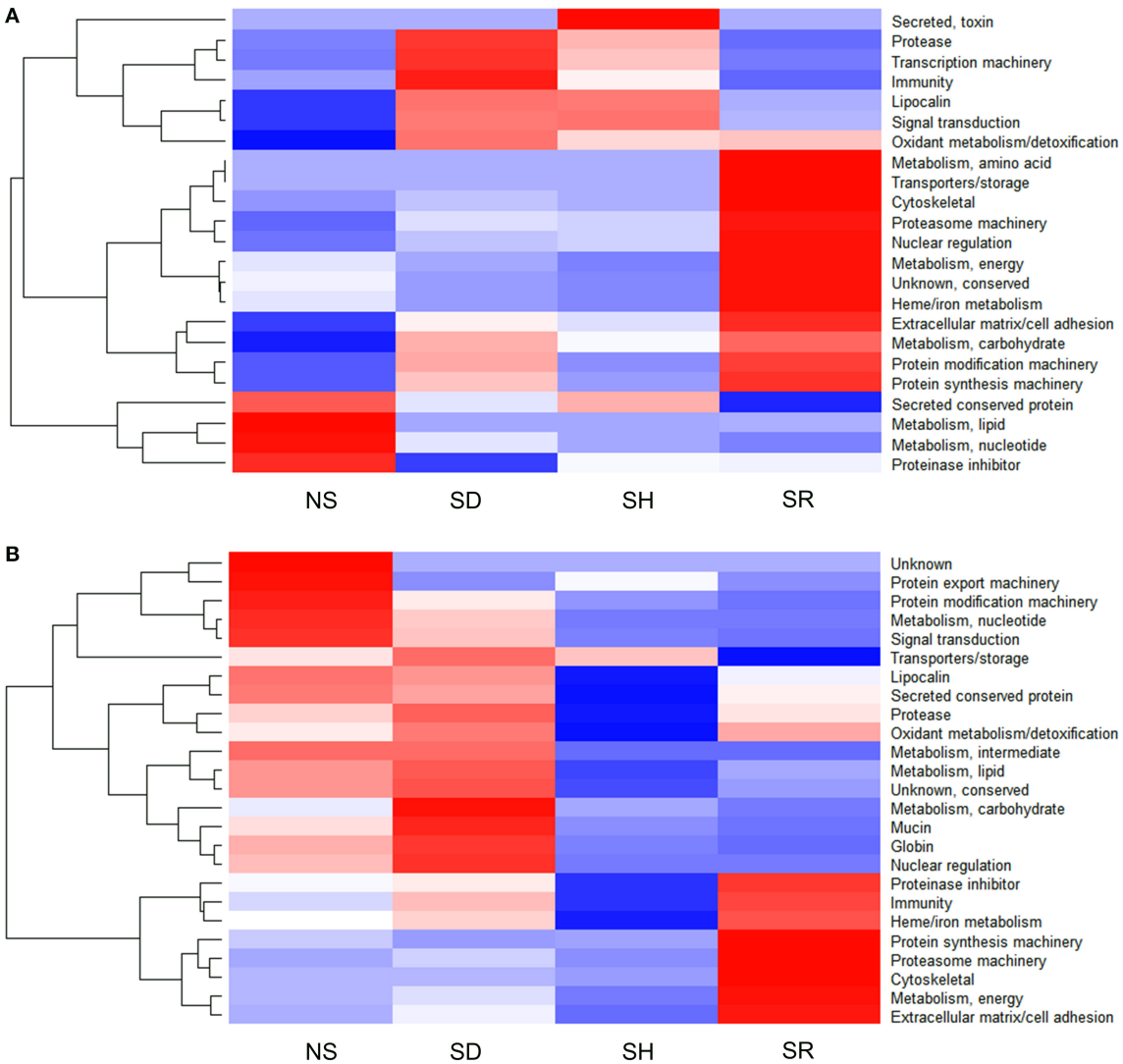

FIGURE 4 | Proteins in saliva of *Ixodes scapularis* **(A)** and *Amblyomma americanum* **(B)** ticks that were not-stimulated (NS) to start feeding, and those that were stimulated to feed on dog (SD), human (SH), and rabbit (SR) are differentially abundant. Normalized spectral abundance factor (NSAF) for each protein is expressed as a percent of total NSAF. Z-scores were calculated and used to generate heat maps as described in Materials and Methods section. Red color indicates proteins of high abundance and blue color indicates proteins of low abundance.

anticipate that if the *A. americanum* genome became available, additional host-dependent *A. americanum* tick saliva protein secretions will be identified.

Despite the fact that we identified a limited number of differentially abundant *A. americanum* proteins, the secretion dynamics of AV422 (Mulenga et al., 2007) was similar in both *A. americanum* and *I. scapularis* (**Tables S4**, **S5**). Our data here show this protein is secreted at high abundance when *A. americanum* is stimulated to feed on humans (FC: 1.7) and dogs (FC: 2.2). Similarly, in *I. scapularis*, AV422 homolog is secreted at high abundance when *I. scapularis* is stimulated to feed on humans (FC: 1.6) and dogs (FC: 1.8). It will be interesting to further investigate the role(s) of *I. scapularis* AV422 homolog in tick feeding were similar to those observed *A. americanum* (Mulenga et al., 2013).

The observation that, *A. americanum* ticks may secrete fatty acid-binding protein at FC of 40.2 (Aam-134, **Table S5**) represents the most abundantly secreted protein in saliva of SR *A. americanum* tick saliva. This protein was identified exclusively in saliva of SR and SD ticks and appears more abundant in the former (**Table S5**). Fatty acid-binding proteins have been identified in helminth secretions (Morphew et al., 2007) and in tick saliva. This protein has been described to modulate human monocyte-derived macrophages and generate M2 macrophages (MΦ) activated by the alternative pathway (Figueroa-Santiago and Espino, 2014). M2 MΦ are characterized by secretion of anti-inflammatory cytokines (Gordon, 2003). Since ticks have to evade the host's inflammation defense, could the fatty acid-binding protein being among tick proteins that modulate host defense? It would be interesting to address if the *A. americanum*

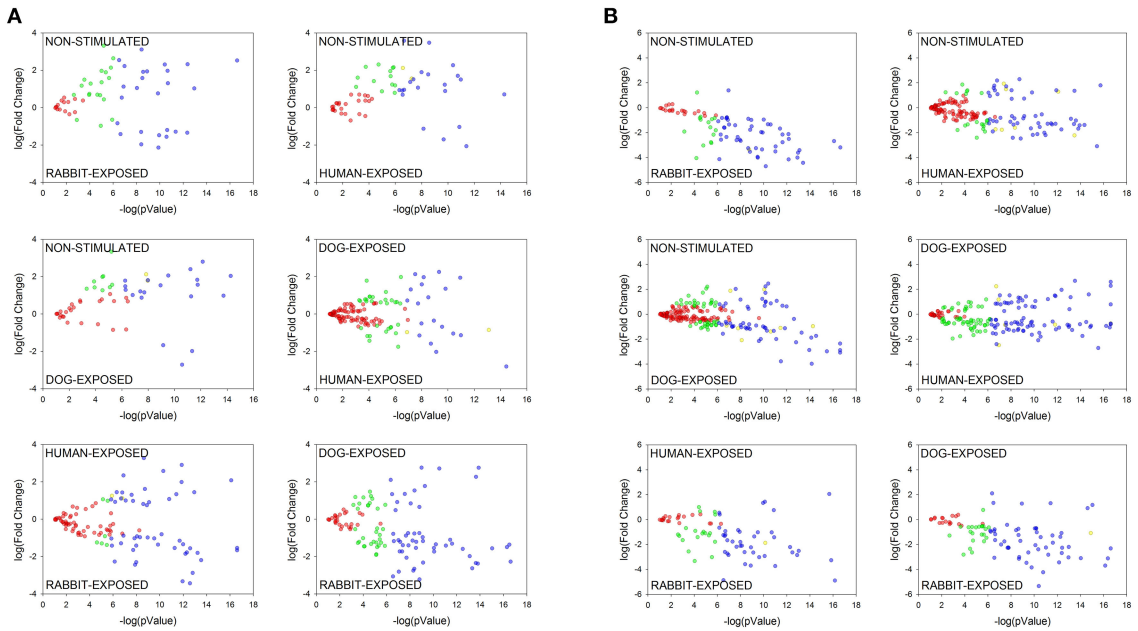

FIGURE 5 | Pairwise comparative analysis showing differentially abundant proteins in saliva of *Ixodes scapularis* **(A)** and *Amblyomma americanum* **(B)** ticks stimulated to feed on different hosts. Normalized spectral abundance factor (NSAF) for each protein was subjected to pairwise analysis as detailed in section Materials and Methods. Each protein is represented as a dot and is mapped according to its fold change on the ordinate axis (y) and *t*-test *p*-value on the abscissa axis (x). Proteins represented by: (i) blue dots are significantly enhanced, (ii) yellow dots are significantly different but not satisfied the fold criteria, (iii) green dots satisfied the fold criteria but not significantly different, (iv) red dots not significantly different (present at equal abundance in both treatments). Details of the computational comparison are available in **Tables S4**, **S5**.

fatty acid-binding protein has similar effects on host immune modulation. Other proteins showing high FC values in saliva of *A. americanum* SR ticks are glycine-rich proteins: Aam-178421 (FC: 29.1), Aam-1227 (FC: 29.0), Aam-327 (FC: 13.1) when pairing with SH; and Aam-179267 (FC: 18.6), Aam-177922 (FC: 14.2), and Aam178421 (FC: 12.8) when pairing with SD (**Table S5**). Glycine-rich proteins are extracellular matrix proteins and/or structural proteins thought to play an important role in attachment to the host, as they are present in cement material secreted by salivary glands during feeding process (Bishop et al., 2002; Maruyama et al., 2010). Since these proteins are secreted in the early stage of tick feeding, we suggest that glycine-rich proteins could be associated with tick cement formation, securely anchoring ticks onto host skin during the prolonged tick-feeding period. Another set of proteins with high FC values include a hemelipoprotein (FC: 21.3), a GAPDH (FC: 16.0), several serpins (FC: 15.9 to 1.5), among others (**Table S5**). More details about the computational comparison are available in **Figure 5**, **Tables S4**, **S5**.

The observation of apparent differential expression is not likely peculiar to this study. In a lone study, tick proteins involved in blood digestion and reproduction were overrepresented in *R. microplus* ticks that fed on cattle when compared to ticks that fed on white-tailed deer (Popara et al., 2013). In a related study, *I. scapularis* displayed variable fibrinogenolytic activities upon feeding on mice with different immune backgrounds (Vora et al., 2017).

CONCLUSIONS AND FUTURE PERSPECTIVES

It is important to note that proteins being discussed here were identified in saliva of unfed adult ticks. Findings that 83 of the 165 proteins found in SR *I. scapularis* tick saliva were also identified in the saliva of *I. scapularis* ticks that were fed on rabbits (**Table S3**) gives us confidence these proteins are also injected into the host during feeding. Of these 83 unique SR tick saliva proteins, 52, 67, 50, 58, 38, 37, and 59 proteins (**Table S3**) were respectively found in saliva of *I. scapularis* that fed on rabbits for 24, 48, 72, 96, and 120 h of feeding as well as those that were engorged but not detached and those that repelete fed and detached (Kim et al., 2016b). Of significance, 47 of the 55 *I. scapularis* proteins that were found in all treatments (**Figure 2A**) were also identified in saliva of this tick during feeding (Kim et al., 2016b; **Tables S1**, **S3**). While, it is apparent that some of the proteins that we found in saliva of SR *I. scapularis* ticks, we cannot confirm at this point if proteins in saliva of unfed SD and SH *I. scapularis* ticks are secreted during feeding as we cannot ethically feed ticks on humans for research purposes. This same limitation applies to our *A. americanum* tick saliva proteins in this study. We may be able to confirm in dogs, however it will be difficult to prove for humans. Despite these limitations, data here provides the first step toward the molecular basis of host range adaptation. To our knowledge, this work is the first report describing the use of LC-MS/MS analysis aimed

at addressing the biologically relevant question of tick saliva plasticity when ticks are stimulated to feed on different hosts. Our data suggest that the tick's molecular preparation to start feeding is likely host-specific, as by differential protein profiles in saliva of both *A. americanum* and *I. scapularis* ticks which were stimulated to start feeding on different hosts. Within these different protein profiles there is a set of proteins that the tick may utilize to feed on all hosts. From the perspective of development of vaccines against tick feeding, data here has practical to the field of the molecular basis of tick feeding physiology, and tick vaccine development in particular. With the exception of anti-*R. microplus* vaccine research, for which its natural host, cattle are used for feeding, various laboratory animals such as lagomorphs and rodents are used as hosts for tick feeding, despite introduction of techniques involving artificial feeding on either animal skins or synthetic membranes (Waladde et al., 1991; Gonsioroski et al., 2012; Hatta et al., 2012). Therefore, contemporary research to develop vaccines against medically important tick species utilizes laboratory animal models in initial screening to identify putative effective antigens (Sugino et al., 2003; de la Fuente and Kocan, 2006; Rodríguez-Mallon et al., 2012; Kim et al., 2016a). Our data here clearly demonstrates that there are potential flaws to the use of laboratory animals to identify putative anti-tick vaccine antigens. Given that the tick might inject different sets of proteins in different hosts, there is potential to focus on irrelevant proteins when model animals are used in initial screens. The core of proteins that the tick might inject into all hosts (**Tables S1**, **S2** and **Figure S1**) or those that the tick might utilize to regulate feeding on both laboratory animal hosts such as rabbits and relevant hosts such as human and dogs (**Figure 5**) could be prioritized for tick vaccine development. New Zealand white rabbits are usually the most accessible and most suitable hosts that are routinely used in tick vaccine research (Troughton and Levin, 2007). However, often there are experimental results obtained using a laboratory model that do not match reality when applied in wildlife animals (Olds et al., 2016). This could potentially be a consequence of targeting proteins that are important to tick feeding success on a lab animal model, but not the relevant in another host. Therefore, the identification of saliva proteins that are secreted in different hosts, including laboratory models such as rabbits, will remove the risk of targeting irrelevant proteins.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: LT, TK, and AM. Performed the experiments: LT, TK, and AP, AM. Contributed reagents, materials, analysis tools: AM and JY. Drafting the article: LT, TK, AP, JY, IdS, and AM. Critical revision of the article: LT, TK, AP, JY, IdS, and AM.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2017.00517/full#supplementary-material

Figure S1 | Protein counts in saliva of *Ixodes scapularis* and *Amblyomma americanum* ticks stimulated to feed on different hosts showing a core set of functionally similar proteins in their saliva. The sum of proteins for each functional class is represented as the percentage of total proteins identified. Details of protein identification are available in **Tables S1**, **S2**.

Table S1 | *Ixodes scapularis* saliva proteins identified by LC-MS/MS.

Table S2 | *Amblyomma americanum* saliva proteins identified by LC-MS/MS.

Table S3 | Comparison of *Ixodes scapularis* tick saliva proteins identified upon stimulation to feed on different hosts and *Ixodes scapularis* tick saliva proteins secreted every 24 h during blood feeding.

Table S4 | Differentially expressed proteins based on *t*-fold analyses comparing *Ixodes scapularis* saliva proteins from tick stimulated to feed on different hosts.

Table S5 | Differentially expressed proteins based on *t*-fold analyses comparing *Amblyomma americanum* saliva proteins from tick stimulated to feed on different hosts.

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REVIEW
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Gene Duplication and Protein Evolution in Tick-Host Interactions

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Ticks modulate their hosts' defense responses by secreting a biopharmacopiea of hundreds to thousands of proteins and bioactive chemicals into the feeding site (tick-host interface). These molecules and their functions evolved over millions of years as ticks adapted to blood-feeding, tick lineages diverged, and host-shifts occurred. The evolution of new proteins with new functions is mainly dependent on gene duplication events. Central questions around this are the rates of gene duplication, when they occurred and how new functions evolve after gene duplication. The current review investigates these questions in the light of tick biology and considers the possibilities of ancient genome duplication, lineage specific expansion events, and the role that positive selection played in the evolution of tick protein function. It contrasts current views in tick biology regarding adaptive evolution with the more general view that neutral evolution may account for the majority of biological innovations observed in ticks.

Keywords: tick evolution, gene duplication, protein family evolution, salivary gland, blood-feeding evolution, hematophagy

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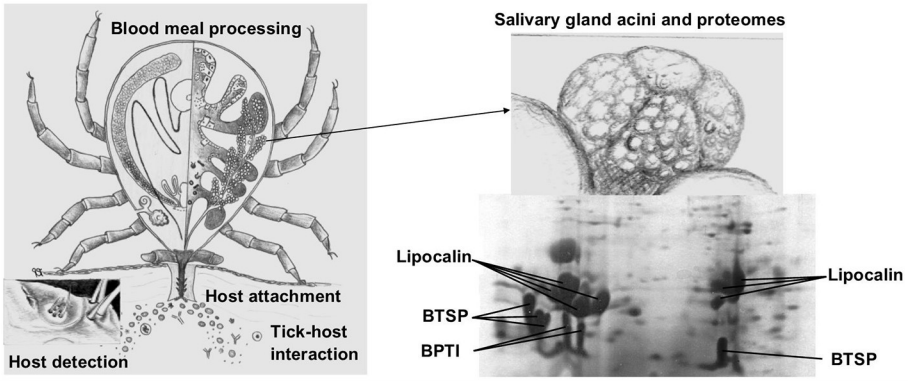
EVOLUTION OF HEMATOPHAGY IN TICKS

Ticks (Ixodida) are obligate blood-feeding ecto-parasites that evolved a blood-feeding lifestyle >250 million years ago (MYA) (Mans et al., 2011, 2012, 2016). The ancestral hematophagous lineage diverged into extant families: Argasidae (soft ticks ~200 species), Ixodidae (hard ticks ~700 species), and the monotypic Nuttalliellidae (*Nuttalliella namaqua*) (Guglielmone et al., 2010). A parasitic blood-feeding lifestyle entails interaction with the vertebrate host, necessitating the evolution of mechanisms to ensure successful acquisition of a blood meal, described as the four stages of blood-feeding evolution (**Figure 1**), namely: host-detection, host-attachment, host-interaction, and blood meal processing (Mans, 2014). For the current study, tick-host interactions and lineage specific innovation that occurred after divergence of the main tick families are of interest. This is most apparent in the comparison of the salivary gland repertoires secreted into the host during feeding. The evolution of salivary gland protein families and protein function is mediated by gene duplication and a major issue is whether tick-host evolution is adaptive or neutral. The current review will examine these issues and aim to illuminate the general theories of gene duplication and functional evolution with tick specific examples.



Major Salivary Gland Protein Families Prone to Gene Duplication

Lipocalins: 34-1132 genes per tick species. **Functions:** Histamine, Serotonin, LTB4, LTC4, TXA2 scavengers, Complement C5 inhibitors.

BPTI/Kunitz: 21-990 genes per tick species. **Functions:** Blood-clotting inhibitors, Platelet aggregation inhibitors, Tryptase inhibitors.

BTSP: 19-716 genes per tick species. **Functions:** Clotting inhibitors.

Metalloprotease: 38-991 genes per tick species. **Functions:** Fibrinogenases.

FIGURE 1 | Gene duplication in the evolution of blood-feeding in ticks. The four main stages in the evolution of blood-feeding includes host detection, host attachment, tick-host interaction, and blood meal processing (Mans, 2014). Tick salivary glands play a central role in tick-host interactions by secreting the bioactive molecules that modulates host defense mechanisms. Some of these secreted proteins belong to large protein families expanded by gene duplication that can count from tens to hundreds of different genes per tick species. BPTI (Basic pancreatic trypsin inhibitor), BTSP (Basic tail secretory proteins), LTB4 (leukotriene B4), LTC4 (leukotriene C4), TXA2 (thromboxane A2). Information on protein families summarized from Mans et al. (2016).

COMPARATIVE TRANSCRIPTOMICS BETWEEN HEMATOPHAGOUS ARTHROPODS

Independent evolution of hematophagy is evident given the paraphyletic nature of blood-feeding arthropod lineages that originated at least 20 times (Ribeiro et al., 2010; Mans, 2011; Mans et al., 2016). Convergent evolution is apparent from the various solutions that blood-feeders found for their common problem, i.e., how to control the vertebrate host's defense mechanisms. A number of generalizations regarding salivary gland transcriptomes suggest that evolutionary mechanisms may be universal for all blood-feeders (Mans, 2011). As such, all blood-feeders secrete bioactive components from their salivary glands into the feeding site that target similar key host defense mechanisms. This underscores the universality of vertebrate host inflammatory and hemostatic mechanisms, since these evolved (>350 MYA), before most arthropod lineages adapted to blood feeding (Delvaeye and Conway, 2009; Conway, 2015). Unique salivary gland protein families in different arthropod lineages, suggests that salivary gland repertoires differed before adaptation to blood feeding depending on the specific lifestyle (scavenger, parasites, plant feeder, or predator) of the non-hematophagous ancestral lineage. Closely related hematophagous lineages have similar salivary gland protein families, even if they do not have similar functions and these same families are prone to gene duplication (Mans, 2011). These generalizations have predictive value for the study of uncharacterized blood-feeding lineages, where we expect targeting of key host processes, unique salivary gland repertoires and limited numbers of protein families that have been expanded by gene duplication.

TICK SALIVARY GLAND TRANSCRIPTOMES

Hundreds to thousands of proteins synthesized in the salivary glands of ticks target key host defense mechanisms involved in inflammation and hemostasis (Francischetti et al., 2009). Extensive tables summarizing hemostatic and immune inhibitors, host targets, protein family affiliations, and distribution of orthologs in genera and lineages were recently updated (Mans, 2016; Mans et al., 2016). Summaries of sequenced transcriptomes, secretory, and house-keeping proteins, protein family members, and largest expanded families were also tabulated (Mans, 2016; Mans et al., 2016). All tick families possess the same major salivary gland secretory protein families (**Figure 1**). These include lipocalins, kunitz-basic pancreatic trypsin inhibitors (BPTI), metalloprotease, serpin, trypsin inhibitor-like (TIL), cystatin, and basic tail secretory (BTSP) families (Mans et al., 2008a, 2016). However, few orthologs are shared between the different tick families and lineage specific expansions of these gene families are common (Mans et al., 2008a, 2016; Dai et al., 2012; Schwarz et al., 2014a). Of interest, is the sudden rise in described secretory family members after introduction of next-generation sequencing technologies (NGS), with an ∼10-fold increase for secretory protein family members from conventional cDNA libraries (10–100s family members) to NGS (100–1,000s family members)

sequenced transcriptomes. The numbers of secretory family members found in the *Ixodes scapularis* genome is closer to that observed for cDNA libraries. Whether the high levels of lineage specific expansion observed in NGS transcriptomes are real due to alternative splicing or gene duplication, or artefactual due to transcriptome assembly, taxonomic sampling, or sequence depth bias cannot be determined yet (Mans, 2016; Mans et al., 2016). However, functional analysis of the major host interacting proteins confirm that they belong to different protein families in the different tick families and therefore evolved these functions independently (Mans et al., 2002, 2016; Mans and Neitz, 2004a; Mans, 2011). The current study will discuss scenarios and evolutionary mechanisms by which these novel and different functions evolved.

SPECIATION, GENE DUPLICATION, AND THE FATES OF GENES

Members of protein families are homologous, i.e., share a common ancestor (Koonin et al., 2002). Homology may be due to vertical descent (speciation) and such genes are orthologs or orthologous. Homology may also be due to gene duplication resulting in two or more members of the same gene within the genome and such genes are paralogs or paralogous (Fitch, 1970, 2000; Koonin, 2005; Gabaldón and Koonin, 2013). During subsequent speciation, these paralogs will become orthologs in the descendant lineages. Gene loss may occur in orthologs and paralogs, or gene duplication may occur in selective descendant lineages and can lead to phylogenetic relationships that are complex and difficult to resolve, given our dependence on information from extant lineages. Attempts to classify these relationships led to creation of designations such as co-orthologs (a gene from one species is collectively orthologous to duplicated genes in other species), inparalogs (lineage-specific gene duplications occurring after speciation), and outparalogs (lineage-specific gene duplications occurring before speciation) (Sonnhammer and Koonin, 2002; Koonin, 2005). It becomes very difficult to delineate these without a broad taxonomic sampling of closely related and divergent lineages and for most discussions, the general concepts of orthologous and paralogous genes are sufficient (Jensen, 2001). Confounding factors in ortholog identification includes domain-shuffling, acquisition/loss of new domains and alternative splicing (Gabaldón and Koonin, 2013). Most small tick proteins involved at the tick-host interface belong to single domain families (BPTI, cystatin, lipocalin, serpin, TIL), or when multi-domains are present they are generally oligomers of the same domain (e.g., BPTI, BTSP) (Francischetti et al., 2009). Even so, given the fact that extensive lineage-specific expansions occur in ticks (existence of co-orthologs and inparalogs), the identification of orthologs remains problematic.

ORTHOLOGOUS GENES IN TICKS

Orthologs generally possess similar molecular structure, function, mechanism of action, conserved residues involved in molecular interaction and domain architecture across species or lineages and can be traced to the last common ancestral lineage where this function originated (Gabaldón and Koonin, 2013). This is the basis for the universality of general cell biological processes such as transcription, translation, cellular localization, secretion, transport, metabolism, and our ability to annotate genes by homology as encapsulated in the ortholog conjecture (Gabaldón and Koonin, 2013; Rogozin et al., 2014). Since most orthologs perform vital functions in general cell biology or development, they are fixed in populations or species by negative selection. Even so, gene losses occur, orthologs acquire new or additional functions, domains are exapted for a new function (Gabaldón and Koonin, 2013). Exaptation of house-keeping functions at the tick-host interface have occurred as seen for glycolytic enolase, that also function as plasminogen activator (Díaz-Martín et al., 2013). Numerous orthologs specifically involved in tick-host interaction exist. Apyrases, biogenic amine binding proteins (BABP), enolase, metalloproteases, and defensins evolved in the last common ancestor to all ticks (Mans et al., 2016). BPTI-thrombin inhibitors, BPTI-fibrinogen receptor antagonists, and cysteinyl leukotriene scavengers evolved in the last common ancestor to soft ticks (Mans and Ribeiro, 2008a; Mans et al., 2008b). Ixodegrins, serpin-thrombin inhibitors, BPTI-thrombin inhibitors, and immunoglobulin-binding proteins evolved in the last common ancestor to hard ticks (Mans et al., 2016). Similarly, orthologs restricted to different genera exist, for example, the moubatin-clade thus far restricted to the genus *Ornithodoros* that are paralogous to the biogenic amine binding clade (Mans and Ribeiro, 2008b).

PARALOGOUS GENES IN TICKS

Once a gene duplicates several possibilities exist for its fate (**Figure 2**). One copy may rapidly acquire deleterious mutations and become pseudogenized (non-functionalization) (Innan and Kondrashov, 2010). Paralogs may retain the same function and if maintained by negative selection, are considered to be due to requirements of a dosage effect in house-keeping proteins, i.e., high concentrations are necessary to fulfill the requirements of the cell (Innan and Kondrashov, 2010; Rogozin et al., 2014). Genes in this category include histone and 18S, 5.8S, and 28S ribosomal RNA cassettes that occur in hundreds of copies in metazoans, including ticks (Mans et al., 2015). All copies are highly similar and maintained via concerted evolution, gene conversion or strong purifying selection (Nei and Rooney, 2005; Innan and Kondrashov, 2010). Examples of paralogs maintained for dosage effects at the tick-host interface are the savignygrins and monogrins secreted at high concentrations to target the abundant fibrinogen receptor on platelets (Mans et al., 2003a, 2008b).

Mutations may accumulate in one copy resulting in loss of the old function and evolution of a new function (neo-functionalization) and leads to the generalization that paralogs have different functions (Gabaldón and Koonin, 2013). Functions may be similar in a general sense, i.e., members of the Ran-GTP or karyopherin families all function in cellular transport and trafficking (Mans et al., 2004); tick lipocalins function

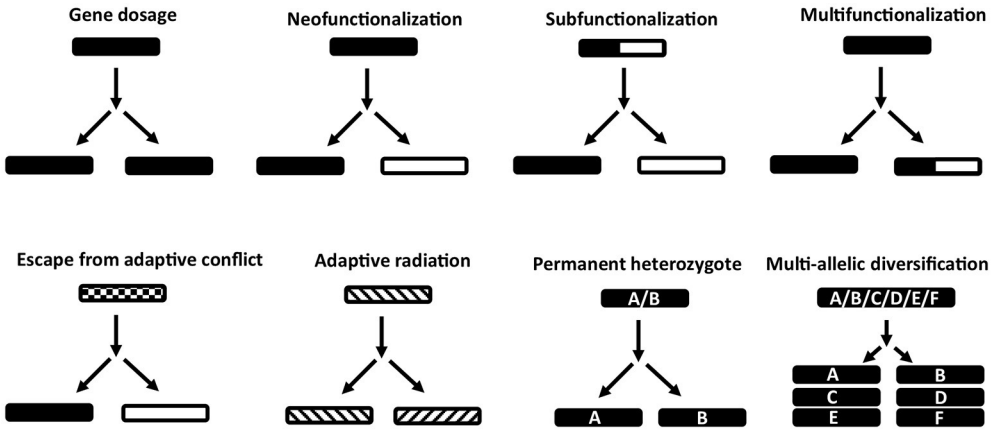


FIGURE 2 | Models of gene duplication and evolution of function. Indicated are various models for the evolution of function before and after gene duplication events. Gene dosage refers to maintenance of gene duplicates with the same function. Neofunctionalization refers to the acquisition of a new function and loss of the old function in one gene duplicate. Subfunctionalization refers to the segregation of two functions in different gene duplicates that was present in the ancestral gene. Multifunctionalization refers to acquisition of a new function in one gene copy, while retaining the original function. Escape from adaptive conflict refers to an ancestral gene with functions that are overlapping or sub-functional due to exclusion effects. These functions are optimized in respective gene duplicates after duplication. Adaptive radiation refers to ancestral genes that have pre-adapted functions, which allow the evolution of similar functions in gene duplicates. Permanent heterozygote refers to heterozygotes that have better fitness than homozygotes and where gene duplication leads to fixation of both alleles in paralogs. Multi-allelic diversification refers to the case where the highest number of heterozygous individuals in a population is advantageous. Gene duplications leads to many divergent genes coding for the same function.

as scavengers of diverse bioactive molecules such as biogenic amines (Mans et al., 2008c), cysteinyl leukotrienes (Mans and Ribeiro, 2008a), thromboxane A2 (TXA2), and leukotriene B4 (LTB4) (Mans and Ribeiro, 2008b); or BPTI inhibitors that target different serine proteases such as fXa and thrombin (Mans et al., 2002), but differ in target specificity. Functions may also differ completely, such as the platelet aggregation inhibitors that belong to the BPTI protein family, but target integrin receptors (Mans et al., 2008b).

Some genes may have multiple functions and after duplication, each gene loses one function but retains the other (sub-functionalization) (Force et al., 1999; Lynch and Force, 2000). An example in ticks may be the histamine and serotonin binding proteins, monotonin and monomine in *Argas* ticks that probably derived from a lipocalin capable of binding both histamine and serotonin in the same site (Mans et al., 2008c).

Some paralogs may also retain the original function and evolve new functions, thereby becoming multifunctional (multi-functionalization). An example in ticks would be the complement C5 inhibitors from *Ornithodoros* ticks that retained the ancestral function of scavenging LTB4, but evolved C5 complement binding activity on their βH-α2 loop (Mans and Ribeiro, 2008b).

If multiple functions are overlapping in mechanism, hindering functional optimization, gene duplication, and subsequent divergence can optimize functions; the escape from adaptive conflict model (Hughes, 1994; Des Marais and Rausher, 2008). In ticks, an example of this specific model has not yet been found, although candidates exist that would certainly benefit from this, e.g., moubatin that binds LTB4 (inhibition of neutrophil migration) and TXA2 (inhibition of platelet aggregation) in the same binding pocket at similar affinities (Mans and Ribeiro, 2008b). This increases the risk of competitive exclusion and neutralization of both functions if both antagonists were to be present in the feeding site at the same time.

Proteins with multiple functions or broad specificities may already be predisposed to evolve a new function once duplicated, the adaptive radiation model (Francino, 2005). Tick inhibitors with similar general functions may fall in this class. Positive selection has originally been promoted as the driving force for gene-level adaptive radiations, but it should be noted that neutral or even mildly deleterious mutations can also accumulate in genes and become fixed within a population through processes such as drift. It may be argued that neutral evolutionarily processes play a particularly important role for generating gene diversity and novel functions in genes with broad specificities because mutations in genes with promiscuous activity are more likely to be neutral than they are to have either a positive or negative effect on fitness.

Where heterozygous alleles have better fitness than homozygotes, gene duplication can create a permanent heterozygote (Proulx and Phillips, 2006). An extension of this model, the multi-allelic diversifying selection model, occurs when heterozygosity is advantageous at the population level (Innan and Kondrashov, 2010). In such instances, gene duplication leads to fixation of different heterozygotes under positive selection, accumulation of new alleles and gene expansion. For both these models, hyper-variable genes in ticks that possess the same function would fit in these models as proposed for genes evading the host immune system (Chmelař et al., 2016). However, even where related extant taxa have been well-sampled, in most instances it is challenging to determine

which model best accounts for specific instances of family expansions because while the underlying causes for family diversification may differ the outcomes may appear similar. For example, when considering the multi-allelic diversifying model, the gene dosage model may instead be considered for gene duplicates where the active or binding site is internally located or the functional residues are restricted to a small area of the protein structure and where purifying selection only acts on these regions to conserve functionality and protein fold structure. Equally, neutral evolution and accumulation of mutations also lead to extremely diverse families that have the same or similar functions that would resemble the multi-allelic diversification model, but without the need for positive selection.

RATES OF GENE DUPLICATION

Gene duplication gives rise to paralogs and is considered the major mechanism to generate genetic diversity and new functions (Lynch, 2002). The general rate of gene duplication is ∼1% per gene per million years, while 50% of duplicated genes are lost every 4 million years (Lynch, 2002). In a species with ∼20,000 genes (such as ticks), ∼1,000 genes will become fixed over 10 million years. With regard to ticks, *I. scapularis* is the only genome available (Gulia-Nuss et al., 2016), with 2–3% predicted genomic and 7–22% transcriptomic paralogs (Van Zee et al., 2016). Paralog pairs (two duplicated genes) make up ∼80% of paralogs found in the genome, indicating a single duplication event for these genes. The majority of these duplications occurred <6 MYA (Van Zee et al., 2016), which fit the expected gene duplication rate. The transcriptome data suggest a higher rate of gene duplication than expected. Genome and transcriptome differences are partly due to a higher number of genes in salivary gland transcriptomes (Valenzuela et al., 2002; Ribeiro et al., 2006), not present in the genome assembly, which only extracted gene annotations from ∼57% of the genome (Gulia-Nuss et al., 2016). Conversely, other measures of completeness such as the core eukaryotic genes mapping approach (CEGMA, 248 conserved genes) and benchmarking universal single-copy orthologs (BUSCO, 2,675 conserved genes), indicate ∼80 and ∼69% genes represented in the *I. scapularis* genome, respectively (Hoy et al., 2016). A lack of gene coverage alone cannot account for these differences.

The only related tick species with extensive genetic data is *I. ricinus* for which salivary, midgut, and hemocyte transcriptomes were sequenced and a draft genome assembled (Chmelař et al., 2008; Schwarz et al., 2013, 2014b; Cramaro et al., 2015, 2017; Kotsyfakis et al., 2015a,b; Perner et al., 2016). Molecular clock analysis based on the mitochondrial proteins, suggests that *I. scapularis* and *I. ricinus* diverged ∼9 MYA, suggesting the majority of the gene duplications detected by Van Zee et al. (2016) occurred after their divergence. It is therefore of interest that ticks exhibit extensive duplicated gene families in their salivary gland transcriptomes which seem to be lineage or species specific expansions, i.e., phylogenetic analysis indicate protein family clades that effectively consist of genes from only one species (Mans et al., 2008a, 2016; Schwarz et al., 2013). Reciprocal best hit analysis of non-redundant datasets (clustered at 95% protein identity to remove possible alleles, resulting in 20,869 *I. scapularis* and 30,641 *I. ricinus* proteins), indicates that 10,105 best hits (orthologs) occur between *I. scapularis* and *I. ricinus* ($<E^{-10}$) (Mans et al., 2016). This corresponds with ∼10,000 orthologs found between *I. scapularis* and other parasitiform mites (Dong et al., 2017). Unique proteins from each dataset with significant hits ($<E^{-10}$) in their respective orthologous sets, comprised 23% (4,738 proteins) and 53% (16,000 proteins), respectively for *I. scapularis* and *I. ricinus*, indicating that these paralogs duplicated after speciation, or were lost in different species. In the case of *I. ricinus*, it indicates a larger gene duplication rate than *I. scapularis*. This could be due to genes missing from the *I. scapularis* assembly, true duplications in *I. ricinus* or artifacts from *de novo* assembled transcriptomes derived from NGS (Mans et al., 2016). The genome size of *I. ricinus* (∼2.71 Gbp) is larger than *I. scapularis* (∼2.2 Gbp) (Geraci et al., 2007; Cramaro et al., 2017), and may explain some of the paralog differences. However, for both species the number and rate of gene duplications are higher than expected, differing by ∼5–16-fold from the expected number of gene duplications. With regard to major secretory protein families, the Kunitz-BPTI, BTSP, and lipocalin protein family members of *I. scapularis* possess 69 (39%, 176 total), 49 (40%, 123 total), and 47 (39%, 119 total) unique genes (duplicated per species), respectively. For *I. ricinus* there are 846 (80%, 1,058 total), 682 (82%, 834 total), and 1,011 (79%, 1,283 total) unique genes for the Kunitz-BPTI, BTSP, and lipocalin family members, respectively (Mans et al., 2016). This ranges from 40 to 80% of the total members for each family and is higher than the overall percentage of duplicated genes (7–22%) observed in the genome of *I. scapularis* (Van Zee et al., 2016). This differs by orders of magnitude from the average rate of gene duplication observed in other arthropods (Gulia-Nuss et al., 2016; Hoy et al., 2016; Dong et al., 2017), and suggests elevated gene duplication rates in ticks and most especially for secretory proteins. The question raised, is whether this number of gene duplications could have occurred since speciation, with the implication that these duplicates are an evolutionary response of the tick to a blood-feeding environment.

MOLECULAR ARMS RACES

The diversity in gene family members may be an adaptive response to escape the host immune system described as an escalating host-parasite arms race that leads to innovation from either side to counter-act new functions (Mans, 2011; Chmelař et al., 2016). This may take the form of new functions evolving, making defense systems on both sides more redundant (i.e., the Red Queen hypothesis). It could take the form of expansion of the same protein family to yield antigenic variants that retain the same function. These variants, secreted concurrently at low levels during feeding, evade the immune system, while still achieving a concentration necessary for immune system inhibition: the varying epitope hypothesis (Couvreur et al., 2008; Chmelař et al., 2016). The varying epitope hypothesis is a variant of the multi-allelic diversifying selection model (Innan and Kondrashov, 2010). It may also take the form of an expanded family that escapes the host's immune system by differential

expression during feeding: the antigenic shift during feeding hypothesis (Chmelař et al., 2016). Supporting evidence for this may be heightened non-synonymous substitution rates (Kn/Ks > 1, positive selection), indicative of adaptive selection (Kotsyfakis et al., 2015b; Ribeiro et al., 2017).

Paralogous proteins from the same clade with similar functions such as BABPs, LTB4 scavengers or GPIIbIIIa inhibitors have appreciable sequence similarity, excluding antigenic variation to escape the immune system of the host (Mans et al., 2003a, 2008b,c; Mans and Ribeiro, 2008b). These paralogs probably fulfill a dosage requirement, since functions like scavenging of bioactive molecules or targeting abundant platelet receptors, require high concentrations of inhibitors. The only study that characterized an extensive multigene family that shows the same function, is the Salp20 group of complement inhibitors that disrupts the active C3 convertase (C3bBbP) complex by binding properdin (Valenzuela et al., 2000; Daix et al., 2007; Tyson et al., 2007, 2008; Couvreur et al., 2008). Different Salp20 genes are expressed in different individuals across the time course of feeding (Couvreur et al., 2008). This family would represent a mixed model of antigenic shift during feeding and varying epitopes hypotheses. This family has a number of gene members in both *I. scapularis* and *I. ricinus* and shows overall sequence identity of 35–75%. The members of this family are all highly N- and O-linked glycosylated, while thrombospondin repeats of properdin specifically bind sulfated glycoconjugates and glycosaminoglycans (Couvreur et al., 2008; Tyson et al., 2008). The mechanism of action among the various gene duplicates may in certain instances be sequence independent (i.e., glycosylation dependent), relaxing purifying selective constraints on the protein sequences, while retaining their function. This family would then be a very specific case of functional conservation due to post-translational modification, and not necessarily an example of antigenic shift or varying epitopes and may not be good support in favor of these hypotheses. Similarly, scavengers of bioactive molecules such as the lipocalins, may show extensive sequence variation, since their conserved binding sites are internally located and antibody responses would not necessarily block activity. Their sequence diversity may therefore not be due to antigenic variation, but may be due to genetic drift and relaxation of purifying selection.

Co-evolution of tick and host in an escalating arms race was proposed to explain the diversity of tick salivary gland proteins (Mans, 2011; Chmelař et al., 2016). In this, positive selection of secretory proteins plays an important role as a means to evade the host's immune system (Kotsyfakis et al., 2015b; Ribeiro et al., 2017). The Red Queen hypothesis was also invoked to explain gene duplication and protein evolution in salivary glands as part of an escalating arms race (Schwarz et al., 2014a). The Red Queen hypothesis is an extension of co-evolution that specifically indicates that as ticks evolve numerous functions, the host should respond in kind. Whereas, extensive gene duplication and genetic diversity is observed for ticks, the same cannot be said for the host hemostatic or even immune systems. The basic hemostatic and immune functions of the host were evolved before adaptation of ticks to a blood-feeding lifestyle and are generally conserved in mammals (Delvaeye and Conway, 2009; Conway, 2015). The Red Queen hypothesis cannot therefore be strictly applied to tick-host interactions.

POSITIVE SELECTION IN THE FACE OF NEUTRAL EVOLUTION AND GENETIC DRIFT

The current dominant view in evolutionary biology is that neutral evolution and genetic drift may have been responsible for many evolved functions and that neutral evolution should be considered as the null hypothesis that must first be rejected before adaptive explanations are considered (Lynch, 2007; Nielsen, 2009; Koonin, 2016). Positive selection, where the rate of non-synonymous substitutions in orthologs (i.e., different species) is greater than synonymous substitutions (Kn/Ks > 1), is the gold standard measure of adaptive signal (Yang and Bielawski, 2000; Nielsen, 2005; Kondrashov, 2012). When comparing closely related taxa, the majority of genes found in eukaryotic genomes have a Kn/Ks ratio $\leq$1, which is indicative of neutral evolution or negative/purifying selection. Overall, few genes have been identified that exhibit signatures of positive selection (Yang and Bielawski, 2000; Kondrashov, 2012). In ticks, positive selection has been detected in many secretory proteins supporting the hypothesis that ticks have adapted to a blood-feeding environment (Daix et al., 2007; Couvreur et al., 2008; Dai et al., 2012; Kotsyfakis et al., 2015b; Van Zee et al., 2016; Ribeiro et al., 2017). All of these studies calculated Kn/Ks ratios between paralogous proteins within single species or used mapping and variant detection of NGS data, where higher Kn/Ks ratios may reflect non-specific mapping of reads to paralogs. These approaches are similar to measures of volatility and liable to suffer from the same drawbacks from which no conclusion can be made regarding positive selection (Nielsen and Hubisz, 2005). In contrast, if orthologs and paralogs between *I. ricinus* and *I. scapularis* from the Salp20 families are compared, ortholog Kn/Ks ratios are below 1 (Daix et al., 2007). Detection of positive selection is complicated by the presence of paralogs in a dataset (Brieuc and Naish, 2011), and increased Kn/Ks ratios may be expected between paralogs with different functions, since their divergence entails non-synonymous substitutions. Relaxation of purifying selection in recently duplicated genes (i.e., increased rates of mutation) is also observed, but is not necessarily due to positive selection (Kondrashov et al., 2002). In ticks, the average Kn/Ks obtained for secreted proteins ranges from Kn/Ks of 1.1–1.3 (Kotsyfakis et al., 2015b; Ribeiro et al., 2017), which may be interpreted as slightly above neutral signal. However, this may be due to relaxation of purifying selection rather than positive selection, with the caveat that differences between paralogs may still be more extensive than between orthologs. An alternative definition of neutral evolution, where mutations that do not alter gene function appreciably are considered neutral, Kn/Ks-values slightly above or below 1 are still considered neutral

(Nei, 2005). As yet, we cannot conclude that positive selection is evident in secretory proteins at genome or transcriptome scale for ticks until the Kn/Ks ratios for orthologs between closely related species such as *I. scapularis* and *I. ricinus* have been determined. Therefore, the null hypotheses of neutral evolution cannot be rejected in favor of an adaptive interpretation for tick-host interactions.

NEUTRAL EVOLUTION AND GENETIC DRIFT AS DRIVERS OF BLOOD-FEEDING EVOLUTION

If neutral evolution and genetic drift are major causes of diversity in ticks and adaptive selection is limited, the questions remain how ticks evolved the impressive array of functions exhibited at the feeding site and why elevated levels of gene duplication are observed in salivary gland families (Mans et al., 2016). One possibility lies in the opportunity that salivary gland expression offers for the evolution of new functions (Mans, 2011, 2016). Proteins destined for secretion need to maintain signals for secretion and sorting, i.e., signal peptides and sorting signals for secretory granules (Nielsen et al., 1997; Gomez-Navarro and Miller, 2016). Once a protein enters the secretory pathway, deleterious effects on cellular activities or its own function will be negated by containment within cellular and extracellular compartments, such as the secretory granule where it is packaged with other secretory proteins before being secreted into the external environment of the feeding site. Since the energetic cost of maintaining gene duplicates is relatively inexpensive (Lynch and Marinov, 2015), and deleterious mutations will not affect other cellular processes, ticks can maintain such sub-functional or even non-functional genes until they are fixed by neutral evolution and genetic drift. Neutral mutations may accumulate in these secreted sub-functional (or even non-functional genes), and be fixed as long as the secretory signals or structural determinants are maintained (Lynch et al., 2016). Once a mutation arises that is beneficial, followed by subsequent mutations that enhances this new functionality, this function may be maintained by purifying selection. This is the general case of neofunctionalization, where new functions evolve due to random neutral mutations (Innan and Kondrashov, 2010). Evolution of new function may even be more subtle, when redundant pathways or processes are targeted, starting with maintenance of the original function in the new gene duplicate until a mutation arises that changes its functional specificity slightly and allows binding to related targets with related functions. Subsequent optimization of function will lead to loss of the original function and gain of a new function. This will be a combination of the multi-functionalization, neofunctionalization, subfunctionalization, escape from adaptive conflict, and adaptive radiation models. Another possibility is for genes with broad target specificities that duplicate and subfunctionalize due to loss-of-function mutations, which result in maintenance of gene duplicates with different functions (Lynch and Force, 2000). These scenarios may explain why so many members of the same protein family target similar members in a host family (BPTI➔serine proteases, serpins➔serine proteases, cystatins➔cysteine proteinases, evasins➔chemokines). It also implies that many proteins in salivary transcriptomes may have sub-optimal or no functions, but are not pseudogenes in the classical sense (Mudge and Harrow, 2016), since they are still transcribed, expressed and secreted. In this regard, pseudogenes may compose a large part of multigene families in genomes ranging from 25 to 60% (Nei and Rooney, 2005). It may also explain in part the lineage specific expansions observed in salivary gland protein families, since duplicated genes may rarely be lost as observed for house-keeping duplicates. The birth and growth of multigene families approximate power law behavior (Koonin et al., 2002; Koonin, 2011), with those families that start to expand due to gene duplication, accumulating more gene duplications over time. In this scenario, multigene families can rapidly expand without recourse to adaptive selection. Instead of an adaptive arms race with the host, on the battlefield of the tick-feeding interface (Chmelař et al., 2016), the salivary glands and their secretory proteins become a vast experimental playground, where the building blocks can be tested and changed by neutral evolution without being discarded. The latter scenario posits the null hypothesis of neutral evolution in terms of tick-host interactions and the general argument may hold for blood-feeding arthropods in general, since all show expansion of lineage specific protein families (Mans, 2011).

DATING OF MULTIGENE FAMILIES

Gene duplication linked with molecular sequences and phylogenetic analysis offers the promise of dating gene duplication events (Kumar, 2005). This could allow determination of gene duplication rates and estimation of the emergence time of new functions. However, without accurate constraints on nodes, the high level of divergence in salivary gland paralogs could lead to over-estimation of divergence times using molecular clock models. While this is an acknowledged problem in molecular clock dating techniques (Kumar, 2005), scenarios arise where divergence estimations for *I. ricinus* and *I. scapularis* based on analysis of the BPTI family indicated multiple divergence dates for these species in different clades of the same protein family (Schwarz et al., 2014a). As such, dates ranged from 10 to 66 MYA for divergence of *I. ricinus* and *I. scapularis*, and from 8 to 60 MYA for clades that were clearly lineage specific expansions in *I. ricinus* (Schwarz et al., 2014a). This implies non-clock-like behavior in different clades (Mans et al., 2016). Since *I. scapularis* and *I. ricinus* are genetically closely related and estimated to have diverged ∼9 MYA, only one divergence date is possible for these species, while lineage specific expansion likely occurred <6 MYA in both species (Van Zee et al., 2016). This negates most of the evolutionary conclusions made in the Schwarz et al. (2014a) study. It was suggested that clade G6 is fundamental in understanding evolution of the BPTI inhibitors in *I. ricinus* (Schwarz et al., 2014a). This clade is the largest monophyletic clade (lineage specific expansion) and the fastest evolving clade (diverging around 70 MYA as estimated with the molecular clock). However, as indicated, the divergence observed

could only have occurred <9 MYA and therefore represents an interesting view on the gene duplication rate in this family. It was suggested that accelerated evolution is evident from this analysis based on the number of members (Schwarz et al., 2014a). However, counts of the numbers of orthologs (15), paralogs (150) and gene duplications per orthologous clade (Average = 4.9, Range = 1–22), indicate that any given orthologous clade did not expand excessively (≤25 duplications/paralogous clade in 5 million years). Given power law behavior this number of gene duplications may be well within the expected norm for the major secretory protein families. If it is taken into account that some genes may be pseudogenes (Nei and Rooney, 2005), that some orthologous groups may be underrepresented in the *I. scapularis* genome, or that the *I. ricinus* transcriptome may be artefactually inflated, then the proposed accelerated evolution is not obvious. What this section highlights is the need for robust and careful analysis when multigene families are analyzed, specifically with regard to divergence time estimates. Efforts to establish which genes are truly expressed as proteins and functional at the feeding site would also assist in making phylogenetic analysis more robust. Taxonomic sampling of closely related lineages to assist in the identification of orthologous clades to prevent artificial interpretations of species specific expansions is necessary and important to correlate divergence dates for clades expected to have diverged at the time of speciation. Conversely, inclusion of highly divergent sequences from distantly related species does not increase nodal confidence, since small families such as the BPTI fold are likely to have accumulated saturated substitutions. In these cases phylogenetic trees may be obtained that given the low nodal support are basically non-informative (Dai et al., 2012). Given the expansion of tick sequences, future analyses of multigene families may need to focus exclusively on specific lineages or genera, with the emphasis that adequate taxon sampling in these lineages or genera is necessary to delineate orthologous relationships and lineage specific expansions. Inclusion of soft and hard tick sequences in the same analysis leads to the interesting conclusion that argasid and prostriate ticks diverged 155 or 100 MYA, while metastriate ticks diverged 193 MYA (Schwarz et al., 2014a), or that hard and soft tick sequences are interspersed throughout the tree with low nodal support and no evidence for orthology (Dai et al., 2012). This may be expected when divergent paralogous genes are analyzed, which beyond sharing a common ancestor in the ancestral tick lineage, may be separated by multiple gene duplication events. Analysis of multigene families should therefore be performed within a biological context, taking into account recognized phylogenetic relationships (Null hypothesis) and where phylogenetic data deviate from this, data that is not robust should be discarded. Conversely, multigene families and paralogous clades offer the opportunity to reduce uncertainty in molecular clock estimates by using the principle that divergence of paralogous clades from different species by definition occur at the same time. Different nodes can then be constrained by cross-calibration or cross-bracing to ensure that they will have similar ages, thereby improving time estimates across the whole tree (Shih and Matzke, 2013; Zhaxybayeva et al., 2013).

PHYLOGENETIC ANALYSIS OF FUNCTIONAL EVOLUTION

While adaptive selection may not be a major phenomenon in the evolution of blood-feeding behavior, ancestral reconstruction of functional evolution allows for a historical description of "adaptation to a blood-feeding environment." This approach aims to answer the how, when and where functions evolved in different tick lineages (Mans et al., 2016). Phylogenetic analysis linked with functional annotation allow for the reconstruction of the evolution of functions in paralogous genes or testing of orthologous relationships to define the origins of functions in ancestral lineages. However, most phylogenetic analyses of salivary gland protein families are used to summarize or visualize transcriptome data and not to construct explicit functional evolution scenarios. Little information regarding evolution of function is extracted from these analyses beyond confirmation of lineage specific expansion and assignment of proteins to clades (Valenzuela et al., 2002; Francischetti et al., 2005, 2008a,b, 2011; Couvreur et al., 2008; Mans et al., 2008a; Anatriello et al., 2010; Karim et al., 2011; Ribeiro et al., 2011, 2012; Garcia et al., 2014; Tan et al., 2015). This is partly due to the paucity in available functional information and, given the increase in transcriptome studies and the lag in functional studies are likely to continue into the future. However, it is also due to the extensive lineage specific expansions observed in protein families, making identification of orthologs in different genera difficult, since the families are now becoming so large that accurate multiple alignments and well-supported phylogenetic trees are not feasible goals anymore (Mans et al., 2016). On the other hand, phylogenetic analyses have also been performed to analyze protein families during their functional characterization. This was done in the BPTI family for hemostatic (Mans et al., 2002, 2003a, 2008b; Francischetti et al., 2004), ion channel (Paesen et al., 2009), and tryptase inhibitors (Valdés et al., 2013). Analysis of the BPTI family in general was performed for *I. ricinus* (Schwarz et al., 2014b; Valdés and Moal, 2014), *I. scapularis* (Dai et al., 2012), and *R. microplus* (Louw et al., 2013). For the lipocalin family it has been performed for the biogenic amine binding proteins (Mans et al., 2003b, 2008c; Mans and Neitz, 2004b; Mans and Ribeiro, 2008a,b; Díaz-Martín et al., 2011; Valdés et al., 2016), LTB4 and TXA2 binding proteins (Mans and Ribeiro, 2008b), LTC4 binding proteins (Mans and Ribeiro, 2008a; Manzano-Román et al., 2016), japanin (Preston et al., 2013), and savicalin (Cheng et al., 2010). Lipocalins have also been analyzed in general for *I. ricinus* (Beaufays et al., 2008; Konnai et al., 2011), *Ornithodoros savignyi* (Mans et al., 2003b; Mans and Neitz, 2004b), and *R. microplus* (Rodriguez-Valle et al., 2013). Evolution of the serpin family has been studied in *A. americanum* (Mulenga et al., 2007; Porter et al., 2015), *I. scapularis* (Mulenga et al., 2009), and *R. microplus* (Tirloni et al., 2014). Cystatins were analyzed in *I. scapularis* (Kotsyfakis et al., 2006; Ibelli et al., 2013) and novel families described using phylogenetic analysis (Mulenga et al., 2013). It is clear that phylogenetic analysis has become a central part of salivary gland protein family characterization. However, the majority of these studies still use phylogenetic analysis to place their proteins of

interest within a context of other proteins with known function, predict function by homology, or attempt to describe domain evolution in general. Reconstruction of functional evolution of paralogous clades is still limited and mainly due to the lack of knowledge regarding the functions and functional mechanisms of the majority of secretory proteins.

Studies that addressed scenarios for the evolution of function between paralogs, include the clotting and platelet aggregation inhibitors from soft ticks (Mans et al., 2002, 2008b), evolution of biogenic amine binding (Mans et al., 2008c) and evolution of LTB4, TXA2, and complement C5 inhibitors in the moubatin clade of soft tick inhibitors (Mans and Ribeiro, 2008a). For BPTI proteins it has been proposed that soft tick thrombin, fXa and fibrinogen receptor inhibitors share a common evolutionary pathway (Mans et al., 2002, 2008b). Both thrombin and platelet aggregation inhibitors have orthologs in the *Argas* and *Ornithodoros* genera, implying that blood-clotting and platelet aggregation inhibitors evolved in the last common ancestor to soft ticks, which was dated at $\sim$234 $\pm$ 25 MYA using mitochondrial protein analysis (Mans et al., 2008b, 2012).

The biogenic amine binding clade represents an interesting model for functional evolution since members exist that can bind histamine in an upper binding pocket (or not) and histamine and/or serotonin in a lower binding pocket (Paesen et al., 1999, 2000; Sangamnatdej et al., 2002; Mans et al., 2008c). Several models for evolution of biogenic amine binding therefore exist that cannot yet be adequately resolved (**Figure 3**). Parsimony analysis of the BABPs suggested that the ancestral biogenic amine bound in the lower binding pocket of the lipocalin barrel was serotonin and that histamine binding originated several times independently, both in the lower and upper binding sites (Mans et al., 2008c). This would represent the neofunctionalization model of biogenic amine binding. Alternatively, the ancestral BABP possessed both an upper histamine-binding and a lower histamine/serotonin-binding site as retained in lipocalins from *Ornithodoros* and *Dermacentor* (Sangamnatdej et al., 2002; Mans et al., 2008c). Subsequent gene duplication and subfunctionalization (or escape from adaptive conflict, since histamine and serotonin shares the same lower binding pocket) led to loss of the upper binding site and lipocalins that either binds histamine or serotonin in the lower binding site as observed for monotonin and monomine from *Argas*, or the *Ixodes* serotonin binding lipocalins (Mans et al., 2008c). In the case of *I. scapularis*, the histamine-binding gene may have been lost after subfunctionalization, although a much larger screening of genes is necessary to confirm this. A similar scenario exists for the histamine-binding lipocalins from *Rhipicephalus appendiculatus* that seem to bind only histamine (Paesen et al., 1999). These alternative scenarios may be resolved once more members are empirically verified from different genera, emphasizing the need for a broad taxon sampling in evolutionary studies. The evolutionary models presented here aim to distill our current knowledge on BABP evolution from experimentally verified data. It should be noted that the evolutionary pathways may be more complex, since an expansion of proteins with the biogenic amine binding motif is observed in all tick transcriptomes, leading to potentially tens to hundreds of biogenic amine binders in each species. It is likely that all different models of functional evolution after gene duplication are present in this family, making it a particularly interesting study model.

Another clade with detailed functional analysis that allows for dissection of evolutionary scenarios are the moubatin-clade of LTB4, TXA2 and complement C5 inhibitors (Mans and Ribeiro, 2008a). This clade shares a sister relationship with the BABP clade and is found only in the Ornithodorinae. It therefore represents paralogous gene duplication that occurred in the last common ancestor of the Ornithodorinae. The residues conserved for TXA2 and LTB4 binding are conserved across the family, suggesting that this is the ancestral function of this clade. Interestingly enough, this is a potential case for escape from adaptive conflict. In this regard, glycine 85 located inside the lipocalin beta-barrel is important for TXA2 binding, since other more bulky residues such as arginine interferes sterically with binding. TSGP2 that possesses an arginine at this position is unable to bind TXA2, but can be rescued by a R85G mutation. The same R85G mutation occurs in *Ornithodoros moubata* complement inhibitor (OMCI) and would suggest that LTB4 binding has been optimized in these two members of the clade. Both of these lipocalins (and TSGP3) target complement C5 using a very specific βH-α2 loop (Mans et al., 2008a; Jore et al., 2016). This would suggest multi-functionalization and escape from adaptive conflict by specialization of LTB4 and TXA2 binding. Recent research suggests that targeting of the complement cascade, neutrophils and platelet aggregation are closely interlinked (Deppermann and Kubes, 2016). Multi-functional proteins that target related mechanisms may evolve, not because of adaptive selection, but rather because of neutral evolution resulting in redundancy ultimately leading to beneficial mutations.

WHOLE GENOME DUPLICATION IN TICKS

Gene duplication may occur for individual genes in a stochastic manner across the genome. On the other hand, large-scale gene duplication events that include chromosome or whole genome duplications give rise to many gene duplicates at once and are an important source for the generation of gene copies and as a catalyst for adaptation to new environments and speciation (Nei, 2005; Van de Peer et al., 2009). In ticks, the extensive duplication events observed in transcriptomes were suggested to be due to possible genome duplications in hard ticks (Ribeiro et al., 2006; Dai et al., 2012). This may be partly supported by the increase in chromosome numbers observed in ticks when compared to other parasitiform mites, with mesostigmatid mites having 2- to 3-fold fewer chromosomes ($2n = 8 \pm 0.3$), compared to soft ($2n = 22 \pm 4$) and hard ($2n = 22 \pm 2$) ticks (Oliver, 1977; Mans and Neitz, 2004a). Analysis of the *I. scapularis* genome found no evidence for a genome duplication event (Van Zee et al., 2016). However, the analysis focused on recent gene duplication events dated <6 MYA. Given the similarities in chromosome numbers of hard and soft ticks, genome duplication in ticks may have occurred in the ancestral tick lineage (>290 MYA) and given that the majority of duplicated genes go extinct, may not be easily detectable since detection of whole genome duplication becomes increasingly difficult with age (Vanneste et al., 2013). The major contributor to genome size in ticks are repetitive elements with

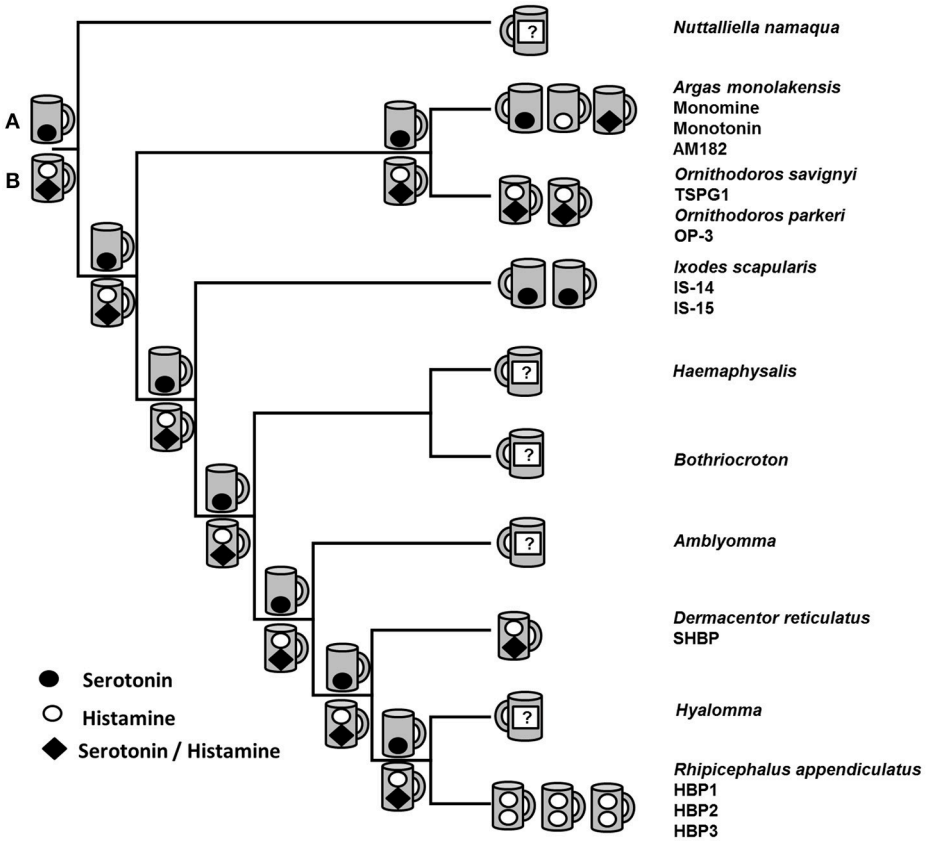


FIGURE 3 | Evolutionary scenarios in the reconstruction of biogenic amine binding function. Indicated are the phylogenetic relationships of various tick genera as determined with mitochondrial analysis (Mans et al., 2012). Terminal nodes represent extant lipocalins for which empirical histamine or serotonin binding data exist. The number of lipocalins for which experimental data have been generated are indicated as cups (lipocalin∼calyx∼cup) that bind histamine in the upper binding site and serotonin and/or histamine in the lower binding pocket. Names of characterized lipocalins are indicated below their genus names. Internal nodes represent two possible ancestral binding site hypotheses: **(A)** The ancestral BABP had a single lower serotonin-binding site, or **(B)** the ancestral BABP had an upper histamine and lower histamine/serotonin-binding site.

66% contributing to the genome size of *I. scapularis* and 69% for *R. microplus* (Ullmann et al., 2005). It is not yet clear what proportion of the expanded genome size of *I. ricinus* may be attributed to repetitive elements (Cramaro et al., 2017), but it is likely that most of the genome size differences observed between tick species are not due to gene duplications, but rather the universal abundance of repetitive elements and non-coding DNA (Mans et al., 2016). Larger genome sizes of ticks compared to other mites is not evidence of genome duplication *per se* (Geraci et al., 2007). We may tentatively propose that chromosome numbers suggest a whole genome duplication in the ancestral lineage of ticks and this may have been associated with adaptation to blood-feeding. Subsequent divergence and stochastic gene duplication may explain more recent differences observed in tick transcriptomes.

CONCLUSIONS

Gene duplication remains a major factor in the evolution of blood-feeding behavior of ticks, even if most new functions and the evolution of secretory families in ticks may undergo neutral evolution. Hypotheses on adaptive evolution should first reject the null hypothesis that evolution did not occur via neutral evolution. More rigorous analysis is also necessary to characterize paralogous gene families, especially where molecular dating is concerned. Analysis of the literature indicates that our understanding of functional evolution is still limited, since few studies aim to reconstruct evolutionary pathways. Even so, the increase in taxon sampling of salivary gland transcriptomes holds the promise that we will be able to get an accurate estimate of the number of orthologous clades distributed across various tick lineages and the number of functions encoded in the tick sialoverse.

AUTHOR CONTRIBUTIONS

BM, JF, MdC, and RP conceptualized the manuscript. BM and JF wrote the manuscript. BM and RP prepared the figures. BM, JF, MdC, and RP revised and edited the manuscript.

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REVIEW
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A Roadmap for Tick-Borne Flavivirus Research in the "Omics" Era

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Tick-borne flaviviruses (TBFs) affect human health globally. Human vaccines provide protection against some TBFs, and antivirals are available, yet TBF-specific control strategies are limited. Advances in genomics offer hope to understand the viral complement transmitted by ticks, and to develop disruptive, data-driven technologies for virus detection, treatment, and control. The genome assemblies of *Ixodes scapularis*, the North American tick vector of the TBF, Powassan virus, and other tick vectors, are providing insights into tick biology and pathogen transmission and serve as nucleation points for expanded genomic research. Systems biology has yielded insights to the response of tick cells to viral infection at the transcript and protein level, and new protein targets for vaccines to limit virus transmission. Reverse vaccinology approaches have moved candidate tick antigenic epitopes into vaccine development pipelines. Traditional drug and *in silico* screening have identified candidate antivirals, and target-based approaches have been developed to identify novel acaricides. Yet, additional genomic resources are required to expand TBF research. Priorities include genome assemblies for tick vectors, "omic" studies involving high consequence pathogens and vectors, and emphasizing viral metagenomics, tick-virus metabolomics, and structural genomics of TBF and tick proteins. Also required are resources for forward genetics, including the development of tick strains with quantifiable traits, genetic markers and linkage maps. Here we review the current state of genomic research on ticks and tick-borne viruses with an emphasis on TBFs. We outline an ambitious 10-year roadmap for research in the "omics era," and explore key milestones needed to accomplish the goal of delivering three new vaccines, antivirals and acaricides for TBF control by 2030.

Keywords: tick-borne flavivirus, *Flaviviridae*, *Ixodidae*, genomics, genetics, vaccine, anti-viral, acaricide

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INTRODUCTION

Ticks (subphylum Chelicerata, subclass Acari, suborder Ixodida) are ectoparasites of humans and animals, and vectors of bacteria, protozoa, and viruses (Gulia-Nuss et al., 2016). Scientists have documented more than 38 species of viruses comprising members of the families Asfarviridae, Reoviridae, Rhabdoviridae, Orthomyxoviridae, Bunyaviridae, Flaviviridae, and possibly the Arenaviridae that are transmitted by ticks (Labuda and Nuttall, 2004). Reports of tick-borne viruses are increasing (Mansfield et al., 2017a,b), and new viruses are emerging such as the Heartland and Bourbon viruses identified in the U.S.A. (https://www.cdc.gov/heartland-virus/index.html;

https://www.cdc.gov/ncezid/dvbd/bourbon/index.html). The geographic ranges of tick species are expanding (Medlock et al., 2013), yet the implications for disease epidemiology are not well understood. There is growing appreciation of the complexity of the tick "microbiome," defined as the complement of pathogens, commensals and symbionts carried in or on a tick, variation at spatial and temporal scale (Narasimhan and Fikrig, 2015; Van Treuren et al., 2015), and the prevalence of tick co-infections (Diuk-Wasser et al., 2016; Moutailler et al., 2016). Knowledge regarding virus species transmitted by ticks is limited, and many pathogenic viruses may go unnoticed or undiagnosed (Hubalek and Rudolf, 2012; Lani et al., 2014). Research is now pivoting to determine the complement of viruses acquired by ticks during blood feeding, and the role of these viruses in pathogenesis, with data-driven research likely to facilitate a precision medicine approach to the diagnosis and treatment of TBFs.

Of the viruses transmitted by ticks, the tick-borne flaviviruses (TBFs) are considered the most important affecting human health globally. TBFs are transmitted by multiple species of ixodid ticks in the families Ixodidae (hard ticks) and Argasidae (soft ticks) (**Table 1**). In the past two decades there has been a notable increase in the incidence of TBF disease (Lasala and Holbrook, 2010). Among TBFs, tick-borne encephalitis virus (TBEV) is regarded as one of the most dangerous human neuroinfections in Europe and Asia where it causes between 10,000 and 15,000 human cases every year, respectively (Gritsun et al., 2003a,b; Dobler, 2010; Rumyantsev et al., 2013). Other members of the TBF complex of importance to public health include Louping-ill virus (LIV) in the United Kingdom, Omsk hemorrhagic fever virus (OHFV) in parts of Russia, Kyasanur Forest Disease virus (KFDV) in parts of India, Alkhurma hemorrhagic fever virus (AHFV) in Saudi Arabia, and Powassan encephalitis virus (POWV), including deer tick virus Powassan lineage II, the only human pathogenic TBF detected in North America to date (Dobler, 2010).

Next generation sequencing (NGS) technologies have allowed the generation of new resources for tick-borne disease research. Genomics has enabled reverse genetics to identify tick proteins and biochemical pathways that could be targeted to disrupt virus transmission. The assembly of the *Ixodes scapularis* (black-legged tick) genome (Gulia-Nuss et al., 2016), a vector of POWV, is the first such resource for a tick and a nucleation point for tick genome research. Draft genome assemblies are available for the castor bean tick, *Ixodes ricinus* (Cramaro et al., 2015), also a TBF vector, and for the southern cattle tick, *Rhipicephalus* (*Boophilus*) *microplus* (Guerrero et al., 2006, 2010; Barrero et al., 2017). These resources will enable investigations of tick-pathogen relationships in a "genome-wide" context and comparative genomic research between lineages comprising major tick vectors. Progress in gene discovery for species of hard and soft ticks has been extensive (Meyer and Hill, 2014), with an emphasis on elucidating gene products associated with tick-host-pathogen interactions. Whole genome computational analyses have revealed duplication events involving large numbers of genes in *I. scapularis* and other species of hard ticks that may be associated with the evolution of parasitic strategies (Van Zee et al., 2016). Transcriptome and proteome studies have examined the molecular response of *Ixodes* cells to viral infection (Villar et al., 2015; Weisheit et al., 2015; Grabowski et al., 2016; Mansfield et al., 2017a) and functional analyses have investigated proteins that exhibited differential expression post infection with virus (Schnettler et al., 2014; Ayllon et al., 2015a; Weisheit et al., 2015; Grabowski et al., 2017a).

Despite these achievements, there remain challenges to the identification of protein targets for vaccine, drug, and acaricide development. Deliberate investment in resources for forward and reverse genetics with an emphasis on major tick vectors and pathogenic virus strains is required. Metabolomics and structural genomics represent new frontiers. When coupled with sequence-based genetic mapping and tools for genetic transformation, these fields have the potential to identify molecular targets and guide the rational design of transmission blocking vaccines and acaricides. The scope of genomic resources required is substantial given the biological complexities of TBF transmission. Here we present a 10-year roadmap for research to expand the arsenal of TBF control technologies and deliver three new antiviral, vaccine, and acaricide products by a proposed target date of 2030. The roadmap and associated milestones are intended as a framework to guide discussions between the research community and funding agencies. While ambitious, the importance of TBFs necessitates commitment to strategic research priorities to ensure the timely achievement of public health goals.

Tick-Borne Flaviviruses

TBFs are enveloped, positive-strand RNA viruses in the family Flaviviridae that includes dengue (DENV), hepatitis C (HCV), Japanese encephalitis (JEV), West Nile (WNV), and Zika (ZIKV) viruses. Many TBFs cause significant human and animal disease worldwide (**Table 1**) and are transmitted primarily via the bite of an infected tick. In nature, TBFs are maintained in a cycle between small mammal reservoirs and ticks. However, the complex transmission cycles of many TBFs have not been resolved and studies to incriminate tick species in virus transmission are needed. Most TBFs are classified Biosafety-level (BSL) 3 and 4 (**Table 1**). In humans, symptoms of TBF infection range from febrile illness to more serious encephalitis and hemorrhagic complications. Case fatality rates as high as 20% have been recorded for the most pathogenic TBFs (e.g., far-eastern form of TBEV). Multiple vaccines are available in Europe for TBEV, although no TBF-specific antivirals or transmission-blocking vaccines have been developed. At present, TBF treatment and prevention options are considered lacking (Lani et al., 2014).

The focus of tick-borne disease research is shifting from a "one pathogen-one disease" mindset toward an understanding of disease in the context of the "pathobiome" (Vayssier-Taussat et al., 2015). Genomic studies have emphasized high consequence pathogens and their impact on the human host, as well as flavivirus biodiversity and evolution, but there is need to determine the complement of virus species that circulate in host and reservoir populations. NGS involving 454 and Illumina-based 16S rRNA pyrosequencing has been used to explore bacterial communities associated with *I. ricinus* (Carpi et al., 2011), *I. scapularis* (Van Treuren et al., 2015), and

TABLE 1 | Summary of tick-borne flaviviruses associated with disease in humans, geographic location, proposed tick vectors and vaccine approaches.

TBF/Subtypes	Species of ticks that serve as potential vectors	Estimated no. human cases per annum	Geographic distribution	Vaccine(s) available	Biosafety level in USA
Kyasanur Forest Disease Virus (KFDV)	*Hemaphysalis spinigera* *Hemaphysalis turturis*	400–500[a]	India	No[h]	4
Alkhurma Hemorrhagic Fever Virus (AHFV)	*Ornithodoros savignyi* *Hyalomma dromedari*	77[b]	Arabian Peninsula	No	4
Omsk Hemorrhagic Fever Virus (OHFV)	*Dermacentor reticulatus* *Dermacentor marginatus* *Ixodes persulcatus* *Ixodes apronophorus*	24[c]	Russia	No	4
Tick-Borne Encephalitis Virus (TBEV) European/Western Siberian Far-eastern	*Hemaphysalis concinna* *Hemaphysalis punctata* *Dermacentor reticulatus* *Ixodes ricinus* *Ixodes persulcatus*	10,000-15,000[d]	Europe and Asia	Yes	4
Powassan Virus (POWV)	*Haemaphysalis longicornis* *Dermacentor andersoni* *Ixodes marxi* *Ixodes cookei* *Ixodes scapularis (for POWV lineage II: deer tick virus)*	5[e]	North America and Russia	No	3
Louping Ill Virus (LIV)	*Ixodes ricinus*	No confirmed cases since early 1990s[f]	United Kingdom	No	3
Langat Virus (LGTV)	*Ixodes granulatus* *Hemaphysalis papuana*	Unknown; cases recorded only during use of virus in anti-TBEV vaccination trials[g]	Southeast Asia	No	2

Kyasanur Forest disease virus, KFDV; Alkhurma hemorrhagic fever virus, AHFV; Omsk hemorrhagic fever virus, OHFV; Tick-borne encephalitis virus, TBEV; Powassan virus, POWV; Louping ill virus, LIV; Langat virus, LGTV.

[a] *(Holbrook, 2012; Kasabi et al., 2013; Lani et al., 2014; Grabowski et al., 2016).*

[b] *Average from 2009–2011 (Alzahrani et al., 2010; Memish et al., 2014).*

[c] *Average from 1946–2000 (Gritsun et al., 2003a; Grabowski et al., 2016).*

[d] *(Mansfield et al., 2009; Dobler, 2010).*

[e] *Average from 2000–2013 in USA (Paddock et al., 2016).*

[f] *(Jeffries et al., 2014).*

[g] *(Gritsun et al., 2003a,b).*

[h] *A vaccine is licensed and available in endemic areas of India (Holbrook, 2012; Kasabi et al., 2013).*

Amblyomma americanum (Ponnusamy et al., 2014; Williams-Newkirk et al., 2014; Trout Fryxell and DeBruyn, 2016). Pyrosequencing of DNA enriched for bacteria/arachaea has also been used to evaluate the microbiome of seven hard tick species (Nakao et al., 2013). RNAseq revealed that the Flaviviridae infect a wider range of invertebrate hosts and exhibit greater diversity in genome structure than previously anticipated (Shi et al., 2015), but the implications for pathogenesis remain unclear. The relatively small size of TBF genomes (∼10–15 Kb) makes viral whole genome sequencing (WGS) feasible. Future studies must emphasize viral metagenomics using WGS to define the viral phyla associated with ticks (Brinkmann et al., 2016). Information from these studies will guide the development of comprehensive, region-specific molecular diagnostic tools and healthcare guidelines.

De-convoluting the systems biology of the tick bite—that is determining the impact of virus, vertebrate host, and tick genetics (i.e., genome-by-genome-by-genome or GxGxG studies) on pathogenesis, is a priority. The diagnosis and treatment of tick-borne disease could be advanced by considering each tick bite as a "unique" molecular encounter between tick salivary proteins, the microbial flora delivered by the tick and host factors produced at the feeding wound (**Figure 1**). Molecular analyses support a human genetic component to the severity of TBF disease. Complete genome sequencing identified amino acid residues associated with severity of the Far-Eastern subtype (FE) strains of TBEV isolated from patients with encephalitic (Efd), febrile (Ffd), and subclinical (Sfd) forms of the disease (Belikov et al., 2014). Molecular studies revealed polymorphism in the salivary proteins secreted by individual unfed and feeding ticks (Wang et al., 2001), and specific combinations of vector and virus genotype were reported to affect vector competence (Lambrechts, 2011; Fansiri et al., 2013).

Control of TBFs

Options to control TBFs (summarized in **Figure 2**) are limited and rely largely on personal protective measures, acaricides, vaccines against TBEV, and management of the symptoms of infection. Treatment of TBF infections in the human population focuses on palliative care and management of complications. There are currently no chemotherapies developed against TBFs. Viral infection may be treated with the antivirals (ribavirin,

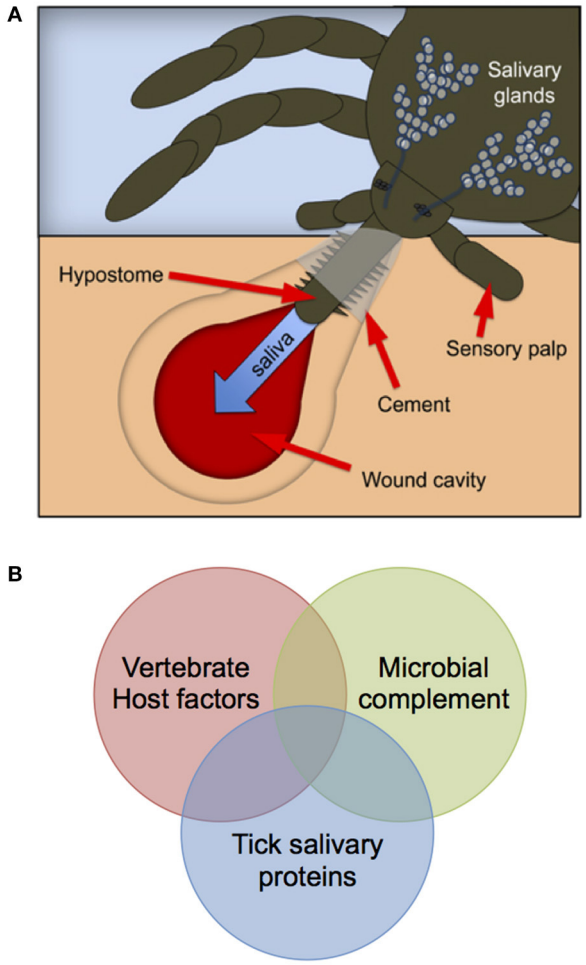

FIGURE 1 | Schematic depicting the concept of the "systems biology" of a tick bite. Each tick bite **(A)** comprises a unique combination of host-derived factors, tick salivary proteins and the microbial flora delivered to the feeding site **(B)**, thus underpinning the need for "personalized" approaches to pathogen detection and treatment. **(A)** reproduced from Figure 1 of Gulia-Nuss et al. (2016) and reprinted by permission from Macmillan Publishers Ltd. ©copyright 2016.

realdiron, larifan, and rifastin) developed to control a variety of human viral pathogens. Clinical studies to determine the effectiveness of these chemotherapies against TBF infection (Loginova et al., 2002; Lani et al., 2014) could have value.

Protective human vaccines are available for TBEV and KFDV. Currently, five products are considered safe and efficacious for protection against TBEVs. These are FSME-Immun and Encepur, manufactured in Austria and Germany respectively, and based on European strains of the virus, TBEV-Moscow, and EnceVir manufactured in the Russian Federation and based on FE strains (WHO), and the SenTaiBao vaccine manufactured in China (Xing et al., 2017) and also based on the FE subtype. These inactivated vaccines require multiple doses to induce and maintain immunity. The development of novel and more effective vaccines remains a high priority (Wang et al., 2016).

There is broad interest in transmission blocking vaccines for control of tick-borne diseases (reviewed below), although there are currently no products registered to prevent transmission of TBFs. The candidate 64TRP transmission blocking vaccine, based on a recombinant form of the 15 kDa cement protein of the African brown ear tick, *Rhipicephalus appendiculatus,* was associated with a reduction in TBEV transmission and disease in an *in vivo* mouse model (Labuda et al., 2006) and could have potential as a broad-spectrum anti-tick vaccine (Trimnell et al., 2005; Havlikova et al., 2009).

Acaricides are used to control ticks of public health and veterinary importance. Unfortunately, continued tick control is complicated by widespread resistance of tick populations to several classes of acaricides, most notably organophosphates (OPs) and carbamates (George, 2000; George et al., 2004; Abbas et al., 2014). The situation is most acute with respect to *R. microplus.* Large-scale application of chemicals has been effective (Ostfeld et al., 2006) for tick control in urban, rural and recreational areas but can also contribute to resistance and effects on vertebrates and other non-target species. Microbial insecticides based on the fungi *Metarhizium anisopliae* and *Beauveria bassiana* have been proposed as environmentally benign alternatives (Benjamin et al., 2002; Hornbostel et al., 2004, 2005; Ostfeld et al., 2006), and other "green" technologies are under consideration (Benelli et al., 2016). Insecticides based on plant-derived extracts are attracting attention as new classes of tick repellants and toxicants, and are the subject of ongoing mode of action studies (Gross et al., 2015, 2017).

Approaches to reduce transmission via management of either the vertebrate reservoir or the tick vector have been investigated. In the U.S., the topical application of acaricides delivered via baited applicators reduced densities of hard ticks on deer and small rodents in the field (Pound et al., 2000; Brei et al., 2009; Carroll et al., 2009; Miller et al., 2009). However, logistics and cost, including the need for constant maintenance of baited-field devices, suggest lack of feasibility at broader scale (Harmon et al., 2011). Passive acaricide applicators remain an option to reduce local tick burden when used in combination with other tick control strategies.

Prospects for TBF-Protective and Anti-Tick Vaccines

Vaccines offer a cost-effective, sustainable, and environmentally friendly approach to control of arthropod-borne diseases, and in combination with drugs and insecticides, are the backbone of global disease control and eradication campaigns. Existing and developmental products suggest prospects for novel anti-TBF vaccines. Effective human vaccines for the prophylaxis of yellow fever (17D live attenuated virus), JEV (live attenuated and inactivated whole virus), and TBEV (inactivated whole virus; Heinz and Stiasny, 2012) are available. The live attenuated Dengvaxia® product developed by Sanofi Pasteur provides moderate protection against DENV1-4 strains and is approved for use in 11 countries. Efforts are also underway to develop vaccines against ZIKV (Abbink et al., 2016; Marston et al., 2016). Chimeric, recombinant, attenuated vaccines for TBEV

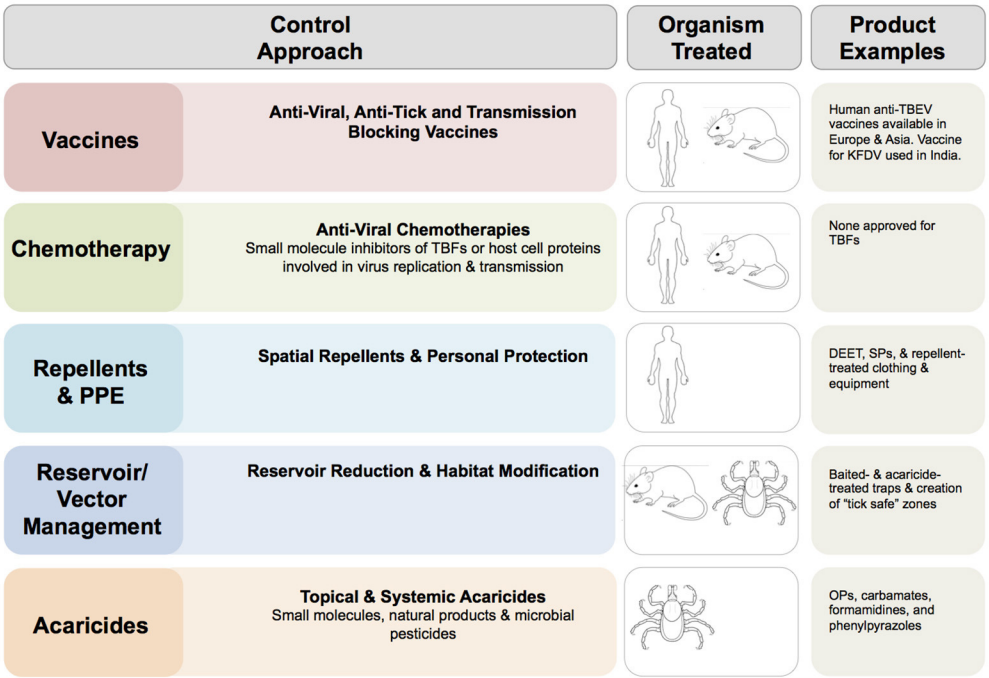

FIGURE 2 | Options for control of tick-borne flaviviruses (TBFs). Common approaches for control of TBFs and examples of commercially available products are shown. DEET, N,N-Diethyl-meta-toluamide; OP, Organophosphate; POWV, Powassan virus; PPE, personal protective equipment; SP, Synthetic pyrethroid, TBEV, tick-borne encephalitis virus. The images of human, mouse and tick indicate the dead-end (human) host, non-human vertebrate reservoir and arthropod vector, as appropriate for virus in question.

have been investigated (Pletnev and Men, 1998; Pletnev et al., 2001; Wang et al., 2016). Live attenuated TBEV vaccines based on a replication-defective (single-cycle) flavivirus platform that provide efficacy after a single dose may be feasible (Rumyantsev et al., 2013). The development of recombinant, live vaccine candidates incorporating microRNA (miRNA) sequences may increase the effectiveness of live anti-TBF vaccines (Tsetsarkin et al., 2016, 2017). However, optimism for vaccine development is tempered by the theoretical risk of vaccine-related adverse events such as immune enhancement of infection and the requirement to induce a long-lasting protective immune response against multiple serotypes (Heinz and Stiasny, 2012).

In addition to novel anti-TBF vaccines, options may exist to "repurpose" existing vaccines and exploit cross reactivity for control of multiple virus species. Several studies suggest that TBEV vaccines may provide cross protection against other members of the TBF complex. There is evidence that immunization with the European/Western-based TBEV vaccine can reduce OHFV infection in mice and humans (Chidumayo et al., 2014), while immunization with the Russian-Spring-Summer Encephalitis virus (RSSEV) form of the TBEV vaccine was associated with a reduction in KFDV infection in mouse models (Aniker et al., 1962; Holbrook, 2012). Unfortunately, preliminary human vaccination studies with the RSSEV-based anti-TBEV vaccine suggested insufficient protection against KFDV (Pavri et al., 1962; Shah et al., 1962; Holbrook, 2012). Similarly, vaccination of mice with the TBEV-Moscow strain did not protect against POWV (Chernokhaeva et al., 2016; Doughty et al., 2017), suggesting limited potential for cross protection.

Anti-tick vaccines represent an effective and environmentally benign approach to control ticks and the pathogens they transmit (de la Fuente et al., 2016). The vaccines TickGARD and Gavac used to control *R. microplus*, a serious pest of cattle in the southern hemisphere and the vector of bovine babesiosis, are based on the Bm-86 midgut protein antigen of the tick. During tick feeding on an immunized host, the ingestion of host immunological factors is thought to induce lysis of tick midgut cells, thus reducing feeding and ultimately tick burden (Willadsen et al., 1989; Willadsen and Jongejan, 1999; Valle et al., 2004; Londono-Renteria et al., 2016). There is need to explore the potential of TickGARD and Gavac to reduce tick infestations on other vertebrate hosts. Vaccination against recombinant Bm-86 has been suggested as a strategy to reduce *R. microplus* infestations in white-tailed and red deer (Carreon et al., 2012), but there remain questions as to feasibility. TickGARD reduced transmission of TBEV from infected *I. ricinus* ticks to mice, but did not provide protection against infection (Labuda et al., 2006). TickGARD and/or Gavac may have efficacy against other vectors of TBFs, although cross-species activity has not been determined (Londono-Renteria et al., 2016). The 64TRP candidate may have potential as a vaccine to prevent TBEV transmission; mice immunized with the recombinant protein and exposed to infected *I. ricinus* were protected against lethal challenge with TBEV (Labuda et al., 2006) but the potential scope of protective

immunity provided by the vaccine (i.e., the tick species and TBFs controlled) requires investigation.

Strategies for *de novo* development of anti-tick vaccines are under investigation. Vaccines against concealed or exposed tick antigens could reduce TBF pathogen load in vector and reservoir, host exposure to ticks, and tick populations (Nuttall et al., 2006; de la Fuente and Merino, 2013). Theoretically these products could be delivered via the vertebrate host (e.g., via oral bait to wildlife or by vaccination of humans and domestic animals). Proteins associated with feeding, reproduction, development, immune response, subversion of host immunity, and that are vital for pathogen infection and transmission have been suggested as candidate protective antigens (Contreras et al., 2016). Multiple recombinant tick proteins are under investigation as candidates for vaccines to control ticks and are described in recent reviews (de la Fuente and Contreras, 2015; de la Fuente et al., 2016). The aquaporin trans-membrane proteins involved in transport of solutes and water, ferritin 2 (Fer2) iron regulating proteins and 64TRP are considered some of the most "promising" candidate antigens (Hussein et al., 2015; de la Fuente et al., 2016). These proteins are associated with a variety of physiological functions; in challenge studies they provided protection against multiple species of ticks, and the potential for immunogenic protection against pathogen transmission is now under investigation. Combinatorial products have also been proposed that would deliver multiple antigens to control transmission of several pathogens (de la Fuente et al., 2016).

High-throughput vaccine discovery platforms have been proposed. Transcriptomic and proteomic data provide a starting point for identification of candidate protective antigens from *I. scapularis* (Contreras et al., 2016). Bioinformatics-based reverse vaccinology approaches (i.e., *in silico* predictions of antigenic epitopes based on *ab initio* gene models or "omics" datasets) are described in a recent review (Lew-Tabor and Rodriguez Valle, 2016).

Prospects for TBF Antivirals

Some progress has been made toward the development of antivirals (Patkar and Kuhn, 2006). Nucleoside analogs have been studied for control of arthropod-borne flaviviruses (Yin et al., 2009) and could help to expand the toolbox of small molecule inhibitors of TBEV. Structure-activity relationship (SAR) studies have identified nucleoside moieties that may inhibit entry of the virus to the host cell or interaction with the non-structural protein 5 methyltransferase and the RNA-dependent RNA polymerase domains of TBEV (Orlov et al., 2017). Drug "repurposing" could expand chemical control options for TBFs. The NITD008 adenosine analog active against mosquito-borne flaviviruses and POWV (Yin et al., 2009) exhibited antiviral activity against KFDV, AHFV, and OHFV *in vitro*, while the BCX4430 analog suppressed WNV, TBEV, LIV, and KFDV *in vitro*, suggesting the potential to suppress "pan-flaviviral activity" (Lo et al., 2016; Eyer et al., 2017).

Small molecule chemistries that target the envelope proteins (E proteins) of TBFs have potential as antivirals (Zhou et al., 2008; Mayhoub et al., 2011a,b). E proteins are involved in virus infection of the host cell, and virus assembly and morphogenesis. The crystal structure of the soluble ectodomain of the DENV type 2 E protein revealed a hydrophobic pocket lined by residues that influence the pH threshold for virus fusion with host cells. Features of the pocket point to a structural pathway for the fusion-activating transition and a mechanism that could be targeted by small-molecule inhibitors of flaviviruses (Modis et al., 2003). The phenylthiazole ring system has emerged as a template for design of antivirals. Virtual screening of the National Cancer Institute (NCI) drug database combined with medicinal chemistry strategies identified small molecules that may be active at this target (Li et al., 2008). Analogues that preserve antiviral activity while reducing adverse effects could provide a new class of antivirals against TBFs.

Prospects for Novel Acaricides

Insecticides are effective tools for control of vector-borne diseases. Unfortunately, widespread resistance among pest populations represents a threat to continued disease control. The identification of pesticide chemistries that operate via novel modes of action (MoA) by binding at alternative sites on existing insecticide targets) or via disruption of novel molecular targets in the arthropod, is a high priority (Van Zee and Hill, 2017). Disease control is expected to rely on insecticides for the next several decades and new acaricides that operate via targets distinct from acetylcholinesterase (the main target of OPs and carbamates) and the voltage-gated sodium channel (the main target of SPs) are sought. The Innovative Vector Control Consortium (IVCC) has issued a call for three new MoA insecticides by 2023 to control mosquito vectors of malaria (Hemingway et al., 2006). We suggest that a similar challenge would also be appropriate for control of ticks and TBFs.

The availability of genome data permits target-based approaches to acaricide discovery. For example, the "genome-to-lead" approach (Meyer et al., 2012) was employed to identify small molecule antagonists of an *I. scapularis* dopamine receptor (DAR). The target was selected from several hundred G protein-coupled receptors (GPCRs) predicted from the IscaW1.1 assembly (Gulia-Nuss et al., 2016). High throughout chemical screening (HTS), followed by "hit-to-lead" and structure-activity studies (SAR) were used to discover several chemistries with high *in vitro* potency for the receptor (Meyer et al., 2011; Ejendal et al., 2012) that may provide leads for new pesticides. Research has also focused on pharmacological characterization of the *R. microplus* octopamine receptor, a suspected target of botanical insecticides (Gross et al., 2015, 2017), and an *I. scapularis* ligand-gated chloride channel considered the target of ivermectin (Gulia-Nuss et al., 2016). These proteins could be used in small molecule screens and targeted by genetic control strategies based on dsRNA/siRNA-mediated RNAi knock-down or Crispr/Cas9 knock out, although protocols for efficient tick transformation would be required for success of the latter.

Tick-Virus "Interactomics"; Understanding Pathogenesis, and Identifying New Vaccine and Acaricide Targets

The identification of protein targets is a major roadblock to development of novel anti-TBF and transmission blocking

vaccines and acaricides; it is here that "omics" research may have greatest impact. Genomics has aided understanding of tick-pathogen interactions and rapid identification of multiple candidate protein targets *en-masse*. Systems biology studies have identified metabolic pathways and enzymes perturbed during viral infection of cells. These studies could help to pinpoint proteins critical to cellular invasion, replication, and transmission of the virus. Transcriptomic and proteomic analyses have focused on the mosquito-DENV (Behura et al., 2011; Bonizzoni et al., 2012; Chauhan et al., 2012; Chisenhall et al., 2014) and tick-*Anaplasma phagocytophilum* (bacterium that causes human granulocytic anaplasmosis) (Ayllon et al., 2015b; Villar et al., 2015; Alberdi et al., 2016; Cabezas-Cruz et al., 2017) "interactomes". The limited concordance between these studies highlights the value of equivalent research in tick-virus systems.

Several transcriptome and proteome studies have analyzed the global response of tick cell lines to infection with TBFs and identified protein candidates for vaccine and acaricide development (Weisheit et al., 2015; Grabowski et al., 2016, 2017a). These studies were conducted using TBEV or the less pathogenic Langat virus (LGTV; **Table 1**; McNally et al., 2012; Weisheit et al., 2015; Grabowski et al., 2016). The involvement of multiple biochemical pathways was suggested following viral infection, with perturbation of pathways for protein folding and degradation, and metabolic processes (**Figure 3**). Some of these pathways have also been implicated in studies of mammalian cells exposed to HCV, DENV, and JEV (Table S1), suggesting the involvement of common cellular responses to flavivirus infection and potential for vaccines with cross-protective immunity. Metabolic pathways have also been investigated in studies of other host-flavivirus (Diamond et al., 2010; Perera et al., 2012; Fischer et al., 2013; Merino-Ramos et al., 2015) and tick-pathogen (Cabezas-Cruz et al., 2017) systems. Proteomic and metabolomics studies also have the potential to uncover proteins and pathways that are unique to the infectious state, an area of research that deserves further attention.

RNAi was used to investigate the role of tick proteins in LGTV infection of the ISE6 cell line derived from *I. scapularis* (Grabowski et al., 2017a) (**Figure 3**, Figure S1). Results suggest involvement of proteins that mapped via *in silico* methods to pathways for amino acid, carbohydrate, lipid, cofactor and vitamin, terpenoid, and polykeytide metabolism. Proteins associated with processing in the endoplasmic reticulum (ER) may also function to facilitate or suppress virus infection (**Figure 3**, Figure S2; Weisheit et al., 2015; Grabowski et al., 2017a). Future work must distinguish metabolic changes associated with direct manipulation of the host cell by the virus versus the generalized cellular stress response. The involvement of orphan proteins reported in LGTV infected *I. scapularis* ISE6 cells (Grabowski et al., 2017a), highlights the need to characterize "hypothetical" proteins predicted by "omic" studies.

One priority is to understand the biology of TBFs in the context of the tick tissues and cells associated with primary and secondary cycles of virus infection and replication. *In vivo* studies have validated several protein targets in tick

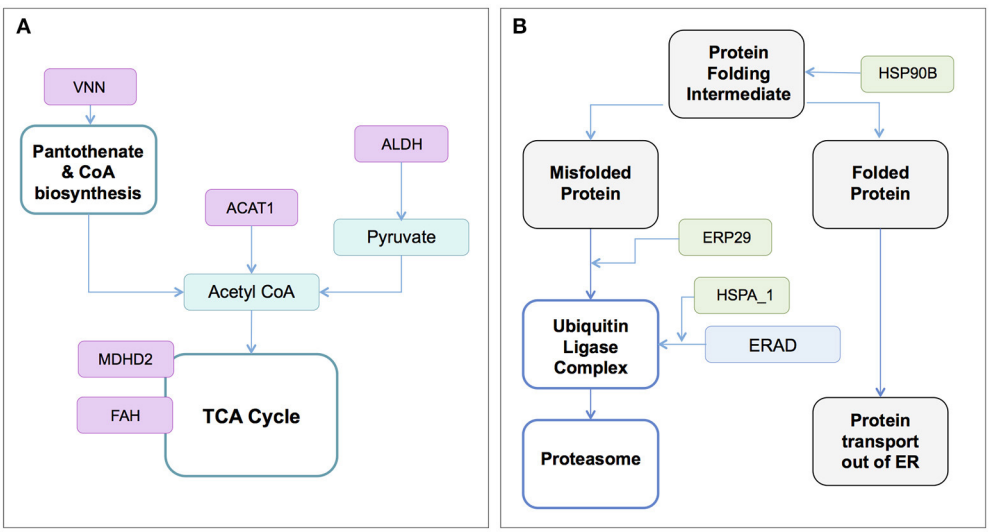

FIGURE 3 | Enzymes and biochemical/metabolic pathways associated with the infection and replication of the tick-borne flavivirus, LGTV (Weisheit et al., 2015; Grabowski et al., 2017a). RNAi-induced knockdown of transcripts for proteins identified to **(A)** the pantothenate and CoA biosynthesis, and TCA cycles, and **(B)** Protein folding and degradation processes was associated with reduced LGTV infection in *Ixodes scapularis* ISE6 cells. Viral infection was assessed by the end points of viral genome replication and infectious virus release. Biosynthetic pathways (teal or blue rectangles), protein states (gray shaded rectangles) and enzymes/proteins (magenta or green rectangles) are shown. VNN and ACAT1 reduced LGTV genome replication and viral replication, while ALDH, MDH2, and FAH reduced LGTV replication only. HSP90B (ISCW022766); ERP29 (ISCW18425); HSP1_8 (ISCW024057, ISCW024910); VNN, (ISCW004822); ACAT1 (ISCW016117); ALDH (ISCW015982); MDH2 (ISCW003528); FAH (ISCW020196). ACAT, acetyl-CoA acetyltransferase; ALDH, aldehyde dehydrogenase; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; ERP29, endoplasmic reticulum protein 29; FAH, fumarylacetoacetate hydrolase; HSPA1_8, heat shock protein 70 family A members 1-8; HSP90B, heat shock protein 90 beta family; MDH2, malate dehydrogenase 2; TCA, tricarboxylic acid.

tissues and whole ticks (Narasimhan et al., 2004; de la Fuente et al., 2005; Karim et al., 2010; Kocan et al., 2011). Electron tomography studies have investigated the three-dimensional architecture of structures derived from host cell membranes that form during DENV infection and replication in mosquito and human cells (Junjhon et al., 2014), and multiple studies suggest virus manipulation of host lipid pathways and cellular membranes (Heaton et al., 2010; Perera et al., 2012; Jordan and Randall, 2016). Similar studies have been performed in tick cells exposed to TBFs (Senigl et al., 2006; Offerdahl et al., 2012; Hirano et al., 2014; Bily et al., 2015) and investigations focused on tissues such as the midgut and salivary glands are needed. TBF infection and spread has been demonstrated in short-term culture of *I. scapularis* organs, providing a platform for tissue-specific studies of virus infection (Grabowski et al., 2017b). Phosphorylation and acetylation of host proteins has been associated with viral infection (Liu et al., 2014; Jeng et al., 2015; Oberstein et al., 2015; Ohman et al., 2015). Metabolomic studies are expected to improve understanding of how post-translational modification (PTM) of host proteins affects viral replication and transmission, consider another area of research priority.

Review of tick-virus "interactome" studies reveals several gaps and impediments to the research goals outlined in this manuscript. Firstly, "omic" research must expand beyond the *Ixodes*-TBF model to other tick-TBF systems (see **Table 1**), emphasizing major vectors and high consequence pathogens. Unfortunately, the biosafety level of the more pathogenic TBFs such as TBEV and POWV restricts research to institutions with appropriate containment facilities. To ensure that data are relevant in a biological context, the field must develop community resources and *in vivo, ex vivo,* and *in vitro* research tools reflective of the vector species and viruses involved. Multiple tick cell lines derived from vectors of TBFs are available for *in vitro* studies via the Tick Cell Biobank (Bell-Sakyi et al., 2007) and in-bred laboratory colonies of ticks competent for TBF transmission must be established. To provide frameworks for resource development, the role of tick species in virus transmission must be addressed via natural history and vector incrimination studies (Nuttall and Labuda, 2003).

The Next Frontier: Structural Genomics and Paradigm Shifts in HTP Vaccine, Drug, and Acaricide Discovery Platforms

The selection of suitable antigens is a major constraint to vaccine development (Havlikova et al., 2009). Target-based antiviral and acaricide discovery also requires validated protein targets amenable to high-throughput (HTP) virtual (i.e., *in silico*) or compound library screening. Advances in structural genomics could facilitate radical changes in HTP discovery platforms for new technologies to control TBFs. Structural genomics is enabling experimental characterization of the three dimensional (3-D) atomic structure of proteins and other molecules having an important biological role in human infectious diseases. Experimental 3-D protein structures and protein-ligand complexes have been generated for organisms causing emerging and re-emerging diseases, including CDC Category A-C priority agents, by the techniques of X-ray, nuclear magnetic resonance (NMR), and cryo-electron microscopy (cryo-EM). These technologies have enabled molecular screening of proteins in complex with inhibitors, cofactors, and substrate analogs, with data from structural studies used to guide virtual screening (Wang et al., 2015).

The past decade has seen an explosion of studies to determine the structure of arthropod-borne flaviviruses in both mature and immature state (**Table 2**). Knowledge of virion structure, assembly and cellular entry mechanisms can support prediction of antigenic epitopes for rational design of vaccines and *in silico*, structure-based discovery of drugs that interfere with viral entry and replication (Patkar and Kuhn, 2006). Cryo-EM has enabled resolution of virion architecture for DENV, WNV, and ZIKV (Heinz and Stiasny, 2012) (**Table 2**). Structural investigations involving TBFs are limited to X-ray crystallography and NMR studies of the TBEV and LGTV E glycoproteins (Rey et al., 1995; Mukherjee et al., 2006), considered the most important immunogen. Homology modeling and molecular docking have been used to identify inhibitors of TBF reproduction, with several compounds showing inhibition of POWV and TBEV *in vitro* (Osolodkin et al., 2013). The structure of human antibodies in complex with ZIKV (Hasan et al., 2017) and DENV (Pokidysheva et al., 2006; Lok et al., 2008) has been determined, suggesting potential for development of neutralizing antibodies.

TABLE 2 | Summary of structural studies of Flaviviridae transmitted by arthropods.

Virus and Strain	Year	Resolution (Å)	Reference(s)	Protein Data Bank (PDB) Accession(s)
DENGUE VIRUS (DENV)				
DENV2 S1 strain[1]	2002	24	Kuhn et al., 2002	1K4R
DENV2	2003	9.5	Zhang et al., 2003	1JCH/1P58 /1SVB
DENV2	2013	3.5	Zhang et al., 2013	3J27
DENV1	2013	4.5	Kostyuchenko et al., 2013	4B03/4AZX
DENV4	2014	4.1	Kostyuchenko et al., 2014	4CBF
WEST NILE VIRUS (WNV)				
NA[2]	2007		Zhang et al., 2007	2OF6
NY 1999[1]	2003		Mukhopadhyay et al., 2003	–
ZIKA VIRUS				
H/PF/2013[1]	2016	3.8	Sirohi et al., 2016	5IRE
NA[2]	2017	9	Prasad et al., 2017	5U4W

Crystal structure of virus E glycoprotein available; DENV (Modis et al., 2003), JEV (Luca et al., 2012), LGTV (Mukherjee et al., 2006), TBEV (Rey et al., 1995) and ZIKV (Prasad et al., 2017) and virus structure in complex with other proteins (Zhang et al., 2015).

[1]Denotes structure of mature virus.

[2]Denotes structure of immature virus.

Greater knowledge of virion structure will enable equivalent studies for TBFs.

Structural genomic studies of tick proteins could generate data for the rational design of next generation transmission blocking vaccines and acaricides (**Figures 4**, **6**). Crystal structures are available for a salivary cystatin from the soft tick, *Ornithodorus moubata* (Salat et al., 2010) and a thrombin from the Tropical Bont tick in complex with S-variegin (Koh et al., 2011). The development of HTP platforms for protein expression and purification could permit atomic level resolution of structures for soluble tick proteins. Advances in techniques for the genetic manipulation of arthropods such as the Crispr/Cas9 gene editing technology could facilitate HTP validation of protein targets *in vivo*. Paradigm shifts in the approach to vaccine and acaricide discovery are expected. Future efforts are likely to incorporate (1) systems biology studies to identify novel protein targets *en masse*, (2) *in vitro* validation of multiple protein targets in parallel via RNAi or Crispr/Cas9 screens, (3) structure-based and virtual screening, and (4) *in vivo* functional studies in tick tissues and whole ticks (see **Figure 4**).

Forward Genetics to Understand Tick Vector Competence and Identify Genetic Elements Associated with TBF Transmission

Forward genetics (i.e., "phenotype to gene studies") represents a powerful approach to identify loci associated with phenotypes such as acaricide resistance, tick host preference and vector competence (Meyer and Hill, 2014). Reverse genetics (i.e., the "gene to phenotype studies" described above) has advanced understanding of the function of tick gene products, yet the "major players"—those gene products critical to viral infection, replication, and transmission, remain elusive. The feasibility and cost of developing genetic resources has stymied forward genetics of ticks. Below, we discuss the potential of forward genetics for tick-virus research, and the resources required to support this work.

Genetic mapping and genome wide association studies (GWAS) are techniques employed to identify quantitative trait loci (QTL) associated with key phenotypes. Genetic studies have been used to investigate mosquito-virus systems. Genetic

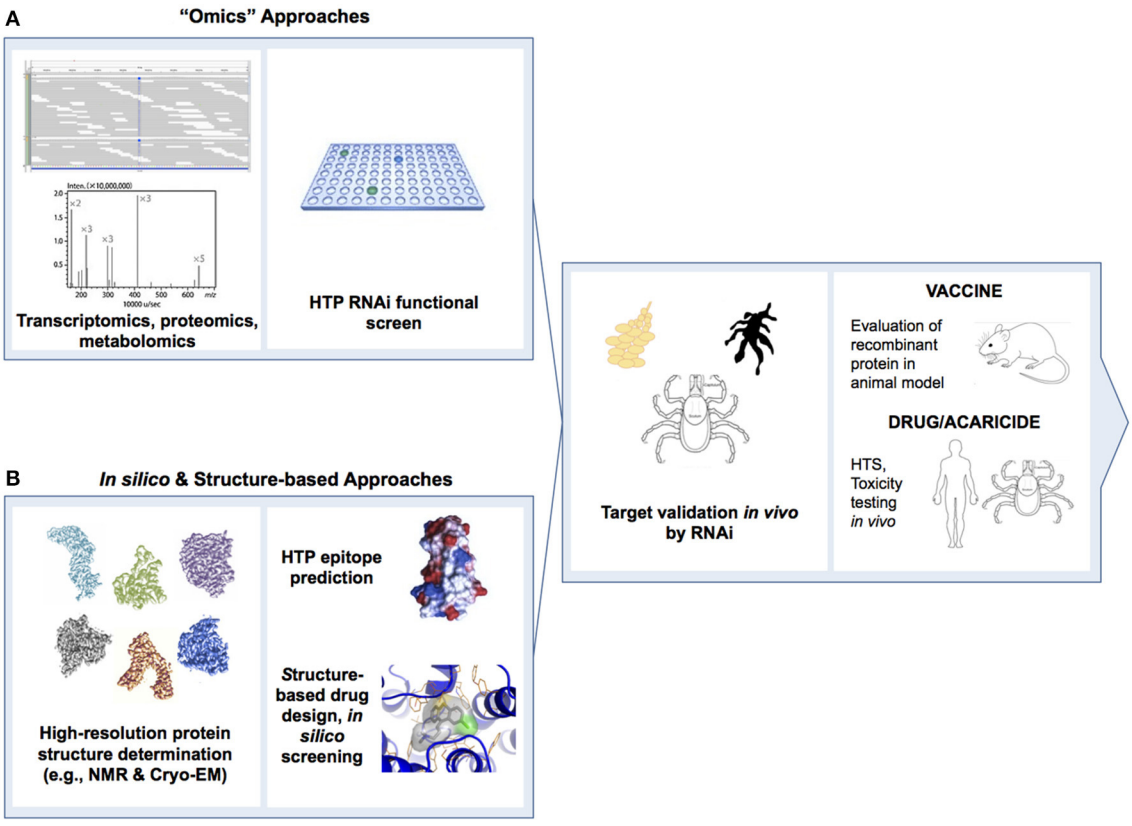

FIGURE 4 | Schematic depicting the major steps in **(A)** wet-lab "omic" and **(B)** *in silico*-processes to identify tick protein targets for development of transmission blocking vaccines, antivirals and acaricides to control TBFs. Antigenic virus or tick proteins identified in **(A,B)** would proceed to vaccine clinical trial. Virus and tick proteins identified in **(A)** would proceed to high-throughput screen (HTS) development and identification of small molecule drugs and acaricides. Tick proteins identified in **(B)** would proceed to pharmacological assays and development of additional HTS. Third panel from left depicts RNAi functional studies in tick salivary glands and midgut, and whole ticks. Cryo-EM, cryo-electron microscopy; HTP, high-throughput; HTS, high-throughput screen; NMR, nuclear magnetic resonance; RNAi, RNA interference.

differences among populations of the *Aedes aegypti* mosquito vector of DENV, ZIKV, yellow fever, and CHIKV were correlated with vector competence for flavivirus transmission (Black et al., 2002). For example, QTL for the "midgut infection barrier" phenotype associated with reduced DENV2 serovar infection of *Ae. aegypti* were mapped to several chromosomes and found to account for a significant percentage of the phenotype (Bosio et al., 2000; Gomez-Machorro et al., 2004). Fine-scale mapping, map-based positional cloning and functional studies are typical next steps to identify genes associated with QTL.

Assembled genomic sequence coupled with expression data, genetic (linkage) maps, and physical maps (**Figure 5**) represent key resources for genomic research. Currently, the *I. scapularis* IscaW1 assembly (ABJB010000000) is the only genome assembly for a tick that comprises sequence scaffolds of Mb length. The assembly consists of 369,495 scaffolds that provide ∼ 3.8X coverage of the 2.1 Gbp haploid genome. Annotation of scaffolds representing ∼57% of the genome, revealed 20,486 protein-coding genes and expansions of gene families associated with tick–host interactions. Improvement of the *I. scapularis* assembly and the generation of draft assemblies for other tick species are high priorities. However, haploid genome size and complexity make this a costly and challenging goal (Meyer and Hill, 2014). The haploid genomes of multiple hard and soft tick species are estimated to exceed 1 Gb, and typically comprise relatively high levels of repetitive DNA sequence as compared to many arthropods (Ullmann et al., 2003; Geraci et al., 2007; Meyer and Hill, 2014; Gulia-Nuss et al., 2016). Third generation genomic technologies such as long-read sequencing (PacBio and Hi-C) and optical mapping (Jiao and Schneeberger, 2017) are expected to enable chromosome-level assemblies for ticks. Optical mapping is ideally suited for the improvement of fragmented genome assemblies and scaffolding of *de novo* assemblies from high throughput sequence reads (Howe and Wood, 2015). These technologies have been used to generate an improved assembly for *Ae. aegypti* (Dudchenko et al., 2017) and will likely be useful to generate genome assemblies for tick species.

Future genome sequencing targets identified by the Tick and Mite Genomes Consortium are described in a white paper (Hill, 2010; Van Zee and Hill, 2017). This project, approved by the National Institutes of Health, is a community-ratified guide for genomic and genetic research on ticks and mites of medical and veterinary importance. The whitepaper proposes sequencing of species representing the major lineages comprising the subclass Acari (ticks and mites). Beachhead species include (1) the prostriate vectors of TBFs in Europe and Asia, *I. ricinus* and *I. persulcatus*, (2) the metastriate ticks *Dermacentor variabilis* (American dog tick), the vector of the *Rickettsia rickettsia* bacterium that causes Rocky Mountain Spotted Fever (RMSF) and *A. americanum* (lone star tick), the vector of erlichiosis and *Borrelia* spp, (3) the soft tick *Ornithodoros moubata* (family Argasidae), and (4) the *Leptotrombidium deliense* mite vector of scrub typhus (Superorder Acariformes). These species represent key phylogenetic nodes, and were selected based on their significance as vectors and potential to nucleate additional genomic research.

Forward genetics requires mapping populations (i.e., inbred laboratory lines with quantifiable traits), large numbers of molecular markers for coarse and fine-scale mapping, and high-density genetic maps. The development of mapping populations of ticks has been stymied by the relatively long lifecycle of many species and the costs associated with colony maintenance. Multiple types of molecular markers have been produced for species of tick vectors (Meyer and Hill, 2014;

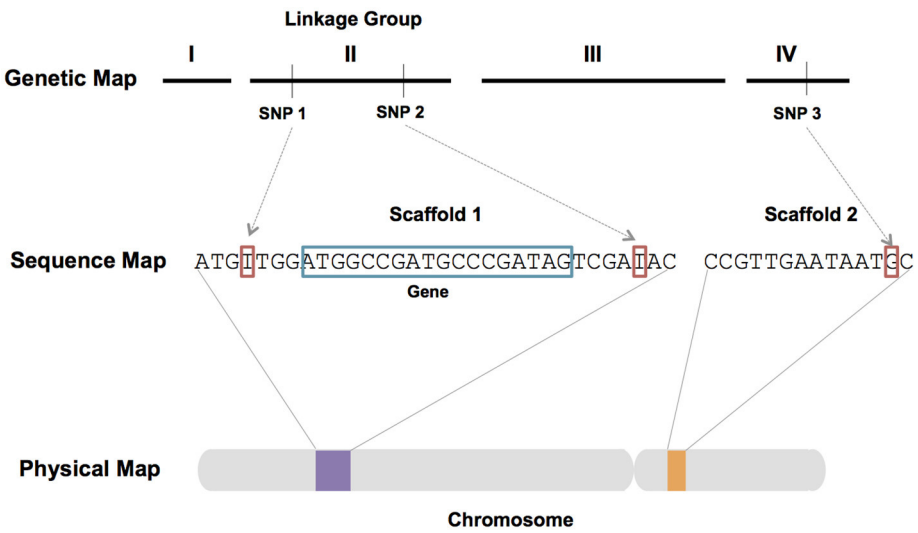

FIGURE 5 | Schematic diagram showing the integration of genetic, sequence and physical maps. Genetic markers such as single nucleotide polymorphism (SNP) markers enable the association of assembled sequence reads with genetic linkage groups. Sequence can be oriented on chromosomes via physical mapping. Integrated maps and fine scale genetic mapping techniques can be used to identify regions of the genome associated with quantitative trait loci (QTL) and genes associated with phenotypes of interest.

Araya-Anchetta et al., 2015). Notably, thousands of single nucleotide polymorphism (SNP) markers have been identified from populations of *I. scapularis* using the technique of *R*estriction Site-*A*ssociated *D*NA sequencing (RADseq) (Gulia-Nuss et al., 2016) and PCR (Van Zee et al., 2013, 2015). The preliminary *I. scapularis* linkage map, generated according to the segregation of 127 loci in 232 F1 intercross progeny from a single female tick and using a combination of RAPD, sequence-tagged RAPD (STAR), cDNA, and microsatellite markers, represents the only such resource for any tick (Ullmann et al., 2003). Fourteen linkage groups were identified that may correspond to the haploid number of chromosomes in *I. scapularis*. The map of 606 centimorgans (cM) had a marker interval of 10.8 cM and the estimated relationship of physical to genetic distance was ∼663 kb/cM. More than 7 M SNPs identified via the study of Gulia-Nuss et al. (2016) and available at VectorBase (www.vectorbase.org/) provide a basis for development of a high-density linkage map for *I. scapularis*. Such maps should also be the goal for other TBF vectors.

Physical mapping is a complementary technique to assign and orient sequence data on chromosomes, integrate sequence and genetic maps, and improve genome assemblies (**Figure 5**). Physical maps support cytogenetic research, including the development of karyotypes and studies of chromosome synteny. Chromosome number has been determined for multiple species of ticks (Oliver, 1977) providing insights into reproductive strategies among members of the Acari. Physical mapping using species of repetitive DNA and assembled sequence data have enabled investigations of genome organization for pro- and metastriate ticks (Meyer and Hill, 2014). Preliminary physical maps were produced for *I. scapularis* and *R. microplus* using the technique of fluorescent *in situ* hybridization (FISH) to study the chromosomal arrangement of families of tandem sequence repeats (Hill et al., 2009; Meyer et al., 2010; Gulia-Nuss et al., 2016). Physical maps must be developed for additional species of hard and soft ticks to support genome research on a range of TBF vectors.

An understanding of population structure and dynamics is critical for determining the role of ticks in disease transmission and for modeling and managing new control strategies. Studies of genetic diversity have been reported for at least 22 tick species representing six genera and the families Argasidae and Ixodidae. In the last several decades, the development of molecular markers has permitted the resolution of phylogenetic relationships at different taxonomic levels and population genetic analyses for multiple species (reviewed in Araya-Anchetta et al., 2015). Observed levels of population genetic structure range from negligible to high across the Ixodida, and for some species, suggest a correlation to host movement and significant host-race adaptation. Increasingly, research is directed at the contribution of tick population structure to the diversity and phylogeography of the pathogens they transmit, and the implications for disease risk (Qiu et al., 2002; Girard et al., 2009; Humphrey et al., 2010; Swei et al., 2015). Collectively, genetic mapping, GWAS and population genomic studies should enable the identification of loci that contribute to TBF transmission.

Priority Areas for Research Investment

Below, we suggest priorities for "omics" research and outline a proposed roadmap for delivery of new TBF control technologies by a target date of 2030. We challenge the field to develop three or more vaccine candidates and three or more leads for novel antivirals and acaricides within this timeframe. Key deliverables and proposed milestone dates are shown in **Figure 6**.

Research on TBFs

1. Metagenomic studies to define the complement of viral phyla and the prevalence of TBFs in the microbiome of tick vectors at regional scale.
2. Studies to determine the pathogenicity of viruses circulating in tick populations.
3. Studies to understand the systems biology of individual tick bites and the molecular interplay between microbial complement and tick and vertebrate host factors (i.e., GxGxG studies; **Figure 1**).
4. Precision medicine and improved passive surveillance for TBFs; the development of comprehensive molecular diagnostic tools (i.e., wearable devices, point of care diagnostics, and field sensors that detect hundreds rather than tens of pathogens, coupled with disease risk matrices to guide health care delivery).
5. Cryo-EM structural studies of high consequence TBFs and virus-antibody complexes.

Research on Tick Vectors of TBFs

1. Field studies focused on elucidation of natural TBF transmission cycles and incrimination of tick vector and reservoir species.
2. Prioritization of research in biologically relevant "tick-virus" systems; the development of resources including in-bred tick strains and tick cell lines derived from major tick vectors, and pathogenic viral species and strains. Dissemination of resources to the scientific community via resource sharing platforms such as the NIH funded BEI Resources (https://www.niaid.nih.gov/research/bei-resources-repository).
3. Development of resources for genome research on high priority tick vectors, including:
   - Improvement of the exitsing *I. scapularis* IscaW1 reference genome assembly (Gulia-Nuss et al., 2016) using third-gen technologies.
   - Production of high quality draft genome assemblies for "node" species, including representatives of the pro- and metastriate lineages, the major genera of hard (*Ixodes, Dermacentor, Amblyomma, Hyalomma, Rhipicephalus*) and soft (*Ornithodorus*) ticks, and the major vectors of tick-borne diseases in Europe, Asia, and the Americas (see **Table 1** and Hill, 2010).
   - Generation of "omic" (transcriptomic, proteomic, and metabolomic) datasets for major tick vectors to support gene annotation, protein prediction and pathway analyses.
4. Structural genomic studies of the tick proteome via cryo-EM and crystal structures of key tick proteins to support *in silico* research.

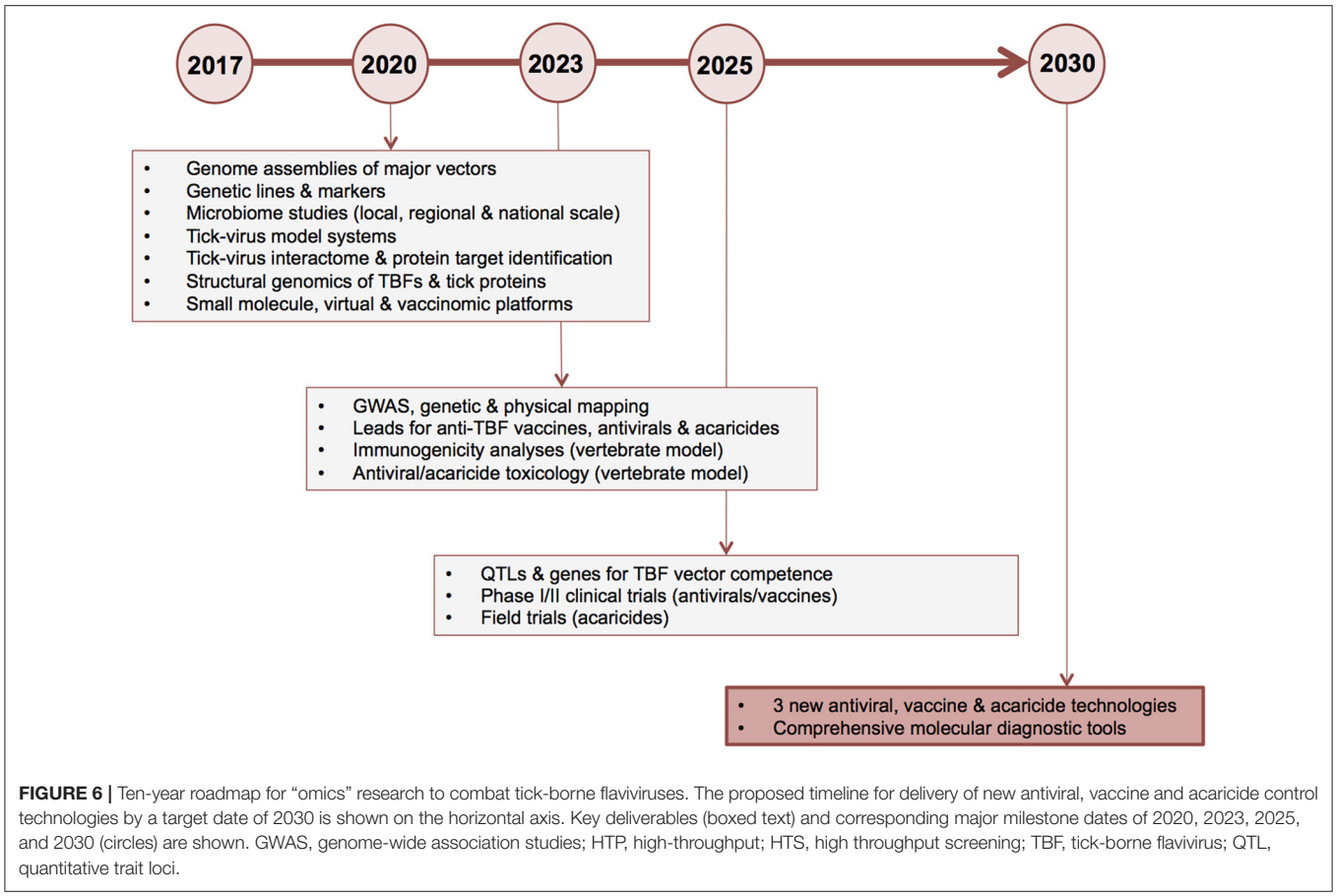

FIGURE 6 | Ten-year roadmap for "omics" research to combat tick-borne flaviviruses. The proposed timeline for delivery of new antiviral, vaccine and acaricide control technologies by a target date of 2030 is shown on the horizontal axis. Key deliverables (boxed text) and corresponding major milestone dates of 2020, 2023, 2025, and 2030 (circles) are shown. GWAS, genome-wide association studies; HTP, high-throughput; HTS, high throughput screening; TBF, tick-borne flavivirus; QTL, quantitative trait loci.

5. Development of resources to support tick genetics and population genomics research, including:
   - Mapping populations of tick species with quantifiable traits, with an emphasis on strains that exhibit differences in vector competence and capacity for transmission of TBFs.
   - Genetic markers (e.g., SNPs) for genetic mapping, GWAS, population genomics and phylogenomics.
   - High density genetic and physical maps for major vector species (**Figure 5**).

Research on Vaccines, Antivirals, and Acaricides to Control TBFs

1. Radical redesign of discovery pipelines incorporating virus and tick protein targets and rational, *in silico* design of vaccines, antivirals and acaricides (see **Figure 4**).

Conclusions: Potential at the Convergence of Forward and Reverse Genetics

Genome assemblies provide an essential framework to support both forward and reverse genetics on ticks. In coming years, the field will witness additional tick genome projects, including assemblies for tick vectors of TBFs. Omic studies must emphasize tick-virus systems and will expand to include metabolomics. Structural studies embracing tick and TBF proteins will enable the redesign of drug discovery pipelines. Finally, it is hoped that forward tick genetics will become a reality, and converge with reverse genetic strategies to permit identification of the gene products associated with transmission of TBFs. Thus positioned, the field can realistically expect a paradigm shift toward precision medicine, and realization of the overarching objective long promised by genomics—the improved control of TBFs.

AUTHOR CONTRIBUTIONS

JG and CH conceived the study, wrote the paper and approved the manuscript.

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SUPPLEMENTARY MATERIAL

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Vaccinomics Approach to the Identification of Candidate Protective Antigens for the Control of Tick Vector Infestations and *Anaplasma phagocytophilum* Infection

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Anaplasma phagocytophilum is an emerging tick-borne pathogen causing human granulocytic anaplasmosis (HGA), tick-borne fever (TBF) in small ruminants, and other forms of anaplasmosis in different domestic and wild animals. The main vectors of this pathogen are *Ixodes* tick species, particularly *I. scapularis* in the United States and *I. ricinus* in Europe. One of the main limitations for the development of effective vaccines for the prevention and control of *A. phagocytophilum* infection and transmission is the identification of effective tick protective antigens. The objective of this study was to apply a vaccinomics approach to *I. scapularis-A. phagocytophilum* interactions for the identification and characterization of candidate tick protective antigens for the control of vector infestations and *A. phagocytophilum* infection. The vaccinomics pipeline included the use of quantitative transcriptomics and proteomics data from uninfected and *A. phagocytophilum*-infected *I. scapularis* ticks for the selection of candidate protective antigens based on the variation in tick mRNA and protein levels in response to infection, their putative biological function, and the effect of antibodies against these proteins on tick cell apoptosis and pathogen infection. The characterization of selected candidate tick protective antigens included the identification and characterization of *I. ricinus* homologs, functional characterization by different methodologies including RNA interference, immunofluorescence, gene expression profiling, and artificial tick feeding on rabbit antibodies against the recombinant antigens to select the candidates for vaccination trials. The vaccinomics pipeline developed in this study resulted in the identification of two candidate tick protective antigens that could be selected for future vaccination trials. The results showed that *I. scapularis* lipocalin (ISCW005600) and lectin pathway inhibitor (AAY66632) and *I. ricinus* homologs constitute candidate protective antigens for the control of vector infestations and *A. phagocytophilum* infection. Both antigens are involved in the tick evasion of host defense response and pathogen infection and transmission, but targeting different immune response pathways. The vaccinomics

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pipeline proposed here could be used to continue the identification and characterization of candidate tick protective antigens for the development of effective vaccines for the prevention and control of HGA, TBF, and other forms of anaplasmosis caused by *A. phagocytophilum*.

Keywords: anaplasmosis, immunology, vaccine, tick, *Ixodes*, *Anaplasma phagocytophilum*

INTRODUCTION

The intracellular bacterium, *Anaplasma phagocytophilum* (Rickettsiales: Anaplasmataceae) is an emerging tick-borne pathogen causing human granulocytic anaplasmosis (HGA), which has emerged as a tick-borne disease of humans in the United States, Europe and Asia, and tick-borne fever (TBF) in small ruminants, most notably in sheep in Europe (Gordon et al., 1932; Foggie, 1951; Dumler et al., 2001; Stuen et al., 2013; Bakken and Dumler, 2015; Dugat et al., 2015; Severo et al., 2015). Clinical presentation of *A. phagocytophilum* infection has been also documented in goats, cattle, horses, dogs, cats, roe deer, and reindeer (Severo et al., 2015). The main vectors of this pathogen are *Ixodes* tick species, particularly *I. scapularis* in the United States and *I. ricinus* in Europe (Stuen et al., 2013; Bakken and Dumler, 2015).

Despite the burden that *A. phagocytophilum* represents for humans and animals, vaccines are not available for prevention and control of pathogen infection and transmission (Dumler et al., 2001; Stuen et al., 2013, 2015; Bakken and Dumler, 2015; Severo et al., 2015; Contreras et al., 2017). One of the main limitations for the development of effective vaccines for the prevention and control of *A. phagocytophilum* infection and transmission is the identification of effective tick protective antigens. Recently, different approaches have been developed for the identification and characterization of candidate tick protective antigens (de la Fuente and Contreras, 2015; de la Fuente et al., 2016a). Vaccinomics is one of the approaches that have been used by our group for the identification of tick-derived and pathogen-derived protective antigens (de la Fuente and Merino, 2013; Merino et al., 2013; Antunes et al., 2014; de la Fuente and Contreras, 2015; Contreras et al., 2016, 2017; de la Fuente et al., 2016a; Villar et al., 2017). Vaccinomics is a holistic approach based on the use of genome-scale or omics technologies integrated in a systems biology approach to characterize tick-host-pathogen interactions for the development of next-generation vaccines (de la Fuente and Merino, 2013; Contreras et al., 2016; de la Fuente et al., 2016a; Villar et al., 2017). In this translational approach, basic biological information on tick-host-pathogen interactions translates into the identification and subsequent evaluation of new candidate protective antigens (de la Fuente and Merino, 2013; de la Fuente et al., 2016a; Villar et al., 2017).

The sequence, assembly and annotation of the *I. scapularis* genome were recently released (Gulia-Nuss et al., 2016), and various genomics, transcriptomics and proteomics studies in *I. ricinus* suggest that these tick species are genetically closely related (Schwarz et al., 2013, 2014; Genomic Resources Development Consortium et al., 2014; Cramaro et al., 2015; Kotsyfakis et al., 2015; Weisheit et al., 2015; Chmelař et al., 2016). These results open new opportunities for research on tick-host-pathogen interactions and the possibility of identifying tick protective antigens for both *I. scapularis* and I. *ricinus* major vectors of *A. phagocytophilum* (de la Fuente et al., 2016b).

Recently, transcriptomics, proteomics and metabolomics datasets have been integrated and used for the characterization of *I. scapularis-A. phagocytophilum* molecular interactions (Ayllón et al., 2015; Villar et al., 2015a,b, 2016; Cabezas-Cruz et al., 2016, 2017a,b; de la Fuente et al., 2016c, 2017; Gulia-Nuss et al., 2016; Shaw et al., 2017). Herein, a vaccinomics pipeline was developed based on quantitative transcriptomics and proteomics data from uninfected and *A. phagocytophilum*-infected *I. scapularis* nymphs, adult female midguts and salivary glands, and ISE6 cells (Ayllón et al., 2015; Villar et al., 2015a). The vaccinomics pipeline was then used for the identification of candidate protective antigens for the control of vector infestations and pathogen infection. The results showed that *I. scapularis* ISCW005600 and AAY66632 and *I. ricinus* homologs constitute candidate protective antigens for the control of vector infestations and *A. phagocytophilum* infection.

MATERIALS AND METHODS

Ticks and Cultured Tick Cells

Ixodes scapularis ticks were obtained from the laboratory colony maintained at the Oklahoma State University Tick Rearing Facility. Nymphs and adult female *I. scapularis* were infected with *A. phagocytophilum* by feeding on a sheep inoculated intravenously with approximately 1×10^7 *A. phagocytophilum* (NY18 isolate)-infected HL-60 human cells (90–100% infected cells) (Kocan et al., 2012; Ayllón et al., 2015). Animals were housed and experiments conducted with the approval and supervision of the OSU Institutional Animal Care and Use Committee (Animal Care and Use Protocol, ACUP No. VM1026). *I. ricinus* ticks were obtained from the laboratory colony maintained at the Freie Universität Berlin. Larvae and nymphs were fed on mice and adults on rabbits. The *I. scapularis* embryo-derived tick cell line ISE6, provided by Ulrike Munderloh, University of Minnesota, USA, was cultured in L-15B300 medium as described previously (Kurtti et al., 1996; Munderloh et al., 1999; Villar et al., 2015a). IRE/CTVM20 embryo-derived tick cells, provided by the Tick Cell Biobank, were maintained as described previously (Bell-Sakyi et al., 2007; Alberdi et al., 2015). Tick cells were first inoculated with *A. phagocytophilum* (human NY18 isolate; Asanovich et al., 1997)-infected HL-60 cells and maintained according to Munderloh et al. (1999). Uninfected and infected cultures ($N = 4$ independent cultures with approximately 10^7 cells each) were

sampled at 7 days post-infection (dpi) (75% infected cells). The percentage of cells infected with *A. phagocytophilum* was calculated by examining at least 200 cells using a 100x oil immersion objective.

Transcriptomics and Proteomics Datasets

The quantitative transcriptomics and proteomics data for uninfected and *A. phagocytophilum*-infected *I. scapularis* nymphs, adult female midguts and salivary glands, and ISE6 cells were obtained from previously published results (Ayllón et al., 2015; Villar et al., 2015a) and deposited at the Dryad repository database, NCBI's Gene Expression Omnibus database and ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002181 and doi: 10.6019/PXD002181.

Sequence Analysis

To find the *I. ricinus* homologs, selected *I. scapularis* sequences were blasted against the *I. ricinus* database using the Blastp tool from BLAST (Altschul et al., 1990; Madden et al., 1996), and the sequences with the lowest *E*-value were selected. Gene ontology (GO) analysis for biological process (BP) was done with Blast2GO software (version 3.0; http://www.blast2go.com) (Villar et al., 2014).

Production of Recombinant Proteins

The coding sequences for *I. scapularis* candidate protective antigens were amplified from synthetic genes optimized for codon usage in *Escherichia coli* (Genscript Corporation, Piscataway, NJ, USA) using sequence-specific primers (**Table 1**). The amplified DNA fragments were cloned into the expression vector pET101 and expressed in *E. coli* strain BL21 using the Champion pET101 Directional TOPO Expression kit (Carlsbad, CA, USA). Recombinant proteins were fused to Histidine tags for purification by affinity to Ni (Merino et al., 2013; Moreno-Cid et al., 2013). Transformed *E. coli* strains were induced with IPTG for 4.5 h to produce recombinant proteins, which were purified to >85% of total cell proteins by Ni affinity chromatography (Genscript Corporation) as previously described (Merino et al., 2013; Moreno-Cid et al., 2013) using 1 ml HisTrap FF columns mounted on an AKTA-FPLC system (GE Healthcare, Piscataway, NJ, USA) in the presence of 7 M urea lysis buffer. The purified antigens were refolded by dialysis against 1,000 volumes of PBS, pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) for 12 h at 4°C.

Production of Rabbit Polyclonal IgG Antibodies

For each recombinant tick protein and total ISE6 tick cell proteins, two New Zealand white rabbits (*Oryctulagus cuniculus*) were subcutaneously injected at weeks 0, 4, and 6 with 50 μg protein in 0.4 ml Montanide ISA 50 V adjuvant (Seppic, Paris, France). Blood was collected before injection and 2 weeks after the last immunization to prepare pre-immune and immune sera, respectively. Serum aliquots were kept at 4°C for immediate use or at −20°C for long-term storage. The IgG were purified from serum samples using the Montage antibody purification kit and

TABLE 1 | Oligonucleotide primers used in this study for cloning, RNAi and RT-PCR.

GenBank accession No.	Oligonucleotide sequence (5′-3′ for forward and reverse primers)
CLONING	
ISCW024685	CACCATGAAAAGCAGCGCACTGCTG
	GCGTTTACCACGAACGCACC
ISCW024295	CACCATGCCGAAACAAGGCGAAAC
	TCCAGAGTCACCACACAAAACG
ISCW022212	CACCATGTGGGGTCAGATTGCGCT
	ACAGATGAATTTTTTCAGGC
ISCW020900	CACCATGAACAAAGCGATCTTCAT
	CACTTCACCGAAAAAGCCGC
ISCW000326	CACCATGCCGGCGTCAATGAAAAG
	CAGAGAACCCAGATTCGGAA
ISCW008146	CACCATGGATTTTGATGACCTGTT
	GAAGCTCAGGGTGTTCTGTT
ISCW008641	CACCATGCAACGTGACATTTTTAG
	CCAACAGCCCGGCTGCGATT
ISCW024499	CACCATGTGCCTGGTGTTTGCAAC
	GCGCAGAAAGGAACTCGTAC
ISCW005600	CACCATGATTCGTCAGGTTCGCGA
	CGAACCTGAGATCGATGAGG
ISCW013709	CACCATGTTTCGTACCAGCTCTGG
	CACAATATAATCCGGTGCAC
ISCW017117	CACCATGCTGAGTGTGCTGCTGGG
	CGTGGTGGCGTCCGGCGGCG
ISCW013574	CACCATGTATCAGCTGCGCGATTT
	GCAACGGGATTTGCGAACAC
ISCW018900	CACCATGGGCCCGTTTATTGGTCT
	GCCGATAATGCGACCGATAA
ISCW023907	CACCATGCCGGTCAATCGCCTGAT
	AACTTTACGAAAGAAAAACA
ISCW024682	CACCATGATTCATGAACCGGTGAT
	ATACGGACAGTACAGTTTGCA
ISCW015453	CACCATGATGAAAAGCCCGCTGTTTAT
	ACCGAAAAAGCCGTGGCCGA
ISCW021670	CACCATGTGGGAACTGCATGCCGA
	CTCCTGGGTAATATTACGCGT
ISCW017271	CACCATGTGCAGCGATTCTAAACC
	CGGCAGATAGGAACCGTGCG
AAY66632	CACCATGGGCCTGACCGGTACCAC
	GTTGTCTTTGGTTTTCTTGG
RNAi AND RT-PCR	
ISCW005600	TCCCCTTCTCAAAGGAGGAT*
	ATCCACAGGCGGATATGAAG*
AAY66632	ACCCGTTCATGGGACAAATA*
	TTCTTGGGCTTCTCAGTTGG*
DQ066214 (*rpS4*)	GGTGAAGAAGATTGTCAAGCAGAG
	TGAAGCCAGCAGGGTAGTTTG

**The same oligonucleotide primers were used to determine gene expression levels by RT-PCR and for the generation of dsRNA for RNAi. To produce dsRNA, the T7 promoter sequence 5′-GAATTAATACGACTCACTATAGGGAGA-3′was added to the 5′-end of each primer.*

spin columns with PROSEP-A media (Millipore, Billerica, MA, USA) following the manufacturer's recommendations.

Western Blot Analysis

Ten micrograms of each recombinant protein or 20 μg total proteins from ISE6 tick cells were loaded onto a 12% SDS-polyacrylamide pre-cast gel (Life Science, Hercules, CA, USA) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MI, USA) for 2 h at room temperature (RT), and washed four times with TBS (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Tween 20). Purified rabbit IgG were used at a 1:500 dilution in TBS, and the membrane was incubated overnight at 4°C and washed four times with TBS. The membrane was then incubated with an anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (Sigma-Aldrich) diluted 1:1,000 in TBS with 3% BSA. The membrane was washed five times with TBS and finally developed with TMB (3,3′, 5,5′- tetramethylbenzidine) stabilized substrate for HRP (Promega, Madrid, Spain) according to the manufacturer recommendations.

Immunofluorescence Assay (IFA) in Adult Female Ticks

Adult *I. scapularis* females were infected with *A. phagocytophilum* (NY18) as described above. Female ticks were removed from the sheep 10 days after infestation, held in the humidity chamber for 4 days and fixed with 4% paraformaldehyde in 0.2 M sodium cacodylate buffer, dehydrated in a graded series of ethanol and embedded in paraffin (Ayllón et al., 2015). Sections (4 μm) were prepared and mounted on glass slides. The paraffin was removed from the sections with xylene and the sections were hydrated by successive 2 min washes with a graded series of 100, 95, 80, 75, and 50% ethanol. The slides were treated with Proteinase K (Dako, Barcelona, Spain) for 7 min, washed with PBS and incubated with 3% BSA (Sigma-Aldrich) in PBS for 1 h at RT. The slides were then incubated for 14 h at 4°C with primary rabbit IgG antibodies diluted 1:100 in 3% BSA/PBS and, after 3 washes in PBS, developed for 1 h with goat-anti-rabbit IgG conjugated with phycoerythrin (PE) (Sigma-Aldrich) (diluted 1:50 in 3% BSA/PBS). The slides were washed twice with PBS and mounted in ProLong Antifade with DAPI reagent (Molecular Probes, Eugene, OR, USA). The sections were examined using a Zeiss LSM 800 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). Sections of uninfected ticks and IgG from pre-immune and anti-ISE6 sera were used as controls.

Antibody Inhibition Assay

The inhibitory effect of rabbit IgG antibodies on *A. phagocytophilum* (NY18) was conducted as described previously (Villar et al., 2015b). ISE6 and IRE/CTVM20 tick cells were pooled and used to seed 24-well plates for each assay. Each well received 1×10^6 cells in L-15B300 (ISE6) or L-15/L-15B (IRE/CTVM20) medium 24 h prior to inoculation with *A. phagocytophilum*. Infected cultures for inoculum were harvested when infection reached 80% and host cells were mechanically disrupted with a syringe and 26-gauge needle. Purified IgG (100 μg/ml) were added to the culture media and incubated with the cells for 48 h. Then, the medium with antibodies was removed and the *A. phagocytophilum* inoculum (100 μl) was added to the cell monolayers and incubated at 31°C for 60 min. The inoculum was removed from the wells and cell monolayers washed three times with PBS. Complete medium (1 ml) was added to each well and the plates were incubated at 31°C. The control included inoculum incubated with rabbit pre-immune and anti-ISE6 IgG. Four replicates were done for each treatment. After 72 h, cells from all wells were harvested and processed for *A. phagocytophilum* detection by real-time PCR after DNA extraction. Results were compared between treatments by the Student's *t*-test with unequal variance ($P = 0.05$; $N = 4$ biological replicates).

Flow Cytometry of Tick Cells Incubated with Rabbit IgG Antibodies

Approximately 5×10^5–1×10^6 of *A. phagocytophilum*-infected ISE6 and IRE/CTVM20 tick cells were collected after incubation with rabbit IgG. Purified IgG (2.2–2.4 mg/ml) were mixed with *A. phagocytophilum* and incubated with tick cells as described above in the antibody inhibition assay. Apoptosis was measured by flow cytometry using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Immunostep, Salamanca, Spain) following the manufacturer's protocols. The technique detects changes in phospholipid symmetry analyzed by measuring Annexin V (labeled with FITC) binding to phosphatidylserine, which is exposed in the external surface of the cell membrane in apoptotic cells. Cells were stained simultaneously with the non-vital dye propidium iodide (PI) allowing the discrimination of intact cells (Annexin V-FITC negative, PI negative) and early apoptotic cells (Annexin V-FITC positive, PI negative). All samples were analyzed on a FAC-Scalibur flow cytometer equipped with CellQuest Pro software (BD Biosciences, Madrid, Spain). The viable cell population was gated according to forward-scatter and side-scatter parameters. The percentage of apoptotic cells was determined by flow cytometry after Annexin V-FITC and PI labeling and compared between treated and untreated uninfected cells by Student's *t*-test with unequal variance ($P = 0.05$; $N = 4$ biological replicates).

RNA Interference (RNAi) for Gene Knockdown in Tick Cells

RNAi was used to characterize the effect of gene knockdown on tick cell pathogen infection. Oligonucleotide primers homologous to selected *I. scapularis* ISCW005600 and AAY66632 genes containing T7 promoters (**Table 1**) were used for *in vitro* transcription and synthesis of dsRNA as described previously (Ayllón et al., 2013), using the Access RT-PCR system (Promega, Madison, WI, USA) and the Megascript RNAi kit (Ambion, Austin, TX, USA). The unrelated *Rs86* dsRNA was synthesized using the same methods described previously and used as negative control (Ayllón et al., 2013). The dsRNA was purified and quantified by spectrophotometry. RNAi experiments were conducted in cell cultures by incubating ISE6 tick cells with 10 μl dsRNA (5×10^{10}–5×10^{11} molecules/μl) and 90 μl L15B300 medium in 24-well plates using 5 wells per treatment (Ayllón

et al., 2013). Control cells were incubated with the unrelated *Rs86* dsRNA. After 48 h of dsRNA exposure, tick cells were infected with cell-free *A. phagocytophilum* (NY18) obtained from approximately 5×10^6 infected HL-60 cells (90–100% infected cells) (Thomas and Fikrig, 2007) and resuspended in culture medium to use 1 ml/well. Cells were incubated for an additional 72 h, harvested and used for DNA and RNA extraction. RNA was used to analyze gene knockdown by real-time RT-PCR with respect to *Rs86* control. DNA was used to quantify the *A. phagocytophilum* infection levels by real-time PCR.

Determination of *A. phagocytophilum* Infection by Real-Time PCR

A. phagocytophilum DNA levels were characterized by *major surface protein 4* (*msp4*) real-time PCR normalized against tick *ribosomal protein S4* (*rpS4*) as described previously (Ayllón et al., 2015). Normalized Ct-values were compared between untreated and treated cells by Student's *t*-test with unequal variance ($P = 0.05$; $N = 4$ biological replicates).

Determination of Tick mRNA Levels by Real-Time RT-PCR

Total RNA was extracted from ISE6 tick cell cultures using TriReagent (Sigma-Aldrich) following manufacturer's recommendations. The expression of selected *I. scapularis* ISCW005600 and AAY66632 genes was characterized using total RNA extracted from infected and uninfected ISE6 tick cells. Real-time RT-PCR was performed on RNA samples using gene-specific oligonucleotide primers (**Table 1**) and the Kapa SYBR Fast One-Step qRT-PCR Kit (Kapa Biosystems, Wilmington, MA, USA) and the Rotor-Gene Real-Time PCR Detection System (Qiagen, Madrid, Spain). A dissociation curve was run at the end of the reaction to ensure that only one amplicon was formed and that the amplicons denatured consistently at the same temperature range for every sample. The mRNA levels were normalized against tick *rpS4* using the genNorm method (Delta-Delta-Ct, ddCT) as described previously (Ayllón et al., 2015). Normalized Ct-values were compared between infected and uninfected tick cells by Student's *t*-test with unequal variance ($P = 0.05$; $N = 4$ biological replicates).

Artificial Tick Feeding

Artificial tick feeding was conducted as previously described for *Dermacentor reticulatus* (Krull et al., 2017). Briefly, 17–19 female and 3 male *I. ricinus* ticks were placed on each feeding unit. The feeding unit was subsequently closed by the insertion of a pierced plastic lid (PE-LD Stopfen 26 mm, Brimon Laborbedarf, Hamburg, Germany) wrapped in gauze fabric into the feeding unit, leaving approximately one cm between the silicone membrane and lid. The feeding unit was then hung into a glass beaker (50 ml, Simax, Czech Republic) containing the bovine blood using a rubber ring with an inner diameter of 32 mm (Lux, Wermelskirchen, Germany). Blood was supplemented with ATP and gentamycin (Krull et al., 2017), and 5 ml blood per feeding unit was pipetted into a sterile beaker and preheated to 37°C on a hot plate. The blood was changed twice daily at 12 ± 2 h intervals. During each blood change, the outside of the feeding unit and underside of the silicone membrane were rinsed with sterile 0.9% NaCl solution, pre-heated to body temperature. The number of attached, dead and fed ticks was counted after which the feeding unit was transferred to a new sterile beaker with fresh blood. Males stayed inside the feeding unit until the end of the experiment, to provide them with sufficient opportunity and time to fertilize any females present. Feeding units were placed in an incubator (ICH 256C, Memmert GmbH, Schwabach, Germany), where the blood was maintained at a constant temperature of 37°C using a heating plate (Hot Plate 062, Labotect, Göttingen, Germany). Environmental conditions were set at 20°C, 80% relative humidity, 5% CO_2 and 15 h light/9 h dark. Once ticks were partially engorged, the feeding units were transferred to a six-well plate (Sarstedt, Nümbrecht, Germany) and ticks were fed for 36 h with 3 ml blood supplemented with 1 mg/ml of pre-immune or antigen-specific purified IgG. Dead and detached engorged ticks were removed, and engorged females that detached were weighed and stored individually in 2 ml Eppendorf tubes with pierced lids, which were kept in desiccators with approximately 90% relative humidity at RT. Ticks were assessed for egg mass 8 weeks post-feeding. The number of dead/fed ticks, ticks and eggs weight, and ticks with or without oviposition were compared between groups by a Fisher's exact test ($P = 0.05$; http://www.socscistatistics.com/tests/fisher/Default2.aspx).

RESULTS AND DISCUSSION

Selection of Candidate Tick Protective Antigens

A vaccinomics pipeline was developed for the selection and characterization of candidate tick protective antigens for the control of vector infestations and pathogen infection (**Figure 1**). The vaccinomics pipeline included the use of quantitative transcriptomics and proteomics data from uninfected and *A. phagocytophilum*-infected *I. scapularis* ticks (Ayllón et al., 2015) for the selection of candidate protective antigens based on the variation in tick mRNA and protein levels in response to infection, their putative biological function, and the effect of antibodies against these proteins on tick cell apoptosis and pathogen infection (**Figure 1**). The characterization of selected candidate tick protective antigens included the identification and characterization of *I. ricinus* homologs, functional characterization by different methodologies including RNAi, IFA, gene expression profile, and artificial tick feeding on rabbit antibodies against the recombinant antigens to select the candidates for vaccination trials (**Figure 1**). This process could be repeated as many times as needed to cover all potential candidate antigens or until the desired number of candidate antigens for vaccination trials is reached (**Figure 1**). The vaccinomics pipeline included some of the algorithms previously proposed (de la Fuente and Merino, 2013; Contreras et al., 2016) and validated (Merino et al., 2013; Antunes et al., 2014) for the selection and characterization of candidate protective antigens, but for the first time it was applied to integrated transcriptomics and proteomics data of tick-pathogen interactions.

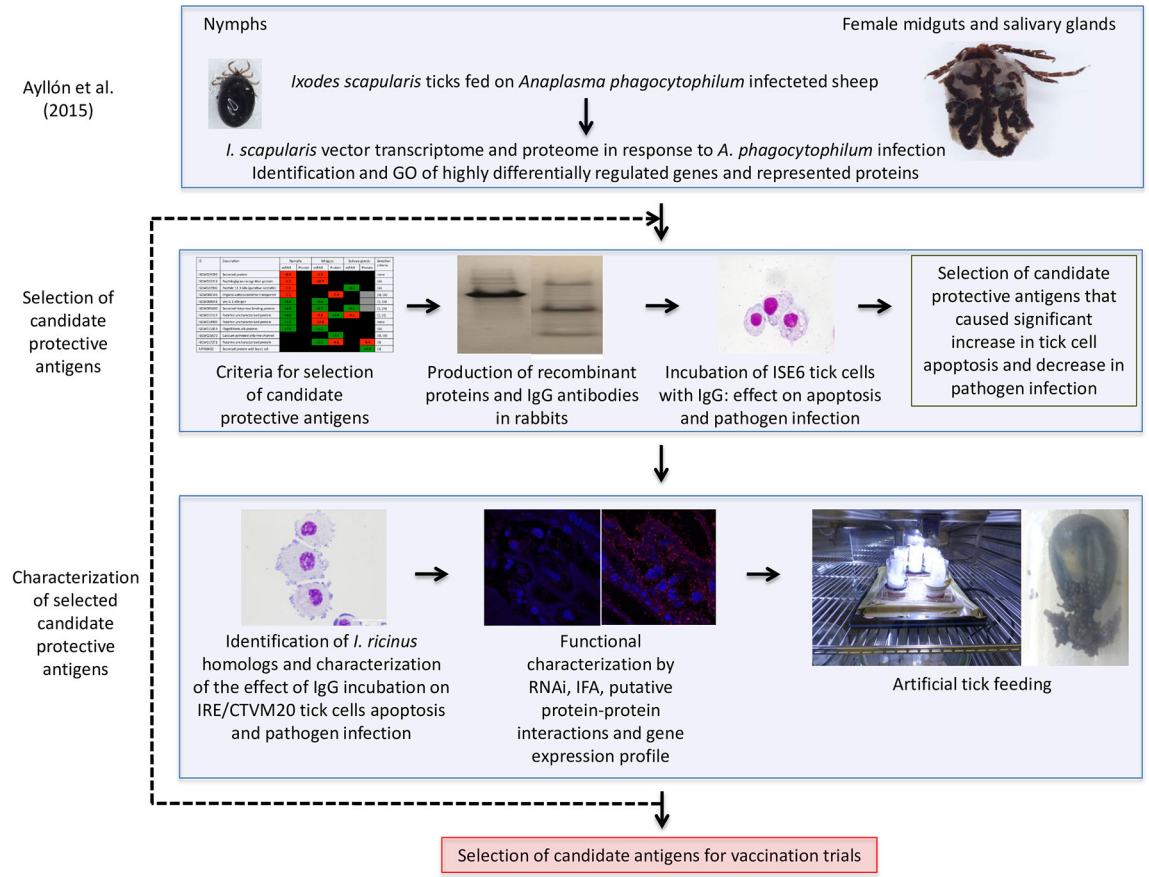

FIGURE 1 | Vaccinomics pipeline for the selection and characterization of candidate tick protective antigens for the control of vector infestations and pathogen infection. The quantitative transcriptomics and proteomics data for uninfected and *A. phagocytophilum*-infected *I. scapularis* nymphs, adult female midguts and salivary glands were obtained from previously published results (Ayllón et al., 2015). The selection of candidate tick protective antigens included criteria based on the variation in tick mRNA and protein levels in response to infection, their putative biological function, and the effect of antibodies against these proteins on tick cell apoptosis and pathogen infection. The characterization of selected candidate protective antigens included the identification and characterization of *I. ricinus* homologs, functional analyses by different methodologies, and artificial tick feeding on rabbit antibodies against the recombinant antigens to select the candidates for vaccination trials.

The characterization of tick-pathogen molecular interactions was based on the previous work by Ayllón et al. (2015) of the *I. scapularis* transcriptome and proteome in response to *A. phagocytophilum* infection in nymphs and female midguts and salivary glands. The highly differentially regulated genes were selected as those with more than 50-fold (log2 normalized fold change >5.64) difference between infected and uninfected tick samples ($P < 0.00003$) (**Figure 2A**). The highly differentially represented proteins were selected as those with more than 15-fold (log2 normalized fold change >3.90) change between infected and uninfected tick samples ($P < 0.00003$) (**Figure 2A**). Of the highly differentially regulated/represented genes/proteins, between 0 and 50% were identified at both mRNA and protein levels in the different samples (**Figure 2B**). The analysis of highly differentially expressed/represented genes/proteins in response to *A. phagocytophilum* infection evidenced tissue-specific differences in response to infection (Ayllón et al., 2015), which were taken into consideration for the selection of candidate protective antigens (**Figures 2A–D**). The candidate protective antigens were selected by using the criteria (i) highly differentially up-regulated genes in at least two samples, (ii) highly down-regulated genes in at least one sample, (iii) highly differentially over-represented proteins and identified in the *I. scapularis* proteome, (iv) highly differentially under-represented proteins and identified in the *I. scapularis* proteome, and/or (v) putative BP in tick-pathogen and tick-host interactions (**Figure 3A**). The rationale behind the selection criteria for candidate protective antigens was based on their putative relevance in (i, iii) tick response to infection (de la Fuente et al., 2016c,d, 2017), (ii, iv) manipulated by *A. phagocytophilum* to decrease tick protective mechanisms and increase infection (de la Fuente et al., 2016c,d, 2017), and (v) tick-pathogen and tick-host interactions (**Figure 3B**).

By using these criteria, a total of 12 candidate tick protective antigens were initially selected, and 7 of them fulfilled two of the selection criteria (**Figure 3A**). The recombinant antigens were

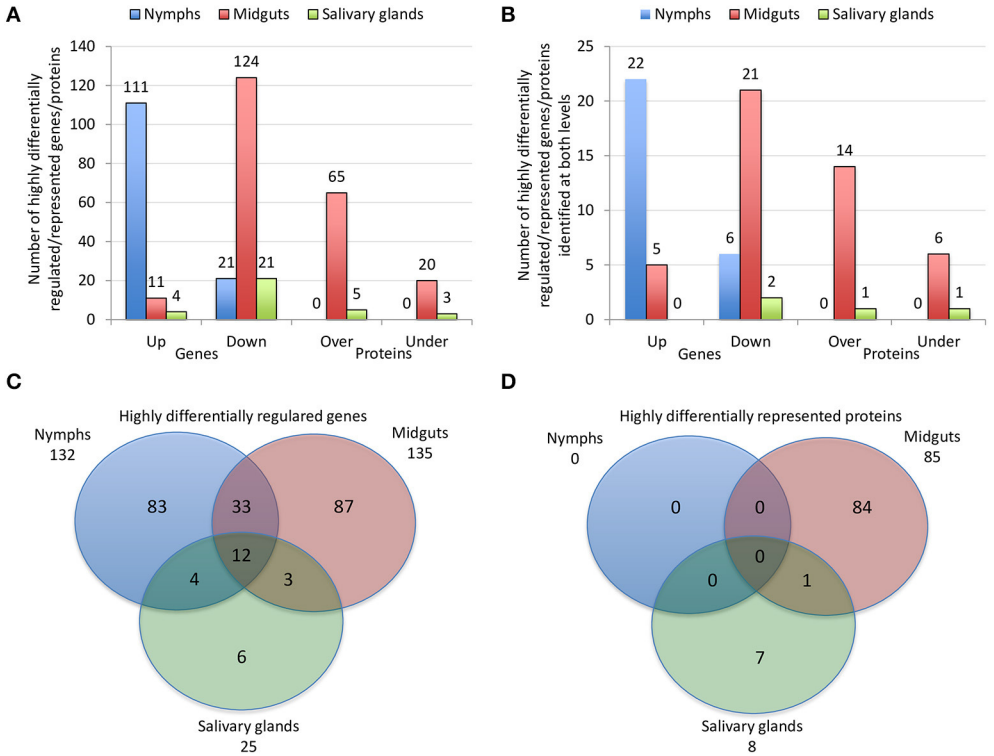

FIGURE 2 | Effect of *A. phagocytophilum* infection on *I. scapularis* highly differentially regulated genes and represented proteins. **(A)** Number of tick highly differentially regulated genes and differentially represented proteins that were up-regulated (Up), down-regulated (Down), over-represented (Over) and under-represented (Under) in response to pathogen infection. **(B)** Number of tick highly differentially regulated genes and differentially represented proteins that were up-regulated (Up), down-regulated (Down), over-represented (Over) and under-represented (Under) in response to pathogen infection, and identified at both mRNA and protein levels. **(C)** Venn diagram with highly differentially regulated genes that were identified in tick nymphs, midguts and salivary glands. **(D)** Venn diagram with highly differentially represented proteins that were identified in tick nymphs, midguts and salivary glands. The highly differentially regulated genes were selected as those with more than 50-fold (log2 normalized fold change > 5.64) difference between infected and uninfected tick samples ($P < 0.00003$). The highly differentially represented proteins were selected as those with more than 15-fold (log2 normalized fold change > 3.90) change between infected and uninfected tick samples ($P < 0.00003$). The quantitative transcriptomics and proteomics data for uninfected and *A. phagocytophilum*-infected *I. scapularis* nymphs, adult female midguts and salivary glands were obtained from previously published results (Ayllón et al., 2015).

produced in *E. coli* and used for the preparation of antigen-specific IgG antibodies in immunized rabbits (**Figures 4A,B**). These IgG antibodies were then used for the incubation with *I. scapularis* ISE6 cells before infection with *A. phagocytophilum* to characterize the effect on cell apoptosis (**Figure 5A**) and pathogen infection (**Figure 5B**). The results showed that anti ISCW005600 and AAY66632 IgG significantly increased the percentage of apoptotic cells when compared to negative control cells incubated with pre-immune IgG (**Figure 5A**). The incubation of ISE6 cells with rabbit IgG against recombinant antigens significantly decreased pathogen infection for 7 antigens when compared to the negative control (**Figure 5B**). The positive control cells were incubated with rabbit IgG antibodies against total ISE6 tick cells proteins, which significantly increased cell apoptosis but did not affect pathogen infection when compared to the negative control (**Figures 5A,B**). The anti-ISE6 antibodies did not affect pathogen infection of tick cells, which as previously discussed (Stuen et al., 2015) was due to the presence of not protective dominant antigens in the protein extract used to immunize rabbits for antibody production. Nevertheless, these results showed that incubation of ISE6 tick cells with IgG antibodies against ISCW005600 and AAY66632 antigens affected both cell apoptosis and pathogen infection, and were therefore selected as the candidate tick protective antigens for further characterization (**Figures 5A,B**).

Characterization of Selected Candidate Tick Protective Antigens

The first step in the characterization of selected candidate tick protective antigens was the identification of *I. ricinus* homologs to evaluate their protective potential in both major tick vector species for *A. phagocytophilum*. The *I. ricinus* homologs for *I. scapularis* ISCW005600 and AAY66632 antigens corresponded to putative salivary gland secreted proteins lipocalins (Beaufays et al., 2008; Schwarz et al., 2013; Valdés et al., 2016) and a lectin pathway inhibitor (Ribeiro et al., 2006; Schuijt et al., 2011), respectively (**Figure 5C**). At the amino acid level, over 70% sequence identity was obtained for both antigens (**Figure 5C**), suggesting that these proteins are highly conserved in *I. scapularis*

A

ID	Description	Nymphs		Midguts		Salivary glands		Selection criteria
		mRNA	Protein	mRNA	Protein	mRNA	Protein	
ISCW024295	Secreted protein	**-8.0**		-2.9				(ii)
ISCW022212	Peptidoglycan-recognition protein	-3.3		**-10.7**				(ii), (v)
ISCW020900	Peptide 11.3 kDa (putative ixostatin)	-1.8				**+8.0**		(v)
ISCW008146	Organic cation/carnitine transporter	-1.5			**-7.9**			(iv), (v)
ISCW008641	Ves G 1 allergen	**+6.0**		**+8.6**				(i), (v)
ISCW005600	Secreted histamine binding protein	**+6.4**		**+5.7**		+0.4		(i), (v)
ISCW017117	Putative uncharacterized protein	**+8.9**		**-7.1**	+6.4	**-7.1**		(ii), (iii)
ISCW018900	Putative uncharacterized protein	+1.6		**-13.9**				(ii)
ISCW015453	Flagelliform silk protein	**+7.6**		+3.2				(v)
ISCW021670	Calcium activated chlorine channel				**+7.5**			(iii), (v)
ISCW017271	Putative uncharacterized protein			+0.7	**-4.5**		**-4.4**	(iv)
AAY66632	Secreted protein with basic tail						**+4.4**	(iii), (v)

Up in infected ticks (+) | Down in infected ticks (-) | No difference | Not found

B

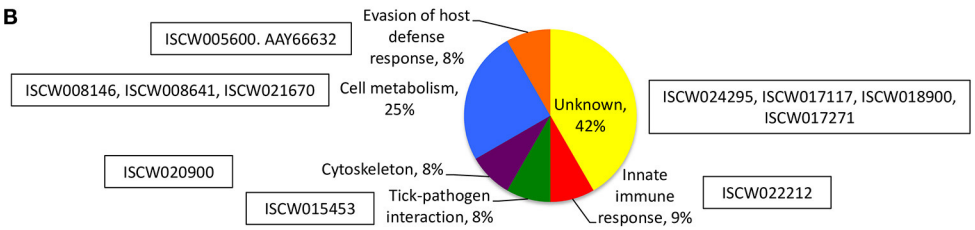

FIGURE 3 | Criteria for the selection of candidate tick protective antigens. **(A)** Annotation and criteria used for the selection of candidate tick protective antigens. The log2 normalized fold change between infected and uninfected tick samples ($P < 0.00003$) is shown. Bold numbers indicate highly differentially regulated genes (log2 normalized fold change > 5.64) and highly differentially represented proteins (log2 normalized fold change > 3.90) between infected and uninfected tick samples. The selection criteria were (i) highly differentially up-regulated genes in at least two samples, (ii) highly down-regulated genes in at least one sample, (iii) highly differentially over-represented proteins and identified in the *I. scapularis* proteome, (iv) highly differentially under-represented proteins and identified in the *I. scapularis* proteome, and/or (v) putative BP in tick-pathogen and tick-host interactions. **(B)** The GO for BP of selected candidate protective antigens was done with Blast2GO software (version 3.0; http://www.blast2go.com).

and *I. ricinus*, and may be protective in vaccine preparations against both tick vector species.

Experiments were then conducted to characterize the effect of rabbit IgG antibodies against ISCW005600 and AAY66632 antigens in heterologous *I. ricinus* IRE/CTVM20 cells as described before in the homologous *I. scapularis* ISE6 cells (**Figures 5D,E**). As in ISE6 tick cells, the results showed that incubation of IRE/CTVM20 tick cells with IgG antibodies against ISCW005600 and AAY66632 antigens affected both cell apoptosis (**Figure 5D**) and pathogen infection (**Figure 5E**), supporting the putative effect of vaccination with these antigens in both tick vector species.

Functional analyses were conducted to gain additional insight into the possible protective mechanisms for these antigens. The expression of ISCW005600 and AAY66632 was determined by RT-PCR and did not change in response to *A. phagocytophilum* infection of ISE6 tick cells (**Figure 6A**), a result that agreed with previous results of transcriptomics analysis (Villar et al., 2015a; **Figure 6B**). The IFA in uninfected and *A. phagocytophilum*-infected *I. scapularis* females showed that as expected, a negative and positive staining was obtained with pre-immune and anti-ISE6 IgG in infected ticks, respectively (**Figures 6Ca–d**). The ISCW017271 antigen, which protein levels were highly under-represented in response to infection in both midguts and salivary glands (**Figure 3A**), was used to validate proteomics results. The IFA using anti-ISCW017271 IgG antibodies showed a positive staining in uninfected (**Figure 6Ce**) but not infected cells (**Figure 6Cf**), thus corroborating the proteomics results.

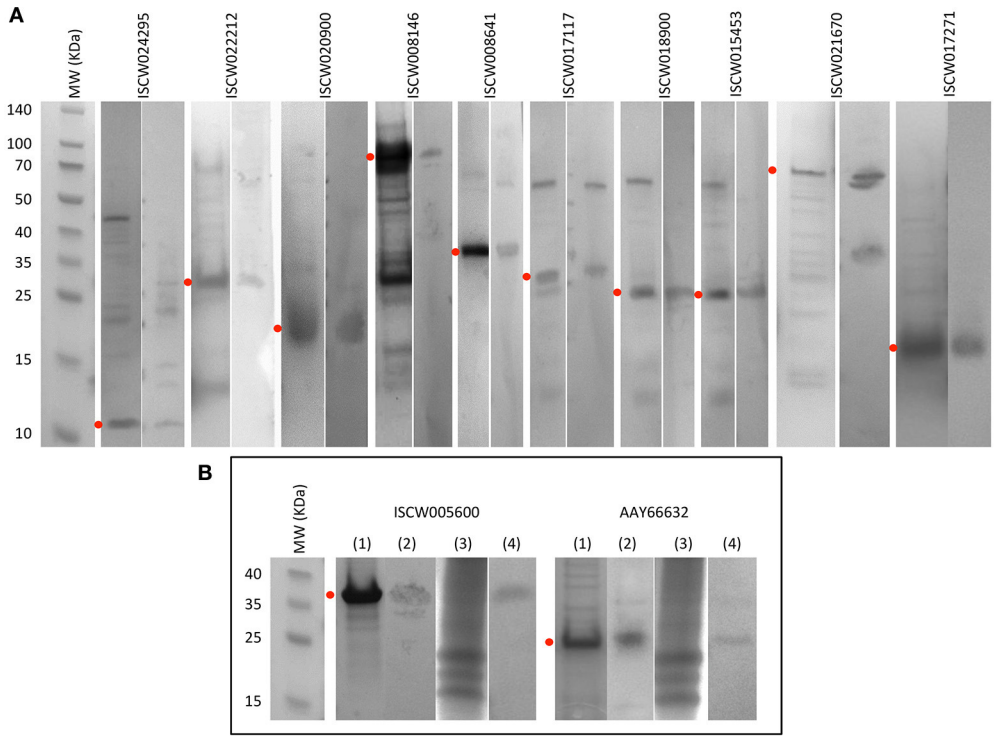

FIGURE 4 | Production of recombinant proteins and rabbit IgG antibodies. **(A)** SDS-PAGE (left panel) and Western blot analysis (right panel) of selected candidate tick protective recombinant antigens. **(B)** Antigens selected for further characterization. (1) SDS-PAGE of recombinant antigens. (2) Western blot analysis of recombinant antigens. (3) SDS-PAGE of total proteins from ISE6 tick cells. (4) Western blot analysis of total proteins from ISE6 tick cells. Western blots were performed with IgG antibodies from rabbits immunized with recombinant antigens. Red dots denote the position of the recombinant antigen. Other protein bands in some of the samples correspond to *E. coli* contamination proteins, and aggregation or degradation products of the recombinant antigens.

For the selected candidate tick protective antigens, the IFA with anti-ISCW005600 IgG did not produce any positive staining (**Figures 6Cg–h**), in accordance with proteomics results (**Figure 3A**). However, for the AAY66632 antigen, a positive staining was obtained in salivary glands from infected ticks after IFA with anti-AAY66632 antibodies (**Figures 6Ci,j**). The positive staining in infected (**Figure 6Cl**) but not uninfected (**Figure 6Ck**) ticks formed a membrane-like structure in salivary glands (arrows in **Figure 6Cl**), and also corroborated the proteomics results for this antigen (**Figure 3A**).

Gene knockdown by RNAi in ISE6 tick cells resulted in significantly lower *A. phagocytophilum* infection levels for both antigens when compared to control cells using the unrelated Rs86 dsRNA (**Figure 6D**). These results suggested that although ISCW005600 and AAY66632 mRNA levels did not change in response to infection of ISE6 tick cells, which constitute a model for tick hemocytes involved in pathogen infection and immune response (Villar et al., 2015a; Alberdi et al., 2016), they may play a role in *A. phagocytophilum* infection.

These results encouraged a final experiment to evaluate the potential effect of ISCW005600 and AAY66632 as vaccination antigens to reduce tick infestations and reproduction. An artificial tick feeding system using silicone membranes was used in this experiment (Kröber and Guerin, 2007; Krull et al., 2017). Although the development of standardized *in vitro* feeding methods for ixodid ticks has been hampered by their complex feeding behavior and the long duration of their blood meal, recent developments provide a valuable tool for the study of tick physiology, tick-host-pathogen interactions and the discovery of drugs and other control interventions without the use of experimental animals (Kröber and Guerin, 2007; Bonnet and Liu, 2012; Sojka et al., 2015; Tajeri et al., 2016; Krull et al., 2017; Trentelman et al., 2017). *I. ricinus* ticks were selected for artificial feeding and the results shown here supported an effect of antibodies against *I. scapularis* antigens (**Figure 4B**) on *I. ricinus* ticks (**Figures 5D,E**).

On the artificial feeding device, the number of attached ticks was similar between groups, but the number of dead ticks increased after feeding on anti-antigen IgG and was significantly higher in ticks fed on anti-AAY66632 antibodies when compared to control ticks fed on pre-immune IgG (**Figure 7**). Significant differences were not observed between groups in tick weight, number of ticks with oviposition and egg weight, but a tendency in the reduction in the number of ticks with oviposition was also observed in ticks fed on anti-AAY66632 IgG (**Figure 7**). Although the number of ticks used for artificial feeding was limited due to experimental conditions, the results suggested an effect of anti-ISCW005600 and anti-AYY66632 antibodies on tick

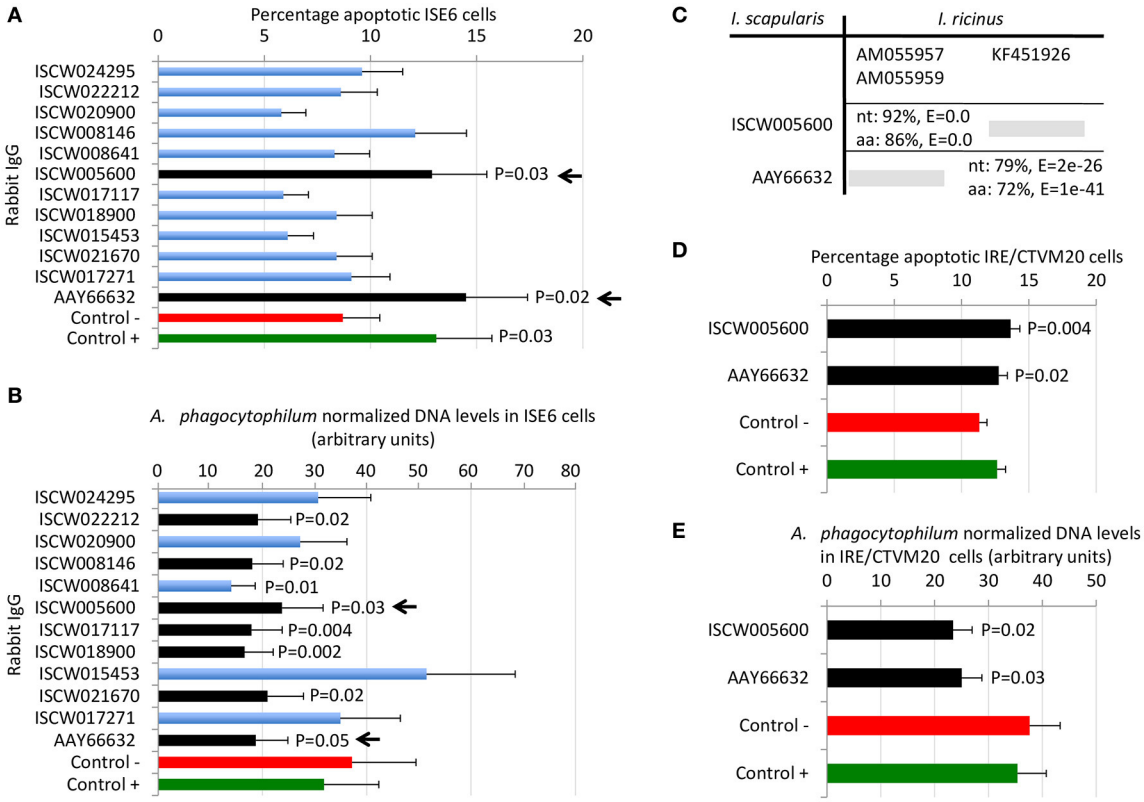

FIGURE 5 | Selection of candidate tick protective antigens. Purified rabbit IgG against recombinant candidate tick protective antigens were incubated with *I. scapularis* ISE6 and *I. ricinus* IRE/CTVM20 tick cells before infection with *A. phagocytophilum*. **(A)** Characterization of the effect of rabbit IgG antibodies on ISE6 tick cells apoptosis. **(B)** Characterization of the effect of rabbit IgG antibodies on pathogen infection of ISE6 tick cells. **(C)** Sequence identity between *I. scapularis* and *I. ricinus* homologs. The accession numbers are shown together with corresponding percent identity for nucleotide (nt) and amino acid (aa) sequences and *E*-values. The selected *I. scapularis* sequences were blasted against the *I. ricinus* database using the Blastp tool from BLAST, and the sequences with the lowest *E*-value were selected. **(D)** Characterization of the effect of rabbit IgG antibodies on IRE/CTVM20 tick cells apoptosis. **(E)** Characterization of the effect of rabbit IgG antibodies on pathogen infection of IRE/CTVM20 tick cells. The percentage of apoptotic cells was determined by flow cytometry after Annexin V-FITC and PI labeling. *A. phagocytophilum* DNA levels were determined by *msp4* real-time PCR normalizing against tick *rpS4*. Control cells were incubated with rabbit pre-immune IgG (negative control, Control -) or rabbit anti-ISE6 IgG (positive control, Control +). Results were presented as average + S.D. normalized Ct-values and compared between each treatment and negative control by Student's *t*-test with unequal variance ($P \leq 0.05$; $N = 4$). The selected candidate protective antigens are shown with arrows.

mortality and a reduction in the number of ticks with oviposition for anti-AYY66632 antibodies.

These results suggested that the selected candidate tick protective antigens might constitute effective vaccine antigens to control tick vector infestations and prevent or control pathogen infection, and therefore could be selected for future vaccination trials.

Putative Mechanisms of Protection for Vaccines Based on Selected Candidate Tick Protective Antigens

After the successful completion of the main objective of this study, which was the identification of tick candidate tick protective antigens for the control of vector infestations and *A. phagocytophilum* infection, a question arose about the putative protective mechanisms of the selected candidate protective antigens. The answer to this question may assist in the selection of additional candidate protective antigens following the vaccinomics pipeline (**Figure 1**), and the evaluation of possible combinations of antigens with different functions to enhance vaccine efficacy (de la Fuente and Merino, 2013).

Both selected candidate tick protective antigens were grouped into the evasion of host defense response BP (**Figure 3B**). The ISCW005600 secreted histamine binding protein appears to be a salivary lipocalin (Beaufays et al., 2008; Schwarz et al., 2013). Lipocalins are a family of salivary gland secreted proteins that play a role in evasion of host immune and inflammatory responses by competing for histamine or serotonin binding (Paesen et al., 2000; Mans, 2005; Beaufays et al., 2008; Valdés, 2014; Valdés et al., 2016). Therefore, these proteins play an important role during tick feeding. The genes encoding for these proteins are up-regulated during tick feeding (Kim et al., 2016; Valdés et al., 2016; Ribeiro et al., 2017) and pathogen infection (Ayllón et al., 2015; Valdés et al., 2016). Additionally, lipocalins were also produced in tick midguts and up-regulated

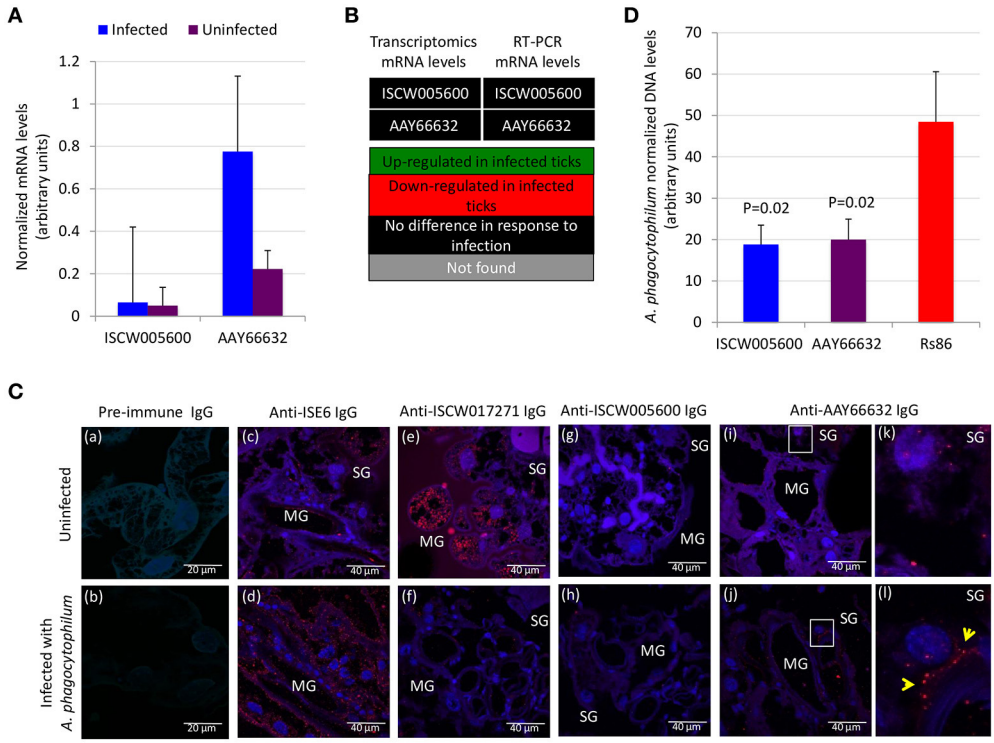

FIGURE 6 | Functional characterization of selected candidate tick protective antigens. **(A)** Results of the RT-PCR analysis of the expression of ISCW005600 and AAY66632 genes in uninfected and *A. phagocytophilum*-infected ISE6 tick cells. Results were presented as average + S.D. normalized Ct-values and compared between infected and uninfected cells by Student's *t*-test with unequal variance ($P \leq 0.05$; $N = 4$). **(B)** Comparison of the transcriptomics and RT-PCR results for mRNA levels of ISCW005600 and AAY66632 genes in ISE6 tick cells in response to *A. phagocytophilum* infection. Transcriptomics results were obtained from Villar et al. (2015a). **(C)** Representative images of imunofluorescence analysis of uninfected **(a,c,e,g,i,k)** and *A. phagocytophilum*-infected **(b,d,f,h,j,l)** adult female *I. scapularis* midguts (MG) and salivary glands (SG). Tick tissues were stained with rabbit pre-immune control IgG **(a,b)**, anti-ISE6 tick cells IgG **(c,d)**, or anti-tick antigens IgG **(e–l)** labeled with RFP (red) and DAPI (blue). Yellow arrows illustrate a positive staining for AAY66632 in the SG sections in white squares in infected **(l)** but not uninfected **(k)** ticks. **(D)** The *A. phagocytophilum* DNA levels were determined after RNAi in infected ISE6 tick cells treated with ISCW005600 and AAY66632 dsRNAs or control *Rs86* dsRNA. *A. phagocytophilum* DNA levels were determined by *msp4* real-time PCR normalizing against tick *rpS4*. Results are shown as average + S.D. normalized Ct-values and compared between treated and control groups by Student's *t*-test with unequal variance ($P < 0.05$; $N = 5$ biological replicates).

in response to *A. phagocytophilum* infection (Ayllón et al., 2015; **Figure 3A**), suggesting as reported in other organisms (Cassidy and Martineau, 2014; Abella et al., 2015) a role for these proteins in tick innate immune response to infection. Therefore, lipocalins may have a dual role in tick-pathogen interactions. These proteins may facilitate pathogen transmission by reducing host inflammatory responses (Valdés et al., 2016), but control tick infection by depleting strategic compounds for pathogens (Ferreira et al., 2015). In humans, lipocalins have also been shown to regulate apoptosis by inducing or inhibiting this process under different physiological conditions (Chakraborty et al., 2012; Abella et al., 2015). Based on the results obtained here with anti-ISCW005600 antibodies and RNAi (**Figures 5A,B,D,E**, **6D**, **7**), ISCW005600 may function to inhibit tick cell apoptosis and facilitate *A. phagocytophilum* infection with a possible role during tick feeding (**Figure 7**). Therefore, the proposed protective mechanisms for vaccines containing this antigen may include reduction of tick infestations by increasing cell apoptosis and reducing protective capacity to host response while reducing pathogen infection and transmission. Tick lipocalins have been proposed before as vaccine antigens for the control of tick infestations (de Castro et al., 2016; Manzano-Román et al., 2016), but only low partial protection have been reported in soft ticks, *Ornithodoros moubata* fed on immunized rabbits (Manzano-Román et al., 2016).

The AAY66632 antigen is a secreted lectin pathway inhibitor (Ribeiro et al., 2006; Schuijt et al., 2011), which is involved in the inhibition of the innate immune response complement lectin pathway (CLP). The CLP is involved in host response to infection with different pathogens (Evans-Osses et al., 2013). The CLP is activated when mannan-binding lectins or ficolins bind to patterns of carbohydrates or acetyl groups on the surface of protozoan, virus, fungi, or bacteria (Runza et al., 2008; Héja et al., 2012; Evans-Osses et al., 2013). In ticks, the inhibition of the complement system during and after blood feeding is critical for tick feeding success and development by minimizing damage to the intestinal epithelium as well as avoiding inflammation and opsonization of salivary molecules at the bite site (Wikel and Allen, 1977; Franco et al., 2016). Therefore, complement inhibitors are present in both tick saliva and midgut (Barros et al.,

Number of attached ticks	19	17	17
Number of dead ticks	4	6*	0
Number of fed ticks	15	11	17
Tick weight (mg) (average ± S.D.)	193±39	134±81	177±44
Number of ticks with oviposition	13	5	10
Egg weight (mg) (average ± S.D.)	39±25	48±29	24±18

FIGURE 7 | Artificial tick feeding. Artificial tick feeding was conducted as previously described (Krull et al., 2017) with 17–19 female and 3 male *I. ricinus* ticks per feeding unit. Environmental conditions were set at 20°C, 80% relative humidity, 5% CO_2 and 15 h light/9 h dark. Feeding units containing partially engorged ticks were placed on a six well plate and ticks were fed for 36 h with blood supplemented with 1 mg/ml of pre-immune or antigen-specific purified IgG. Dead and detached engorged ticks were removed, and engorged females that detached were weighed and stored individually in 2 ml Eppendorf tubes with pierced lids, which were kept in desiccators with approximately 90% relative humidity at RT. Ticks were assessed for egg mass 8 weeks post-feeding. The number of dead/fed ticks and thus with oviposition were compared between groups by a Fisher's exact test (*$P = 0.02$).

2009; Mendes-Sousa et al., 2013; Ayllón et al., 2015) (**Figure 3A**). The presence and activity of salivary anti-complement molecules has been well characterized in *Ixodes* spp. ticks including the *A. phagocytophilum* vectors, *I. scapularis* (Valenzuela et al., 2000; Tyson et al., 2007, 2008, Schuijt et al., 2011) and *I. ricinus* (Lawrie et al., 1999, 2005, Daix et al., 2007; Couvreur et al., 2008). Moreover, tick lectin pathway inhibitors have been shown to facilitate *Borrelia burgdorferi* pathogen infection and transmission (Schuijt et al., 2011; Wagemakers et al., 2016). Our results supported a role for AAY66632 in tick feeding success (**Figure 7**), the inhibition of tick cell apoptosis (**Figures 5A,D**) and facilitation of *A. phagocytophilum* infection (**Figures 5B,E**, **6D**). Therefore, the proposed protective mechanisms for vaccines based on this antigen may include reduction of tick infestations by affecting tick attachment and/or feeding, while reducing pathogen infection and transmission. The protective capacity of vaccines containing this antigen has not been reported.

CONCLUSIONS

The main objective of this study was to apply a vaccinomics approach to the identification and characterization of candidate tick protective antigens for the control of vector infestations and *A. phagocytophilum* infection. The vaccinomics pipeline developed in this study was applied to tick-*A. phagocytophilum* interactions and resulted in the identification of two candidate tick protective antigens that could be selected for future vaccination trials. The results showed that *I. scapularis* ISCW005600 and AAY66632 and *I. ricinus* homologs constitute candidate protective antigens for the control of vector infestations and *A. phagocytophilum* infection. Both lipocalin (ISCW005600) and lectin pathway inhibitor (AAY66632) are involved in the tick evasion of host defense response and pathogen infection and transmission, but targeting different immune response pathways. Therefore, based on the putative function of these antigens, vaccine protective mechanisms were proposed that supported antigen combination to improve vaccine efficacy. The vaccinomics pipeline proposed here could be used to continue the identification and characterization of candidate tick protective antigens for the development of effective vaccines for the prevention and control of HGA, TBF, and other forms of anaplasmosis caused by *A. phagocytophilum*.

AUTHOR CONTRIBUTIONS

JD conceived the study. MC, PA, IF, MV, CK, and AN performed the experiments. MC, PA, MV, CK, AN, and JD performed data analyses. JD, MC, and PA wrote the paper, and

other coauthors made additional suggestions and approved the manuscript.

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OPINION
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Tick Bioactive Molecules as Novel Therapeutics: Beyond Vaccine Targets

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Tick-pathogen-host interactions have been closely studied to understand the molecular mechanisms of pathogen transmission for tick-borne diseases, including Lyme disease, babesiosis, spotted fever diseases, and Tick-borne encephalitis, among others. Such studies have yielded insights into disease processes and have identified promising candidates for vaccines against tick-borne diseases (Dai et al., 2009; Schuijt et al., 2011; de la Fuente et al., 2016). In addition to these vaccine targets, the advent of "omics" technologies, such as transcriptomics and proteomics, has opened the doors for discovery of a wide variety of tick bioactive molecules (Francischetti et al., 2005, 2008, 2011; Untalan et al., 2005; Aljamali et al., 2009; Kongsuwan et al., 2010; Karim et al., 2011; Diaz-Martin et al., 2013; Oliveira et al., 2013; Egekwu et al., 2014; Radulovic et al., 2014; Tirloni et al., 2014; Karim and Ribeiro, 2015; Oleaga et al., 2015; Bullard et al., 2016; Kim et al., 2016; Moreira et al., 2017). While some of these bioactive molecules may be applicable for the treatment of tick-borne diseases, many are promising candidates for the treatment of other pathogens or human diseases. Therefore, we propose that careful study of tick bioactive molecules, such as those discovered in "omics" studies, is a promising rich source of novel therapeutics.

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TICK-PATHOGEN INTERACTIONS

Tick-borne pathogens have a complex lifecycle that involves both a tick and vertebrate host. Within the natural cycle, uninfected ticks acquire pathogens when taking a blood-meal on an infected host. The microbes enter with the blood into the tick's gut. At this point, some pathogens, such as *Anaplasma phagocytophilum* (the causative agent of human granulocytic anaplasmosis), migrate to the salivary glands (Hodzic et al., 1998). Others, such as *Borrelia burgdorferi* (the etiologic agent of Lyme disease) remain in gut (De Silva and Fikrig, 1995). The pathogens are then maintained within the tick organs during molting (De Silva and Fikrig, 1995; Hodzic et al., 1998). Upon the next blood meal, the infectious microbes exit into a vertebrate host with the tick saliva, which is made in the salivary glands (De Silva and Fikrig, 1995; Hodzic et al., 1998). Therefore, microorganisms that remain in the gut through molting must migrate to the salivary glands during the next blood meal.

The complex processes of acquisition and transmission of tick-borne pathogens require specific interactions between the tick, microbe, and host. Indeed, disruption of some tick-pathogen interactions has been shown to decrease transmission (Ramamoorthi et al., 2005; Dai et al., 2009; Zhang et al., 2011; Narasimhan et al., 2014; Coumou et al., 2016). Likewise, vaccination against some tick saliva or salivary gland proteins decreases the ability of the tick to feed on a mammalian host (Gomes et al., 2015; Contreras and de la Fuente, 2016, 2017), which could reduce transmission of pathogens. Therefore, tick proteins that interact with pathogens or facilitate tick feeding have been studied as potential vaccine targets for tick-borne diseases. However, many of these proteins perform biological functions that could also be exploited for therapeutic development.

TICK BIOACTIVE MOLECULES

Perhaps the best-studied source of tick bioactive molecules is tick saliva. Tick saliva includes a cocktail of potent proteins that aid in the feeding of the tick on a mammalian host and improve pathogen transmission from a tick to a mammalian host. These proteins are known to act as anticoagulants, immunosuppressants and immunomodulators, platelet inhibitors, vasodilators, inhibitors of wound healing, and facilitators of tick attachment (Reviewed in Kazimírová and Štibrániová, 2013). Many of these functions have potential uses in the treatment of disease.

For example, coagulation is an important process in many cancers, as it supports tumor growth, angiogenesis, and metastasis (Rickles et al., 2001). Additionally, cancer patients often have complications related to coagulation, such as venous thromboembolisms (Karakatsanis et al., 2016). Treatment of some cancers and cancer complications with anticoagulants has been shown to be effective (Rickles et al., 2001; Karakatsanis et al., 2016). Tick saliva is a rich source of novel anticoagulants that could be exploited for the development of anticoagulants for the treatment of diverse cancers. Indeed, Ixolaris and Amblyomin-X, anticoagulant and antiangionenic proteins from *Amblyomma cajennense*, have shown promising results for the treatment of glioblastoma (Carneiro-Lobo et al., 2009; Barboza et al., 2015), renal cell carcinoma (de Souza et al., 2016), and melanoma (Chudzinski-Tavassi et al., 2010; de Oliveira Ada et al., 2012) in mice. Additionally, complement inhibitors may be useful for disorders of inappropriate complement activation (Baines and Brodsky, 2017) or diseases exacerbated by the complement system, such as cardiovascular disease (Shields et al., 2017). Indeed, *Ornithodoros moubata* Complement Inhibitor (OmCI) has shown promising results in an *in vitro* model of the complement disease paroxysmal nocturnal hemoglobinuria (Kuhn et al., 2016) and a porcine model of myocardial infarction (Pischke et al., 2017). Additional uses for salivary gland proteins include treatment of microbial infections (Cabezas-Cruz et al., 2016; Abraham et al., 2017), autoimmune disease (Sá-Nunes et al., 2009; Soltys et al., 2009), and cardiovascular diseases (Abendschein et al., 2001).

Recently, tick—tick microbiome—pathogen interactions have begun to be studied to understand the implications of the tick microbiome in pathogen transmission. Indeed, perturbing the *Ixodes scapularis* tick microbiome decreases transmission of *B. burgdorferi* (Narasimhan et al., 2014) and increases transmission of *A. phagocytophilum* (Abraham et al., 2017). Study of such interactions can lead to the discovery of novel mechanisms of interaction and potential therapeutics. For example, further work into *A. phagocytophilum* - microbiota interactions determined that *A. phagocytophilum* modulates the tick microbiome during colonization of *I. scapularis*, which facilitates its migration from the tick gut to the salivary glands (Abraham et al., 2017). This occurs through the bacterium inducing expression of the tick gut protein *I. scapularis* antifreeze glycoprotein (IAFGP) (Neelakanta et al., 2010; Abraham et al., 2017), which decreases microbiota biofilms in the tick gut (Abraham et al., 2017). The antibiofilm activity of IAFGP makes it a promising candidate for the treatment of antimicrobial-resistant bacterial pathogens that form biofilms. Indeed, IAFGP expression in flies and mice increases their resistance to bacterial pathogens, such as *Staphylococcus aureus* (Heisig et al., 2014). Additionally, testing in a catheter model demonstrated that IAFGP coatings can inhibit bacterial biofilm formation on medical devices (Heisig et al., 2014). These studies on IAFGP function and potential highlight that other interactions within the tick, such as those between the ticks, pathogens, and microbiomes, are another rich source of bioactive molecules.

"OMICS" STUDIES FOR THE DISCOVERY OF BIOACTIVE MOLECULES

The advent of "omics" technologies, including transcriptomics, proteomics, and genomics, has opened the door for the discovery of new microbial consortium members, host-microbe interactions, and bioactive molecules. Such studies have led to the discovery of many new promising therapeutic candidates, such as animal venom peptides from mollusks (Verdes et al., 2016) and antibiotics from bacteria (Wecke and Mascher, 2011).

The use of proteomic and transcriptomic analyses has uncovered many novel tick-microbe interactions. Additionally, these studies have yielded a multitude of predicted tick bioactive molecules, such as anticoagulants, platelet aggregation inhibitors, vasodilators, antimicrobials, immunosuppressants, immunomodulators, and inhibitors of wound healing (**Table 1**; Francischetti et al., 2005, 2008, 2011; Untalan et al., 2005; Aljamali et al., 2009; Kongsuwan et al., 2010; Karim et al., 2011; Diaz-Martin et al., 2013; Oliveira et al., 2013; Egekwu et al., 2014; Radulovic et al., 2014; Tirloni et al., 2014; Karim and Ribeiro, 2015; Oleaga et al., 2015; Bullard et al., 2016; Kim et al., 2016; Moreira et al., 2017). These studies have also identified new classes of protein families as well as many proteins of unknown function (**Table 1**; Francischetti et al., 2005, 2008, 2011; Untalan et al., 2005; Aljamali et al., 2009; Kongsuwan et al., 2010; Karim et al., 2011; Diaz-Martin et al., 2013; Oliveira et al., 2013; Egekwu et al., 2014; Radulovic et al., 2014; Tirloni et al., 2014; Karim and Ribeiro, 2015; Oleaga et al., 2015; Bullard et al., 2016; Kim et al., 2016; Moreira et al., 2017). The vast majority of these bioactive proteins have not been studied in detail, and it is likely that many may be homologs or overlap in function. Therefore, the actual number of discovered bioactive proteins with divergent mechanisms of action is likely less than the total of these studies. However, these studies highlight that there is a vast array of potential bioactive molecules within tick-microbe interactions awaiting further study.

DEVELOPMENT OF BIOACTIVE MOLECULES INTO THERAPEUTICS

Although "omics" studies have identified a plethora of potential therapeutics, these studies have not led to FDA approval of any novel drugs. In fact, at the time of this publication, no arthropod compound identified by proteomics, transcriptomics, or genomics is in clinical trials in the United States. As mentioned

TABLE 1 | Proteomic and transcriptomic studies that have predicted novel tick proteins.

Tick Source[a]	Analysis[b]	Total identified[c]	Bioactive function[d]	Protease inhibitor[e]	Protease[f]	Unknown function[g]	Other predicted function[h]	Citation[i]
Amblyomma americanum cement cone	Proteomics	33	0	1	4	18	10	Bullard et al., 2016
Amblyomma americanum saliva	Transcriptomics and proteomics	895	7	23	18	517	330	Radulovic et al., 2014
Amblyomma americanum salivary glands	Transcriptomics and proteomics	5,792	81	98	37	2,608	2,968	Karim and Ribeiro, 2015
Amblyomma americanum salivary glands	Transcriptomics	2,002	14	13	2	1,674	299	Aljamali et al., 2009
Amblyomma maculatum salivary glands	Transcriptomics and proteomics	15,914	800	379	311	5,389	9,035	Karim et al., 2011
Amblyomma sculptum midguts, ovaries and salivary glands	Transcriptomics	27,308	285	79	132	2,312	24,500	Moreira et al., 2017
Hyalomma marginatum rufipes	Transcriptomics and proteomics	2,084	35	62	3	722	1,262	Francischetti et al., 2011
Ornithodoros coriaceus salivary glands	Transcriptomics and proteomics	726	60	6	13	127	520	Francischetti et al., 2008
Ornithodoros erraticus midgut	Proteomics	555	8	0	15	79	453	Oleaga et al., 2015
Ornithodoros moubata saliva	Proteomics	193	9	2	7	51	124	Diaz-Martin et al., 2013
Rhipicephalus (Boophilus) microplus midgut	Proteomics	142	0	0	3	8	131	Kongsuwan et al., 2010
Rhipicephalus (Boophilus) microplus saliva	Proteomics	187	57	29	4	60	35	Tirloni et al., 2014
Rhipicephalus (Boophilus) microplus whole ticks	Proteomics	20	0	0	0	12	8	Untalan et al., 2005
Rhipicephalus sanguineus saliva	Proteomics	19	2	0	0	4	13	Oliveira et al., 2013
Ixodes pacificus salivary glands	Transcriptomics	557	46	21	1	463	26	Francischetti et al., 2005
Ixodes scapularis saliva	Proteomics	582	33	43	33	112	361	Kim et al., 2016
Ixodes scapularis synganglion	Transcriptomics	41,249	140	0	0	12,660	28,449	Egekwu et al., 2014

[a] *Source of the tick sample including species name and organ.*
[b] *Type of analysis performed on the tick sample.*
[c] *Total number of proteins or transcripts identified by the study.*
[d] *Total number of predicted proteins that were classified by the study as having a potential bioactive activity, including anticoagulants, platelet aggregation inhibitors, vasodilators, antimicrobials, immunosuppressants, immunomodulators, and inhibitors of wound healing.*
[e] *Total number of predicted proteins that were classified by the study as potential protease inhibitors. Some protease inhibitors can have bioactive functions of interest, such an immuosuppressant activity.*
[f] *Total number of predicted proteins that were classified by the study as potential proteases, which can have bioactive functions of interest.*
[g] *Total number of predicted proteins that were classified by the study as having an unknown function.*
[h] *Total number of predicted proteins that were classified by the study as having other functions, such as cell junction, energy metabolism, and cytoskeletal functions.*
[i] *Citation for the study.*

above, this is partially due to lack of follow-up studies on the mechanisms, uses, and optimization of the drug candidates. However, this is likely also due to issues specific to arthropod compounds.

Arthropod compounds often have high cytotoxicity and/or are unstable (Ratcliffe et al., 2014). Therefore, the development of some compounds will require basic research into optimization of the compound, dosage, synthesis methods, and delivery

mechanism. For example, Cantharidin, a small molecule toxin from beetles in the Meloidae family, has potent anti-cancer activities and has been shown to be effective against a large variety of cancers (Reviewed in, Deng et al., 2013; Puerto Galvis et al., 2013). However, this compound also has significant toxicity in mammals related to its anticancer activity (Deng et al., 2013; Puerto Galvis et al., 2013; Ratcliffe et al., 2014). Extensive studies have been undertaken to reduce this toxicity through modification of the compound (Deng et al., 2013; Puerto Galvis et al., 2013), alternative production and delivery methods (Chang et al., 2008; Han et al., 2013; Yu and Zhao, 2016), or combination therapies (Wu et al., 2015). These efforts highlight that the resolution of issues, such as toxicity, will require the investment of time and money into basic scientific research for the development process.

Additionally, there are concerns with developing individual compounds from a complex mixture, such as tick saliva. Tick saliva contains a cocktail of potent proteins, and the production of these proteins changes throughout tick feeding (Kim et al., 2016). This suggests that saliva proteins may work synergistically within the context of tick feeding for differing functions or similar functions (e.g., various immunosuppressants could work in concert for greater immunosuppression) at specific time points. Additionally, it is possible that separately encoded proteins or subunits may be necessary for proper function. Therefore, studying individual genes or proteins may miss potential therapeutics. In these cases, it would be necessary to consider co-expression of proteins and/or identify interacting partners within the tick saliva to capture the optimal combinations.

It is worth noting that is some instances the lack of progress toward a viable therapeutic candidate is due to the high cost of drug development rather than a lack of follow-up research. For these compounds, investing in the approval process is not attractive for pharmaceutical companies (Shlaes et al., 2004; Kinter and DeGeorge, 2016). This is the case for many antimicrobials, such as arthropod-derived antimicrobial peptides that target bacterial and fungal pathogens (Ratcliffe et al., 2011).

CONCLUSIONS

Tick-derived bioactive molecules are a promising source of new therapeutics. However, the discovery and development of such compounds is in its infancy. Although some drug candidates have shown promising pre-clinical results, these compounds could fall into the so-called "Valley of Death," the gap between basic research and translation into treatments. For some therapeutics, this is due to the broad issues common to potential therapeutics: lack of funding for translational research and/or lack of viable pathways for clinical development (Butler, 2008; Collins et al., 2016). However, as discussed in this article, this can also be due to a lack of basic research assessing biological function, potential uses, or optimization of the compound. For tick bioactive compounds to be successfully developed into therapeutics, it will require the investment of basic researchers into the discovery and approval of therapeutic candidates.

AUTHOR CONTRIBUTIONS

KEM and EF contributed to the writing and editing of the manuscript.

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REVIEW
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Metazoan Parasite Vaccines: Present Status and Future Prospects

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Eukaryotic parasites and pathogens continue to cause some of the most detrimental and difficult to treat diseases (or disease states) in both humans and animals, while also continuously expanding into non-endemic countries. Combined with the ever growing number of reports on drug-resistance and the lack of effective treatment programs for many metazoan diseases, the impact that these organisms will have on quality of life remain a global challenge. Vaccination as an effective prophylactic treatment has been demonstrated for well over 200 years for bacterial and viral diseases. From the earliest variolation procedures to the cutting edge technologies employed today, many protective preparations have been successfully developed for use in both medical and veterinary applications. In spite of the successes of these applications in the discovery of subunit vaccines against prokaryotic pathogens, not many targets have been successfully developed into vaccines directed against metazoan parasites. With the current increase in -omics technologies and metadata for eukaryotic parasites, target discovery for vaccine development can be expedited. However, a good understanding of the host/vector/pathogen interface is needed to understand the underlying biological, biochemical and immunological components that will confer a protective response in the host animal. Therefore, systems biology is rapidly coming of age in the pursuit of effective parasite vaccines. Despite the difficulties, a number of approaches have been developed and applied to parasitic helminths and arthropods. This review will focus on key aspects of vaccine development that require attention in the battle against these metazoan parasites, as well as successes in the field of vaccine development for helminthiases and ectoparasites. Lastly, we propose future direction of applying successes in pursuit of next generation vaccines.

Keywords: parasites, vaccines, parasite control, antigen identification, systems biology, OMICS techniques, vaccine development

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INTRODUCTION

It is projected that the combined populations of Asia and Africa will constitute 9 billion of the 11 billion global population by 2100 (FAO, 2017). With ever increasing urbanization, there is an apparent concomitant shift from low agricultural labor productivity to higher labor productivity in services and manufacturing for many developing countries of sub-Saharan Africa, Asia and Latin America (Nations, 2015; Diao et al., 2017). At the same time, the global demand for animal and animal-derived products will increase with sustainable and efficient production practices becoming

paramount to ensure food security and food safety (Godfray et al., 2010; Herrero and Thornton, 2013; Allen and Prosperi, 2016; Little et al., 2016; OECD/FAO, 2016). This is, however, a challenge when sustainable growth in agricultural productivity is hindered by the inevitable degradation of natural resources (i.e., land and water), losses in biodiversity, as well as the spread of trans-boundary plant and animal pests and diseases (Garnett et al., 2013; Fry et al., 2016; Pekel et al., 2016; FAO, 2017; Watts et al., 2017). Introduction and/or resurgence of pests and diseases within endemic and non-endemic regions of the world will not only threaten public health and welfare directly, but also indirectly through veterinary health that affects animal-derived commodities needed for nutrition (improving health and growth) and generation of wealth (to relieve poverty) (De Magalhaes and Santaeulalia-Llopis, 2015; Cable et al., 2017). Moreover, as human and animal contact increases through urbanization the potential for transmission/contraction of debilitating or lethal zoonotic diseases is a serious global concern (Polley, 2005; Mackenstedt et al., 2015; Cable et al., 2017).

Pathogens (including some parasites) continue to cause some of the most detrimental and difficult to treat diseases (or disease states) in both humans and animals. Around 17 neglected tropical diseases (NTD) that affect more than a billion people globally have been identified by the World Health Organization (WHO) as priority strategic areas for development of effective control, elimination or eradication programs (WHO, 2012). Of these, 11 are caused by internal eukaryotic parasites or pathogens (http://www.who.int/neglected_diseases/en/)[1]. (Hotez et al., 2016). In addition, around 116 animal (terrestrial and aquatic) diseases, infections or infestations are currently listed as notifiable by the World Organization for Animal Health (OIE) (http://www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2017), of which some 23 involve eukaryotic parasites or pathogens of agro-veterinary importance (Stentiford et al., 2014). Many of the most important medical and veterinary diseases are vector-borne and/or transferred through animal reservoir hosts (Gubler, 2009; Torgerson, 2013; Wilson et al., 2017), which is exaggerated by poor socioeconomic stability and anthropogenic factors leading to the persistence and/or expansion of parasites into non-endemic areas and associated increases in the agro-developmental burden of developing and poverty stricken regions of the world (Torgerson, 2013; Nii-Trebi, 2017).

In the case of metazoan parasites, prophylactic prevention and treatment relies heavily on antiparasitic drugs and/or chemical control strategies (such as topical dips and sprays) to relieve parasite burden and vector-borne infection rates (Torgerson, 2013; Andrews et al., 2014). Unfortunately, abuse and misuse of such antiparasitics can often have unwanted side effects and prolonged persistence in treated hosts leading to contamination of animal-derived products and environmental resources (Boxall, 2004; Aktar et al., 2009). Though resistance to chemotherapeutics varies widely depending on the biological and ecological complexity of each metazoan parasite, there is enough indication of step-wise gains in resistance for several endo (e.g., helminths) and ectoparasites (e.g., arthropods such as ticks and other biting insects) of global importance (Benz et al., 1989; Jones et al., 1992; Rust and Dryden, 1997; Coles, 1998; Sibley and Hunt, 2003; Vercruysse et al., 2007; Abbas et al., 2014; Liu et al., 2014; Benelli, 2015; Geurden et al., 2015; McNair, 2015; Mougabure-Cueto and Picollo, 2015; Genta et al., 2016; Van Wieren et al., 2016). Moreover, whether existing compounds are used correctly, new drugs are developed and/or restricted use is implemented, eventual selection of resistant populations are inevitable (Molento, 2009). Therefore, complimentary strategies are needed to limit the selection pressures leading to resistance. To date, vaccines have been included successfully as part of integrated pest and disease management programs for both humans and animals representing a promising approach for the future control of endo- and ectoparasites (Lombard et al., 2007; Vercruysse et al., 2007; Mariner et al., 2012; Joice and Lipsitch, 2013; Bottazzi, 2015; Hussein et al., 2015).

This review aims to summarize and highlight some of the successful approaches in developing metazoan parasitic vaccines directed at both endo- and ectoparasites, and to garner lessons from these endeavors that can guide us in the development of the next generation of vaccines.

ENDOPARASITE VACCINES: HELMINTHIASES

The largest complement of disease causing parasites of medical and veterinary importance are endo-parasitic, with helminthiases being a major contributor (Hotez et al., 2016), causing a number of chronic and/or debilitating diseases that can impede normal physical and cognitive development, to which children especially are susceptible (Briggs et al., 2016; Hotez et al., 2016). Mass anti-helminthic drug administrations (MDA), such as praziquantel for schistosomiasis and albendazole or mebendazole for soil-transmitted helminthiases (STH), are the main public health interventions prescribed to control morbidity in endemic countries (WHO, 2012), but treatment is marred by the specter of acquired chemical resistance (Sutherland and Leathwick, 2011; Keenan et al., 2013; Geurden et al., 2015). Vaccination as part of an integrated veterinary and public health strategy has been proposed for helminth control and several vaccine candidates are currently in development and/or available for treating helminthic infections (Supplementary Table 1) (Matthews et al., 2016; Molehin et al., 2016; Tebeje et al., 2016). In the following sections selected current lead antigens used in medical and veterinary vaccines against helminthiases will be discussed.

Trematodes and Soil-Transmitted Helminths (STH)

Schistosomiasis

Schistosoma trematodes are freshwater snail-transmitted dimorphic parasitic flatworms that circulate in the bloodstream of mammalian hosts causing a debilitating intravascular disease

[1]Organization, W. H. Available: http://www.who.int/neglected_diseases/diseases/en/ [Accessed 2017].

known as schistosomiasis (or bilharzia) with *Schistosoma haematobium* and *S. mansoni* as the main etiological agents (Adenowo et al., 2015; Haçariz and Sayers, 2016; Molehin et al., 2016; Nii-Trebi, 2017). Currently, some 219 million people are exposed to schistosomiasis in 52 countries of which ∼90% of the estimated cases in 2003 occurred in Africa (Wikel, 1999; Steinmann et al., 2006; Adenowo et al., 2015). To date, a number of studies have been conducted and candidate proteins for novel vaccines and diagnostic tools identified (Fonseca et al., 2012; Ludolf et al., 2014; Van Der Ree and Mutapi, 2015; Haçariz and Sayers, 2016; Molehin et al., 2016; Hinz et al., 2017; Homann et al., 2017).

Proof-of-concept for vaccination against Schistosoma trematodes was established in mice and non-human primates immunized with native irradiated cercariae, conferring some 80% protection against schistosomula challenge (Hagan et al., 1995; Coulson, 1997; Coulson and Wilson, 1997). Attenuated preparations were deemed unfeasible as a human cercarial vaccine and subunit vaccine development was pursued (Molehin et al., 2016). Targets for current lead subunit vaccines were primarily identified using classical biochemical techniques from parasite protein fractions (e.g., *S. mansoni* 28 kDa glutathione S-transferase and 14 kDa fatty-acid-binding protein antigens) (Hagan et al., 1995). These targets are mostly excretory/secretory (ES) surface molecules from tegumental membranes of the migrating schistosomulum stages (affecting parasite invasion), as well as the adult females (affecting parasite survival and fecundity) (Hagan et al., 1995; Ricciardi and Ndao, 2015). Current advances in high-throughput technologies have expedited antigen identification for important schistosome species, but translation from basic research to clinical and field evaluations is still lacking. Only a few recombinant targets are currently in varying stages of clinical and commercial development (Merrifield et al., 2016; Molehin et al., 2016; Tebeje et al., 2016).

A common *S. haematobium* 28 kDa glutathione S-transferase (Sh28GST) protein, with a wide distribution in parenchymal tissues and tegumental structures of immature and adult worms (Balloul et al., 1985, 1987a; Porchet et al., 1994), is currently being evaluated in phase III clinical trials as Bilhvax for treating urinary schistisomiasis (Supplementary Table 1) (Riveau et al., 2012; Ricciardi and Ndao, 2015; Molehin et al., 2016). This antigen was originally cloned, crystallized and tested with promising results against *S. mansoni* (Balloul et al., 1987b; Trottein et al., 1992; Hagan et al., 1995). The precise biological role of this antigen is however not fully elucidated, but evidence for its involvement in parasite cellular detoxification and host immune regulation has been presented (Huang et al., 2012). Since GST proteins also play essential roles in parasite resistance to chemotherapy (Huang et al., 2012; Joice and Lipsitch, 2013), a combinatorial approach using a GST-directed vaccine and chemotherapies targeting parasite detoxification processes (e.g., Praziquantel or PZQ) could have excellent therapeutic potential (Huang et al., 2012). Unfortunately no further data is publically available for phase III clinical evaluations since 2009 and protection efficacy studies in human subjects remain elusive. This antigen has however been patented and applications for evaluation in separate phase II clinical trials against Crohn's inflammatory autoimmune disease is in progress (Supplementary Table 1).

For intestinal schistosomiasis, a recombinant *S. mansoni* 14 kDa monovalent fatty acid-binding protein (Sm14) (Moser et al., 1991; Tendler and Simpson, 2008; Coler et al., 2011), is currently in the final stages of phase II field trial testing in adult male subjects from endemic regions in Africa and Brazil (Supplementary Table 1) (Ricciardi and Ndao, 2015; Tendler et al., 2015; Santini-Oliveira et al., 2016). This antigen also shows promise as a dual-purpose helminthic vaccine against veterinary fasciolosis by conferring complete protection in mice challenged with *Fasciola hepatica* (67% for *S. mansoni*) (Tendler et al., 1996) and vice versa with a similar native protein isolated from *Fasciola hepatica* (nFh12) that cross-protected mice against *S. mansoni* infection (>80% efficacy) (Vicente et al., 2016). This study is currently active and promising results could eventually lead to the first human antihelmintic vaccine.

An additional *S. mansoni* 9 kDa tetraspanin surface protein (Sm-TSP-2) is also showing promise in phase I human clinical trials (Supplementary Table 1) (Tran et al., 2006; Pearson et al., 2012). Previous pre-clinical immunization studies in mice indicated a reduction in adult parasite and liver egg burdens of 57 and 64%, respectively, following challenge with *S. mansoni* (Tran et al., 2006). A similar tetraspanin loop protein (TSP-LEL) has also been successfully evaluated in mice and non-human primates (∼95% and >88% protection, respectively) as a multivalent fusion protein (rBmHAT vaccine) as a treatment against human lymphatic filariasis caused by the nematode *Brugia malayi* (Dakshinamoorthy et al., 2013, 2014; Chauhan et al., 2017a). A membrane surface calpain (Sm-p80) involved in parasite membrane renewal, is also showing promise as a lead candidate (Siddiqui et al., 1993; Ahmad et al., 2009) with proof-of-concept studies completed in animal models (including non-human primates) showing significant cross-species protection against *S. haematobium* and *S. japonicum* (Zhang et al., 2001; Karmakar et al., 2014a,b; Molehin et al., 2016). A recombinant Sm-p80/GLA-SE vaccine is currently trademarked as SchistoShield®, and is going through process development, formulation, stability and potency testing for final international patenting and phase I/II human clinical trials (Supplementary Table 1) (Molehin et al., 2016).

For the zoonotic parasite, *S. japonicum*, mostly transmission blocking vaccines are being pursued to limit parasite transmission and persistence in reservoir animal hosts consequently lowering infection rates in humans (Molehin et al., 2016; Tebeje et al., 2016). A number of candidate antigens (Sj97, Sj23 and SjTPI) shown promise for treatment of *S. japonicum* in domesticated animals (Ohta et al., 2004; Zhu et al., 2004, 2006; Dai et al., 2014, 2015; Jiz et al., 2015; Molehin et al., 2016). However, extensive field evaluations are needed for a commercial vaccine to be developed.

Soil-Transmitted Helminths (STH): Hookworm Disease

Soil-transmitted helminths (STH) are a group of medically important endoparasites that include: roundworms (e.g., *Ascaris lumbricoides*); whipworms (e.g., *Trichuris trichiura*) and

hookworms (*Necator americanus* and *Ancylostoma duodenale*) (WHO, 2016). Currently, approximately two-thirds of the global human disease burden from soil-transmitted nematode infections can be ascribed to hookworm infections and as such, much effort has been expended in the pursuit of a vaccine (Murray et al., 2013). Human hookworm disease, with *N. americanus* and *A. duodenale* as principal disease causing parasites, is an affliction causing severe anemia affecting some 440 million people globally in Asia, sub-Saharan Africa, the Caribbean and Latin America (Hotez et al., 2013; Pullan et al., 2014). Hookworm vaccine development was initially evaluated in the veterinary field by Miller et al. using a native preparation derived from whole irradiated *A. caninum* L3 larvae to immunize 3–4 month old dogs (Miller, 1964). This seminal work indicated a 37% overall vaccine protection with a 91% decrease in fecal egg output that was further optimized to confer more than 80% protection depending on the route of administration (Miller, 1964, 1965) resulting in a commercialized vaccine in the United States in 1973 for canines. But this vaccine was discontinued 2 years later due to various limitations recorded at the time that included cost, storage and stability, as well as the lack of sterilizing immunity (Miller, 1978; Schneider et al., 2011).

For further subunit vaccine development, the infective larval L3 stage was explored, since many stages-specific targets are produced that are essential for host invasion, modulation of host immunity and parasite establishment. The latter includes all of the released excretory-secretory (or ES) products (Hawdon et al., 1996; Bethony et al., 2008; Bottazzi, 2015), of which a tissue invasion-related metalloprotease (Zhan et al., 2002; Williamson et al., 2006) and two Ancylostoma secreted proteins (ASP-1 and ASP-2) were identified from the canine hookworm *A. caninum* (Hawdon et al., 1996, 1999). ASPs are cysteine-rich proteins of unknown function that could be linked to pathogenesis via screening of hyper immune serum from humans living in endemic countries (Brazil and China) detected ASP-2 (Bethony et al., 2005). The ASP-2 antigen was considered a lead candidate for human hookworm vaccine development and promising results were obtained in both animal models and preliminary human clinical vaccination trials (Bethony et al., 2005, 2008; Mendez et al., 2005). However, further human clinical trials using recombinant *N. americanus* ASP-2 (*Na*-ASP-2) antigen were halted in 2008 due to adverse reactions (generalized urticaria) observed in a Brazillian cohort of chronically infected subjects (Schneider et al., 2011; Diemert et al., 2012; Bottazzi, 2015).

Severe immediate-type allergic reactions observed in previously exposed individuals caused investigators to focus more on excreted/secreted adult gut antigens (Hotez et al., 2013). Two *N. americanus* antigens, the aspartic protease haemoglobinase APR-1 and GST-1, shown promise in animal studies (Plieskatt et al., 2012; Hotez et al., 2013; Curti et al., 2014; Bottazzi, 2015).

Other Veterinary Helminthiases

Gastrointestinal Nematodes (GINs)

Both *Ostertagia ostertagi* and *Cooperia oncophora* are the most prevalent gastrointestinal nematodes (GINs) of grazing cattle that often occur as co-infections together and/or with other GINs (Matthews et al., 2016). For *O. ostertagi*, several native vaccine preparations based on excretory/secretory or membrane targets have been shown to reduce worm fecundity by ±50% (Geldhof et al., 2002, 2004; Vercauteren et al., 2004; Claerebout et al., 2005; Meyvis et al., 2007; Geldhof and Knox, 2008; Halliday and Smith, 2010). Activation-associated secreted proteins (ASPs) of unknown function are currently evaluated against both *O. ostertagi* and *C. oncophora* (Geldhof et al., 2003, 2004; Borloo et al., 2013; Vlaminck et al., 2015). Homologous challenge with a double-domain ASP (dd-ASP) protein from *C. oncophora* was shown to reduced cumulative fecal egg counts by 91%, while field trials achieved a 58.8% reduction overall (Vlaminck et al., 2015). Despite promising results for dd-ASP, ASP1 and the polyprotein allergen OPA, a commercial vaccine targeting GINs is hindered by limitations in antigen production and formulation to deliver reproducible results (e.g., ASP1) (Vercauteren et al., 2004; Matthews et al., 2016).

In small ruminants such as sheep, the nematode *Teladorsagia circumcincta* is considered a dominant intestinal parasite within the temperate regions of the world, causing parasitic gastroenteritis (PGE) that has a severe impact on productivity by reducing live weight gain (Nisbet et al., 2016). A multivalent recombinant subunit vaccine was developed by Nisbet and co-workers (Nisbet et al., 2013), consisting of: 4 antigens (an ASP-1, cathepsin F, astacin-like metalloproteinase and an uncharacterized 20 kDa protein) identified from larval excreted/secreted protein extracts; a *T. circumcincta* homolog of an *A. caninum* protective antigen Tc-SAA-1 and 3 larval immunosuppressive molecules (macrophage migration inhibitory factor-1, calcium-dependent apyrase-1 and a TGF beta homolog Tci-TGH-2) (Redmond et al., 2006; Nisbet et al., 2009, 2010a,b, 2011; Smith et al., 2009). Trials performed on lambs showed a reduction in parasite egg production (70 and 58%), reductions in peak egg shedding (92 and 73%) and total worm burdens (75 and 56%) among vaccinated cohorts in two separate trials (Nisbet et al., 2013). Later trials indicated a 45% reduction of egg outputs from vaccinated pregnant ewes during periparturient relaxation in immunity (Nisbet et al., 2016). This vaccine now requires further field evaluations and refinement of components to simplify protein production and enable cost-effective up-scaling for commercial application (Matthews et al., 2016).

The barber's pole worms, *Haemonchus contortus* and *H. placei*, are pathogenic blood-feeding parasites that attach to the abomasum of ruminants (sheep, goats and cattle) causing production losses due to malaise and mortality of susceptible young animals (Matthews et al., 2016). Several gut membrane antigens have been tested in vaccination trials against *H. contortus*, namely: contortin (Hc-PCP1 and Hc-PCP2) (Munn et al., 1987; Geldhof and Knox, 2008); an unknown 100 kDa gut surface antigen (Jasmer et al., 1993, 1996); a microsomal aminopeptidase H11 (Smith et al., 1993), as well as a Haemonchus galactose-containing membrane glycoprotein complex (H-gal-GP) (Smith et al., 1994; Smith and Smith, 1996; Knox et al., 1999). To date, Barbervax® (WormVax, Australia) that contains purified native H-gal-GP and H11 from the guts of

adult *H. contortus* is available (Supplementary Table 1) (Bassetto et al., 2014) is available. Unfortunately, subunit vaccines based on H11 and H-gal-GP proteins have been largely unsuccessful in conferring protection, even when producing antigens in *Caenorhabditis elegans* (Cachat et al., 2010; Roberts et al., 2013; Matthews et al., 2016).

Cysteine protease-enriched protein fractions (CPFs) from adult *H. contortus* have been tested in both sheep and goats, with a cathepsin B cysteine proteinase (AC-5) showing promise in immunized lambs (up to 50% reduction in burden and fecundity) (Bakker et al., 2004; Ruiz et al., 2004; De Vries et al., 2009; Molina et al., 2012). Additionally, an adult somatic protein, Hc23 (Domínguez-Toraño et al., 2000; Alunda et al., 2003), is also showing promise achieving an overall reduction of more than 80% in fecal egg counts and parasite burdens in vaccinated lambs (Fawzi et al., 2015). These latter antigens are currently being developed further for a next generation subunit vaccine against *H. contortus*.

Of note is that two other native veterinary vaccines have been commercialized that consist of radiation attenuated L3 larvae for protection against lung worm or lung verminosis infections. These are Bovilis Huskvac® (MSD Animal Health, Ireland) and Difil (Nuclear Research Laboratory of the Indian Veterinary Research Institute, India) designed against *Dictyocaulus viviparous* (bovine lung worm) and *D. filaria*, respectively (Supplementary Table 1) (Sharma et al., 2015).

Cestoid Parasites

Zoonotic alveolar (AE) and cystic echinococcosis (CE) are recognized neglected diseases caused by *Echinococcus* spp. with a current global burden of some 188,000 (CE) and 18,200 (AE) new human cases per annum (Torgerson et al., 2010, 2015). However, due to the high costs associated with human vaccine development and the relatively low transmission rates of *E. granulosus* in developed countries, commercial incentive is lacking for the production of a human CE vaccine (Lightowlers, 2006; Craig et al., 2017). Therefore, the production of veterinary transmission blocking vaccines are considered a more practical alternative.

Protection was achieved in sheep studies against *E. granulosus* infection following subcutaneous vaccination with either oncospheres (Heath et al., 1981) or fractionated secretory products derived from *in vitro* cultured oncospheres (Osborn and Heath, 1982). Studies performed by Heath and Lawrence (Heath and Lawrence, 1996) identified a protective antigen called EG95, cloned from oncosphere mRNA (Lightowlers et al., 1996), that has conferred up to a 100% protection in experimental immunization trials conducted in seven countries (i.e., Argentina, Australia, Chile, China, Iran, New Zealand, and Romania) (Lightowlers, 2006, 2012; Craig et al., 2017). It was also evaluated for protection against alveolar echincoccosis in sheep caused by *E. multilocularis* with protection ranging between 78.5 and 82.9% (Gauci et al., 2002). Consequently, this antigen is the principal component of the only commercialized vaccine that is registered for use in China (June 2007) and Argentina (February, 2011) (Providean Hidatil EG95®, Tecnovax, Uruguay) (Supplementary Table 1) (Bowman, 2014; Craig et al., 2017).

For alveolar echincoccosis specifically, several additional protective candidates validated in mice have been described including: a recombinant 14-3-3 antigen rE14ζ (97.35% reduction in parasite load following oral egg challenge) (Siles-Lucas et al., 2003, 2008; Craig et al., 2017); the EG95-like protein from *E. multilocularis* EM95 (Gauci et al., 2002), recombinant tetraspanin transmembrane proteins TSP1 and TSP7 (causing a reduction of lesions by 87.5 and 37.6%, respectively) (Dang et al., 2009, 2012), and a metacestode development protein P29 (i.e., rEmP29) that wasfirst identified from *E. granulosus* (up to 75 and 53% reduction in parasite mass and load) (Wang et al., 2009; Boubaker et al., 2015). Therefore, a specific commercial product may stem from these antigens.

Lastly, cysticercosis is a zoonotic tissue infection transmitted between humans (definitive host) and pigs (intermediate hosts) caused by the cestode parasite, *Taenia solium* that is regarded as the most important foodborne parasitic infection and a leading cause of neurological disease in developing countries (Robertson et al., 2013; Lightowlers et al., 2016). A recombinant stage-specific oncosphere surface antigen TSOL18 has been independently tested in both experimental and field trials with a greater than 90% protection conferred in pigs from various localities in Central America, South America and Africa (Lightowlers, 2013; Lightowlers et al., 2016; Lightowlers and Donadeu, 2017). This vaccine is currently the first and only cysticercosis vaccine licensed for commercial production as Cysvax® (Indian Immunologicals Ltd., India) (Supplementary Table 1) (Lightowlers and Donadeu, 2017). Two additional oncosphere-derived proteins, TSOL16 and TSOL45-1A, have shown promise as next generation porcine cysticercosis transmission blocking vaccine candidates with 99.8 and 97.9% protection in vaccinated pigs against *T. solium* cysticerci challenge (Gauci et al., 2012).

Overall, for human helminthiases, no commercial vaccines are currently available with only four recombinant vaccine candidates related to schistosomiasis (*Sh*28GST, Sm14-FABP, Sm-p81, and Sm-TSP-2) being in various processes of clinical development. For the veterinary market, five vaccines have been successfully commercialized that consists of three native (Barbervax®, Huskvac®, and Difil) and two recombinant (Cysvax® and Hidatil EG95®) products. Currently, for veterinary helminth vaccines, it appears that live, inactivated or attenuated vaccines are more successful than recombinant subunit vaccines. This may be due to the need of complex, multi-antigen vaccines that are needed to confer protection to these complex parasites. Live vaccines do however place limitations on the preparation and safety of these vaccines (requiring host animals to produce the parasite material) and an unbroken cold chain that increases costs. In future, subunit vaccines will offer a better solution in terms of safety, stability and production of vaccines. Surprisingly all the antigens mentioned in section Endoparasite Vaccines: Helminthiases, were identified decades before they were commercialized or tested in field or clinical trials, highlighting the arduousness of vaccine development.

ECTOPARASITE VACCINES: PARASITIC ARTHROPODS

Ectoparasitic arthropods form part of the largest animal phylum of increasing veterinary and medical importance (Mathison and Pritt, 2014; Goddard, 2016). Arthropod-borne pathogens account for more than 20% of all emerging infectious diseases documented between 1940 and 2004 (Jones et al., 2008). However, despite the vast amount of research on vaccine development, there have only been a few commercial successes to date (Supplementary Table 1). Here, we aim to highlight some of the progress and challenges in ectoparasite vaccine development.

Parasitic Flies

Flies of veterinary importance are divided into three major groups, namely the haematophagous biting flies, non-biting nuisance flies and myiasis-causing flies (Pape et al., 2011). Biting flies of veterinary importance include *Haematobia irritans* (horn fly), *H. irritans exigua* (buffalo fly) and *Stomoxys calcitrans* (stable fly) (Pruett, 2002). Horn flies are economically devastating pests of cattle causing severe irritation, reduced milk production, weight loss, substantial blood loss and damage to hides (Byford et al., 1992). Moreover, horn flies are competent vectors of several pathogens including *Stephanofilaria stilesi*, a filarial parasitic nematode of cattle (Hibler, 1966), and several *Staphylococcus* spp. that cause mastitis in dairy heifers (Owens et al., 1998; Gillespie et al., 1999). Stable flies in turn are competent vectors of several pathogenic organisms including viruses [e.g., equine infectious anemia, African horse sickness (AHS) virus and fowl pox], bacteria (e.g., *Brucella* spp. causing brucellosis and *Bacillus anthracis* causing anthrax), protozoa (e.g., *Trypanosoma evansi* the causative agent of Surra), as well as helminths (e.g., nematodes such as *Habronema microstoma* and *Dirofilaria* spp.) (Greenberg, 1973; Turell and Knudson, 1987; Mongoh et al., 2008; Baldacchino et al., 2013).

Research has been conducted to develop new control strategies for biting flies, including vaccines. However, no effective targets have been identified to date (Wijffels et al., 1999; Guerrero et al., 2008; Oyarzún et al., 2008). Only two secreted salivary targets conferring partial protection to horn flies in cattle vaccinations have been identified to date: an anti-thrombin peptide (thrombostasin) (Zhang et al., 2001; Cupp et al., 2004, 2010) and a hematobin (Breijo et al., 2017). With the lack of tools for rapid validation of promising antigens, RNAi has been used despite its inability to translate a phenotype directly into protection. An RNAi study by Torres et al. targeting transcripts of the abdominal tissue of partially fed female horn flies revealed significant mortality and reduced oviposition rates for selected transcript functional groups (Torres et al., 2011). However, off-target effects influenced the results obtained and further optimization is required (Torres et al., 2011; Marr et al., 2014). Currently, no effective candidates are available for biting flies and the rationale for future vaccine development strategies remains to be demonstrated.

Myiasis-Causing Flies

Myiasis infections refer to the infestation of a host with the larvae of a range of species with adverse consequences to the host. Myiasis-causing flies are classified into three different groups based on their pathology: cutaneous myiasis-causing skin infections; bot flies that infect the gastrointestinal tract and body orifices of the host; as well as warbles that infect and migrate subcutaneously (Stevens and Wallman, 2006; Stevens et al., 2006).

The majority of research conducted on myiasis-causing flies focused on understanding the pathology and immune reactions caused by sheep blowflies (*Lucilia cuprina* and *L. sericata*) and cattle warble flies (*Hypoderma lineatum* and *H. bovis*). Larvae from *Lucilla* spp. are responsible for cutaneous myiasis, also known as blowfly strike, with a substantial economic impact on the wool industry (Elkington and Mahony, 2007). The immune responses to blowfly strike and vaccine development have been reviewed extensively (Elkington and Mahony, 2007; Sandeman et al., 2014). Many studies have focused on raising protective antibodies against a range of antigens derived from *L. cuprina* larvae including: cuticle proteins (Barrett and Trevella, 1989); whole larvae extracts (East et al., 1992); fractionated larval extracts (Tellam and Eisemann, 1998); excretory and secretory (ES) products (Bowles et al., 1987; Tellam et al., 1994); purified serine-proteases (Tellam et al., 1994) and larval peritrophin membrane proteins (Casu et al., 1997; Tellam and Eisemann, 1998; Colditz et al., 2002). In many instances larval growth was significantly retarded *in vitro* when using antisera raised from immunized animal models. However, no significant protection was conferred against larval infestations during *in vivo* studies. These results were mainly attributed to insufficient levels of IgG produced *in vivo* (2- to 4-fold lower than that used in *in vitro* studies) (Johnston et al., 1992).

As eliciting protective humoral immune responses is challenging, further studies are now focusing on cellular immunity (Sandeman et al., 1986; Bowles et al., 1987, 1996). In this regard, a vaccine formulation containing native antigens derived from larvae, adjuvant (Montanide™ ISA-25) and recombinant ovine interleukin-1β (rovIL-1β) were used for immunization of sheep resulting in significant levels of protection in two consecutive trials (86–67% reduction in strike incidence and 85–31% reduction in larval weight) (Bowles et al., 1996). A fundamental result from this study was that the humoral response did not correlate to the levels of protection, and more significantly that the antibodies derived from the serum are also unlikely to confer protection. Stimulation of type I (IgE-mediated) and type III (Arthus-type) immediate hypersensitivity responses were instead indicated as involved in protection, which was supported by previous observations in blowfly "resistant" sheep (Sandeman et al., 1986; Bowles et al., 1987, 1996). In addition, a 56 kDa excretory/secretory protein from *L. cuprina* larval tissues was shown to inhibit lymphocyte activation, supporting the notion that antibody-mediated immunity is not always sufficient for control of some parasites (Elkington et al., 2009).

In contrast to blowfly infections, it is well known that cattle develop a protective immunity against cattle grub infections, caused by *H. lineatum* (Gingrich, 1980, 1982; Pruett and Kunz, 1996). Increased mortality in larvae was demonstrated during cattle vaccination studies against *H. lineatum* crude larval extracts and culture-derived antigens with some cross-protection induced against *H. bovis* (Baron and Weintruab, 1986). A later study reported a 100% mortality rate for *H. lineatum* following immunization with soluble extracts of third instar fat body using an adjuvant (Colwell, 2011). Additional secreted serine proteinases (hypodermins HyA, HyB, and HyC) were also tested in cattle trials and showed a significant decrease in the amount of developing pupae (Baron and Colwell, 1991). Hypodermin A appears to be the most promising lead antigen and is implicated in the downregulation of host lymphocyte proliferation (Panadero et al., 2009) and cytokine responses *in vivo* (Nicolas-Gaulard et al., 1995). However, to the best of our knowledge no large-scale evaluations have been performed to test the efficacy of Hypodermin A under field conditions.

Mosquitoes

With almost 1 million human deaths associated with mosquito borne illnesses every year (Caraballo and King, 2014), mosquitoes represent a major threat to human health. Diseases involved include malaria (caused by *Plasmodium* species) and filariasis (cause by filarial nematodes) (Tolle, 2009). Mosquitoes are also well known vectors of viruses including Dengue, West Nile, Chikungunya, Yellow fever and Japanese encephalitis (Tolle, 2009). Current research is focusing on the development of vector-directed vaccines and/or transmission blocking vaccines (targeting the pathogen) to reduce disease occurrence in human and animal hosts. The latter fall outside of the scope of this review. However, vector control is a vital part of disease reduction, since the number of competent vectors can directly affect the incidence of disease.

With regard to blocking of mosquito antigens to control Plasmodium transmission, a lead candidate is the mosquito aminopeptidase N1 (APN1, located on the mosquito midgut luminal surface) that is suggested to be involved in Plasmodium ookinete invasion of the midgut (Dinglasan et al., 2007). Varying transmission blocking activities have been reported with the *Anopheles gambiae* APN1 antigen produced *in situ* using viral vectors, all largely unsuccessful (Kapulu et al., 2015). In contrast, antibodies targeting *An*APN1 in various *Anopheles* species were found to inhibit transmission of several *Plasmodium* species (Dinglasan et al., 2007; Mathias et al., 2012; Armistead et al., 2014). A single protective, highly conserved epitope has consequently been identified for *An*APN1 that has been tested both in murine and non-human primate models with great success (Armistead et al., 2014). A number of additional mosquito antigens have been identified to control Plasmodium transmission, including numerous *A. aegypti* midgut proteases (Shahabuddin et al., 1995; Molina-Cruz et al., 2005; Lavazec et al., 2007), an *An. gambiae* midgut chitinase propeptide (Bhatnagar et al., 2003) a 12 amino acid peptide (SM1- salivary gland and midgut peptide 1) from *An. stephensi* (Ghosh et al., 2001) and a putative transcription factor akrin (or subolesin) (Moreno-Cid et al., 2010, 2013). All of the latter remains to be validated before any definite prediction with regards to their potential as commercial vaccine antigens can be made. One shortfall requiring attention is the need for improved immunogenicity of antigens and longevity of immune responses raised in the respective hosts (Sinden, 2017).

As arthropod saliva is known to mediate host immunity and in doing so aid the transmission of disease causing agents (Titus et al., 2006), it remains a research focus area. A breakthrough with regards to mosquito vaccines has been made in recent years with a multi-antigen vaccine being tested in clinical phase I trials at the moment (Supplementary Table 1). Based on an interview with one of the main researchers, salivary proteins were tested in several groups based on weights with main focus on 20–40 kDa sized proteins with four subsequently chosen based on their occurrence in several types of mosquitoes. The AGS-v vaccine is suggested to not only reduce parasite transmission, but potentially reduce the lifespan of the mosquito itself (Mole, 2017; Unwin, 2017). However, in spite of the significant progress made in recent years, the development of a vaccine targeting mosquito antigens has been slow. Knowledge regarding the mechanism of *Plasmodium* ookinete recognition and invasion of the midgut epithelium, as well as parasite defenses against host and vector immune factors is still lacking considering that successful transmission blocking vaccines are assumed to target accessible vector midgut antigens via antibodies subsequently interfering with their function (Sinden, 2017).

Lice

Lice are apterous obligate ectoparasites, belonging to the order Phthiraptera, of which only a small fraction is of economic importance. Currently, the majority of vaccine research is dedicated to the aquatic sea lice *Lepeophtheirus salmonis* and *Caligus rogercresseyi* (Pike and Wadsworth, 1999; Costello, 2006). A recombinant peptide-based subunit vaccine against *C. rogercresseyi*, Providean Aquatec Sea Lice® (Tecnovax S.A., Argentina), is currently commercially available (Supplementary Table 1) (Villegas, 2015). However, as aquatic parasites falls outside the scope of this review, they will not be discussed further.

Acarines

Mites

Sarcoptes scabiei is a parasitic mite responsible for sarcoptic mange and scabies in both animal and human hosts (Mellanby, 1941, 1944). The invasive nature of this mite enables it to burrow down into the skin of its host resulting in inflammation and intense hypersensitivity responses that can result in severe secondary bacterial infections (Walton et al., 2004). Currently no vaccine against scabies is available and limited advances have been made in the field to date (Arlian and Morgan, 2017). The most promising vaccination studies performed thus far include immunization of rabbits with extracts of the house dust mite which elicited only partial protection (Arlian et al., 1995).

Acquired resistance to *Sarcoptes scabiei var. canis* has been described in canines that was previously infested and then again challenged under laboratory conditions. These animals had

increased IgG1, IgG2, IgM antibodies (Arlian et al., 1996) that can now be used in follow-up studies to gain insight into drivers of protective immunity. In contrast, sheep that were infected with *Sarcoptes Scabiei var. ovis* only acquired partial protection despite them also inducing IgG and IgE antibody responses (Rodríguez-Cadenas et al., 2010).

The sheep scab mite *Psoroptes ovis* is an important ectoparasite of both cattle and sheep. In contrast to *S. scabiei*, it is a non-burrowing mite which feeds off the exudation from skin surfaces (Downing, 1936). Infestation with *P. ovis* results in severe allergic dermatitis in cattle (Stromberg and Fisher, 1986) and scabietic lesions in sheep (Jayawardena et al., 1998). Although vaccination using mite extracts have shown to elicit partial protection in cattle and sheep (Pruett et al., 1998; Smith et al., 2002; Smith and Pettit, 2004), there has been limited success in vaccine development against this species of mite. A subunit cocktail vaccine consisting of seven targets has been tested in sheep with a reduction of more than 55% in mite numbers and lesion size obtained (Burgess et al., 2016).

Dermanyssus gallinae (the poultry red mite) is a species that severely affects the poultry egg-laying industry (Van Emous, 2005). Initial vaccination studies with a crude protein fraction of homogenized mites resulted in significant increase in mite mortality compared to control hens (Wright et al., 2009). *In vitro* feeding assays using serum derived from the latter, led to the identification of a serpin (Deg-SRP-1), vitellogenin (Deg-VIT-1), hemelipoglycoprotein (Deg-HGP-1) and a protein of unknown function (Deg-PUF-1) that significantly increased mite mortality rates (Bartley et al., 2015). Field trials comparing soluble mite extract (SME) vaccine to a recombinant vaccine containing Deg-SRP-1, Deg-VIT-1 and Deg-PUG-1 in 384 hens challenged with *D. gallinae* indicated the former vaccine to offer 78% reduction of mites while the latter did not give any significant results (Bartley et al., 2017). This highlights the need for field trials to firmly establish an antigen as being protective. Additional subunit vaccines have been tested, containing tropomyosin, paramyosin (Wright et al., 2016) or subolesin (Harrington et al., 2009). All offered limited protection against infestation.

Taken together the data does indicate a realistic potential for mite vaccines to protect human and animal hosts. Failures or insufficient protection reported to date for mite vaccine studies have been attributed to problems related to formulation, dose, vaccination regime and vaccine delivery (Arlian and Morgan, 2017). Moreover, the parasite-host interaction is rather complex and these and other parasites have documented abilities to evade the host's immune responses through bioactive salivary molecules (Titus et al., 2006; Arlian and Morgan, 2017). Investigating the immunological response to *D. gallinae* infestation revealed that during feeding there is an inhibition of Th1 inflammatory responses (Harrington et al., 2009), a situation echoed by *S. scabiei* infestations (Lalli et al., 2004). It may therefore be beneficial to design future vaccines to elicit a balanced Th1/Th2 response, which may protect the host against infestation.

Ticks

Ticks are obligate hematophagous ectoparasites that feed on all classes of terrestrial vertebrates (Sonenshine, 1991). Moreover, ticks are considered very important vectors of diseases affecting cattle and pets, as well as the second most important vector of human diseases after mosquitoes (De La Fuente et al., 2008). Moreover, as hematophagous ectoparasites, the damage inflicted by ticks, especially in livestock, includes the damage to hides, anemia, weight loss and secondary infections (de La Fuente and Contreras, 2015).

The concept of experimental vaccination against ticks has been explored since 1939 when William Trager demonstrated that injecting guinea pigs with whole extracts of *Dermacentor variabilis* tick larvae conferred some protection against subsequent infestations (Trager, 1939). The feasibility of vaccinating cattle against ticks was later demonstrated by Allen and Humphreys (1979). This seminal research led to the discovery of Bm86 in 1986, a protective antigen in membrane fractions of *Rhipicephalus microplus*, identified through classical protein fractionation experiments (Willadsen and Kemp, 1988; Willadsen et al., 1989). This antigen is a membrane-bound glycoprotein located on the gut lumen of the tick digestive tract (Gough and Kemp, 1993) and suggested to be involved in cell-cell or pathogen-gut cell interactions (Liao et al., 2007). The Bm86 (and/or its related homolog Bm95) antigen is the basis for the only commercial tick vaccines developed in 90's, including GAVAC® and GAVAC® Plus available in Latin American countries (Herber-Biotec S.A., CIGB, Camagüey, Cuba), as well as the discontinued TickGARD® and TickGARD®PLUS from Australia (Intervet Australia Pty. Ltd., Australia) (De La Fuente et al., 2007), and is still being used effectively on a large scale for vaccination such as performed recently in Venezuela (Suarez et al., 2016). Only one other commercial vaccine is currently produced for the Latin American market by Limor de Colombia® (Bogotá, Colombia) under the product name Go-Tick® or Tick-Vac® that is directed against *R. microplus* infestations (Supplementary Table 1). A probable native vaccine, the manufacturer claims ∼80% protection in field tests, though no research publications based on its use have been disclosed to assess the veracity of protection claims.

Vaccination with Bm86-based vaccines results in a reduction of the number of engorging female ticks, their engorgement weight, but mainly their reproductive capacity (Rodríguez et al., 1994). This eventually leads to reduced larval infestation in subsequent generations. However, due to the vaccine's inefficacy against all tick life stages, its variability of protection against different tick strains and species, across geographical regions, and the requirement of several boosts per year for optimal efficacy, the pressing need for improved tick control is reiterated (García-García et al., 1999). The preliminary research that led to the identification of Bm86 illustrated the advantage of vaccinating with protein extracts, which gave a higher vaccine efficacy than the fractionated proteins, probably due to the synergistic effects of protein combinations, proving the feasibility of combinatorial vaccines (Rand et al., 1989; Willadsen et al., 1989). Several studies using Bm86 (Bm86 homologs) or parts thereof in combination with other tick antigens have been published and show promise (Richards et al., 2015; De La Fuente et al., 2016a; Schetters et al., 2016).

A rational approach toward the identification of protective antigens would be to target proteins crucial for the biological function and survival of the parasite including tick attachment to the host, circumvention of the host's defense mechanisms, feeding and digestion of the blood meal, metabolism, mating, fertility, embryogenesis and oviposition (De La Fuente et al., 2016a). To date, several recombinant vaccine candidates identified from different Rhipicephalus species have been validated *in vivo* with their effectiveness in controlling tick populations ranging from 0 to almost 100% (Richards et al., 2015). Some of the most promising candidates currently under investigation include a mix of six peptides (that were identified using reverse vaccinology followed by *in vitro* feeding of peptide-specific antibodies) and aquaporins (Schetters et al., 2016). The latter have been patented by Dr. F. Guerrero and colleagues (US Patent nr(s).: 2013/0315947; US 2016/0361396) for the purpose of vaccine development against ticks as single (Guerrero et al., 2016), as well as combinatorial antigen formulation with a novel gut antigen (Guerrero and De Leon, 2017). Aquaporins have also been found to be effective against *I. ricinus* larvae with the best aquaporin formulation reaching 80% efficacy (Contreras and de La Fuente, 2017).

Other recently tested promising antigens include subolesin vaccine formulations including subolesin/akirin chimera which resulted in a 99% and a 46.4% vaccine efficacy in rabbits against *I. ricinus* and *D. reticulatus*, respectively (Contreras and de La Fuente, 2016). Additionally, recombinant subolesin tested against *H. anatolicum* and *R. microplus* infestation resulted in 65.4 and 54% protective efficacy, respectively (Kumar et al., 2017). The promise of this antigen is evident by the presence of a patent based on a combinatorial vaccine including Bm86 and subolesin (Patent nr.: PCT/EP2014/056248). An improvement of the current vaccine antigen Bm86 via multi-antigen formulations seems a promising avenue to improve an existing effective vaccine.

Although numerous vaccine candidates have been identified, a vaccine capable of protecting against a range of species remains hypothetical. However, in recent studies conducted by Rodríguez-Mallon and colleagues investigated the acidic ribosomal protein P0 (highly conserved among tick species) against *R. microplus* (Rodríguez-Mallon et al., 2015) and *R. sanguineus* (Rodríguez-Mallon et al., 2012). Immunization of rabbits offered an overall efficacy of 90%, mainly via reducing the number of adults and egg hatching (Rodríguez-Mallon et al., 2012). During bovine immunization studies against *R. microplus* and an overall vaccine efficacy of 96% was obtained (Rodríguez-Mallon et al., 2015). As a consequence of these results a vaccine composition based on the P0 peptide was patented recently due to its potential as protective vaccine antigen against a range of ectoparasites (Mallon et al., 2015).

Since the generation of tick sequence databases (genome and transcriptome), *in silico* vaccinology approaches have been used with success to identify protective antigens, as evident from a next generation multi-peptide tick vaccine that has been developed (Schetters et al., 2016). Future studies are now needed to identify protective antigen epitopes to reduce costs associated with production of a commercially viable vaccine. Several other high-throughput techniques including expression library immunization or ELI, sequence suppression subtractive hybridization or SSH, microarray hybridization, RNAi and proteomics have also been evaluated as screening platforms for candidate tick protective antigens (de La Fuente and Contreras, 2015; De La Fuente et al., 2016a; Lew-Tabor and Valle, 2016), with only subolesin being identified. A major limitation in tick vaccine development for livestock is access to pre-vaccination screening tools to identify promising antigens for large-scale production and evaluation in large animal models. In this regard, artificial feeding methods have been developed (Abel et al., 2016; Tajeri et al., 2016; Krull et al., 2017; Böhme et al., 2018), offering some promise as a pre-screening approach with ticks feeding on serum derived from vaccinated hosts (Lew-Tabor et al., 2014; Lew-Tabor and Valle, 2016).

METAZOAN VACCINE DEVELOPMENT: IDENTIFICATION TO FORMULATION

Significant efforts have been directed toward vaccine development for many metazoan parasites of medical and veterinary importance, since it is generally considered an ideal approach to prevent re-infection/infestation that is generally not achievable with repeated prophylactic chemotherapy (Noon and Aroian, 2017). In successful cases, target product profiles (TPP) or preferred product characteristics (PPC) were developed to establish the value of including a vaccine into public control programs, such as developed for hookworm (Hotez et al., 2013; Bartsch et al., 2016) and schistosomiasis (Molehin et al., 2016) vaccines. These product profiles were refined, not only through successes but also from failures during animal and human trial evaluations. A prime example, comes from hookworm vaccines developed against *A. caninum*, where a native veterinary vaccine based on attenuated L3 larvae failed commercially (Miller, 1978; Schneider et al., 2011) and an abandoned recombinant larval surface protein (ASP-2) that caused undesirable adverse reactions in phase I human clinical trials (Schneider et al., 2011; Bottazzi, 2015). The latter was due to elevated IgE levels elicited within pre-exposed and possibly sensitized test subjects staying within a parasite endemic area causing generalized urticaria (Diemert et al., 2012). It is believed that the IgE axis (including receptors and cellular responses) has evolved to counter infection/infestation by metazoan parasites (helminths and arthropods) that cannot be phagocytosed (Fitzsimmons et al., 2014). Moreover, metazoan parasites can be strong inducers of inflammatory responses (e.g., helminths and mites), and these parasites have also been implicated in human host sensitization and development of various allergies (e.g., tick-induced meat allergies) (Cabezas-Cruz and Valdés, 2014; Fitzsimmons et al., 2014; Steinke et al., 2015; Posa et al., 2017). For helminth infections, a strongly skewed host response toward Th2 immunity is observed and these parasites actively moderate the host Th2 (and other) responses via secreted bioactive compounds that eventually leads to a diminished antiparasitic IgE and cellular response (McSorley et al., 2013; Fitzsimmons et al., 2014; Nutman, 2015). A similar modulation

of host defenses toward Th2-mediated anti-inflammatory effects is observed for tick-derived immunomodulatory compounds (Kazimírová and Štibrániová, 2013; Silva et al., 2016). It would appear that Th2 and IgE related responses are vital for natural host antiparasitic defenses (Fitzsimmons et al., 2014). However, protection conferred by recombinant subunit vaccines does not conform to natural anti-parasite immunity and in most cases IgG responses that block essential antigen function mediate protection (e.g., helminths and ticks) (Jonsson et al., 2014; De La Fuente et al., 2016a; Contreras and de La Fuente, 2017; Noon and Aroian, 2017). In general, it seems a fine line exists between positive vaccine therapeutic effects and unwanted hyper-immunity in sensitized individuals that presents an ongoing challenge for vaccine development (Fitzsimmons et al., 2014). Therefore, care in the selection, production and formulation of targets for especially human vaccines must be taken and homology (i.e., structure and epitope) screening of such antigens against libraries of known allergens (i.e., plant and animal) might be a good stage-gate for pre-selection.

In the case of veterinary vaccines, clear product profiles are not as evident. Effective native (e.g., Bovilis Huskvac®, Barbervax®, and GoTick®) and recombinant (e.g., Providean Hidatil EG95®, Cysvax®, Providean Aquatec Sea Lice®, GAVAC®) vaccine preparations have been commercialized, whereas pure recombinant antigens are preferred for human vaccines (Supplementary Table 1). Native vaccine formulations require production of parasite products, often using animal hosts, on a large enough scale to produce a commercially viable product. Therefore, considering the biological complexity of most metazoan parasites, the use of native vaccines (whole organism or purified protein fractions) are generally unfavorable (relative to recombinant vaccines) for commercial vaccine design due to limitations including: scalability of antigen production, high production costs, low vaccine stability and shelf life as well as safety. In some cases, however, native vaccines such as Barbervax® are so effective that they remain more economical, relative to no intervention or mass drug administration MDA (Noon and Aroian, 2017).

Distilling Antigens of Interest

Prior to commercial considerations, identification of antigenic targets remains the essential rate-limiting step for vaccine development. Moreover, proteins that have essential functions in parasite biological processes can be targeted for development of next generation controls and diagnostics that include: host invasion and evasion of immunological responses (also so-called virulence-related proteins); parasite metabolism, development and fecundity; parasite-host co-evolution; even targets related to parasite acquired resistance (i.e., chemotherapeutic resistance) (de La Fuente and Contreras, 2015; Lv et al., 2015; Haçariz and Sayers, 2016; Kuleš et al., 2016; Arlian and Morgan, 2017). However, in many cases parasite proteins identified to date relate mostly to physiological pathways or are stage specific in the parasite and may not necessarily be directly associated with host-parasite interaction. To fill knowledge gaps, complementary technologies that can assist in expanding our current understanding of parasite-host biology and expedite identification strategies to prevent and control parasitic infections/infestations are available (**Figure 1**). These so-called -omics or systems biology approaches have found fertile ground especially in unicellular parasite/pathogen vaccine discovery and have matured into new emergent fields such as systems vaccinology or immunomics (Hagan et al., 2015; Nakaya and Pulendran, 2015; De La Fuente et al., 2016a; Haçariz and Sayers, 2016; Kuleš et al., 2016; Villar et al., 2017).

In this context, *in silico* comparative approaches are useful for robust identification of additional targets for vaccine development. In the wake of the genomics revolution, sets of sequence data (i.e., genomes, shot-gun, expressed sequence tags and suppression subtractive hybridization or SSH libraries, microarray and RNA sequencing transcriptomic datasets, etc.), representing different life stages and different conditions, have been expanding for many metazoan parasite species of economic importance (Anstead et al., 2015; Greene et al., 2015; Lv et al., 2015; Schwarz et al., 2015; Tyagi et al., 2015; De La Fuente et al., 2016a,b; Kuleš et al., 2016; Arlian and Morgan, 2017; Barrero et al., 2017). These growing repositories of genetic information enable researchers to gain access to a greater complement of molecules involved in parasite and parasite-host biology, also enabling evolutionary analyses to determine parasite diversity and encoded protein conservation/divergence within and between parasitic and non-parasitic species (Lv et al., 2015; Haçariz and Sayers, 2016; Barrero et al., 2017; Mans et al., 2017). Using bioinformatics tools, parasite target sequences can be subjected to a series of analyses such as motif searches (e.g., protein families, domains, conserved catalytic or interaction sites) (Jones et al., 2014), biological pathways and protein interaction network analyses (Kandpal et al., 2009; Khatri et al., 2012; Hernández Sánchez et al., 2016; Nguyen et al., 2016; Rahmati et al., 2017), produce gene ontology (GO) information (Ashburner et al., 2000; Gene Ontology, 2015); predict subcellular localization (Horton et al., 2007) and even predict potential antigens or antigenic regions via reverse vaccinology and immuno-informatics approaches (Maritz-Olivier et al., 2012; Bremel and Homan, 2013; Goodswen et al., 2013, 2017; Maritz-Olivier and Richards, 2014; Lew-Tabor and Valle, 2016) (**Figure 1**).

Combining such *in silico* methods with manual inspection, literature searches and even additional bioassays, will assist functional annotation of transcripts that lack sequence homology to model organisms (De La Fuente et al., 2016a; Haçariz and Sayers, 2016; Lew-Tabor and Valle, 2016). In this regard, additional reverse genetics approaches such as gene knock-down (e.g., RNAi) and gene editing (CRISPR/Cas9) have been successful in describing *in vivo* protein function for parasitic and non-parasitic (e.g., *Caenorhabditis elegans* and *Drosophila melanogaster*) helminths and arthropods, as well as evaluation of potential targets for parasite/vector control (e.g., expressed library immunization or ELI and phage display) (Dalvin et al., 2009; Ellis et al., 2012; Waaijers et al., 2013; Sandeman et al., 2014; Tröße et al., 2014; Aghebati-Maleki et al., 2016; Britton et al., 2016; De La Fuente et al., 2016a; Zamanian and Andersen, 2016; Crauciuc et al., 2017; Gao et al., 2017; Macias et al., 2017; Rahumatullah et al., 2017).

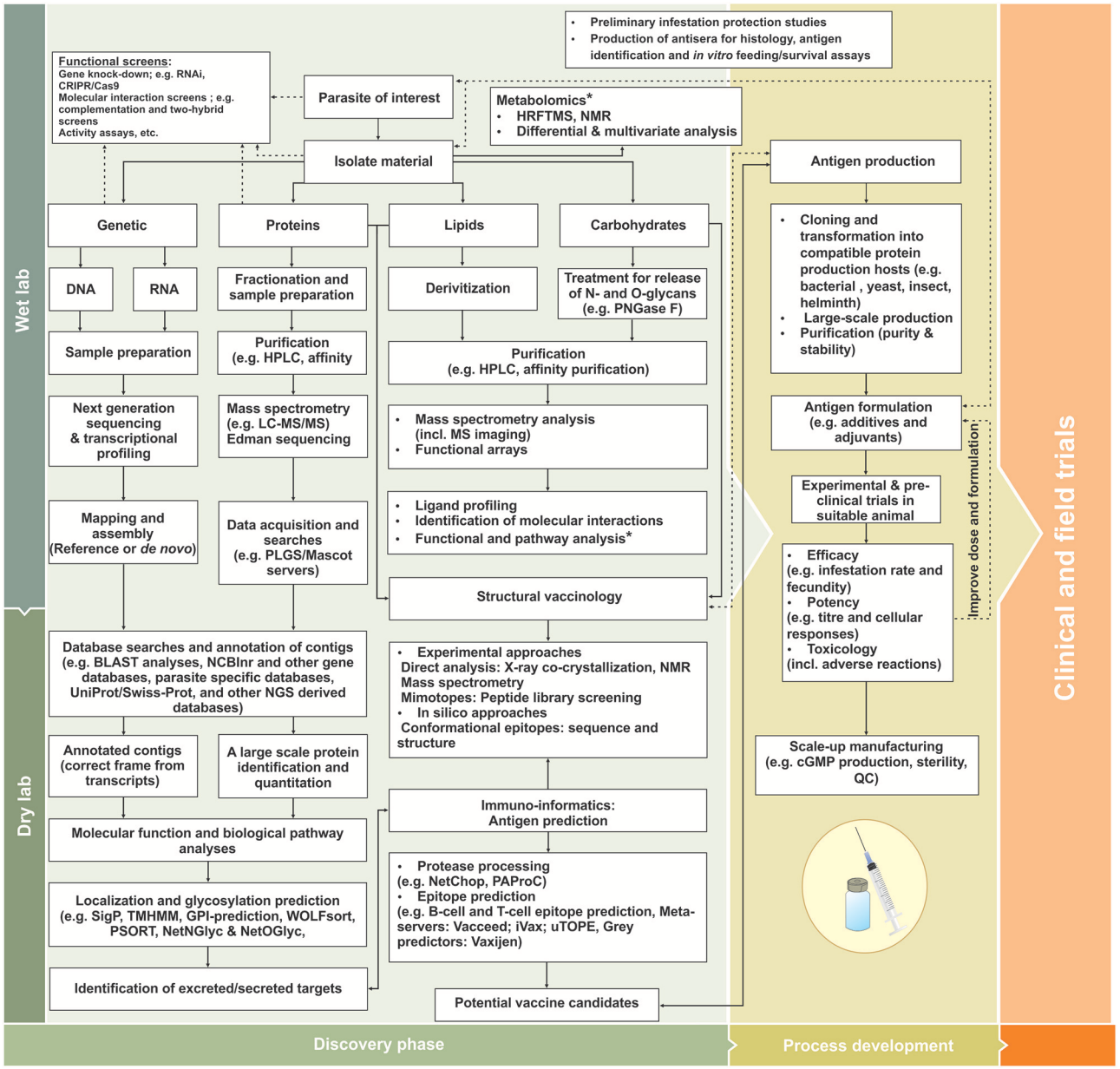

FIGURE 1 | Diagrammatic workflow for identification and evaluation of next generation metazoan vaccine candidates adapted from Haçariz and Sayers (2016). In wet-lab conditions, the parasite of interest is treated to ensure isolation of appropriate factors involved in parasite biology and parasite-host interactions, providing data on genomics, transcriptomics, proteomics, lipidomics, glycomics, and metabolomics levels. During "dry lab" applications, the various parasite components can be analyzed and functionally annotated using various functional and reverse genetics techniques. By employing large-scale techniques and bioinformatics tools, exposed targets able to elicit a host immune response can be preferentially selected and their protective epitopes predicted for improved vaccine design. These targets can enter process developmental stages where antigens are produced and tested in small-scale experimental vaccination trials. Subsequent improvements of protective antigens include vaccine formulation, stability and efficacy during process development, prior to extensive clinical and field trail evaluations. Dashed lines indicate some additional loops for antigen discovery, functional annotation and vaccine improvement and asterisks feedback information from metabolomics studies. cGMP, Current good manufacturing practices; GO, gene ontology; HRFTMS, Fourier transform mass spectrometry; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; MS, mass spectrometry; NMR, Nuclear magnetic resonance; PLGS, ProteinLynx Global; PNGase F, Peptide-N-Glycosidase F; QC, Quality control.

In the tick research field, systems biology approaches are also gaining momentum with new studies combining -omics approaches (e.g., transcriptomics, proteomics and even metabolomics) to not only define parasite biology and vector-pathogen interfaces, but also identify next generation targets for antiparasitics (Chmelar et al., 2016; Ramírez Rodríguez et al.,

2016; Contreras and de La Fuente, 2017). Functional proteomics approaches are currently enjoying a resurgence in parasite research and advances in high-throughput mass spectrometry technologies (including complementary analytical and *in silico* or bioinformatics methods) have improved identification and quantitation of proteins (and protein mixtures), as well as enabled structural analysis of multiprotein complexes (i.e., subunit composition, stoichiometry and topology) (Aebersold and Mann, 2016; Haçariz and Sayers, 2016; Villar et al., 2017). Additional protein and peptide microarrays can provide an *in vitro* platform for functional high-throughput screening of protein targets for protein-protein (i.e., interactomic) and protein-antibody (serodiagnostics and vaccine reactive antigenic epitope) screens (Manzano-Romá et al., 2012; Gaze et al., 2014; Carmona et al., 2015; Driguez et al., 2015; Kassegne et al., 2016). However, this technology remains underutilized in metazoan parasite vaccine research.

A further consequence of proteomic and bioinformatic developments is the emerging field of structural vaccinology (Kulp and Schief, 2013; Donnarumma et al., 2016; Villar et al., 2017; Wang and Chance, 2017). Bespoke antigens can be made that are modeled onto a stabilizing protein scaffold that contains only functional structural epitopes (determined by *in silico* methods and *in vivo* and/or *in vitro* assays) defined from antigen-antibody contacts (via mass spectrometry and crystallographic technologies) that are highly optimized and tailored for specific immune responses (Kulp and Schief, 2013; Malito et al., 2015; Saeed et al., 2017; Simkovic et al., 2017). Such antigens can potentially contain any number of protective epitopes for a number of different strains and species, but this approach has only been investigated for unicellular parasites and pathogens (e.g., Lyme disease vaccine based on the outer surface protein A or OspA) (Kulp and Schief, 2013; Malito et al., 2015). Another major draw-back is the lack of crystal structures (or sufficient homologs) that are available (<1,000, https://www.rcsb.org/) to enable such an approach to vaccine design. In this case *ab initio* molecular modeling could contribute in the absence of defined crystal structures (Khor et al., 2014, 2015, 2017).

A final consideration in metazoan antigen discovery, is the presence of parasites that do not have effective laboratory animal models and/ or require large animal hosts for propagation and challenge, or do not produce comparative responses (including pathological endpoints) between intermediate or definitive hosts (e.g., hookworms) (Schneider et al., 2011; de La Fuente and Contreras, 2015). In these cases, to further antigen discovery, a related parasitic species can be used that have a permissible animal model for vaccine trial evaluations. In this manner homologous target proteins can be identified that may confer cross-species protection such as subolesin/akarin and P0 antigens (Moreno-Cid et al., 2013; de La Fuente and Contreras, 2015; Mallon et al., 2015; Carpio, 2016; Villar et al., 2017). Additional *in vitro* feeding systems for ectoparasites such as ticks, lice, mites and mosquitoes have become valuable tools for parasite study and development of antiparasitics by mitigating the requirement for host animal challenges and affording a platform for high-throughput studies (i.e., infection studies, gene knock-down and chemical assays) (Kessler et al., 2014; Bartley et al., 2015; Sangaré et al., 2016; Agramonte et al., 2017; Kim et al., 2017; Krull et al., 2017; Trentelman et al., 2017). Unfortunately, due to the more intimate and complex association between endoparasitic metazoan helminths, *in vitro* culturing systems are not yet optimal for parasite development of some species (e.g., *H. contortus* and *S. hamatobium*) and culture conditions may not necessarily reflect *in vivo* conditions (a similar case for some ectoparasites) or culture components may interfere with small molecule studies (e.g., peptides and metabolites) (Shepherd et al., 2015; Britton et al., 2016; Driguez et al., 2016). Moreover, success in *in vitro* assays may not guarantee vaccine protection *in vivo* (e.g., poultry red mite) (Bartley et al., 2015, 2017), consequently field trials using the definitive (or equivalent) host animals are still required for final proof-of-concept.

Production of Antigens

Large-scale production of recombinant proteins that maintain immunological activity comparable (or better) to the native parasite protein is another challenge for recombinant vaccine development. Therefore, correct folding and post-translational modification (i.e., glycosylation) will depend upon the protein production host used (prokaryotic vs. eukaryotic). Recombinant protein production in *Escherichia coli* is a popular approach, however, production of insoluble antigens (e.g., *O. ostertagi* rASP1) and unsuccessful application of such proteins in vaccination trials (e.g., OPA and H-gal-GP) have been observed (Vercauteren et al., 2004; Cachat et al., 2010; Matthews et al., 2016). In some cases production of antigens as fusion proteins with maltose binding protein (MBP) and glutathione-S-transferase (GST) in *E. coli* have resulted in excellent protection during vaccination trials, as exemplified by *T. solium* antigens, TSOL16 and TSOL45-1A (in QuilA adjuvant) (Gauci et al., 2012). However, these results might be due to ancillary factors aside from the chosen recombinant antigen that may contribute to the observed protection conferred by native preparations. Moreover, recombinant antigen quality (such as protein solubility, folding and glycosylation) produced by protein production hosts may influence the host immune response (e.g., isoform, specificity and avidity of antibodies produced) (Matthews et al., 2016). To mitigate such outcomes recombinant protein production can be attempted in parasite-derived cells or a closely related species (e.g., *C. elegans* and bacculovirus-insect cell expression systems) in order to produce a protein mimicking the native molecule (Nisbet and Huntley, 2006; Roberts et al., 2013; Hussein et al., 2015; Van Oers et al., 2015). However, for some antigens like *H. contortus* antigen H11, protein recombinantly produced in *C. elegans* was unsuccessful when applied in experimental vaccination trials (Roberts et al., 2013). Moreover, some protective antigens are multiprotein complexes that simply cannot be easily produced using synthetic production hosts. Barbervax® (WormVax, Australia), as an example, is a native vaccine isolated from adult *H. contortis* of which the two major antigenic fractions, H11 and H-gal-GP (∼1,000 kDa), consists of multiple enzymes and protein complexes integral to the parasite gut membrane (Supplementary Table 1) (Salle et al., in review).

Protein glycosylation is also regarded as an important factor when manufacturing vaccines as it is known that polysaccharides

can serve as a first signal for B cell activation, however, limited glycomics studies have been conducted in metazoan parasites (de La Fuente et al., 2006; Maritz-Olivier et al., 2012; Hokke and Van Diepen, 2017). For ectoparasites such as ticks, preliminary evidence for the importance of carbohydrate moieties in protective antigens was demonstrated by Lee et al. (1991), where protective responses in vaccinated cattle were abolished following treatment of *R. microplus* midgut extracts with sodium metaperiodate. Additional studies, using native and recombinant Bm86 (produced in prokaryotic and eukaryotic protein production systems), showed that the carbohydrate determinants of Bm86 contributed to the protective responses observed in vaccinated animals (Willadsen and McKenna, 1991; de La Fuente et al., 2006). It has also been indicated that tick-derived carbohydrates (such as alpha fucosylation of tick glycoproteins by fucosyltransferases) are role-players in tick-pathogen interactions, such as colonization and transmission in vector cells (e.g., *Anaplasma phagocytophilum*) (de La Fuente et al., 2006; Pedra et al., 2010; Cabezas-Cruz et al., 2017). Similar studies in schistosomes have indicated an important role of glycan epitopes in host-parasite interactions including modulation and evasion of host innate and adaptive immunity, as well as infection biology during snail-schistosome interactions (e.g., fucosylated structures produced on larval surfaces and released during larval transformation and sporocyst development) (Mickum et al., 2014; Jurberg and Brindley, 2015; Smit et al., 2015; Nascimento Santos et al., 2017). Investigation of carbohydrate-protein interactions have been revolutionized with the development of functional glycomics tools such as glycan arrays that have been applied successfully in the analysis of glycan binding protein associated biology, host-pathogen interactions and immune recognition (by antigen specific antibodies) (Heimburg-Molinaro et al., 2011; Rillahan and Paulson, 2011; Arthur et al., 2014). Preliminary studies using glycan arrays have been conducted to identify the repertoire of anti-glycan antibodies produced during helminth infections in both humans and animals (Muthana and Gildersleeve, 2014; Hokke and Van Diepen, 2017). Though identification of glycan antigens has proven to be useful for vaccine or biomarker development (i.e., diagnosis, prognosis, risk prediction, and monitoring immune responses) (Bhatia et al., 2014; Luyai et al., 2014; Yang et al., 2017), application of this technology is still lacking for many metazoan parasites. Additional, structural glycomics techniques such as tandem mass spectrometry (MS), nuclear magnetic resonance (NMR) and compositional (and linkage) analyses of glycoproteins can be used to analyse protein-specific glycosylation, along with traditional blotting and microscopy techniques for localization and distribution studies (Jurberg and Brindley, 2015; Hokke and Van Diepen, 2017).

A separate platform for vaccine antigen delivery/presentation is the use of antiparasitic DNA vaccines, especially for veterinary medicine. These types of vaccine platforms have mostly been applied to protozoal and helminth parasites of medical and veterinary importance with some promising evidence for use in ticks (Ghosh et al., 2007; Guerrero et al., 2014; Wedrychowicz, 2015; Qian et al., 2016; Tebeje et al., 2016; Noon and Aroian, 2017). Currently, only a single plasmid vector has been approved for human DNA vaccine design (Halstead and Thomas, 2011), and a limited number of DNA vaccines have been commercialized against viral pathogens and cancer treatment (e.g., Oncept®, Merial Inc.) in animals (Wahren and Liu, 2014; Finocchiaro and Glikin, 2017). For metazoan parasite vaccines, however, these types of vaccine strategies appear only to be used for initial antigen screening purposes (e.g., expression library immunization) or as pre-clinical evaluations (as either sole or prime-boost strategies) in spite of the availability of newer technologies (e.g., minimized non-viral vectors) (Hardee et al., 2017). Consequently, these strategies appear to be largely abandoned toward final commercialization in favor of recombinant protein vaccines with proper formulations (Ghosh et al., 2007; Merino et al., 2013; Wedrychowicz, 2015; Qian et al., 2016; Tebeje et al., 2016).

Formulation of Vaccines

In general vaccine development, a major shortfall requiring attention is the need for improvement of vaccine immunogenicity and longevity of the immune responses raised (Sinden, 2017). Adjuvants have been employed extensively in vaccine formulations to: reduce the amount of antigen per dose and number of doses required; induce a more rapid immune response; induce broad antibody responses via expansion of B cell diversity; increase the magnitude and functionality of antibodies produced; improve antigen stability; product safety; improve biodegradability, lower costs by improving effectiveness and ease of use (Mohan et al., 2013; Reed et al., 2013; Chauhan et al., 2017b). And with well over 30 defined adjuvant molecules in use today the choice for co-administration of any adjuvant with a chosen antigen is based on a balance between obtaining a higher level of immunogenicity and lesser side effects in the vaccinated host. Some of the adjuvants developed include water-in-oil (e.g., Montanide™), oil-in-water emulsions (e.g., MEtastiM®, Zoetis Inc.), as well as emulsions containing agonists/ligands such as imidazoquinolines (e.g., R848), synthetic oligodeoxynucleotides (i.e., unmethylated CpG motifs), triterpene glycosides or saponins (e.g., Quil-A, ISCOM, QS-21) and monophosphoryl lipid A (MPLA) derivatives (e.g., glucopyranosyl lipid A or GLA) that can tailor host immune responses (i.e., Th1 or Th2) via Toll-like receptor activation (e.g., TLR 7, 9 and 4) (Reed et al., 2013; Chauhan et al., 2017b).

Though many of these additives have been applied successfully in, for example, cattle subunit vaccines (e.g., R848, CpG, and MPLAs) against systemic pathogens and parasites (Rankin et al., 2002; Jones et al., 2013; Reed et al., 2013; Zhou et al., 2014), limited data is available for their use in ectoparasite vaccines. The current commercial helminth vaccines (including formulations used in clinical trials) have been formulated in glucopyranosyl lipid adjuvant either as a stable emulsion (GLA-SE) (i.e., Sm14 and Sm-p80 or SchistoShield®) or as an aqueous formulation (GLA-AF) in combination with alum (i.e., Sm-TSP-2) (Supplementary Table 1). The glucopyranosyl lipid adjuvant is a Toll-like receptor 4 agonist that promotes a strong Th1 (via cytotoxic T lymphocytes) and a balanced IgG1/IgG2 response in vaccinated hosts (Cauwelaert et al., 2016; Dowling and Mansell, 2016). In contrast, for most promising tick-derived vaccine antigens, oil-based emulsions (e.g., Freund's Complete Adjuvant,

Montanide™ and saponin adjuvants) have been used for vaccine formulations (García-Garcí et al., 2000; Andreotti et al., 2002; Patarroyo et al., 2002; Canales et al., 2009; Almazán et al., 2010, 2012; Hajdusek et al., 2010; Parizi et al., 2011, 2012; Ali et al., 2015; Schetters et al., 2016). The Bm86-based vaccine GAVAC® formulated in Montanide™ 888 adjuvant, provided superior protection in calf vaccine trials in comparison to yeast produced antigen formulated in saponin and could provide a long-during protection (5–6 months) (Supplementary Table 1) (Rodríguez Valle et al., 2001; De La Fuente et al., 2007). Moreover, a recent study provided additional evidence that formulation of cement cone extracts of *Hyalomma anatolicum anatolicum* in Montanide ISA-50 lowered the dose of antigen required to confer protection in vaccinated goats (Iqbal et al., 2016). The latter adjuvants have been shown to stimulate an enhanced cytotoxic T lymphocyte and antibody response in vaccinated cattle hosts (Dar et al., 2013; Chauhan et al., 2017b).

Unfortunately, adjuvants such as Montanide™ and saponin can cause systemic side effects including tissue damage (Chauhan et al., 2017b), where severe inflammation can "trap" antigens at the injection site preventing a proper host immune response, as well as cause carcass trim losses in production animals such as cattle (Van Donkersgoed et al., 1999; Spickler and Roth, 2003). A pen study using heifers vaccinated with Bm86, also showed adverse reactions to Montanide™ water-in-oil emulsions (Petermann et al., 2017). But since Willadsen and colleagues indicated a significant positive correlation between the size of the injection site reaction and the resulting antibody titer (Willadsen et al., 1995), the authors suggested that a visible local reaction could be linked to better protection in animals to gain acceptance by producers (Petermann et al., 2017).

Some examples of conjugation of antigens to effector molecules to increase antigenicity are also available for metazoan parasite vaccines currently in clinical evaluations, as well as commercial production (Supplementary Table 1) (Hussein et al., 2015; Molehin et al., 2016; Tebeje et al., 2016; Noon and Aroian, 2017). Vaccination studies with sea lice showed that pP0 and pP0Cr chimeric antigens fused to the T-cell epitopes of tetanus toxin, including a fusion protein of measles virus within the same gene construct, delivered an increased protection in comparison to peptides conjugated with KLH alone (Mallon et al., 2015). The latter indicates the need for testing potential protective antigens in a variety of experimental layouts and formulations, using available adjuvant compounds, to optimize their utility for metazoan parasite vaccine development.

Finally, microencapsulation of parasite antigens is a new promising alternative (or complementary) technology to conventional adjuvants that is currently being tested. In this regard, a limited number of studies have explored the use of poly(d,l)-lactide-co-glycolide (PLGA) microspheres as a vehicle for antigen delivery and include native adult worm extracts for *B. malayi* (BmA), *S. haematobium* glutathione S-transferase (P28GST) and a *R. microplus* Bm86-derived synthetic peptide (SBm7462) (Sales-Junior et al., 2005; Saini et al., 2013; Thi et al., 2017). Such microencapsulation of antigens in biodegradable polymers can improve the profile (safety, specificity, and efficacy) of a vaccine candidate by mediating efficient cellular delivery (targeting and uptake), afford different routes of administration (by promoting mucosal adhesion, penetration, and retention), and provide immunostimmulatory or modulatory effects (Himly et al., 2017). The polyesters derived from lactic and glycolic acids (PLGAs) present additional advantages such as targeted release and better dosing, antigen protection and formation of benign degradation products, as well as a reduction in antigen quantity required for protection (Lima and Rodrigues Junior, 1999; Himly et al., 2017). More studies are, however, needed using different encapsulation techniques for both endo- and ectoparasites, including protection parameters (such as vaccine efficacies) to assess whether microencapsulation will play a beneficial role in future commercial metazoan vaccine development. An interesting development in the field is the transformation of a vector-borne pathogen to produce vector-associated antigens to act as a dual live vector vaccine (e.g., *B. bovis* producing a *Haemaphysalis longicornis* glutathione-S-transferase) (Oldiges et al., 2016). Though this approach is still in its infancy, it could provide a new paradigm in vector-borne disease transmission management.

CHALLENGES AND FUTURE PERSPECTIVES

With such a wide array of metazoan parasites of human and veterinary importance, the impact on the health and productivity of the afflicted cannot be underestimated. This problem is compounded with the co-habitation of pathogens with vector parasites that facilitate transmission and spread of debilitating diseases within and between human and animal hosts. Moreover, humans and animals can play host to multiple parasite co-infections (so-called multiparasitism). These interactions can be synergistic or antagonistic, producing diverse effects on host susceptibility, infection duration, transmission profile and clinical manifestations (Thumbi et al., 2014; Vaumourin et al., 2015; Ahmed et al., 2017). Protection through vaccination has become a key research focus area for parasite control as an alternative or complementary approach to costly drug development and growing concerns regarding resistance and chemical residues (**Figure 2**) (Yadav et al., 2017). However, much information is still lacking for parasitic arthropods such as flies, fleas, lice and mites, but application of new –omics approaches can potentially expand our knowledge on parasite biology and epidemiology, as well as identify new targets for parasite control and serodiagnostic development. For both human and veterinary vaccines, various socio-political (e.g., access, concerns on safety, regulation, and implementation) and scientific (e.g., pathogen and host diversity, host immunosenescence, etc.) challenges remain and will require more attention for the development of future metazoan parasite vaccines (Sheerin et al., 2017). Economics is also a major factor in the deployment of veterinary medicine in particular, as the cost-to-benefit for resource-poor producers may be limited. Therefore, an integrated parasite management strategy is paramount to limit the need and overuse of treatments (i.e., vaccination and chemical prophylactics) that includes: better management practices; selection and breeding

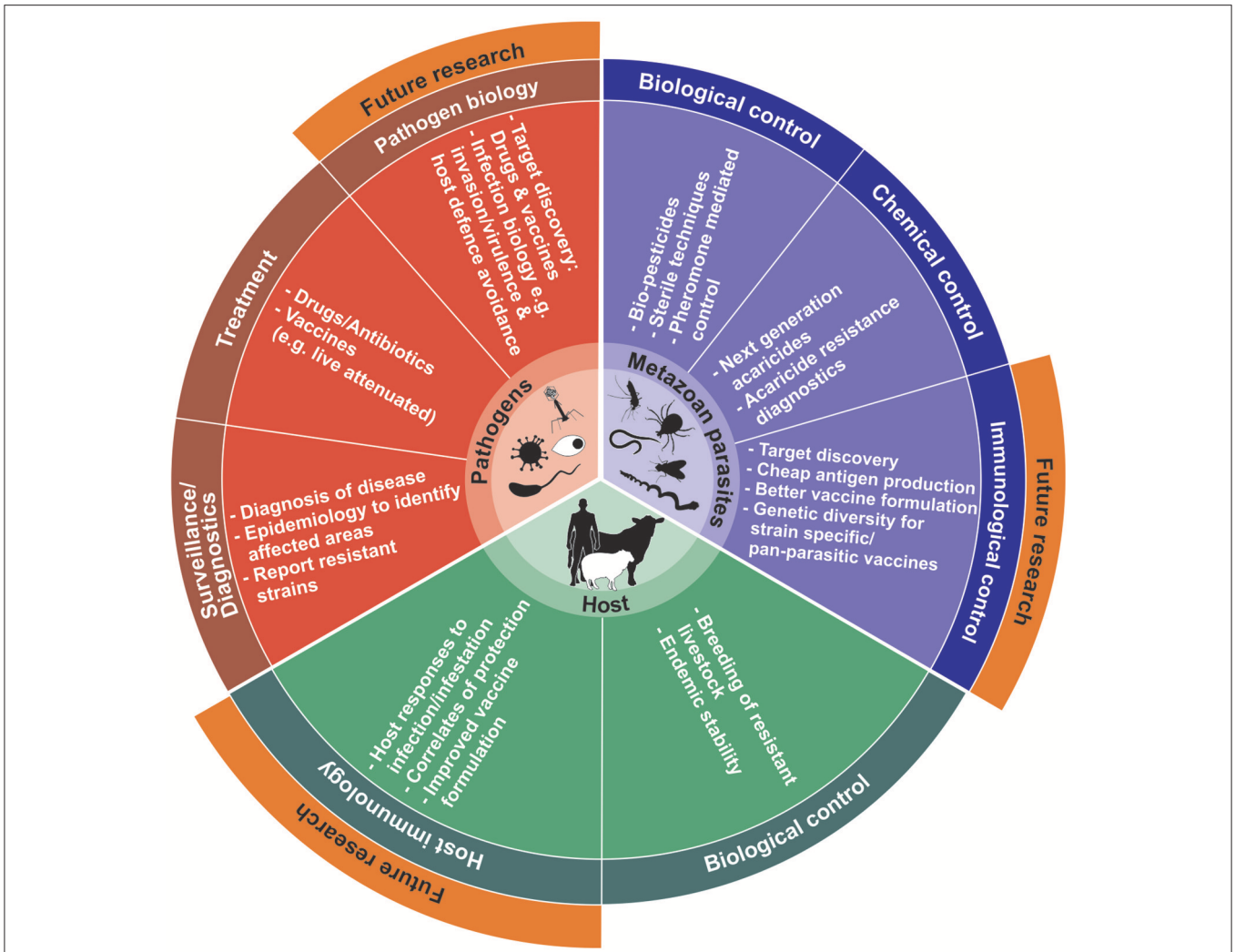

FIGURE 2 | An integrated strategy for parasite and parasite-borne disease control. Several tools are available for management of metazoan parasites (purple), the associated pathogens that can be transmitted (red) and the host animal (green). For metazoan parasite control, pesticides are the most prevalent control measure currently implemented. Resistance to chemical control is a concern that has led to the development of diagnostic tests to identify resistant populations, as well as biological control methods such as bio-pesticides, sterile parasite techniques and pheromones (Yadav et al., 2017). For disease control, treatments are available for most pathogens. Early diagnosis and reports of new and/or resistant pathogenic strains are essential to limit the impact of outbreaks. Some control measures for hosts (human and animal) has involved breeding of resistant stock and/or maintaining endemic stability. Several key areas are currently a priority focus of ongoing research efforts (orange tabs) and these include understanding the host defense against infection/infestation, development of the next generation of vaccines (and diagnostics) and understanding the biology of transmitted pathogens to identify new targets for treatment and diagnosis.

of robust or resistant animals, improved biosecurity to limit host exposure, better intervention through diagnostics and therapy, maintenance of herd immunity, etc. (Rashid et al., 2012; Fitzpatrick, 2013; Vreysen et al., 2013; Maqbool et al., 2017; Robbertse et al., 2017; Tabor et al., 2017) (**Figure 2**).

A combinatorial approach targeting the parasite/pathogen and its arthropod vector via a combination of treatments (including drugs and vaccines), remains a final goal to achieving long-term solutions (Merino et al., 2013; Sinden, 2017). The development of a so-called pan-parasitic vaccine remains hypothetical to date, but if possible can mitigate cost-to-benefit concerns. However, the identification of suitable conserved antigens effective against a multiplicity of metazoan parasitic species is a challenging step and appears to be a product of trial and error. Some proof of principal for such pan-parasitic vaccines have been demonstrated to date for the *Sm*-14, subolesin/akarin and P0 vaccine antigens. As these antigens are all highly conserved intracellular proteins, understanding the mode of action underlying protection via vaccination remains vital. Furthermore, molecular epidemiology and genetic variability studies of parasite populations (i.e., diversity within and between populations) will be needed to assess the conservation of proteins targeted by vaccination and extend vaccine coverage (Sheerin et al., 2017). Moreover, vaccination can influence the life-history traits of pathogens leading to more virulent strains (e.g., *Plasmodium* spp. and viruses) through so-called imperfect

vaccines (Gandon et al., 2001; Read et al., 2015). Though evidence for genetic variation (or diversification) has been observed for many metazoan parasites such as helminths (especially on pesticide resistance), the long-term effects of vaccination on the evolution of metazoan parasite life-history traits are not clear and require further investigation (Kennedy and Harnett, 2013).

As we move into the cosmos of an integrated veterinary and human health paradigm (Yamey and Morel, 2016; Xie et al., 2017), a renewed and concerted effort is needed to create metazoan vaccines that are relatively simple to produce, correctly formulated and structurally stable, containing conserved epitopes for cross-species vaccination, with the right qualities for commercialization and public distribution. Therefore, establishment of cooperatives and consortia (Supplementary Table 2) that combines the skills of a myriad of disciplines (such as economics, mathematics, social sciences, veterinary and medical sciences, chemical and industrial engineering, etc.), as well as buy-in from industry and governmental institutions (including national and international veterinary and human health organizations), is required to address in full the needs of vaccine development in the next millennium.

AUTHOR CONTRIBUTIONS

CS conceived and coordinated the writing of this publication. CS, SR, MF, SB contributed to literature research and writing. CS, SR, CM-O contributed in critical review and revision of the entire manuscript prior to submission.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00067/full#supplementary-material

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