TICKS AND HOST IMMUNITY – NEW STRATEGIES FOR CONTROLLING TICKS AND TICK-BORNE PATHOGENS

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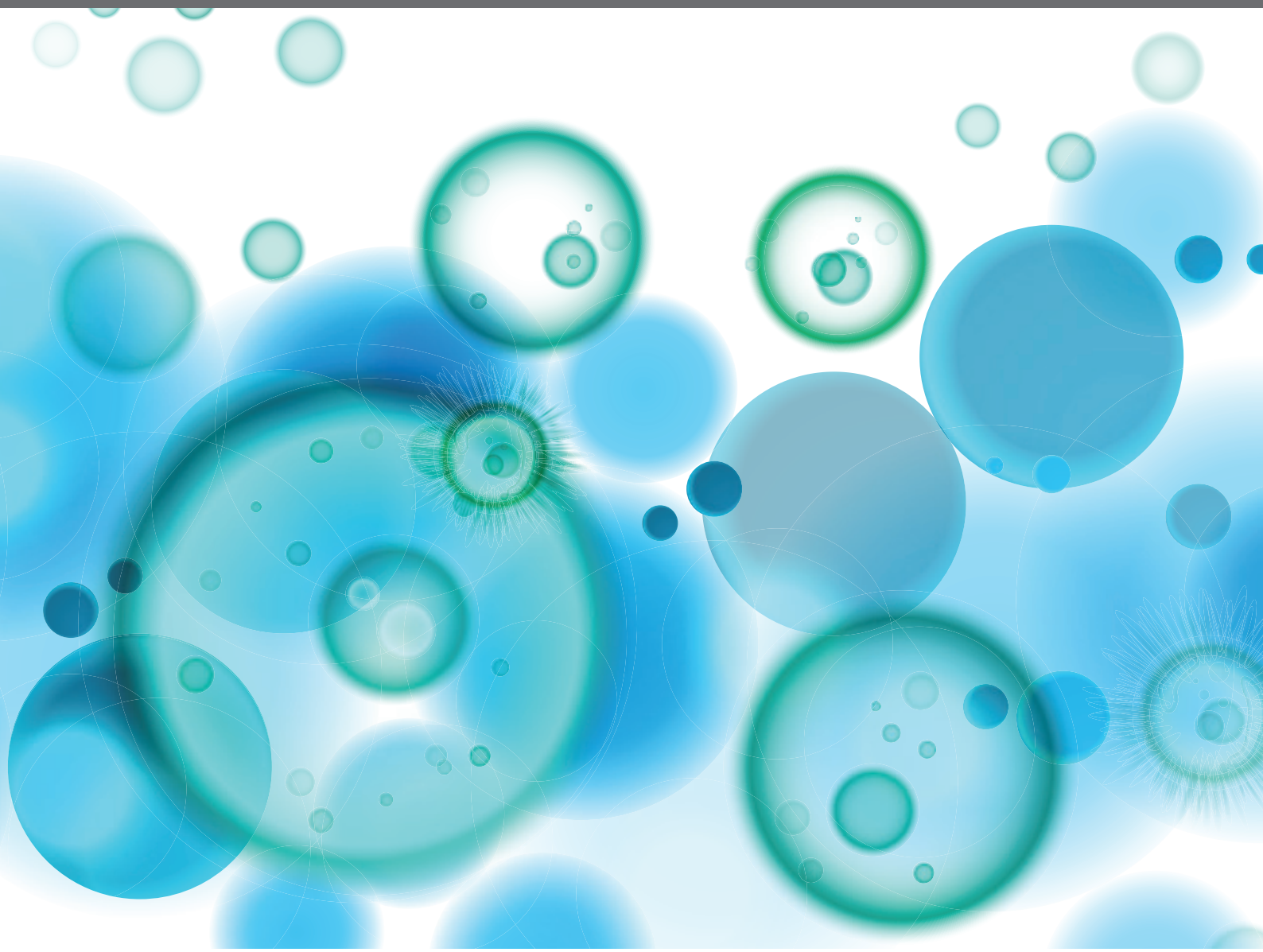

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TICKS AND HOST IMMUNITY – NEW STRATEGIES FOR CONTROLLING TICKS AND TICK-BORNE PATHOGENS

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Table of Contents

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Editorial: Ticks and Host Immunity – New Strategies for Controlling Ticks and Tick-Borne Pathogens

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Editorial on the Research Topic

Ticks and Host Immunity – New Strategies for Controlling Ticks and Tick-Borne Pathogens

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Ticks, as major vectors of human and veterinary diseases, interact with pathogens on two levels: (1) within the tick, where there is a close interaction between the pathogens and the tick's innate immune system, and (2) during transmission when the pathogens take advantage of the tick's saliva to increase virulence. The blood feeding habit of ticks also directly affects the host because the parasitic process results in numerous wounds to the host skin and to complete a blood meal ticks inject relatively large amounts of saliva that contain many pharmacological mediators and, depending on the species of the parasite, even lethal toxins. This Research Topic focuses on the host-tick-pathogen interface, adaptations to different hosts, and how these insights can inform the development of successful vaccines and other sustainable technologies for controlling ticks. The 13 articles of this themed Research Topic highlight the latest discoveries and opinions to strategically address tick and tick-borne disease (TBD) control in the future. **Figure 1** is a representation of the articles described.

Tick loads in cattle have moderate to high levels of heritability, depending on the population studied and the method used to obtain phenotypes. The advent of technologies supporting genome wide association studies has made the genomic selection of cattle for tick resistance a realistic solution. However, Cardoso et al. point out that genomic selection in cattle for tick resistance has been hampered by the difficulty in phenotyping large numbers of animals for the tick loads necessary to obtain the markers needed for this definitive approach. As an alternative, they pooled genotype and phenotype data from reference populations of beef cattle breeds from three countries that were phenotyped with different methods for tick resistance. For most reference populations this approach is feasible for obtaining genomic estimated breeding values, but accurate phenotypes are fundamental to this approach.

In another study with a genetic approach, Jonsson et al. examine if tick loads in cattle were associated with allelic variants for the tyrosine phosphatase receptor type-C (PTPRC) gene (CD45), a cell surface glycoprotein that initiates antigen receptor signaling in lymphocytes. They did not find a significant correlation with this parameter but did find significant associations between variants and total leucocyte counts, red blood cell counts, and IgG antibody responses to extracts of tick tissues. It will be interesting to examine if these parameters affect parasitism downstream from larval

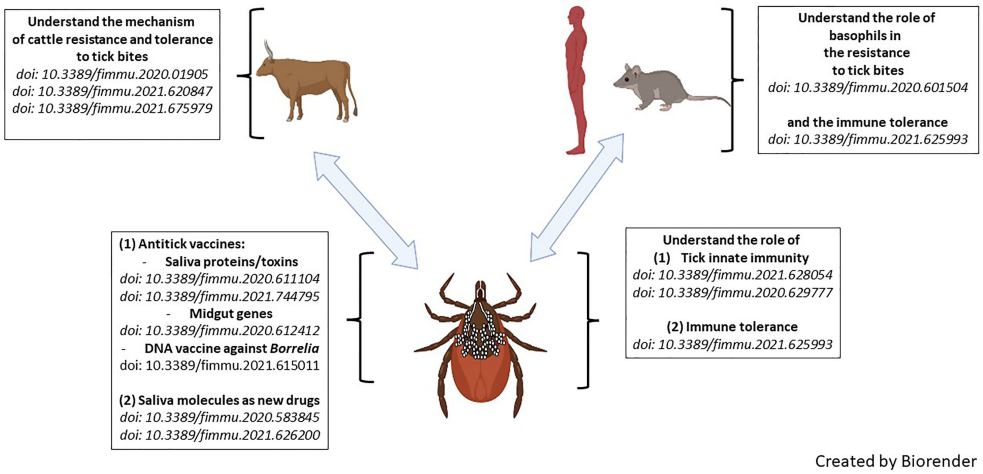

FIGURE 1 | Graphical abstract of articles published in this Research Topic entitled 'Ticks and Host Immunity – New Strategies for Controlling Ticks and Tick-Borne Pathogens'.

attachment and blood feeding, since leukocytes and hemoglobin contain components that are toxic for the tick and may overload the homeostatic mechanisms that deal with them, reflected by damaged females that oviposit smaller egg masses with lower hatching rates.

Ticks are vectors of economically important hemoparasites for cattle. Cavani et al. examine the relation between heritabilities of tick counts and levels of parasitemia caused by one of these hemoparasites, *Babesia bovis*. As the authors argue, heritability estimates for traits related to infectious diseases are low because the manifestation of the phenotypes relies on many gene products. Indeed, the authors found low heritability (0.077) for parasitemia with *B. bovis*. Nonetheless, among the 42 candidate genes identified with the top 10 SNPs, nine participate in pathways of innate and acquired immune responses. Functional confirmation of their roles in resistance and susceptibility to bovine babesiosis is much anticipated, further to research on genomic selection, they can guide the design of other control measures.

A few articles in this Research Topic explore the identification of novel vaccine targets and modifications in the delivery of vaccines. Klouwens et al. compare the results obtained with recombinant outer-surface protein C (OspC) *Borrelia* vaccination using Freund's adjuvants (Complete Freund's for the first boost and Incomplete Freund's for the second and third boosts) and DNA OspC delivered using a tattoo needle gun. The groups were challenged with *Borrelia burgdorferi* ss (causative agent in human Lyme disease in North America) infected *Ixodes scapularis* nymphs and both were protected from *Borrelia* (except for one positive *Borrelia* culture in the vaccinated group) compared to unvaccinated mice (all positive for *Borrelia*). In contrast, DNA vaccinations for *I. scapularis* genes Salp15, tHRF, TSLPI, and Tix-5 in mice failed to protect against *Borrelia* challenge, despite prior demonstrations of efficacy against tick feeding and *Borrelia* transmission as recombinant vaccines.

Amblyomma sculptum is the main tick associated with human bites in Brazil and the vector of Brazilian spotted fever (*Rickettsia rickettsia*). Costa et al. identified three vaccine antigens involved with hematophagy including a Kunitz domain protein, a 'basic tail' protein (PFAM domain TSGP1), and an 8.9 kDa polypeptide protein (Von-Willebrand factor type c domain) previously shown to be common in tick saliva. All three proteins were found to be abundant within *A. sculptum* salivary transcriptomes. Recombinant proteins were found to inhibit the activities of factor Xa, thrombin, and/or trypsin. A mouse immunization trial demonstrated 59.4-85% efficacy against adult female ticks and 70-100% against nymphs.

Rodriguez-Valle et al. described the first anti-venom vaccine that successfully protected immunized dogs from paralysis caused by tick holocyclotoxins (HTs). The family of tick holocyclotoxins was recently described in greater detail thanks to functional genomics of tissues from the Australian paralysis tick, *Ixodes holocyclus*. This milestone, together with commercial anti-paralysis sera, permitted the design of synthetic peptides for immunization studies, including evaluating the need for correct folding, a feature that is common to many anti-toxin vaccines, as found also for HTs. With an octavalent vaccine, the authors also addressed the large HT family to ensure coverage of the sequence variability it presents.

A borreliosis study examined the mid-gut transcriptome of *Ixodes ricinus* ticks during the transmission of *Borrelia afzelii* -the predominant Lyme disease agent in Europe. Mahmood et al. identified 553 upregulated and 530 downregulated tick genes in unfed, 24h fed, and fully fed nymphs, with 5 validated in RNA interference experiments. An uncharacterized protein delayed the infection progress and decreased infection prevalence in mouse tissues. The identification of tick proteins associated

with *Borrelia* transmission or establishment could help to develop novel preventative strategies for Lyme disease.

Tick saliva is also a key element in tick-borne diseases. Due to its impact on the homeostasis and immunology of the vertebrate host, it contributes to the success of the blood meal but also the transmission of pathogens. Many proteins, peptides, lipids, and non-coding RNA molecules have been identified in saliva. Aounallah et al. review the literature and analyze the immune (i.e. complement system, antibody secretion) and physiological (i.e. itching, pain) mechanisms that are controlled by the saliva. Long non-coding RNAs and miRNAs deserve to be investigated more precisely, especially during secretion into the skin *via* exosomes, given their possible involvement in the regulation of host genes. The authors discuss their potential use in different pathologies and applications in therapeutics. Some saliva proteins are particularly well characterized such as Iripin-3, a serine protease belonging to the serpin superfamily (Chlastáková et al.). It acts at different levels on host adaptive immunity but is also involved with coagulation and macrophage proliferation. (Aounallah et al.).

An extensive review examines the successful synergy between ticks and tick-borne pathogens that leads to host immune tolerance. This facilitates successful tick attachment and feeding, which in turn modulates cutaneous and systemic immune defenses, thus allowing the introduction of the pathogen and contributing to successful long-term infection (Boulanger and Wikel). Gaps identified include the current lack of understanding of skin immunity (including microbiome and non-coding RNAs) to tick-borne pathogens to better unravel the complexity of host-pathogen-tick interactions. Once achieved, we could advance the development of strategies to successfully disrupt both tick feeding and pathogen transmission. Other articles in this Research Topic partly examine these gaps (Mahmood et al.). The resistance of the vertebrate host to ticks has also been studied for many years as a potential way to control ticks, notably the role of basophils in the process of the tick bite (Karasuyama et al.). During this process, the tick saliva proteins are taken up by dendritic cells that migrate to the lymph nodes and induce the activation of B and CD4 memory T cells. IgE antibodies are produced that bind to basophils. During a second infestation, memory CD4 T cells present in the skin release IL-3 that activates basophils and the secretion of histamine. The molecule binds to keratinocyte receptors, which proliferate and form a hyperplasia inhibiting the tick bite.

Many aspects of tick innate immunity have been extrapolated from Drosophila innate immunity studies. Fogaça et al. compare innate immunity mechanisms in insects and ticks highlighting disparities between the two models. Humoral immunity relies upon the activation of antimicrobial peptides, redox metabolism, and the complement system, while cellular immunity is mainly composed of hemocytes involved in encapsulation and nodulation. However, other components of immunity that determine vector competence have been less explored. Rosche et al. reviewed the literature concerning two mechanisms of regulation of homeostasis, notably the "Unfolded Protein Response" and the "Integrated Stress Response". These processes are well described in mammals and also in insects when responding to viral infections. As ticks are vectors of numerous viruses, it is also reasonable to question the presence of these processes in ticks.

In summary, although major progress has been made in the knowledge of host resistance mechanisms to tick bites, the innate immunity of ticks to pathogens, and the identification of tick saliva molecules essential in the transmission of infectious agents, in large part due to the use of "omics" technologies, many questions remain unanswered and require a multidisciplinary approach to better control ticks and TBDs.

AUTHOR CONTRIBUTIONS

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Genomic Study of *Babesia bovis* Infection Level and Its Association With Tick Count in Hereford and Braford Cattle

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Bovine babesiosis is a tick-borne disease caused by intraerythrocytic protozoa and leads to substantial economic losses for the livestock industry throughout the world. *Babesia bovis* is considered the most pathogenic species, which causes bovine babesiosis in Brazil. Genomic data could be used to evaluate the viability of improving resistance against *B. bovis* infection level (IB) through genomic selection, and, for that, knowledge of genetic parameters is needed. Furthermore, genome-wide association studies (GWAS) could be conducted to provide a better understanding of the genetic basis of the host response to *B. bovis* infection. No previous work in quantitative genetics of *B. bovis* infection was found. Thus, the objective of this study was to estimate the genetic correlation between IB and tick count (TC), evaluate predictive ability and applicability of genomic selection, and perform GWAS in Hereford and Braford cattle. The single-step genomic best linear unbiased prediction method was used, which allows the estimation of both breeding values and marker effects. Standard phenotyping was conducted for both traits. IB quantifications from the blood of 1,858 animals were carried using quantitative PCR assays. For TC, one to three subsequent tick counts were performed by manually counting adult female ticks on one side of each animal's body that was naturally exposed to ticks. Animals were genotyped using the Illumina BovineSNP50 panel. The posterior mean of IB heritability, estimated by the Bayesian animal model in a bivariate analysis, was low (0.10), and the estimations of genetic correlation between IB and TC were also low (0.15). The cross-validation genomic prediction accuracy for IB ranged from 0.18 to 0.35 and from 0.29 to 0.32 using k-means and random clustering, respectively, suggesting that genomic predictions could be used as a tool to improve genetics for IB, especially if a larger training population is developed. The top 10 single nucleotide polymorphisms from the GWAS explained 5.04% of total genetic variance for IB, which were located on chromosomes 1, 2, 5, 6, 12, 17, 18, 16, 24, and 26.

Some candidate genes participate in immunity system pathways indicating that those genes are involved in resistance to *B. bovis* in cattle. Although the genetic correlation between IB and TC was weak, some candidate genes for IB were also reported in tick infestation studies, and they were also involved in biological resistance processes. This study contributes to improving genetic knowledge regarding infection by *B. bovis* in cattle.

Keywords: babesiosis, cross-validation, genetic parameters, genomic selection, genome-wide association studies

INTRODUCTION

Bovine babesiosis is a tick-borne disease caused by intraerythrocytic protozoa of the *Babesia* genus leading to substantial economic losses for the livestock industry throughout the world (1–3). In Brazil, bovine babesiosis is caused by *Babesia bovis* and *Babesia bigemina*, which are exclusively transmitted by the one-host tick *Rhipicephalus microplus* (4, 5). *B. bovis* is the most pathogenic species (6). The infective forms, which are in tick saliva, invade the host's erythrocytes, multiply until hemolysis, and invade new erythrocytes until the host dies or develops immunity (7). Calves have an innate age-related resistance to babesiosis. Thus, in regions where there is endemic stability, calves are exposed to babesiosis and develop immunity to the disease (6). On the other hand, in regions of endemic instability, where climatic conditions prevent the survival of ticks during a certain period of the year, calves may not be infected when they are young, and outbreaks of babesiosis may occur when ticks reinfest the pastures (8).

Bock et al. (9) and Jonsson et al. (10) observed that zebu (*Bos taurus indicus*) were more resistant to *B. bovis* than taurine (*Bos taurus taurus*) breeds. More recently, the levels of *B. bovis* infection in bovine blood samples have been successfully quantified through quantitative PCR (qPCR) assays (11–14). Differences in these levels were observed between zebu (*B. taurus indicus*) and taurine (*B. taurus taurus*) breeds (12), corroborating previous findings. Phenotypic variation of the level of *B. bovis* infection has been reported (12, 15). However, no quantitative genetic studies have been found in the literature, and little is known regarding its association with tick resistance.

Advances in molecular genetic techniques have allowed the incorporation of genetic markers such as single nucleotide polymorphisms (SNPs) into the breeding analysis, enabling earlier accurate predictions (16). Genomic selection is a powerful strategy to increase the rate of genetic gain, especially in traits where selection based on phenotypic records is difficult, such as disease resistance traits and low heritability traits (17, 18). Cardoso et al. (19) showed that it is possible to control tick infestation through the genomic selection of tick-resistant animals. Therefore, the genetic improvement could be an important tool for *B. bovis* infection control. Moreover, using SNP information allows the detection of genomic regions associated with *B. bovis* infection level through genome-wide association studies (GWAS) (20) and, thus, contributes to a better understanding of the genetic basis of this economically important and complex trait. Regarding a tick resistance trait in cattle, many studies have identified genomic regions through association studies (21–26).

The objective of this study was to estimate the genetic correlation between *B. bovis* infection level (IB) and tick count (TC), evaluate predictive ability and application of genomic selection, and perform GWAS for IB in Hereford and Braford cattle to better understand the biological mechanisms underlying IB and its association with tick resistance.

MATERIALS AND METHODS

Phenotype Data

The data set was provided by the Delta G Connection breeding program (Gensys Associated Consultants, Porto Alegre, RS, Brazil), which included Hereford and Braford cattle raised on pastures in southern Brazil. The Braford breed is a combination of 3/8 indicine breeds and 5/8 Hereford; however, in Brazil, the breeders are allowed to vary the relative proportion of the component breeds. In addition to phenotypic records on IB and TC, pedigree information for the last three generations and genotype data were included. A total of 5,867 (1,915 Hereford and 3,952 Braford) animals provided TC records, and 1,858 animals (225 Hereford and 1,633 Braford) provided IB records, between the years 2010–2013. The average age of the animals during the evaluation period was 17.5 months (10.9–23.1 months).

Babesia bovis Infection Level

The IB was assessed by determining the number of copies of *B. bovis* target DNA sequence (cytochrome b gene). For that, DNA was extracted from blood samples of each animal collected on FTA cards using the Gensolve DNA recovery kit (Gentegra, Pleasanton, CA, USA). The concentration and quality of this DNA were determined in a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, Delaware, USA). The DNA samples were kept at −80°C until further analysis. After that, the qPCR was performed using the CFX^{TM} Real-Time PCR Detection System (BioRad, Hercules, CA, USA), according to Giglioti et al. (14). The primers cbosg 1 (F): 5′ -TGTTCCTGGAAGCGTTGATTC-3′ and cbosg 2 (R): 5′-AGCGTGAAAATAACGCATTGC-3′ amplify an 88-bp fragment from the cytochrome b gene of *B. bovis* (11, 27). The standard curves were plotted using 10-fold dilutions of synthetic DNA gBlocks® Gene Fragments (IDT, Coralville, IA, USA), which contain known concentrations of the *B. bovis* target sequence. To

estimate the number of copies of the target DNA sequence (NC), the samples and controls were submitted to qPCR tests together with dilutions of synthetic DNA gBlocks®. Then, using software native to the CFX96 system, NC values for dilutions were utilized as a reference for the estimation of NC in the samples. Default settings were used for all parameters, except for the threshold line that was set to the same value, at 200 relative fluorescence unit, for all qPCR tests. All samples and controls were tested in duplicate, and samples with a standard deviation >0.5 were retested. Samples presenting NC > 0 and specific temperature melting (Tm) ($77.5 \pm 0.5^{\circ}$C) were considered positive.

Tick Count

The animals were naturally exposed to ticks, and after weaning, when the visual estimate of the average infestation across all animals in a management group (animals raised together, receiving the same feeding and sanitary management) exceeded about 20 engorged female ticks, counts were performed by manual counting adult female ticks (4.5–8 mm in length) on the right side of each animal (28). This process was carried out one to three times in each management group. Tick counts were performed in late spring, summer, and early fall, and the minimum period between counts was 30 days.

For the analyses, the IB and TC records were transformed in log10(x + 1) due to normality assumptions of the models used in this study. The descriptive statistics are shown in **Table 1**.

Genotype Data

A total of 4,496 animals were genotyped with the Illumina BovineSNP50 BeadChip (50K; Illumina, San Diego, CA). Genotype quality control was performed using the R *snpStats* package (29) to remove samples with a call rate < 0.90, heterozygosity 3.0 SD above or below the observed mean, mismatching sex, and duplicated records. Only SNPs mapped to the autosomes, with call rates > 0.98, minor allele frequencies > 0.03, and not in a highly significant deviation from the Hardy–Weinberg equilibrium ($P > 10^{-7}$), were considered for the analyses. Additionally, only the SNP with the highest minor allele frequency was retained when two SNPs were highly correlated ($r > 0.98$). After quality control, 39,919 SNP markers and 4,388 samples remained for the statistical analysis.

Statistical Models

The genetic parameter estimations for IB and TC were performed by Bayesian inference in a bivariate analysis using an animal model. The Bayesian approach was chosen because the sample size was not large, and, to our knowledge, this is the first quantitative genetic study for IB, which is a new phenotype. The advantage of Bayesian methods, in this case, is that interpretation of the results and uncertainties about the estimates are facilitated, as all results are presented in terms of probabilities (30).

The model can be represented as follows:

$$\mathbf{y}=\mathbf{X}\boldsymbol{\beta}+\mathbf{Z}_1\mathbf{a}+\mathbf{Z}_2\mathbf{p}+\mathbf{e}$$

with the joint distribution of vectors **a**, **p**, and **e** as:

$$\begin{bmatrix}\mathbf{a}\\ \mathbf{p}\\ \mathbf{e}\end{bmatrix} \sim N\left\{\begin{bmatrix}0\\0\\0\end{bmatrix}, \begin{bmatrix}\mathbf{G_0}\otimes\mathbf{H} & 0 & 0\\ 0 & \mathbf{P_0}\otimes\mathbf{I} & 0\\ 0 & 0 & \mathbf{R_0}\otimes\mathbf{I}\end{bmatrix}\right\},$$

where **y** is a vector of observations; **β** is a vector of systematic (fixed) effects; **a** is a vector of random additive genetic direct effects; **p** is the vector of random permanent environmental effects; **e** is a vector of random residuals; **X**, $\mathbf{Z_1}$, and $\mathbf{Z_2}$ are known incidence matrices; $\mathbf{G_0}$ and $\mathbf{P_0}$ are the additive genetic direct and environmental permanent effects (co)variance matrices, respectively; **H** is the additive genetic relationship matrix; $\mathbf{R_0}$ is the residual (co)variance matrix; and **I** is an identity matrix with suitable dimensions. Only the permanent environmental effects were included in the model for TC; therefore, **p**, $\mathbf{Z_2}$, and $\mathbf{P_0}$ were not considered for IB.

The **H** matrix combines genotype and pedigree information (31, 32), and its inverse ($\mathbf{H^{-1}}$) can be described in matrix notation as:

$$\mathbf{H}^{-1} = \mathbf{A}^{-1} + \begin{bmatrix}0 & 0\\ 0 & \mathbf{G}^{-1}-\mathbf{A}_{\mathbf{22}}^{-1}\end{bmatrix},$$

where **G** is a genomic relationship matrix constructed as in VanRaden (33) using current allele frequencies, and $\mathbf{A_{22}}$ is a numerator relationship matrix only for genotyped animals.

Concerning the systematic effects, contemporary groups (CGs) were included for IB and TC, as well as the effect of racial composition (zebu proportion, heterozygosity, and recombination loss computed from pedigree information). Linear and quadratic effects of animal age were considered only for TC. For IB, the linear effect of total DNA concentration available for qPCR assays was considered. The CG was composed of the animal from the same farm, sex, year and season of birth, and management group. For TC, the date of the phenotypic evaluations was also included in the CG. CGs with less than three observations were excluded from the data set. The total numbers of CGs for IB and TC were 15 and 227, respectively.

TABLE 1 | Descriptive statistics for *Babesia bovis* infection level (IB) and tick counts (TC) in Braford and Hereford cattle.

Traits	N[a]	Mean[b]	SD[b]	Minimum	Median[b]	Maximum[b]
IB	1,858	719.9 (1.6)	5,920.68 (1.14)	0	79.2 (1.9)	154,199.5 (5.2)
TC	13,874	38.9 (1.4)	48.46 (0.47)	0	25 (1.4)	600 (2.8)

[a] *Number of records.*

[b] *Log-transformed scale data is shown in parentheses.*

The GIBBS2F90 program (34) was used to obtain samples from the posterior distributions of genetic, permanent environmental, and residual (co)variances. A Gibbs sampling chain with 500,000 samples was generated, with the initial 50,000 samples discarded as burn-in based on visual inspection of trace plots and the convergence tests of Gelman and Rubin (35) and Geweke (36) as well as of Heidelberg and Welch (37) using the *coda* package (38) of the R software (39). The posterior distributions of the variance and covariance components were approximated based on the remaining 450,000 samples.

Genomic selection and GWAS were performed just for IB using the single-step genomic best linear unbiased prediction (ssGBLUP) approach, and the effects considered in the model were the same as previously described for the IB trait. The ssGBLUP method allows estimation of both breeding values and marker effects and combines genomic and pedigree relationships using the **H** matrix, as described earlier.

To estimate the genomic selection accuracy, cross-validation was applied. Furthermore, two animal grouping methods were used: k-means and random. More details are described later.

The predictive ability of genomic selection for IB was assessed by cross-validation, where 1,855 animals (1,631 Braford and 224 Hereford) with genotypes and IB phenotypes were divided into three groups by two strategies using R software (39). The strategies to divide the groups were k-means clustering of marker relationship distances and replicated 10 times at random. Average genomic relationships of each animal with all others within and between groups were calculated to characterize relatedness between training and validation sets (40). For each grouping strategy, 3-fold cross-validations were performed by alternately using records of two groups as training sets to derive genomic predictions for the third (validation) group, whose data were omitted in the analyses for marker effect estimation. Prediction accuracies, within a cluster *c*, were estimated as the correlation between predicted ($\hat{\mathbf{a}}$) and estimated true ($\mathbf{a}$) breeding values ($\hat{r}_{\mathbf{a}\hat{\mathbf{a}}c}$), as proposed by Legarra et al. (41):

$$\hat{r}_{\mathbf{a}\hat{\mathbf{a}}c} = PA / \sqrt{h^2}$$

in which PA is the predictive ability defined as the correlation between IB of animals from group *c* adjusted for the fixed effects and predicted values from cross-validation, represented by direct genomic value. Moreover, h^2 is the heritability for IB.

The GWAS for IB were performed using the method proposed by Wang et al. (42), which is based on the ssGBLUP. The effects of the SNPs ($\hat{\mathbf{u}}$) were obtained using the equation described as:

$$\hat{u} = \lambda \mathbf{D}\mathbf{Z}'\mathbf{G}^{*-1}\hat{\mathbf{a}}_{\mathbf{g}}$$

where $\hat{\mathbf{u}}$ is the vector of estimated SNP effects; λ is the variance ratio calculated according to VanRaden et al. (43); $\hat{\mathbf{a}}_{\boldsymbol{g}}$ is the animal effect of genotyped animals; $\mathbf{Z}'$ is a transpose matrix that relates the genotypes of each locus; $\mathbf{G^*}$ is the weighted genomic relationship matrix; **D** is a diagonal matrix of the weights of SNP variances obtained by the algorithm with the following steps, where *t* is an iteration number, *p* is the allele frequency of the second allele, and *i* is the *i-th* SNP:

1. $t = 0$; $\mathbf{D}_{(t)} = \mathbf{I}$; $\mathbf{G}_{(t)} = \mathbf{Z}\mathbf{D}_{(t)}\mathbf{Z}'\lambda$
2. Compute $\hat{\mathbf{a}}_{\boldsymbol{g}}$ by ssGBLUP;
3. Calculate $\hat{\mathbf{u}}_{(t)} = \lambda \mathbf{D}_{(t)}\mathbf{Z}'\mathbf{G}^{-1}_{(t)}\hat{\mathbf{a}}_{\boldsymbol{g}}$;
4. Calculate the weight for each SNP: $d^*_{i_{(t+1)}} = \hat{u}^2_{i_{(t)}}\, 2p_i(1 - p_i)$ (44);
5. Normalize $\mathbf{D}_{(t+1)} = (\mathrm{tr}(\mathbf{D}_{(0)})/\mathrm{tr}(\mathbf{D^*}_{(t+1)}))\mathbf{D^*}_{(t+1)}$;
6. Calculate $\mathbf{G}_{(t+1)} = \mathbf{Z}\mathbf{D}_{(t+1)}\mathbf{Z}'\lambda$;
7. Loop to step 3 for 3 times.

The analyses were carried out using the BLUPF90 family of programs (34). The results of GWAS are reported as the proportion of variance explained by a single SNP. A Manhattan plot was created using the R package "ggplot2" (45). Once the SNPs that explain the largest amount of IB genetic variance were identified, they were assigned to the candidate genes. The candidate genes were identified through the Ensembl genome database project, available at https://useast.ensembl.org/index.html, based on the *Bos taurus* ARS.120 reference assembly. For that, the genomic coordinates were expanded by 500 kb upstream and downstream; in this sense, an SNP was assigned to a candidate gene if it was located within or near to the gene. Gene ontology and biological pathway annotations of the genes were retrieved using the biomaRt package (46) and Reactome Pathway Knowledgebase (47), respectively.

RESULTS AND DISCUSSION

Genetic Parameters

Estimates of heritability and repeatability for TC were low (0.127) and moderate (0.267), respectively (**Table 2**). The proportion of phenotypic variance explained by genetic variance of TC evaluated in the population of Hereford and Braford cattle was lower than other studies in Brazil with the same breeds that reported a heritability of 0.19 (19, 48). These differences between estimated heritability can be explained by population sample differences and by the model. The main model difference is the matrix relationships; in this study, we used genomic information (SNPs) to build the matrix relationships (H matrix). Lower

TABLE 2 | Posterior mean and 95% highest posterior density intervals (within parentheses) of (co)variance components for *Babesia bovis* infection level (IB) tick counts (TC), and genetic correlation between IB and TC in Braford and Hereford cattle performed by Bayesian animal model in bivariate analysis.

Parameter	IB	TC
Additive genetic variance, σ^2_a	0.088 (0.040, 0.141)	0.012 (0.009, 0.016)
Permanent environmental variance, σ^2_p	–	0.014 (0.010, 0.071)
Residual variance, σ^2_e	1.048 (0.168, 0.890)	0.072 (0.070, 0.074)
Heritability, h^2	0.077 (0.037, 0.124)	0.127 (0.093, 0.160)
Repeatability, r^2	–	0.267 (0.245, 0.289)
Genetic correlation, $r_{IB,TC}$	0.152 (−0.147, 0.445)	

estimates of genetic variance based on genomic relationships compared with those using pedigree relationships may occur; however, the estimates based on genomic relationships are frequently more accurate (49). Further, higher heritabilities would be expected in experimental stations' environmental conditions, as shown by Burrow (50), who reported a heritability of 0.42 for TC in a composite breed, as the conditions of the collection are more controlled. In the present study, the tick counts were carried out through collection by several technicians, and although all were trained for collection, this can be a factor in increasing experimental error. From this perspective, Ayres et al. (51) estimated heritabilities of 0.12 and 0.11 for TC in Brazilian Nellore × Hereford crossbred cattle, and Budeli et al. (52), in South African Bonsmara breed cattle, found heritabilities ranging from 0.03 to 0.17 in groups of animals divided according to mean tick count.

Despite the low posterior mean for IB heritability (0.077), there is additive genetic variability for this trait, and therefore, selection responses may be obtained. No previous information on quantitative genetics for IB was found in the literature; however, low to moderate values for repeatability of IB in Angus (13) and Canchim (14) have been reported. Usually, the heritability estimates for disease traits are low mainly because of the complexity behind these phenotypes (53, 54). Moreover, the genetic correlation between IB and TC was weak (0.152). This suggests that selection for TC would not change IB considerably in the population of this study. Although no quantitative genetic studies were found for levels of babesiosis infection in cattle, Giglioti et al. (14) found the phenotypic correlation between tick count and *B. bovis* levels ranging from 0.02 to 0.17, in different ages. It is important to note that the number of records for IB is much lower than for TC; besides, IB is a new phenotype related to a disease.

Genomic Selection for *Babesia bovis* Infection Level

The k-means clustering yielded three unbalanced groups with 830, 770, and 255 animals in groups 1, 2, and 3, respectively (**Table 3**). A multidimensional scaling bidimensional scatter plot according to the k-means groups is presented in **Figure 1**. Groups 1 and 2 were composed mainly by Braford breed with an average of 35% zebu contribution, whereas group 3 contained primarily Hereford breed (11% zebu contribution). As expected, the average genomic relationship was larger within than between groups. The average number of animals for the groups divided at random replicated 10 times was 618.33 animals (74.67 ± 6.18 Hereford, 12.33 ± 3.36 1/2 Braford, 496.67 ± 6.51 3/8 Braford, and 34.67 ± 3.51 1/4 Braford). Random groups had a similar average genomic relationship within groups (0.011 ± 0.045), and between groups, with the average close to zero.

The accuracy of prediction for groups divided at random was higher than k-means clustering for groups 1 and 2 (**Table 4**). The groups generated by the k-means method had a larger number of crossbred animals, mainly in group 1 (800 animals of Braford breed, **Table 3**). For group 3, the accuracy of the predictions for k-means clustering and random methods was almost the same (0.3). Although, to our knowledge, there is no published genomic predictive study for *B. bovis* in cattle, the superiority of prediction accuracy using random clustering compared with k-means was also observed by Bock et al. (19) for tick resistance in

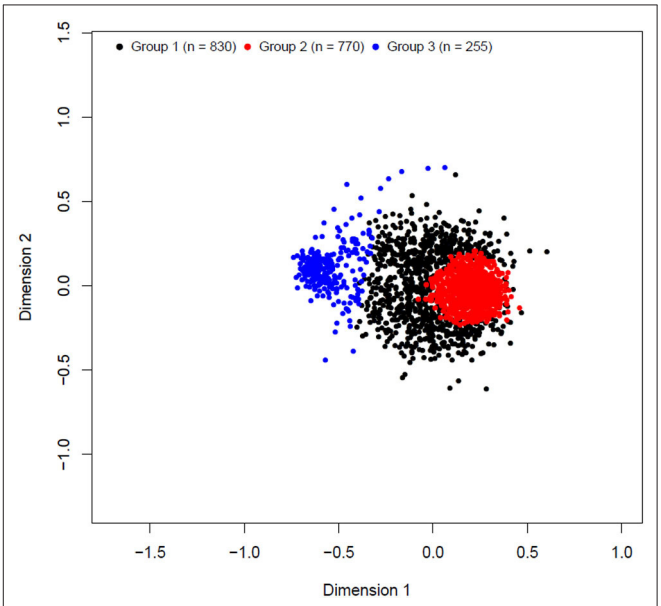

FIGURE 1 | Multidimensional scaling bidimensional scatter plot of k-means clustering cross-validation groups.

TABLE 3 | Number of individuals (N) and averages (±SD) of genomic relationship (*Gij*) within and between-group of Hereford breed and Braford composition breed for k-means clustering groups.

Groups; *N*	Hereford	Braford			*Gij* within group	*Gij* between group
		1/2[a]	3/8[a]	1/4[a]		
1; 830	30	18	734	48	0.009 ± 0.035	0.000 ± 0.030
2; 770	30	18	696	26	0.054 ± 0.042	−0.008 ± 0.049
3; 255	164	1	60	30	0.070 ± 0.054	−0.003 ± 0.056

[a]*Zebu proportion.*

TABLE 4 | Prediction accuracy of direct genomic value predictions for each k-means clustering and random cross-validation group using the ssGBLUP method for *Babesia bovis* infection level.

	Prediction accuracy		
	Group 1	**Group 2**	**Group 3**
k-means	0.180	0.225	0.346
Random	0.293	0.317	0.316

Braford and Hereford cattle. Based on these results, the number of animals used for training the model is more important to achieve better prediction accuracy than the difference between the breed composition of group 3 and the other two groups. This is in agreement with a previous study that reported that a large number of animals in the training population is an important factor that improves the accuracy of genomic selection (18). The lower number of animals could explain the low accuracy for group 1 under k-means clustering in the training population and also the larger genetic relationship distance of groups 2 and 3. Accuracies of genomic predictions are related to the training data size and genetic relatedness between training and validation individuals (55, 56). Furthermore, the low IB heritability (**Table 2**) could be a determinant to the low prediction accuracies found in this study, as genomic selection reliability also depends on trait heritability (57). In this same population, Sollero et al. (58) found much higher accuracy for tick resistance (0.27 to 0.44), even when a specific SNP panel for TC with very low density was used. In dairy cattle, some traits related to resistance to infectious diseases have already been included in genetic evaluations and selection programs using genomic data (59), for example, the overall immune response (60, 61) that has higher heritability and prediction accuracy than results in the present study and the incidence of clinical mastitis (62) showing accuracies and heritability values for the predicted breeding values comparable with those found here. Other traits such as *Mycobacterium avium* subspecies paratuberculosis infection (63) or bovine respiratory disease in Holstein (64) also present low heritability and predictive accuracy. Despite low accuracy, genomic predictions could still be used as a viable tool to obtain a selection response for IB for replacement candidates. However, the expected genetic progress for this trait would be slow.

Genome-Wide Association Studies for *Babesia bovis* Infection Level

Figure 2 shows the Manhattan plot with the percentages of additive genetic variance explained by each SNP for the IB trait. The top 10 SNPs (**Table 5**) explained 5.05% of IB additive genetic variance and identified 42 candidate genes involved in biological mechanisms that may underlie *B. bovis* resistance in cattle. Defense against parasites is mediated by sequential and coordinated immune responses called innate and adaptative (65), and several of the candidate genes participate in immune system pathways (*ATP8A1*, *LCP1*, *LRCH1*, *QSOX1*, *FGF2*, *DSC1*, *DSC3*, *FGFR2*, and *CEBPG*), which include adaptive and innate immune systems, and cytokine signaling pathways, indicating that genetic variations in these genes can alter the immune response and consequently, influence susceptibility and outcome of babesiosis in cattle. An essential aspect of *B. bovis* infection is that young calves have strong innate immunity, which lasts until about 6 months of age (66). Animals that survive infection with *B. bovis* become persistently infected and resistant to the clinical form of the disease, a phenomenon known as concomitant immunity (67). Adaptative immunity mechanisms are responsible for the absence of clinical signs in persistently infected animals.

LCP1 is a protein of the plastin family. This family is composed of actin-binding proteins that are conserved evolutionarily and expressed in different types of plants and animals (68). In mammals, three isoforms have been identified: T, I, and L-plastin. This latter group includes LCP1 that is expressed in hematopoietic cell lines, with essential functions in the activation of macrophages (69), lymphocytes, and granulocytes (70). According to Brown (71), the immune response against babesiosis depends on the activation of CD4+ T lymphocytes in the development of acquired protein antigen-specific responses. CD4+ T cells are essential for coordinating high-affinity IgG production and activating macrophages through the production of IFN-ɤ.

LRCH1 also encodes proteins that influence the migration of CD4+ T cells (72). These cells play a regulatory role in the immune response and result in higher resistance to *R. microplus* in cattle, although other genes have been reported as mediators for T cell regulation (73, 74). Piper et al. (75) reported that Brahman animals (*B. indicus*) had higher percentages of T cells than did the Holstein–Friesians (*B. taurus*). Constantinoiu et al. (76) observed an increase of T cells in the skin around the site of *R. microplus* larvae attachment in both *B. indicus* and *B. taurus* cattle. Moré et al. (23) observed the participation of CD4+ T cell subtypes in Braford animals classified as tick-resistant. Another type of cell that influences the immune system is the B cell, which plays a role in the humoral immunity component of the adaptive immune system by secreting antibodies (77). The B cells are activated by the proteins encoded by *FGF2* and *FGFR2* genes through the signaling process, and *CEBPG* genes are involved in B cell differentiation. An increase of B lymphocytes in the dermis of tick-resistant cattle breeds was also observed, and differential B-lymphocyte regulation in lymph node tissue was associated with tick susceptibility (78).

The *FGF2* and *FGFR2* genes are involved in interleukins, fibroblast growth factor receptor, cytokine, and MAPK signaling pathways. The *SPRY1* gene also participates in fibroblast growth factor receptor and MAPK signaling pathways. Inflammatory interleukins, growth factors, and cytokines activate the MAPK signaling pathway, which regulates the immune response against intracellular parasites (79, 80). Moreover, cytokines stimulate natural killer cells to produce interferon-gamma (IFN-γ) during the chronic phase of *B. bovis* infection. The IFN-γ activates macrophages that synthesize and release nitric oxide, which inhibits *B. bovis* replication (81–84). The *CEBPG* gene also influences the natural killer cell process.

DSC1, *DSC2*, and *DSC3* are involved in the keratinization pathway and have been reported in tick resistance studies.

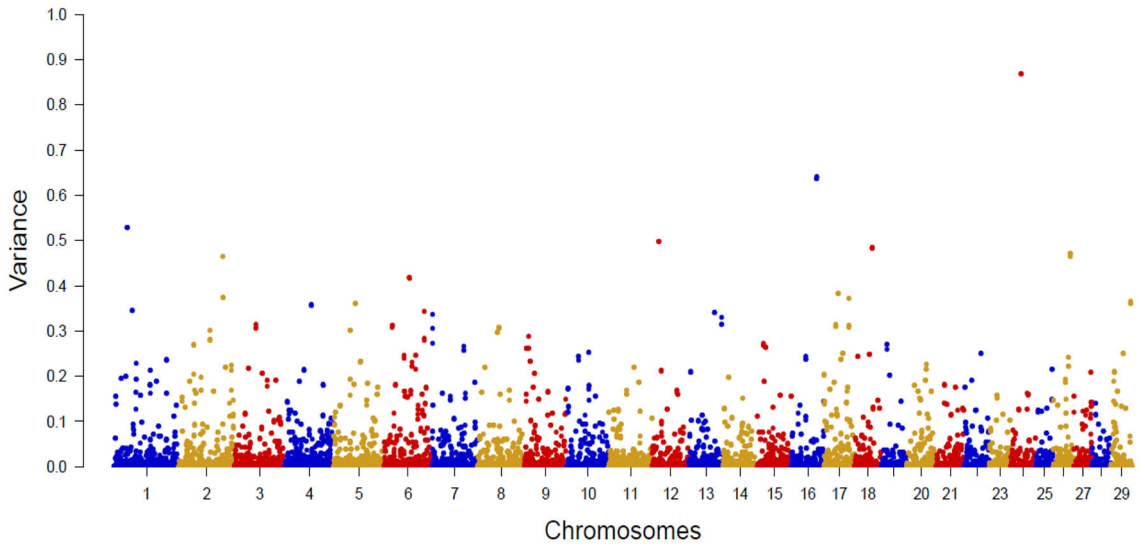

FIGURE 2 | Manhattan plot of additive genetic variance explained by single SNP for *Babesia bovis* infection level.

TABLE 5 | Description of the SNPs with the largest effects on *Babesia bovis* infection level in Hereford breed and Braford composition breed.

BTA	SNP position	Genes[a]	Var (%)
24	26,698,515	*DSC1, DSC2, DSC3*	0.853
16	62,734,784	*CEP350, QSOX1, LHX4, ACBD6, TOR1AIP1, TOR1AIP2, FAM163A*	0.640
1	32,883,377	*CADM2*	0.548
12	16,641,931	*LRCH1, ESD, HTR2A, RUBCNL, LRRC63, LCP1*	0.508
18	44,019,061	*PEPD, CEBPG, CHST8, KCTD15, SLC7A10, LRP3, WDR88, GPATCH1, FAAP24*	0.487
26	42,178,883	*ATE1, NSMCE4A, TACC2, BTBD16, FGFR2*	0.479
6	62,979,121	*ATP8A1, SHISA3, BEND4, SLC30A9, TMEM33, GRXCR1*	0.408
2	109,327,881	*Intergenic region*	0.377
17	34,752,485	*SPRY1, SPATA5, NUDT6, FGF2*	0.377
5	53,704,130	*SLC16A7*	0.367

[a]*Genomic coordinates for each gene based on the Bos taurus ARS.120 reference assembly were expanded by 500 kb upstream and downstream.*
BTA, Bos taurus autosome; Var, proportion of additive genetic variance explained by the single SNP.

Terminal differentiation of keratinocytes is important for the renewal of the stratum corneum, which plays an essential role in defense against the pathogen (85). According to the authors, tick bite lesions led to an increase of keratinocyte differentiation and the promotion of stratum corneum formation. Wang et al. (86) suggested that a dramatic reduction in keratin transcripts may occur in response to tick infestation. Also, *DSC1*, *DSC2*, *DSC3*, and *CADM2* genes participate in the cell adhesion process, which plays a critical role in initiating and sustaining the immune response against foreign pathogens (87). Piper et al. (88) reported that *DSC2* was detected as differentially expressed between tick-infested Holstein–Friesian and Brahman animals at the tick-attachment site. Moreover, a gene with an important biological function in controlling cellular adhesion and migration was associated with tick burden in cattle (89).

Genes involved in the hemostasis pathway, such as *SLC7A10* and *QSOX1*, were also found to harbor the regions identified as influencing IB. Sustained heavy *R. microplus* infestation has been shown to alter host hemostatic mechanisms by inhibiting platelet aggregation and coagulation functions (90). Several putative genes (*SPRY1*, *NUDT6*, *FGF2*, *FGFR2*, and *TACC2*) influencing IB participate in the cell proliferation process. In response to a heavy tick burden, many different types of cells proliferate to present exogenously derived antigens to the immune system (75). The authors identified genes differentially expressed between tick-infested Holstein–Friesian and Brahman animals that were involved in the cell proliferation process.

HTR2A is involved in inflammatory mediator regulation of transient receptor potential (TRP) channels and calcium signaling pathways. Modifications in intracellular calcium concentrations represent a fundamental mechanism in the

control of inflammatory and immune cell functions (91). Intracellular calcium influx is a key process for lymphocyte activation and proliferation and cytokine synthesis (92, 93). Cytokine is involved in the immune process against *B. bovis* infection and replication, as discussed previously. As TRP channels favor intracellular calcium permeability, it is conceivable that, in association with other prominent molecular pathways, TRP channels could contribute to immune and inflammatory responses (91). Bagnall et al. (85) demonstrated that genes involved in the intracellular calcium regulation pathway are upregulated in response to cattle tick infestation in bovine skin. Also, the *HTR2A* gene participates in the ERK1 and ERK2 cascade process, which has control over inflammatory mediator synthesis and survival of innate immune cells (94).

CONCLUSIONS

Predictive accuracies are related to the size of training populations, and despite its low heritability, genomic predictions could be used as a tool to improve genetics for *B. bovis* infection level in Hereford and Braford cattle. The effectiveness of this process would rely on generating a larger reference population than that used in the present study. Moreover, some candidate genes that participate in immunity system pathways were identified and could contribute to improving the genetic knowledge regarding *B. bovis* infection in cattle. Although the genetic correlation between *B. bovis* infection level and tick count was weak, some candidate genes for IB were also reported in tick infestation studies, and they were also involved in biological resistance processes.

DATA AVAILABILITY STATEMENT

The raw data cannot be made available because it is the property of the Braford and Hereford producers, Embrapa, and GenSys Consultants and this information is commercially sensitive.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee for Ethics in Animal Experimentation (CEEA) from the Federal University of Pelotas (Pelotas, RS, Brazil; process number 6849 and 9409). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

LC: conception, analysis and interpretation of data, writing the draft, and approval of the version to be published. CB: analysis and interpretation of data and approval of the version to be published. RG and CO: sample preparation, laboratory analysis, interpretation of results, and approval of the version to be published. CG-G: experimental planning and design, data collection, sample preparation, and approval of the version to be published. AC: experimental planning and design, laboratory analysis, and critical revising of the manuscript. MO: technical and conceptual guidance, supervision of laboratory analysis, critical revising of the manuscript, and approval of the version to be published. FC: experimental planning and design, data collection, and approval of the version to be published. HO: experimental planning, critical revising of the manuscript, and approval of the version to be published. All authors contributed to the article and approved the submitted version.

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REVIEW
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Tick Salivary Compounds for Targeted Immunomodulatory Therapy

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Immunodeficiency disorders and autoimmune diseases are common, but a lack of effective targeted drugs and the side-effects of existing drugs have stimulated interest in finding therapeutic alternatives. Naturally derived substances are a recognized source of novel drugs, and tick saliva is increasingly recognized as a rich source of bioactive molecules with specific functions. Ticks use their saliva to overcome the innate and adaptive host immune systems. Their saliva is a rich cocktail of molecules including proteins, peptides, lipid derivatives, and recently discovered non-coding RNAs that inhibit or modulate vertebrate immune reactions. A number of tick saliva and/or salivary gland molecules have been characterized and shown to be promising candidates for drug development for vertebrate immune diseases. However, further validation of these molecules at the molecular, cellular, and organism levels is now required to progress lead candidates to clinical testing. In this paper, we review the data on the immunopharmacological aspects of tick salivary compounds characterized *in vitro* and/or *in vivo* and present recent findings on non-coding RNAs that might be exploitable as immunomodulatory therapies.

Keywords: tick saliva, salivary glands, host immunity, immunomodulation, drug discovery

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INTRODUCTION

The vertebrate immune system is a sophisticated and highly developed network of cells, tissues, and organs that together identify and neutralize foreign and endogenous threats. Immunodeficiencies represent a breakdown in these highly organized processes caused by a lack or dysfunction of specific immune cell subpopulations or soluble effectors (1). Immune disorders also arise due to

Abbreviations: AAS, *Amblyomma americanum* serpin; BIF, B cell inhibitory factor; BIP, B cell inhibitory protein; BMDCs, mouse bone marrow-derived dendritic cells; BMDMs, mouse bone marrow-derived macrophages; CD, cluster of differentiation; CFA, complete freund's adjuvant; DC, dendritic cells; EAE, experimental autoimmune encephalomyelitis; HIV, human immunodeficiency viruses; HL-p36, *Haemaphysalis longicornis* p-36; IFN-γ, interferon gamma; IL, interleukin; Irac, *Ixodes ricinus* anticomplement; IRS-2, *Ixodes ricinus* Serpin- 2; Isac, *Ixodes scapularis* salivary anticomplement; LPS, lipopolysaccharide; MBL, mannose-binding lectin; MC, Mast cells; MCP-1 monocyte chemoattractant protein 1; MHC-I, -II, major histocompatibility complex-I, -II; MIF, macrophage migration inhibitory factor; MS, mass spectrometry; NGS, next-generation sequencing; OmCI, *Ornithodoros moubata* complement inhibitor; PDAC, pancreatic ductal adenocarcinoma; PGE_2, prostaglandin E2; Ra-HBPs, *Rhipicephalus appendiculatus* histamine-binding proteins; RmS-3, *Rhipicephalus microplus* serpin 3; Salp, salivary protein; SHBP, serotonin and histamine-binding protein; TCR, T cell receptor; TdPI, Tick-derived protease inhibitor; TGF-β, transforming growth factor-beta; Th, T helper; TLR, toll-like receptors; TNF, tumor necrosis factor; TSLPI, tick salivary lectin pathway inhibitor.

excessive immune responses, such as in allergic reactions or autoimmunity (2), usually as a result of dysregulation or overexpression of specific cytokines and their related immune signaling pathways (3). The human immune system is also affected, and often weakened, by stress, malnutrition, and age-related changes, which increase susceptibility to infectious diseases and other pathologies such as cancer (4–6). Current therapies for immune-related illnesses usually have side-effects or suboptimal efficacy (7, 8), leading to efforts to identify alternative therapies that might naturally inhibit and/or modulate specific immune system targets without significant off-target effects (9), including bioactive molecules derived from natural sources (10). Compounds with therapeutic potential have traditionally been extracted from plants (phytotherapy) (11), but other sources have included scorpion and snake venom (12, 13) and arthropods (14). However, substances extracted from natural sources and applied in practice remain limited, and their clinical utility is hampered by the risk of contamination with impurities (15). More positively, drug discovery has been assisted by the development of new protein production methods using various expression systems (16–18), and advances in new technologies such as next-generation sequencing (NGS) and mass spectrometry (MS) have revolutionized screening for novel natural substances (19, 20).

Tick salivary glands are now recognized as a rich source of pharmaco-active molecules (21). Tick saliva contains a rich cocktail of bioactive molecules including protein and lipid derivatives with a remarkable binding affinity, avidity, and selectivity for their targets in various host defense systems (22). Ticks are obligatory hematophagous that, in order to feed, must overcome the evolutionarily sophisticated immune defense systems of their vertebrate hosts. To achieve this, they secrete a wide variety of molecules in their saliva with immunomodulatory, anti-inflammatory, anti-clotting, and anti-platelet effects (23, 24).

Despite the identification of a large number of bioactive tick salivary molecules, their investigation for therapeutic purposes remains in its outset. Only a limited number of tick salivary compounds, mostly proteins, have been tested pre-clinically (25, 26). Here we discuss the literature on therapeutically valuable tick salivary molecules with function(s) known to be directly related to host immune responses. In doing so, we identify the most promising salivary candidates with drug development potential, including newly discovered non-coding (nc) RNAs.

TICK SALIVA TARGETS AT THE TICK-HOST INTERFACE

Ticks are obligate hematophagous ectoparasites of amphibians, reptiles, birds, and mammals that cause host blood loss and skin damage (27). Compared to other blood feeders, tick feeding behavior is unique. Hard ticks discretely and solidly attach to their hosts for several days to weeks to complete their blood uptake (28), with the skin representing the main interface where tick salivary compounds meet host defense systems (29, 30) (**Figure 1**). Furthermore, the skin is the site of multiplication and persistence of several tick-borne pathogens (31, 32). In the local environment of the skin, the tick-host molecular interaction can be viewed as a competition between host defense mechanisms against the ectoparasite and tick evasion strategies (30). To ensure an uninterrupted blood meal, ticks have developed myriad strategies to overcome the complex homeostatic and immune responses that are raised against them (22). As long-term pool feeders, they create an immunologically privileged micro-environment in the host's skin, in which they secrete an impressive mixture of proteins, peptides, and non-peptide molecules (**Figure 1**), thereby modulating wound healing, hemostasis, inflammation, and both the innate and adaptive immune responses (33). These molecules interfere with various host molecules including enzymes, cytokines, complement components, antibodies, cell signaling molecules, and immune cell receptors (33).

Hemostasis is triggered within seconds of tissue damage and is the product of the triad of blood coagulation, platelet aggregation, and vasoconstriction, a process mainly controlled by serine proteases (34). The anti-hemostatic properties of tick salivary secretions are reviewed elsewhere (23, 35). In addition to hemostasis, complement components and inflammatory mediators are also initial tick saliva targets (36, 37). When tick mouthparts are inserted into the skin, pre-positioned sentinel leukocytes of the epidermis and dermis, including mast cells (MCs), eosinophils, dendritic cells (DCs), and macrophages, as well as keratinocytes and endothelial cells, are activated by mediators released from damaged skin cells or expressed by pathogens transmitted by ticks (38). The resident macrophages release pro-inflammatory chemokines and cytokines including interleukin-8 (IL-8), tumor necrosis factor (TNF), and IL-1β (38). These chemoattractants recruit blood-borne innate immune cells such as neutrophils and monocytes to the bite site, which intensify the stimulation of local and infiltrating innate immune cells (30, 39). Monocytes secrete growth hormones that induce fibroblast proliferation and extracellular matrix deposition, thus contributing to wound healing (39). Given their parasitic lifestyle, ticks must overcome innate immunity during their primary infestation and both innate and adaptive immunity during secondary or subsequent infestations (22). Following tick feeding, tick-derived antigens may be presented to naïve B and T cells by activated DCs that have acquired foreign antigens and have migrated to skin-draining lymph nodes (40). Both the humoral and cellular branches of host adaptive immunity are activated, thereby resulting in the generation of antigen-specific antibodies and T lymphocytes (40). In subsequent infestations, activated memory T and B lymphocytes secrete cytokines and produce specific antibodies that target tick salivary or mouthpart-derived antigens in an effort to reject the tick (41).

IMPACT OF TICK SALIVARY COMPOUNDS ON HOST IMMUNE RESPONSES

The complex mixture of tick salivary compounds specifically and selectively targets host immune reactions, subverting the rejection and death of the tick (24). This specificity and selectivity

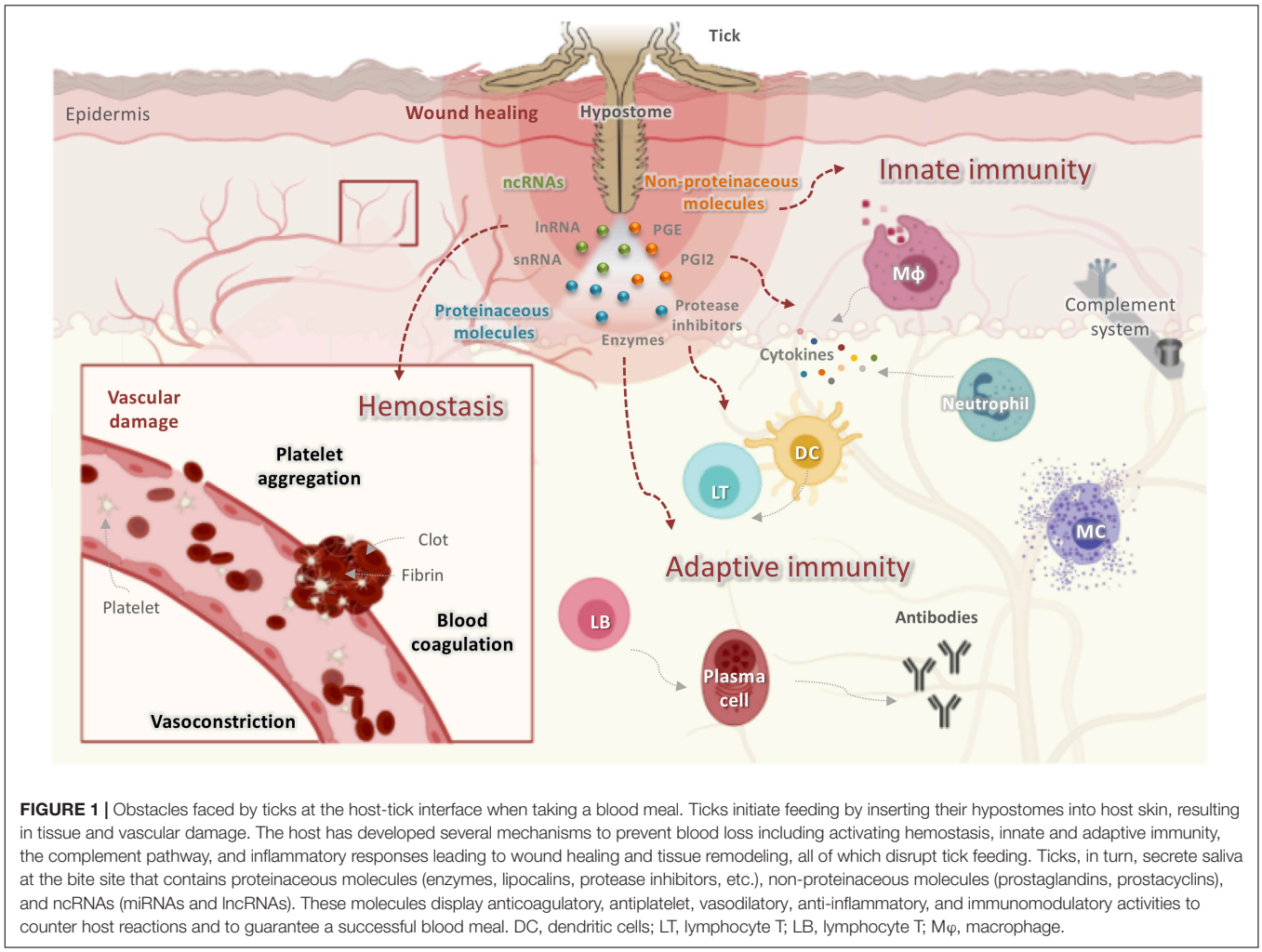

FIGURE 1 | Obstacles faced by ticks at the host-tick interface when taking a blood meal. Ticks initiate feeding by inserting their hypostomes into host skin, resulting in tissue and vascular damage. The host has developed several mechanisms to prevent blood loss including activating hemostasis, innate and adaptive immunity, the complement pathway, and inflammatory responses leading to wound healing and tissue remodeling, all of which disrupt tick feeding. Ticks, in turn, secrete saliva at the bite site that contains proteinaceous molecules (enzymes, lipocalins, protease inhibitors, etc.), non-proteinaceous molecules (prostaglandins, prostacyclins), and ncRNAs (miRNAs and lncRNAs). These molecules display anticoagulatory, antiplatelet, vasodilatory, anti-inflammatory, and immunomodulatory activities to counter host reactions and to guarantee a successful blood meal. DC, dendritic cells; LT, lymphocyte T; LB, lymphocyte T; Mφ, macrophage.

may therefore be exploited for diseases in which dysfunction of the same host immune reactions is implicated in their pathogenesis, for instance the innate immune system in Sjögren's syndrome (42), adaptive immunity in Tregopathies (43) and autoimmune encephalomyelitis (44), and complement activation in local and/or systemic inflammation, tissue damage, and disease (45).

Some tick salivary components have immune system specificity at different steps of immune recognition (21). Despite this specificity, tick salivary component targets often show redundancy at the molecular, cellular, and functional level (i.e., may be targeted by more than one tick salivary molecule) (29). Furthermore, several tick salivary compounds are pleiotropic, targeting both hemostatic and immune system components (29). For instance, Salp15, a multifunctional protein, was shown to bind specifically to dendritic cell, inhibit CD4 + T cell activation and proliferation, and block the liberation of interleukin 2 (IL-2) (46). Apart from its immunomodulatory activities, it has also been described to modulate host coagulation (47). This pleiotropicity could be problematic in targeted immunotherapy, which relies mainly on single target drugs and is often considered as a serious limitation in drug conception. Nevertheless, this issue does not exclude the possibility of using these salivary components in other therapeutic applications. Multi-target drugs, also known as multifunctional drugs or network therapeutics are more suitable as potential therapeutic solutions in diseases of complex etiology, such as Alzheimer's disease, Parkinson's disease, and neglected tropical diseases (48). Single-target drugs, although highly selective and specific, may not necessarily have better efficacy in these cases (49). Indeed, multi- target drugs tend to be beneficial to face complex disorders, multifactorial health conditions and drug resistance issues (50, 51). Some studies trend to changing paradigm from "one target one ligand" toward "multi-target" as they does not provide a complete solution for multifactorial diseases (49). Therefore, tick salivary compounds with broad-spectrum targets might be useful in these diseases mediated by multiple processes.

The immunomodulatory properties of whole tick saliva and/or salivary gland extracts were previously reviewed by Kotál and colleagues (52), and some comprehensive and recent reviews have focused on the composition and role of saliva in tick feeding and tick-host-pathogen interactions (21, 53, 54). These articles have tended to focus on proteinaceous salivary component families including lipocalins, protease inhibitors

such as Kunitz-type domains containing proteins, serpins, and cystatins, metalloproteases, basic tail secreted proteins, small peptide inhibitors, and some protein families unique to ticks. The potential of non-peptide molecules, lipid derivatives, and the recently discovered ncRNAs are rarely mentioned. This review therefore focuses on immunomodulatory substances that have been shown to target both the innate and adaptive host immune systems in *in vitro* and/or *in vivo* animal models of human diseases and in doing so we identify salivary candidates with promise for targeted immunotherapy. A limited number of salivary molecules are progressing in preclinical trials. This includes Amblyomin-X, Ir-CPI, TAP, OmCI, and Ra-HBP, among others. Most of these molecules have been described as anti-hemostatic and anti-tumor candidates (55). Only OmCI and Ra-HBP target immune responses, therefore, their potential applications will be discussed later in this review. Amblyomin-X, a Kunitz-type protease inhibitor, is profoundly exploited in pre-clinical testing and is under development for cancer treatment (26). It selectively induces apoptosis in tumor cells and promotes tumor reduction *in vivo* in melanoma animal models and reduce metastasis and tumor growth in *in vivo* experiments (56). In its pre-clinical evaluation, this protein was proven to significantly decrease lung metastasis in a mice orthotopic kidney tumor model (25). More interestingly, Amblyomin-X does not seem to cause any mortality; and symptoms of toxicity were subtle, reversible, and seen only at higher doses, thus demonstrating a safety profile for injection in mice. More recently, it was investigated on Amblyomin-X-treated horse melanomas showing significant reduction in the tumor size (57). Ir-CPI (*Ixodes ricinus* contact phase inhibitor), an antithrombotic protein, has proven its effectiveness in inhibiting the contact phase of the coagulation cascade in preclinical trials, preventing clotting in catheter and arteriovenous shunt rabbit models during Cardiopulmonary Bypass (58). TAP, a Kunitz domain protease inhibitor from *Ornithodoros moubata*, has been described as an anticoagulant candidate, showing promising results in *in vivo* models of venous and arterial thrombosis (59). Its efficacy on blood coagulation has been approved in preliminary preclinical experiments; however, it has never been inquired in humans mainly due to its antigenicity (59).

Innate Immune Responses

The host innate immune response forms the first and immediate line of defense to tick attachment (52). Activated resident cells in the cutaneous barrier including MCs, macrophages, and DCs stimulate host awareness to the injury followed by removal of feeding ticks (**Figure 2**).

Itch and Pain

Mast cells and basophils degranulate to release soluble mediators such as histamine and serotonin, which cause local itch and pain at the infestation site (60). However, ticks alleviate itch and pain through salivary components that sequester histamine and/or serotonin or modulate MC function. RmS-3, a novel serpin extracted from the salivary glands of *Rhipicephalus microplus* (61), has been shown to modulate MC function by inhibiting chymase and vascular permeability in acute inflammation (**Figure 2**). The hard tick *Rhipicephalus appendiculatus* uses the histamine-binding proteins Ra-HBPs (in the lipocalin family) to bind histamine with high affinity during early feeding (62). Different Ra-HBPs were identified in male and female ticks: male-specific histamine-binding salivary protein Ra-HBP (M) and two female-specific histamine-binding salivary proteins RaHBP(F)-1,2 (**Figure 2**). Ra-HBP2 sequestered two histamine molecules with different affinities, emphasizing its therapeutic potential by targeting multiple effectors (63). Another lipocalin showing histamine binding capacity, HA24, was identified in the salivary glands of *Hyalomma asiaticum* (**Figure 2**) (64). *In vitro* and *in vivo* histamine binding assays showed that recombinant HA24 bound specifically to histamine in a dose-dependent manner and relieved allergic asthma in mice (64). SHBP (serotonin and histamine-binding protein), isolated from *Dermacentor reticulatus* (65), simultaneously interfered with the activity of both serotonin and histamine (**Figure 2**).

In addition to biogenic amine production, MCs contribute to the inflammatory process by releasing a wide range of highly bioactive effectors after degranulation, tryptases being the most abundant (66). Tryptases are implicated in the pathogenesis of allergic inflammatory diseases, cardiovascular disease, lung fibrosis, and even cancer, so their inhibition may be useful therapeutically (66, 67). Interestingly, tick-derived protease inhibitor (TdPI) was identified in the salivary glands of *R. appendiculatus* and was shown to suppress the activity of human β-tryptase and trypsin and, to a lesser extent, plasmin (68). TdPI accumulates in the cytosolic granules of mouse MCs, presumably blocking the autocatalytic activation of tryptase, thereby suppressing inflammation in the host animal's skin (**Figure 2**) (69). Tryptogalinin, an *Ixodes scapularis* salivary Kunitz-type protein, was also found to inhibit β-tryptase (**Figure 2**) and other MC serine proteases such as α-chymotrypsin, plasmin, matriptase, and elastase and participated in inflammation and tissue remodeling (70). β-tryptases are MC-specific serine proteases with roles in inflammation that are used clinically as biomarker of MCs and their activation (67). Both tryptogalinin and TdPI could be engineered as highly specific pharmacological inhibitors of MC tryptases for the treatment of allergic inflammatory disorders like asthma (67–70).

Recruitment of Blood-Borne Innate Immune Cells

Within the first few hours after attachment, blood-borne leukocytes are recruited to the site of injury, triggered by a set of mediators including complement components, eicosanoids, chemokines, and cytokines (71). Neutrophil and monocyte recruitment has been shown to be strongly suppressed by tick salivary compounds. A homolog of the vertebrate macrophage migration inhibitory factor (MIF) was identified in *Amblyomma americanum* (72, 73) and *Haemaphysalis longicornis* (74). In both ticks, functional *in vitro* assays revealed that MIF inhibited the migration of human monocytes, suggesting that it might decrease monocyte recruitment *in vivo*. Ir-LBP from *I. ricinus* inhibited neutrophil chemotaxis and activation *in vitro* by binding specifically and with high affinity to leukotriene B4, an important inflammatory mediator (75). Ir-LBP was also shown to inhibit inflammatory responses in rabbits by decreasing

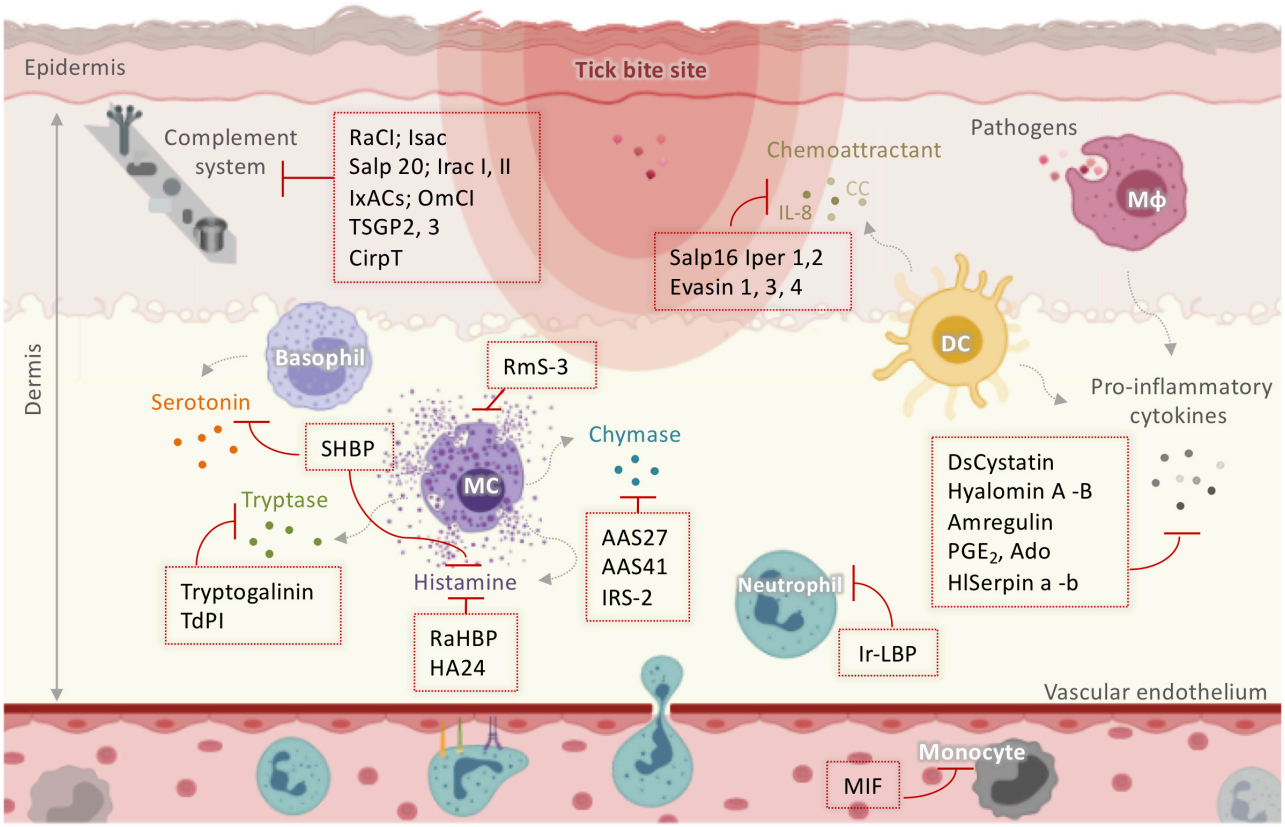

FIGURE 2 | Schematic representation of tick salivary molecules implicated in the modulation of the complement system and innate immunity. Activated resident cells in the dermis stimulate host awareness of injury and removal of the feeding ticks. Tick saliva neutralizes itch and pain through salivary components that sequester histamine and/or serotonin (such as SHBP, Ra-HBPs, and HA24) or modulate MC function (such as RmS-3). TdPI and Tryptogalinin inhibit tryptase released by MCs. AAS27, AAS41, and IRS-2 inhibit chymase liberated by MCs. Neutrophil and monocyte recruitment is suppressed by MIF and Ir-LBP. Moreover, ticks manipulate the host cytokine network by inhibiting cytokines and chemokines using Salp16 Iper 1,2; Evasin 1, 3, 4; DsCystatin; Hyalomin-A, –B; Amregulin; PGE_2; Ado; and HlSerpin-a, –b. Several anti-complement molecules have been identified in tick salivary glands including RaCI; Isac; Salp 20; Irac I, II; IxACs; OmCI; TSGP2, 3; and CirpT. AAS: *Amblyomma americanum* serpin; DC: dendritic cells; IL: interleukin; Irac: *I. ricinus* anticomplement; IRS-2: *I. ricinus* Serpin-2; Isac: *I. scapularis* salivary anticomplement; IxACs: *Ixodes* anticomplement proteins; MIF: macrophage migration inhibitory factor; Mϕ: macrophage; OmCI: *O. moubata* complement inhibitor; PGE_2: prostaglandin E2; Ra-HBPs: *R. appendiculatus* histamine-binding proteins; RmS: *Rhipicephalus microplus* serpin; Salp: salivary protein; SHBP: serotonin- and histamine-binding protein; TdPI: tick-derived protease inhibitor.

the number of neutrophils located at the tick bite site (75). Therefore, Ir-LBP may have therapeutic use in inflammatory diseases or illnesses associated with increased leukotriene B4 production. Moreover, ticks employ salivary inhibitors of CXCL8 (IL-8) and CC chemokines to manipulate the host cytokine network. Salp16 Iper1 and Salp16 Iper2 (**Figure 2**), salivary proteins from *Ixodes persulcatus*, have been shown to have anti-IL-8 activity, thereby impairing neutrophil chemotaxis (76). A very recent review summarized the data on Evasins, which are secreted by hard ticks and bind to host chemokines to inhibit their activation of chemokine receptors (77). Of the Evasins, three from *Rhipicephalus sanguineus* (Evasin-1, -3, and -4) were selective for different chemokines (78). Evasin-1 bound to the CC chemokine members CCL3, CCL4, and CCL18 (78) and inhibited neutrophil, T cell, and macrophage migration and the production of inflammatory cytokines *in vitro* (78). Russo and colleagues demonstrated the high efficiency of Evasin-1 in reducing CCL3-induced influx of neutrophils in murine bleomycin-induced lung fibrosis (79). Evasin-1 has also been shown to reduce graft-versus-host disease in mice, which may be particularly useful in patients undergoing bone marrow transplantation (80). Moreover, the administration of recombinant Evasin-1 reduced skin inflammation and decreased mortality in mice deficient in the chemokine receptor D6, which renders then highly susceptible to inflammation (81). Evasin-3 is specific for the CXC chemokines CXCL8 and CXCL1 and was recently found to disrupt the interaction between CXCL8 and glycosaminoglycans and CXCR2 (**Figure 2**), which modulate neutrophil migration (82). Several studies have demonstrated the effectiveness of Evasin-3 in different neutrophil-dependent disease models. A single administration of Evasin-3 during mouse myocardial ischemia/reperfusion injury effectively reduced infarct size and decreased CXC chemokine-induced neutrophil recruitment (83). Evasin-3 also reduced atherosclerotic vulnerability to ischemic stroke in an *in vivo* murine model (84) and inhibited neutrophil-mediated pancreatic

and lung inflammation in a mouse model of acute pancreatitis (85). Evasin-4 interacts with at least 18 CC chemokines (78). Similar to Evasin-3, Evasin-4 was effective against post-infarction myocardial injury and remodeling (86) and decreased the abundance of macrophages in the lungs without affecting the pancreas in the acute pancreatitis model (85). Due to its broad CC chemokine-binding spectrum, Evasin-4 is considered the most suitable candidate for therapeutic development (86). More recently, Evasin-inspired artificial peptides dramatically reduced inflammation *in vivo* by targeting multiple chemokines (87). These peptides might therefore provide a route to the development of new anti-inflammatory therapeutics for chronic autoimmune diseases such as rheumatoid arthritis and inflammatory diseases such as atherosclerosis. Moreover, the mechanism of action of these peptides suggests that they might also be useful in acute infectious diseases such as influenza or COVID-19, where exuberant cytokine responses are thought to be at least partially responsible for tissue injury (87).

Inflammation

Activated resident cells secrete several pro-inflammatory mediators, which initiate and then reinforce the local inflammatory process at the damaged site. Tick saliva controls inflammation by decreasing or increasing the secretion of pro- and anti-inflammatory cytokines, respectively (29). Two immunoregulatory peptides, Hyalomin-A and –B from *H. asiaticum asiaticum*, overcome host inflammation by modulating cytokine expression, inhibiting the secretion of pro-inflammatory TNF-α, MCP-1, and IFN-γ and stimulating the secretion of the immunosuppressant cytokine IL-10 (88). Amregulin from *Amblyomma variegatum* saliva was found to suppress the *in vitro* production of TNF-α, IL-1, IL-8, and IFN-γ in a dose-dependent manner (89). Hyalomin-A and –B and Amregulin significantly inhibited adjuvant-induced paw inflammation in mouse models *in vivo* (88, 89). HlSerpin-a and –b, novel serpins from the hard tick *H. Longicornis*, suppressed the expression of TNF-α, IL-6, and IL-1β from LPS-stimulated mouse bone marrow-derived macrophages (BMDMs) or mouse bone marrow-derived dendritic cells (BMDCs) (90). Three salivary gland serpins, AAS27 and AAS41 from *A. americanum* (91, 92) and IRS-2 from *I. ricinus* (93), inhibited inflammation by targeting chymase (**Figure 2**), an enzyme produced by activated MCs. IRS-2 also inhibited Cathepsin G, which is involved in tissue remodeling during inflammation and modulates the production of IL-6 by DCs, which subsequently impairs Th17 differentiation and maturation. DsCystatin from *Dermacentor silvarum* salivary glands was shown to impair the expression of the inflammatory cytokines IL-1β, IFN-γ, TNF-α, and IL-6 from mouse BMDMs (94), and the authors proposed that DsCystatin might be useful for the treatment of inflammatory diseases since it suppressed joint inflammation induced by complete Freund's adjuvant (CFA) and *Borrelia burgdorferi* in a mouse arthritis model (95).

Another category of anti-inflammatory compounds are non-proteinaceous substances. It is important to point out that bioactive lipidic salivary component despite being abundant in the saliva of hard ticks; mainly prostaglandins PGE_2 and $PGF_{2\alpha}$, only a few studies describe their pharmaceutical use (96, 97). Cannabinoids have also been detected in *Amblyomma* ticks and have been proposed to act as analgesic and anti-inflammatory compounds (98). However, these lipid derivatives have not been thoroughly investigated and require more rigorous investigation to assess their pharmacological interest. The two most documented non-proteinaceous compounds are purine nucleoside adenosine (Ado) and prostaglandin PGE_2 (**Figure 2**) present in the saliva of *R. sanguineus* (99). Both compounds impaired the production of pro-inflammatory IL-12p40 and TNF-α and stimulated the release of anti-inflammatory IL-10 by murine DCs (99). In humans, Ado is a homeostatic regulator and a "danger signal" for cells and organs, since it is expressed during trauma or stress (100, 101). The therapeutic potential of Ado is well documented elsewhere (100–102). Ado is protective against several pathologies including inflammation and various forms of neuronal hyperexcitability and/or toxicity including hypoxia, seizures, and chronic pain (101). Similarly, PGE_2 has pharmacological proprieties *in vitro* and *in vivo* associated with inflammation (103) and cancer (97) influencing cell proliferation, apoptosis, angiogenesis, inflammation, and immune surveillance (104). Thus, Ado and PGE_2 derived from *R. sanguineus* saliva could be used for therapeutic purposes.

Complement System

The complement system was originally regarded as a support to innate immunity against microbial invaders (45). More recently, complement has been recognized as having functions beyond microbial elimination such as clearance of immune complexes (105), complementing T and B cell immune functions (36), and tissue regeneration (106). Complement links the host innate and adaptive immune responses and is activated via three pathways (alternative, classical, and lectin) (31, 85). The excessive activation of complement components is responsible for a wide range of immune-mediated diseases (107) including autoimmune diseases, Alzheimer's disease, schizophrenia, atypical hemolytic-uremic syndrome, angioedema, macular degeneration, and Crohn's disease (108). Since the introduction of the first complement-specific drug, eculizumab, into the clinic, over 20 candidate drugs are now being evaluated in clinical trials and additional agents are in preclinical development (109). Several molecules with promising anti-complement activities were identified in tick salivary glands. For instance, Isac from *I. scapularis* (110) and its paralogs IRAC I and II from *I. ricinus* (111) specifically blocked binding of complement factor B to complement C3b *in vitro*, inhibiting the formation of the C3 convertase of the alternative pathway. Since convertases mediate nearly all complement effector functions, they are ideal targets for therapeutic inhibition (112). Selective inhibition of complement precursors and regulators is also of great therapeutic interest (109). Salp20 from *I. scapularis* (113) and IxACs from *I. ricinus* (114) inhibit the alternative pathway by binding properdin, a positive regulator of the pathway. Soft ticks have been reported to inhibit the classical complement pathway, with the lipocalins OmCI from *O. moubata* (115, 116) and TSGP2 and TSGP3 from *Ornithodoros savignyi* (117) specifically targeting C5 activation. The recombinant version of OmCI (also known as

Coversin or rEV576) has been tested in several animal models of complement-mediated diseases and has shown efficacy in a clinical trial (115, 118, 119). By inhibiting C5 activation, OmCI significantly reduced excessive inflammatory reactions associated with severe forms of influenza virus A (IAV) in mice (116). In addition to its effect on C5, OmCI significantly decreased leukotriene B4 levels in septic pigs (120). In the same study, combined inhibition of complement C5 by OmCI and CD14 with anti-CD14 antibodies significantly attenuated inflammation, thrombogenicity, and hemodynamic abnormalities (115, 120), suggesting OmCI might be useful for the treatment of sepsis. OmCI was also effective in preventing experimental autoimmune myasthenia gravis induced by passive transfer in normal Lewis rats (118). RaCI, from *R. appendiculatus* salivary glands, bound human C5 and blocked C5a generation and membrane attack complex formation (121). RaCI exhibits cross-species reactivity, allowing it to be used in disease models to test therapeutic effectiveness. An OmCI and RaCI homolog, CirpT, is a new class of complement inhibitor identified in *Rhipicephalus pulchellus* saliva that binds C5 *in vitro* via a unique binding site that does not overlap with other known inhibitors (122). Eculizumab, an antibody against C5, is currently prescribed for diseases characterized by excessive activation of the alternative pathway, including paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome (123, 124). Despite its effectiveness, eculizumab is one of the most expensive drugs in the world (121). Nevertheless, these other proteins derived from tick saliva offer exciting prospects for C5-targeted therapeutics of complement-mediated diseases and could be alternatives to eculizumab. In addition to the alternative pathway, the lectin pathway is also targeted by the tick salivary lectin pathway inhibitor (TSLPI), identified from the salivary glands of *I. scapularis* (125). The lectin pathway is activated in an antibody and C1-independent manner, upon the interaction of Mannose-binding lectin (MBL) with hyper glycosylated pathogen-associated molecular patterns (PAMPs) on microbial surfaces (126). TSLPI was described to directly inhibit MBL complement pathway activation (125). Additionally, both TSLPI and its ortholog from *I. ricinus* were shown to protect *Borrelia burgdorferi* sensu lato from complement-mediated killing, by inhibiting MBL function (127, 128). MBL appears to influence the severity of several diseases including pancreatic ductal adenocarcinoma (PDAC) (129). During a Pancreatic Cancer, MBL is required for oncogenic progression, whereas inhibition of MBL by TSLPI could be protective against tumor growth.

Acquired Immune Responses

During the first encounter with ticks, antigen-presenting cells, mainly DCs, present salivary immunogens to lymphocytes, which trigger cell-mediated and antibody responses (22). Sets of immunoglobulins and long-lived memory T and B cells are induced in the host (**Figure 3**). In subsequent contacts with the same tick, acquired immunity is quickly activated to yield a more specific protective response. It has been reported that tick saliva suppresses adaptive immunity in various ways (33). Some tick salivary compounds interfere with DCs and alter their capacity to present antigens. Other compounds prevent lymphocyte proliferation or T cell cytokine production.

Antigen Processing and Presentation

Several tick salivary molecules target DCs, thereby preventing the initiation of adaptive immunity. PGE_2 has been described as the major DC inhibitor (**Figure 3**) in both *I. scapularis* (130) and *Amblyomma sculptum* saliva (131). RHS2 from *Rhipicephalus haemaphysaloides* can inhibit the differentiation of BMDCs into DCs *in vitro* and promote their differentiation into macrophages (132). RHS2 also inhibits the expression of CD80, CD86, and the major histocompatibility complex class II (MHCII), thereby preventing DC maturation. DC differentiation from monocytes is blocked by Japanin (**Figure 3**), a salivary gland lipocalin from *R. appendiculatus* (133). Moreover, Japanin was found to reprogram DC responses to a broad variety of stimuli *in vitro*, radically altering the expression of co-stimulatory and co-inhibitory molecules and modifying the production of diverse cytokines (134).

Humoral Immunity

Tick salivary components also disarm host humoral immunity by inhibiting B cells responses, including their production of specific anti-tick antibodies (134). BIP (B cell inhibitory protein) (**Figure 3**) derived from *I. ricinus* inhibited B lymphocyte proliferation induced by *B. burgdorferi* lipoproteins (135). Similarly, BIF (B cell inhibitory factor) from *H. asiaticum asiaticum* salivary glands (**Figure 3**) was found to suppress B cell responses (136).

Cell-Mediated Immunity

Numerous inhibitors of T cell functions have been identified in tick saliva and salivary glands. Three immunosuppressant salivary proteins, p36 from *Dermacentor andersoni* (137, 138), HL-p36 from *H. longicornis* (139), and RH36 from *R. haemaphysaloides* (140) suppressed T lymphocyte proliferation *in vitro*. Recombinant HL-p36 and RH36 also directly inhibited the proliferation of several mitogen-stimulated cells *in vivo* and the expression of several cytokines such as IL-2, IL-12, and TNF-α (141, 142). In addition to its effect on DCs, RHS2 can prevent the activation of $CD4^+$ and $CD8^+$ T cells, leading to inhibition of the Th1 immune response (132). Two salivary cystatins from *I. scapularis*, Sialostatin L and Sialostatin L2, have shown promising anti-inflammatory and immunosuppressive activity *in vitro* and *in vivo* (141, 142). Sialostatin L was immunosuppressive in mammalian models of immune-related diseases, suppressing the proliferation of both CD4 and CD8 T cells, neutrophil migration in severe inflammation, and the secretion of cytokines by MCs, DCs, and lymphocytes (130, 141). Sialostatin L reduced the release of IL-9 by Th9 cells, which might explain its suppression of airway hyperresponsiveness and eosinophilia in an experimental asthma model (143). In the experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis, *in vivo* administration of Sialostatin L significantly prevented disease symptoms (144). Furthermore, Sialostatin L effectively altered lysosomal cysteine cathepsins L, C, V, S, X and papain activity, critical elements

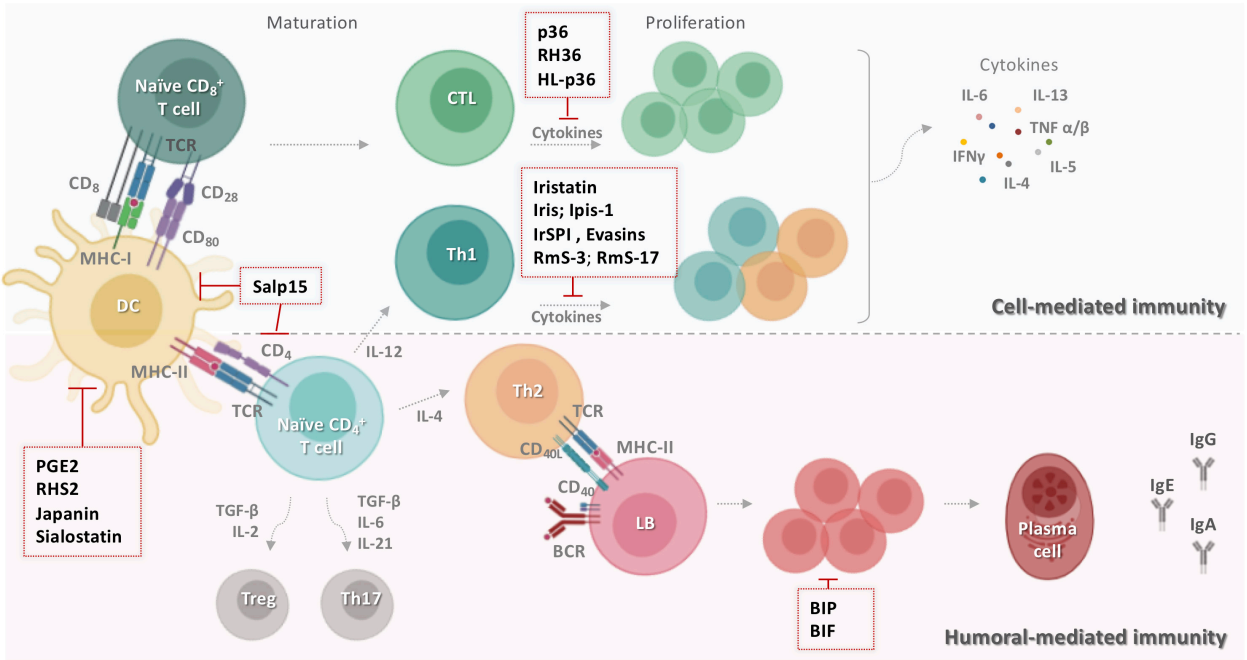

FIGURE 3 | Schematic representation of tick salivary molecules targeting adaptive immunity. During the primary contact with ticks, dendritic cells present salivary antigens to lymphocytes, which trigger cell- and humoral-mediated responses. Some tick salivary molecules prevent the initiation of adaptive immunity by targeting DCs, including Salp15, PGE_2, RHS2, Japanin, and sialostatin. Other compounds block the proliferation of lymphocytes and/or inhibit the production of T cell cytokines such as p36, RH36, HL-p36, Iristatin, Iris, Ipis-1, IrSPI, Evasins, RmS-3, and RmS-17. However, BIP and BIF disarm humoral host immunity by inhibiting B cell responses, including the production of specific anti-tick antibodies. BCR: B cell receptor; BIF: B cell inhibitory factor; BIP: B cell inhibitory protein; CD: cluster of differentiation; CTL: cytotoxic T lymphocyte; DC: dendritic cells; HL-p36: *Haemaphysalis longicornis* p-36; IFN-γ: interferon gamma; Ig: immunoglobulin; IL: interleukin; LB: lymphocyte B; MHC: major histocompatibility complex; PGE_2: prostaglandin E2; RmS: *Rhipicephalus microplus* serpin; Salp: salivary protein; TCR: T cell receptor; TGF-β: transforming growth factor-beta; Th: T helper; TNF: tumor necrosis factor; Treg: regulatory T cell.

in antigen presentation (141). Sialostatin L2 suppressed IFN-β-mediated immune reactions in murine DCs (145), and was also found to inhibit cathepsins C, L, S, and V and decrease IL-1β and IL-18 secretion by macrophages (146). Similar to sialostatins, Iristatin, a novel type 2 cystatin from *I. ricinus*, inhibited the proteolytic activity of cathepsins L and C (147) and diminished IL-2, IL-4, IL-9, and IFN-γ production by different T cell populations, IL-6 and IL-9 production by MCs, and nitric oxide production by macrophages. Iristatin inhibited OVA antigen-induced $CD4^+$ T cell proliferation and leukocyte recruitment *in vivo* and *in vitro* (**Figure 3**). With such a wide spectrum of immunosuppressive activities, Iristatin may be exploitable as an immunotherapeutic.

T cell inhibitory salivary molecules also include Iris and IrSPI from *I. ricinus* (148), Ipis-1 from *I. persulcatus* (149), RmS-3 and RmS-17 from *R. microplus* (61), and Salp15 from *I. scapularis* (47). Iris was found to suppress T cell proliferation, promote a Th2-type response, and bind to monocytes/macrophages, inhibiting their ability to secrete pro-inflammatory cytokines IL-6 and TNF-α (150). Similarly, IrSPI suppressed proliferation of $CD4^+$ T lymphocytes and pro-inflammatory cytokine secretion (**Figure 3**) from splenocytes and macrophages (151). The Iris homolog Ipis-1 inhibited cellular proliferation and the production of IFN-γ in bovine peripheral blood mononuclear cells, suggesting that Ipis could directly interact with T cells and inhibit their functions (149). RmS-3 and RmS-17 inhibited the metabolic activity of lymphocytes, decreasing lymphocyte proliferation (61). One of the most extensively studied components of tick saliva is Salp15, an immunosuppressive cysteine-rich glycoprotein (47). Salp15 binds specifically to CD4 co-receptors on the surface of T lymphocytes, interfering with TCR-mediated signaling transduction and impeding IL-2 secretion in a dose-dependent manner (47, 148, 149). A subsequent study reported that Salp15 interacts with DC-SIGN on DCs, triggering a novel Raf-1/MEK-dependent signaling pathway, thereby impairing cytokine production and T cell proliferation (152). Apart from its effect on adaptive immunity, Salp15 inhibited TLR2-dependent keratinocyte inflammation *in vitro* (153). Together, these findings make this salivary protein a potential candidate for the treatment of T cell-mediated autoimmune diseases. Indeed, the immunomodulatory effect of Salp15 has been investigated in various models *in vivo*. Paveglio and colleagues (154) were the first to show that Salp15 has a therapeutic effect in a mouse model of allergic asthma. The specific binding of Salp15 to CD4 inhibited the proliferation and differentiation of $CD4^+$ T cells toward Th2 cells and suppressed the production of inflammatory cytokines, significantly reducing symptoms of allergic asthma in treated mice. Moreover, Salp15 prevented the association of HIV-1 gp120 and CD4 in an experimental HIV infection (155).

gp120 is an HIV-1 envelope glycoprotein which, by binding to CD4, promotes penetration of HIV-1 into the host cell (156). The authors suggested that Salp15 competes with gp120 for an association with CD4 due to its ability to interact with CD4 on T cells. Another investigation showed the opposite effect in murine EAE/multiple sclerosis (157). The occurrence and progression of EAE is associated with myelin-specific $CD4^+$ T cell activation and secretion of IL-17 and INF-γ by Th17 and Th1 cells, respectively (158). Unexpectedly, Salp15 worsened the pathology in mice upon induction of EAE. A possible reason for this might be that Salp15 promoted Th17 activation, increasing IL-17 levels *in vivo*, and it also enhanced Th17 differentiation in the presence of IL-6 and absence of TGF-β *in vitro* (157). More recently, it was shown that Salp15 binding to CD4 is persistent and induces a long-lasting immunomodulatory effect in a murine model of hematopoietic transplantation (159). Based on these results, Salp15 could provide important opportunities for the development of novel and sophisticated therapies for human disease mainly those mediated by multiple processes.

TICK NON-CODING RNAs: NEW ALTERNATIVES IN TARGETED IMMUNOMODULATORY THERAPY?

Tick salivary molecules are pluripotent (i.e., one molecule may affect more than one host cell population) and show considerable functional redundancy (i.e., the same host cell population may be targeted by more than one tick salivary molecule) (160). Tick saliva not only contains proteins and metabolites but also non-proteinaceous species including nucleic acids (22, 161) and RNA silencing signals, which are now recognized as crucial for cross-species communication across diverse biological niches (162, 163). With respect to the tripartite tick-pathogen-host interaction, recent transcriptomic and proteomic projects, facilitated by rapid developments in high-throughput sequencing, have revealed several tick genes/transcripts of unknown function or transcripts without an open reading frame (ORF) (160). These projects have also revealed a significant number of ncRNAs in tick saliva that are predicted to be functionally involved in the vector-host interaction (164). ncRNAs are important in vector-host interactions at the tick-host interface: ticks use ncRNAs, broadly divided by length into long non-coding RNAs (lncRNAs) (>200 nucleotides (nt) long) and small non-coding RNAs (sncRNAs) (<200 nt long), to hijack host defense mechanisms (165). They are suggested to be secreted via exosomes (166, 167) and released in the host cells, to manipulate gene expression and deregulate host defense pathways (165). LncRNAs are not translated into proteins and include mRNA-like intergenic transcripts (lincRNAs), antisense transcripts of protein-coding genes, and primary RNA polymerase II (Pol II) transcript-derived unconventional lncRNAs (168). LncRNAs are involved in numerous important biological phenomena such as imprinting genomic loci, shaping chromosome conformation and allosterically regulating enzymatic activity (169). On the other hand, sncRNAs represent a diverse set of molecules that control the expression of most vertebrate genes and include small-interfering RNAs (siRNAs), microRNAs (miRNAs), and piwi-interacting RNAs (piRNAs) (170). miRNAs have been extensively studied in several organisms and their function in gene regulation is well understood. The first attempt to identify host targets of tick ncRNAs was performed by Hackenberg and colleagues (171). Their *in silico* study showed that saliva-specific miRNAs from *I. ricinus* might have combinatorial effects on the host targetome: different tick miRNAs may target vertebrate host genes in the same host homeostatic pathway, and the expression of a given host gene may also be regulated by more than one saliva-specific tick miRNA (171). The authors suggested combinatorial effects of vector miRNAs on host target genes, which may be of importance in evolutionary terms to maintain robust regulation of host genes and pathways important in the tick-host interaction. miR-8-3p, bantam-3p, mir-317-3p, and miR-279a-3p from *I. ricinus* were predicted to target host KEGG pathways such as "gap junction" and "inflammatory mediator regulation of TRP channels," which play a role in the host homeostatic response (**Figure 4A**).

The huge number of ncRNA sequences revealed by high-throughput projects suggests that tick ncRNAs might also modulate host gene expression by binding to regulatory host miRNAs involved in host immune cell responses. Tick ncRNAs could regulate protein-coding genes through mRNA cleavage and/or direct translational repression and/or mRNA destabilization. We hypothesize that lncRNAs compete with host mRNAs for host miRNA binding by acting as "sponge" molecules that inhibit host miRNA interactions with host mRNAs, thus affecting host homeostatic responses to tick feeding (**Figure 4B**). *In silico* predictions now need to be validated using systems-based approaches in order to characterize tick ncRNAs and to understand their involvement in tick-host interactions. Various academic and commercial research groups are now exploring ncRNA-based therapies and exploring the potential of miRNA therapeutics, as miRNAs are the most studied ncRNAs to date (172). A few miRNAs have entered preclinical and clinical testing, so might soon be available on the market for use in humans. As tick ncRNAs are likely to be less immunogenic by their intrinsic ability to hijack and bypass host immunity, we strongly believe that this flourishing field might lead to ncRNA therapeutics useful for the treatment of various diseases. Emerging data on the possible regulatory function of ncRNAs, the vector, the pathogen, and the host, the role of ncRNA in the vector or host-pathogen remain quite challenging, due to several biological and technical aspects. For example, the used techniques for ncRNAs study can generate several biases in the results. New scRNA-seq technology that is showing its efficiency to counter this issue, but sufficient read depths of rarely expressed RNAs can also be misleading, and vector, host or pathogen-derived RNAs information could be easily lost. LncRNAs data analyses also remain controversial due to the lack of accurate transcript models in current annotations. LncRNAs could also be pluripotent, and just as the salivary proteins, this would also be a serious limitation in any potential therapeutic applications. Overall, given the arising data on the ability of ncRNAs produced in tick salivary glands and also the

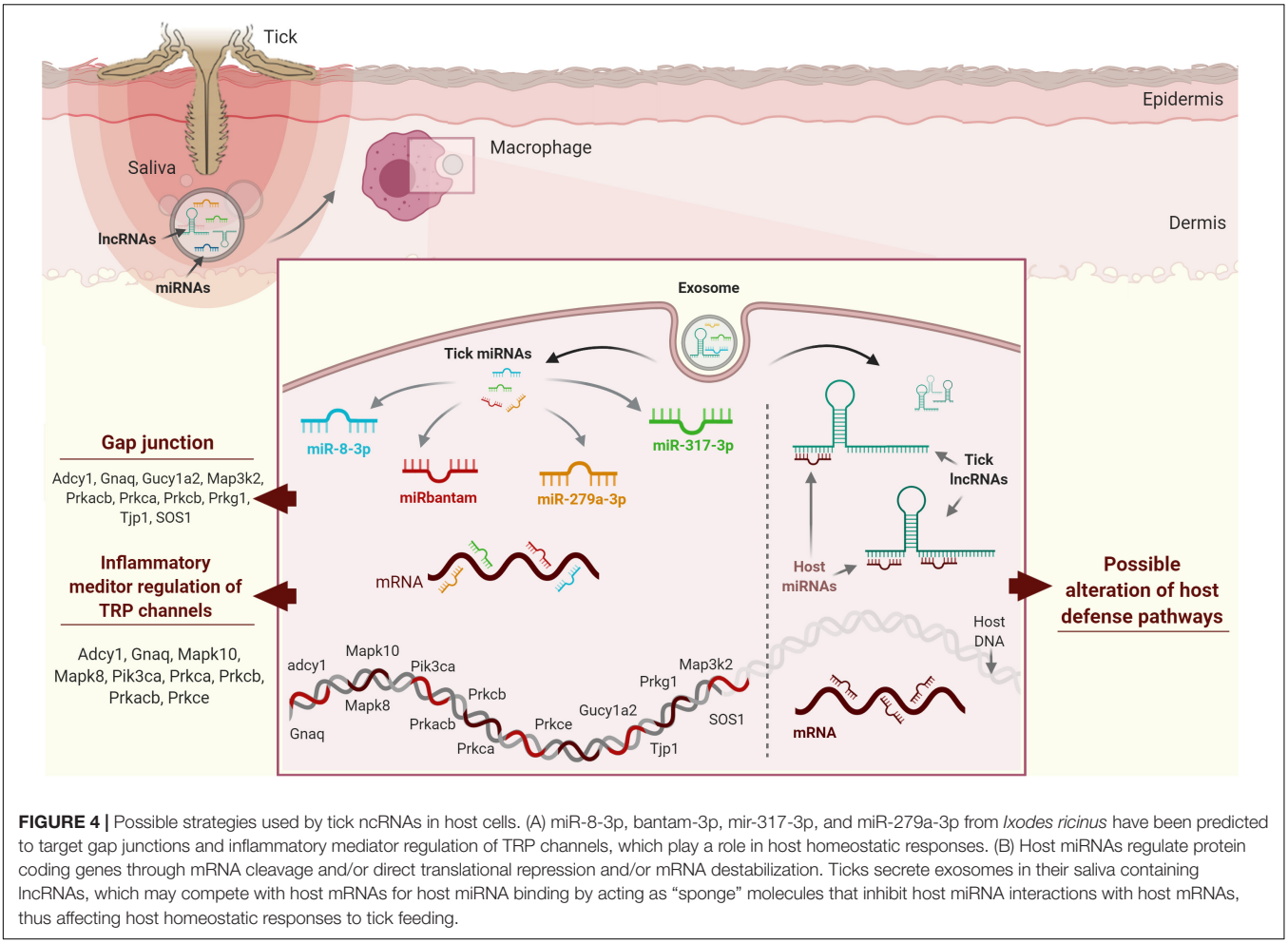

FIGURE 4 | Possible strategies used by tick ncRNAs in host cells. (A) miR-8-3p, bantam-3p, mir-317-3p, and miR-279a-3p from *Ixodes ricinus* have been predicted to target gap junctions and inflammatory mediator regulation of TRP channels, which play a role in host homeostatic responses. (B) Host miRNAs regulate protein coding genes through mRNA cleavage and/or direct translational repression and/or mRNA destabilization. Ticks secrete exosomes in their saliva containing lncRNAs, which may compete with host mRNAs for host miRNA binding by acting as "sponge" molecules that inhibit host miRNA interactions with host mRNAs, thus affecting host homeostatic responses to tick feeding.

possible regulatory function of cellular RNAs on the vector, pathogen or host, the role of ncRNA in the vector or host-pathogen crosstalk might be even more sophisticated than what is expected. Development of research projects that employ single-cell/single molecule sequencing methodologies and do not require nucleic acid amplification will definitely enable less biased and reliable data output.

CONCLUDING REMARKS

Inflammatory, autoimmune, metabolic, and neurodegenerative diseases remain critical health problems worldwide, creating an enormous need for new, effective, and specific medicines. Over the last few decades, ticks have shown their "good side" as promising sources of new drugs targeting distinct pathophysiological pathways in mammals. In this review, we summarized potential drug candidates from tick salivary glands that might have therapeutic use in immune disorders and other related diseases. We have also highlighted that tick ncRNAs are possible new alternatives for targeted immunomodulatory therapy. The molecules described in this review have been tested *in vitro* and/or in various animal models of human disease and have shown encouraging results. However, only a few of them have advanced to (pre-)clinical investigations, and further in-depth molecular and cellular studies are required to further develop candidates for clinical trials. The major challenges to drug development are (i) specificity and efficacy, since broad-spectrum targets can generate unwanted side-effects; and (ii) safety, with drug-induced toxicity and immunogenicity critical limitations of many drug candidates. Addressing these challenges, tick salivary molecules, at least in theory, might have a somewhat higher clinical success rate than molecules from other origins, since many tick-derived products have an intrinsically low risk of toxicity and immunogenicity and high target specificity to execute their intended functions. From the pharmaceutical perspective, some molecules might be unstable or have a short half-lives, so pharmacokinetic, dose optimization, and modification studies are critical research priorities. While experimental validation and ultimately clinical trialing remain the cornerstone of successful therapeutic development, computational methods might play a role as time-saving, cost-effective, and productive approaches to design and discover novel lead compounds for associated disease targets.

Modeling and simulation of preclinical and clinical trials could also be used to bridge the gap between the early stages of drug development and their potential effects on humans.

AUTHOR CONTRIBUTIONS

All authors searched and read the literature and edited the manuscript. HA, CB, and MK wrote the manuscript.

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REVIEW
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Immunobiology of Acquired Resistance to Ticks

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Ticks are blood-sucking arthropods of great importance in the medical and veterinary fields worldwide. They are considered second only to mosquitos as vectors of pathogenic microorganisms that can cause serious infectious disorders, such as Lyme borreliosis and tick-borne encephalitis. Hard (*Ixodid*) ticks feed on host animals for several days and inject saliva together with pathogens to hosts during blood feeding. Some animal species can acquire resistance to blood-feeding by ticks after a single or repeated tick infestation, resulting in decreased weights and numbers of engorged ticks or the death of ticks in subsequent infestations. Importantly, this acquired tick resistance (ATR) can reduce the risk of pathogen transmission from pathogen-infected ticks to hosts. This is the basis for the development of tick antigen-targeted vaccines to forestall tick infestation and tick-borne diseases. Accumulation of basophils is detected in the tick re-infested skin lesion of animals showing ATR, and the ablation of basophils abolishes ATR in mice and guinea pigs, illustrating the critical role for basophils in the expression of ATR. In this review article, we provide a comprehensive overview of recent advances in our understanding of the cellular and molecular mechanisms responsible for the development and manifestation of ATR, with a particular focus on the role of basophils.

Keywords: tick-borne diseases, acquired tick resistance, tick saliva antigens, basophil, skin-resident memory T cells, IgE, histamine, epidermal hyperplasia

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INTRODUCTION

Ticks, especially hard ticks (the *Ixodid* family members), are blood-sucking ectoparasites and serve as vectors for transmission of pathogenic microorganisms, including virus, bacteria and protozoan, that cause serious infectious disorders in animals and humans (1–3). *Ixodid* ticks insert their mouthparts into the host skin and take a blood meal for several days, resulting in increased body weight up to 200-fold. During blood feeding, tick saliva containing a wide range of bioactive substances is injected into host animals to promote successful blood sucking (4–6). During salivation, pathogenic microorganisms can be transmitted from pathogen-infected ticks to host animals. Tick-borne diseases include Lyme disease caused by spirochetes of *Borrelia burgdorferi*, human monocytic ehrlichiosis caused by *Ehrlichia chaffeensis*, Rocky Mountain spotted fever caused by *Rickettsia rickettsii*, virus-mediated encephalitis and sever fever with thrombocytopenia syndrome, and babesiosis caused by protozoa *Babesia* (1–3, 7–9). Apart from tick-transmitted infectious diseases, some people who have experienced tick bites suffer from repeated episodes of

systemic anaphylaxis after eating red meat or treated with monoclonal antibodies for cancer therapy. This particular type of allergy is designated as α-gal syndrome, because patients produce IgE against the carbohydrate Galα1-3Galβ1-4GlcNAc-R (α-Gal) that is shared by tick saliva antigens, red meat, and recombinant antibodies (10–12). Thus, tick infestation and tick-borne diseases constitute a growing burden for human and animal health throughout the world.

Most ticks undergo four life stages, namely egg, six-legged larva, eight-legged nymph and adult, taking 2 or 3 years to complete their full life cycle. After hatching, ticks must feed on the blood of host animals at each stage to survive. Most ticks prefer to target a different host animal at each stage. After feeding, larvae and nymphs drop off from hosts and molt to go to the next stage. Not only ticks but also tick-borne pathogens are maintained in this zoonotic cycle. For example, *Ixodes scapularis* larvae and nymphs feed on small rodents such as *Peromyscus leucopus* (white-footed mouse), the main reservoir host for *B. burgdorferi*, a spirochete causing Lyme disease (13). Larvae acquire the pathogen from infected mice and molt to become infected nymphs that in turn feed on other mice, leading to the pathogen transmission to the mice. Infected nymphs molt to become infected adults that feed on white-tailed deer. Although humans are not natural hosts for *Ixodes* ticks, nymphs accidentally feed on humans, resulting in the pathogen transmission to humans and the development of Lyme disease.

For successful blood feeding, ticks inject saliva containing a wide range of bioactive substances into host animals, including vasodilator, anti-hemostatic, anti-inflammatory, and immunosuppressive reagents (4–6). To counteract these, host animals activate various defense pathways, including innate and acquired immunity against tick infestation. Some animal species, including cattle, rabbits, guinea pigs and mice, have been demonstrated to develop resistance to tick feeding after a single or repeated infestation, depending on the combination of tick species and animal species/strains (14–16). This acquired tick resistance (ATR) is manifested by reduced weights of feeding ticks, reduced numbers of engorged ticks, prolonged duration of feeding, inhibition of molting, death of feeding ticks, diminished production of ova or reduced viability of ova. The expression of ATR is not confined to the skin lesion of previous tick bites and can be induced in uninfested skin of sensitized animals, suggesting the involvement of systemic rather than localized responses. ATR was abolished when guinea pigs were treated with immunosuppressants such as methotrexate and cyclophosphamide (17, 18). Furthermore, ATR can be adoptively transferred to naive syngeneic animals with leukocytes or sera isolated from animals infested previously with ticks (19–22). These findings strongly suggested that ATR is a type of immune reaction. From a clinical point of view, ATR is notable, because it can reduce the risk of pathogen transmission from infected ticks to humans and animals (23–26). Hence, further clarification of mechanism underlying ATR will pave the way for the development of efficient anti-tick vaccines to prevent tick infestation and tick-borne diseases.

Basophils are the least abundant type of granulocytes and account for less than 1% of peripheral blood leukocytes (27, 28). They circulate in the bloodstream under homeostatic conditions and infiltrate peripheral tissues when inflammation occurs there. Although basophils are evolutionally conserved in an array of animal species, their functional roles *in vivo* remained a mystery long after their discovery by Paul Ehrlich in 1879. Basophils are named after basophilic granules in the cytoplasm that stain with basic dyes. In addition to the basophilic granules, blood-circulating basophils share certain phenotypic features with tissue-resident mast cells, including the expression of the high-affinity IgE receptor FcεRI on the cell surface and the release of proallergic mediators such as histamine in response to a variety of stimuli (27, 28). Owing to their phenotypic similarity with mast cells and their rarity, basophils had often erroneously been considered as blood-circulating precursors of tissue-resident mast cells or minor and possibly redundant relatives of mast cells, and therefore neglected in immunological studies (29). Recent development of tools useful for functional analysis, including genetically-engineered mice deficient only in basophils (30–36) (**Figure 1**), has successfully illustrated the nonredundant roles of basophils, distinct from those played by mast cells, in a series of immune responses, including protective immunity to parasitic infections, allergic inflammation,

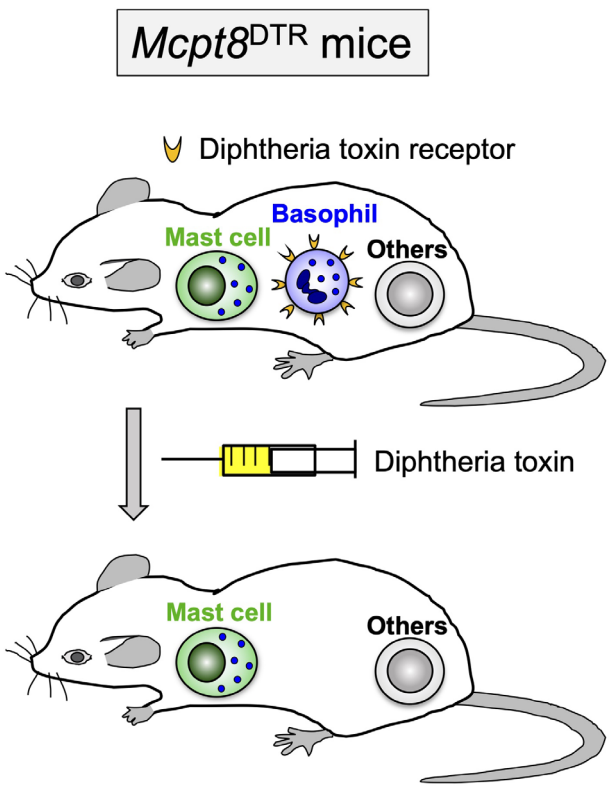

FIGURE 1 | Diphtheria toxin-mediated, conditional depletion of basophils in *Mcpt8*^{DTR} mice. In *Mcpt8*^{DTR} mice, the human diphtheria toxin receptor is genetically engineered to be expressed only on basophils, and therefore the diphtheria toxin administration can induce selective ablation of basophils.

autoimmune diseases, and regulation of innate and acquired immunity (37–39). In this article, we focus on the cellular and molecular mechanisms underlying ATR that have been clarified in animal models of tick infestation.

BASOPHILS PLAY A CRUCIAL ROLE IN THE EFFECTOR PHASE OF ATR

Trager (14) reported in 1939 that when guinea pigs were repeatedly infested with *Dermacentor variabilis* larval ticks, large numbers of larvae engorged in the 1st infestation whereas relatively few larvae did so in the 2nd or subsequent infestations, indicating guinea pigs developed tick resistance after a single infestation. The resistant state developed within 2 weeks after starting the 1st infestation and lasted for at least 3 months. Skin reaction in tick-resistant guinea pigs was characterized by extensive accumulation of basophils and eosinophils, and basophils composed up to 70% of the skin-infiltrating cells (17). The functional significance of basophil accumulation at the tick-feeding site was illustrated by the depletion of basophils in guinea pigs. Brown et al. (40) established rabbit antiserum against guinea pig basophils, and the treatment of resistant guinea pigs with the anti-basophil serum depleted basophils and abolished ATR, demonstrating a crucial role for basophils in the manifestation of ATR (**Figure 2**). In cattle, rabbits and goats, the infiltration of basophils in the tick re-infestation site was also observed (41–45). The frequency of basophils among cellular infiltrates at the tick-feeding sites varied, depending on the combination of host animals and tick species, and the functional role for basophils of these animals in ATR remains to be investigated.

In mice, conflicting and puzzling findings had been reported, regarding to the contribution of basophils to ATR. Matsuda et al. (46–48) demonstrated that mast cell-deficient WBB6F1-*W/W^v* mice failed to manifest ATR when re-infested with *Haemaphysalis longicornis* larval ticks, and that ATR was reconstituted by the adoptive transfer of mast cells. Mast cell-sufficient WBB6F1-+/+ congenic control mice showed ATR even though infiltration of basophils was hardly detected histopathologically at the tick-feeding site during the re-infestation. Therefore, it was concluded that mast cells play an essential role in ATR in mice, in contrast to the case of guinea pigs where basophils do so. On the contrary, DenHollander et al. (49) reported that both WBB6F1-*W/W^v* and WBB6F1-+/+ mice acquired resistance equally well to the infestation with another tick species *D. variabilis* larvae, suggesting that mast cells are dispensable for ATR under these experimental conditions.

It had erroneously been believed for some time that murine basophils either do not exist or are extremely rare. Indeed, it is quite difficult to show their presence in tissue sections by using standard histological methods such as Giemsa stain. The existence of basophils in mice was clearly illustrated by electron microscopic examination (50–52). Steeves et al. (53) used electron microscopy and detected basophils, along with neutrophils and eosinophils, in the tick-feeding skin lesion of both WBB6F1-*W/W^v* and WBB6F1-+/+ mice infested repeatedly with *D. variabilis* larval ticks, suggesting the possible involvement of basophils rather than mast cells in ATR in mice as observed in guinea pigs. Thus, it remained puzzling whether mast cells and basophils differentially contribute to ATR in mice, depending on different species of ticks, either *H. longicornis* or *D. variabilis*.

Wada et al. (30) addressed this issue and revisited the role of mast cells and basophils in mice repeatedly infested with

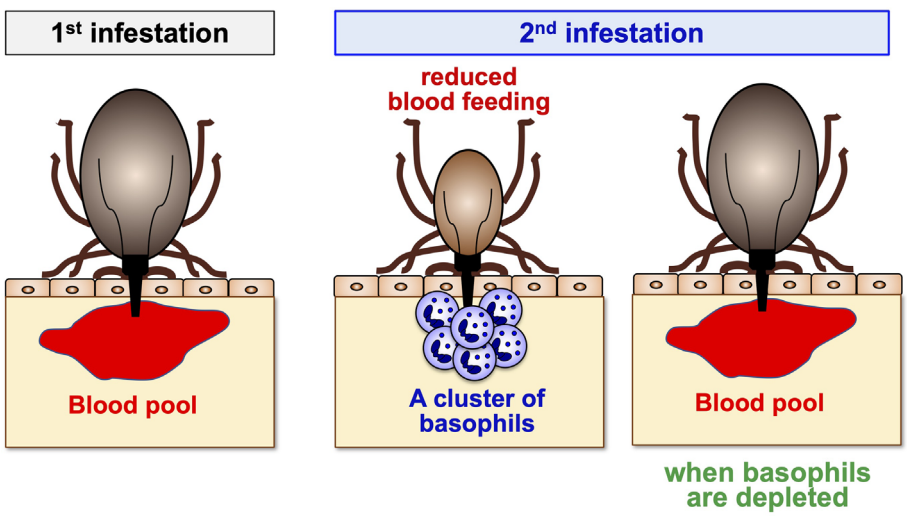

FIGURE 2 | A crucial role for basophils in acquired tick resistance. Some animal species can develop resistance to tick feeding after a single or repeated tick infestation, characterized by reduced body weights of engorged ticks. Basophil accumulation is detected at the tick re-infestation sites of animals showing tick resistance. Basophil depletion just before the 2nd infestation abolishes tick resistance, illustrating a crucial role for basophils in the manifestation of acquired tick resistance.

H. longicornis larval ticks. In accordance with the report by Matsuda (46), mast cell-deficient mice failed to show ATR, and the adoptive transfer of mast cells reconstituted ATR, confirming that mast cells are essential for ATR. Moreover, Giemsa staining of skin sections could not detect basophil infiltration at the tick re-infestation sites of mast cell-sufficient mice showing ATR (30), as reported previously (46), suggesting little or no contribution of basophils to ATR. Importantly, however, RT-PCR analysis detected transcripts of the *Mcpt8* gene encoding the basophil-specific protease mMCP-8 in tick-feeding sites during the 2nd but not 1st infestation (30), implying the possible recruitment of basophils to the 2nd tick feeding site. Indeed, histochemical examination of skin sections using the mMCP-8-specific monoclonal antibody demonstrated the accumulation of basophils to the tick-feeding site and their cluster formation surrounding tick mouthparts during the 2nd but rarely the 1st infestation (30) (**Figure 2**). Ohta et al. confirmed this finding by using intravital imaging of *Mcpt8*^{GFP} mice in that only basophils express green fluorescent protein (GFP) (54). Thus, the recruitment of basophils to the tick re-infested skin lesion was clearly demonstrated in both mice infestated with *H. longicornis* larval ticks (30) and those infested with *D. variabilis* larval ticks (53) as observed in guinea pigs, cattle, rabbits and goats (17, 41–45). Flow cytometric analysis revealed that basophils accounted for less than 5% of leukocytes accumulating at the 2nd tick-feeding site in mice infestated with *H. longicornis* larval ticks, much fewer than in the case of guinea pigs, with monocytes/macrophages, neutrophils and eosinophils being abundant. Nevertheless, basophil ablation by treating mice with basophil-depleting monoclonal antibodies, either anti-CD200R3 (Ba103) or anti-FcεRIα (MAR-1), before the 2nd infestation completely abolished ATR (30) (**Figure 2**). The important role for basophils in ATR was further confirmed by diphtheria toxin-mediated basophil depletion in genetically-engineered *Mcpt8*^{DTR} mice in that only basophils express diphtheria toxin receptors (30) (**Figures 1** and **2**). Collectively, basophils are key effector cells for ATR in mice infested with *H. longicornis* as reported in guinea pigs. Considering that the accumulation of basophils was also detected in tick re-infestation sites of mast cell-deficient mice that showed ATR to the infestation with *D. variabilis* (49, 53), basophils likely play a crucial role in ATR in mice infested with *D. variabilis* as well. Basophil infiltration was also observed in humans at the tick-feeding sites and the skin lesions of scabies (55–57). Although the role for basophils in ATR has not been demonstrated in humans, it was reported that a patient lacking basophils and eosinophils suffered from widespread scabies (58). This suggests the possible contribution of human basophils to protective immunity against ectoparasites, including ticks.

Mast cells, in addition to basophils, contribute to ATR in mice infested with *H. longicornis* but not with *D. variabilis* (30, 46, 49). It remains to be determined what makes this difference. ATR was completely abolished when either basophils or mast cells were absent in mice infested with *H. longicornis* (30), indicating that the function of basophils and mast cells may not be additive. The accumulation of basophils at the 2nd tick feeding site was normally observed even in mast cell-deficient mice (30), demonstrating that mast cells are dispensable for basophil recruitment. Tabakawa et al. (59) took advantage of intravital imaging using confocal fluorescent microscopy and demonstrated that basophils accumulating in the skin lesion are more motile and make a less dense cluster surrounding a tick mouthpart in the absence of mast cells than in the presence of mast cells. This may suggest that mast cells contribute to ATR by modulating the locomotion of basophils directly or indirectly. The contribution of mast cells to ATR has not been clearly demonstrated in animal species other than mice. The exact role for mast cells in ATR awaits further studies.

SKIN-RESIDENT MEMORY T CELLS PLAY AN IMPORTANT ROLE IN BASOPHIL RECRUITMENT TO THE SKIN LESION OF TICK RE-INFESTATION

Basophils are not tissue-resident cells and circulate in the blood stream under homeostatic conditions. Basophils infiltrate and accumulate at the tick-feeding sites of some animal species during re-infestation, but hardly during the 1st infestation, to execute ATR (**Figure 2**). Of note, the recruitment of basophils during re-infestation is detected even in previously un-infested skin, distant from the 1st infestation site. This suggests that in response to the 1st infestation, host animals induce some alteration in the skin throughout the body in order to attract basophils to the tick re-infestation site anywhere in the body at any time. Ohta et al. (54) demonstrated in mice infested with *H. longicornis* larval ticks that skin-resident, memory CD4$^+$ T cells are critically involved in the recruitment of basophils to the re-infestation site, leading to ATR. In response to the 1st infestation, tick saliva antigen-specific CD4$^+$ effector T cells are activated and expand in draining lymph nodes and are distributed to the skin throughout the body, and a fraction of them stay there as skin-resident, memory T cells (**Figure 3**). In the 2nd infestation, tick saliva antigens injected into the tick-feeding site activate these memory T cells to secrete IL-3 that in turn promotes the recruitment of basophils to the tick-infested skin (54) (**Figure 4A**), probably through facilitation of basophil adhesion to endothelium (60–62), leading to transendothelial migration of basophils.

In guinea pigs, complements have been shown to play a part in ATR. Cobra venom factor-mediated depletion of complements blocked ATR in guinea pigs re-infested with *Dermacentor andersoni* larvae, in parallel with reduced numbers of basophils infiltrating the epidermis below the tick attachment site (63). Guinea pigs deficient for the C4 component of complement could acquire and display tick resistance as observed in C4-sufficient guinea pigs (64), suggesting the involvement of the alternative rather than classical pathway of complement activation in ATR. Basophils are chemotactically attracted by fragments of complement components C3 and C5 (65). The deposition of complement components was observed at the dermo-epidermal junction near tick attachment sites and in the basophil-packed epidermal vesicles of resistant guinea pigs (66), suggesting that the complement activation at these sites might contribute to the recruitment of basophils to these sites.

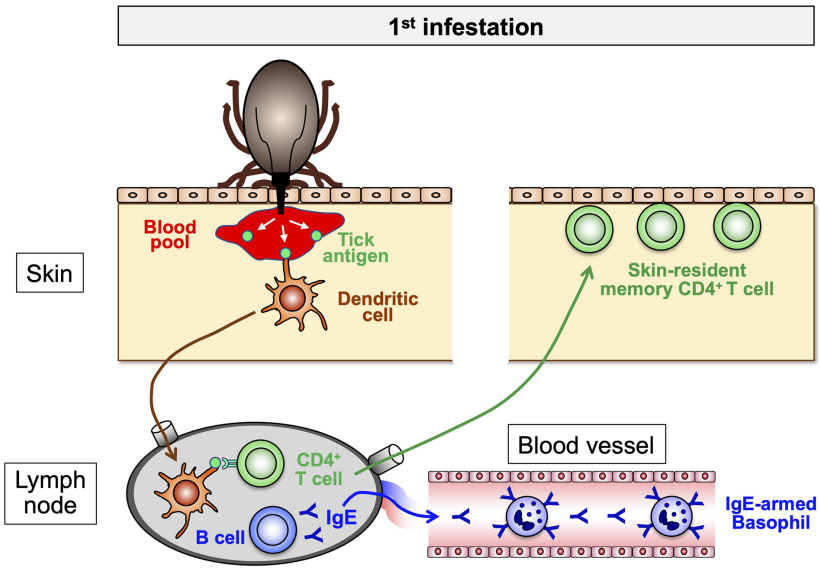

FIGURE 3 | The sensitization phase of acquired tick resistance. In the 1st tick infestation, tick saliva antigens injected into the host skin are taken up by dendritic cells and delivered to draining lymph nodes in that tick antigen-specific B cells and $CD4^+$ T cells are activated to expand. The collaboration of these B and T cells promotes the production of tick antigen-specific IgE that enters the blood stream and binds to the surface of blood-circulating basophils through FcεRI. Some of tick antigen-specific $CD4^+$ T cells migrate into the skin all over the body and remain as skin-resident, memory T cells.

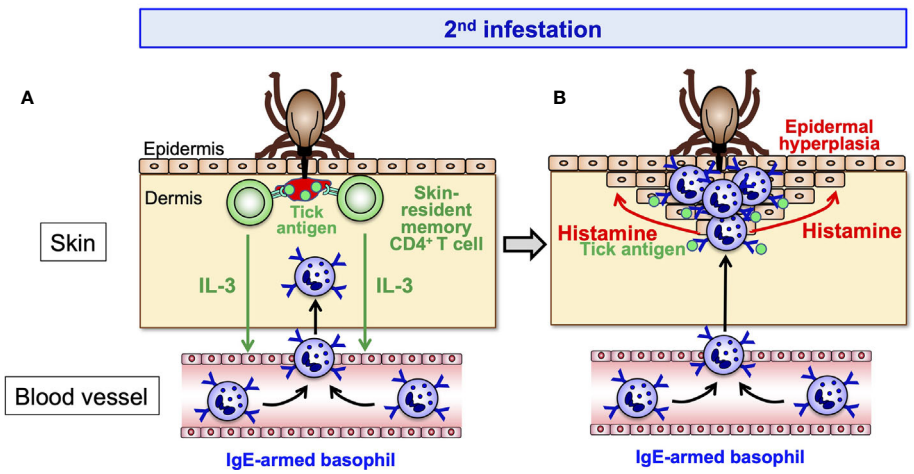

FIGURE 4 | The effector phase of acquired tick resistance. In the 2nd tick infestation, tick saliva antigens injected into the tick-feeding site stimulate the skin-resident, memory $CD4^+$ T cells to secrete IL-3 that in turn acts on endothelial cells and promotes transendothelial migration of basophils toward the tick-feeding site **(A)**. Skin-infiltrating basophils make a cluster surrounding the tick mouthpart and are stimulated by tick saliva antigens to release histamine **(B)**. Basophil-derived histamine acts on keratinocytes, leading to epidermal hyperplasia that hampers tick attachment or blood-feeding.

TICK SALIVA ANTIGENS ACTIVATE IGE-ARMED BASOPHILS *VIA* FCεRI IN THE TICK RE-INFESTATION SITE

In both guinea pigs and mice, the transfer of serum from animals previously infested with ticks has been shown to confer ATR on naive animals (20–22, 48), indicating the contribution of tick antigen-specific antibodies to ATR. In mouse experiments, the heat treatment of the serum at 56°C for 2 h abolished its activity of ATR transfer (48), indicating that heat labile IgE antibodies are responsible for the ATR transfer. In accordance with this, both antibody-deficient mice and *Fcer1g*$^{-/-}$ mice deficient in FcεRI expression showed no ATR (30), suggesting the following steps toward the manifestation of ATR in mice. The

1st tick infestation elicits the production of tick saliva antigen-specific IgE by B cells, and blood-circulating basophils bind such IgE on the cell surface through FcεRI (**Figure 3**). In the 2nd infestation, tick saliva antigens injected into the tick re-infestation site bind to IgE on basophils, resulting in the activation of basophils (**Figure 4B**). Wada et al. (30) demonstrated that both basophils and mast cells critically contribute to ATR in mice infested with *H. longicornis* larval ticks (30), and both of them express FcεRI on the cell surface. Therefore, tick saliva antigens can activate both types of cells through cross-linking the complex of tick antigen-specific IgE and FcεRI on the cell surface. Notably, the adoptive transfer of FcεRI-deficient mast cells conferred ATR on mast cell-deficient mice (30), demonstrating that FcεRI on mast cells is not essential for the manifestation of ATR. In sharp contrast, the adoptive transfer of FcεRI-deficient basophils failed to reconstitute ATR in basophil-depleted mice. Thus, basophils but not mast cells appear to play an important role in IgE-dependent ATR *via* FcεRI-mediated activation in mice, in spite of the fact that both types of cells contribute to ATR. A possible explanation for this will be discussed in the next section.

In guinea pigs, Brown et al. (22) demonstrated that intravenous transfer of immune serum from host animals infested twice with *Amblyomma americanum* larval ticks to naïve animals conferred a significant level of tick resistance. The heat treatment of the serum at 56°C for 4 h had no effect on the serum activity, suggesting little or no contribution of IgE to ATR. The fractionation of the immune serum revealed that IgG1 antibodies are responsible for it. Therefore, IgG1 rather than IgE appears to contribute to ATR in guinea pigs (22).

HISTAMINE RELEASED FROM ACTIVATED BASOPHILS PROMOTES EPIDERMAL HYPERPLASIA, LEADING TO TICK RESISTANCE

Tick salivary glands contain several histamine-binding proteins, including lipocalins, that are injected into host animals during tick-feeding (4–6). These proteins efficiently compete for histamine with its native receptor such as H1 histamine receptor, implying that histamine produced by host animals could be a threat to successful blood feeding by ticks and therefore must be counteracted. Willadsen et al. (67) reported that the amount of histamine in the skin lesion of individual *Bos taurus* cattle that had received extensive exposure to *Boophilus microplus* ticks correlates with the degree of resistance to tick infestation. The treatment of cattle with antihistamine lead to higher tick numbers (68) while the injection of histamine into the cattle skin promoted tick detachment (69). Similar observations were reported in guinea pigs infested with *D. andersoni* (70) and rabbits infested with *Ixodes ricinus* (71), suggesting that histamine is an effector molecule involved in ATR in general. Nevertheless, it remained to be determined until recently what types of cells produce histamine and how histamine executes ATR.

Tabakawa et al. (59) addressed these issues by analyzing mice infested with larval *H. longicornis* ticks. In accordance with the previous findings in cattle, rabbits and guinea pigs, the treatment of mice with antihistamine, particularly histamine H1 receptor antagonist, during the 2nd tick infestation abolished ATR. Repeated intradermal administration of histamine or an agonist of histamine H1 receptor beneath the tick-infested site during the 1st infestation significantly reduced the tick feeding as if it were in the second infestation, demonstrating a crucial role of histamine and histamine H1 receptor in the execution of ATR. Wada et al. (30) demonstrated that both basophils and mast cells play key roles in the manifestation of ATR in mice infested with *H. longicornis* larvae, and both types of cells are well-known producers of histamine. Adoptive transfer of histamine-sufficient but not histamine-deficient basophils reconstituted ATR in basophil-depleted mice whereas adoptive transfer of mast cells regardless of histamine sufficiency or deficiency conferred ATR on mast cell-deficient mice (59), indicating that histamine released from basophils but not mast cells is important for the manifestation of ATR. Confocal microscopic examination revealed that basophils accumulated in the epidermis of the 2nd tick-feeding site and formed a cluster that surrounded a tick mouthpart. In contrast, most of mast cells were scattered in the dermis and located away from the tick mouthpart. Considering the fact that histamine has a short half-life, the accumulation of basophils closer to the tick mouthpart, when compared to mast cells, appears to make histamine released from basophils more effective than that from mast cells.

Histamine released from activated basophils appears to contribute to the manifestation of ATR through several different modes of action. Histamine induces itching and grooming responses in the skin, leading to removal of ticks from cattle (72). Paine et al. (73) demonstrated, by using an *in vitro* model of tick feeding through artificial membrane, that the addition of histamine and serotonin to the feeding medium reduced blood feeding and salivation by ticks, suggesting direct effects of histamine on ticks attached to the skin nearby. Epidermal hyperplasia is a characteristic feature at tick-feeding sites of guinea pigs showing ATR (14). Tabakawa et al. (59) reported the epidermal hyperplasia and the cluster of basophils in the thickened epidermis at the 2nd but not 1st tick-feeding site in mice (**Figure 4B**). In this study, the influence of host grooming on tick feeding and skin architecture was negligible, because ticks were confined inside of an acryl ring attached to the skin. Notably, neither histamine-deficient nor basophil-depleted mice showed the epidermal hyperplasia. Repeated administration of histamine beneath the tick infestation site during the 1st infestation induced epidermal hyperplasia, together with the manifestation of tick resistance. These observations suggested that histamine released from basophils is responsible for epidermal hyperplasia. Given that keratinocytes express functional H1 receptor (74) and histamine promotes keratinocyte proliferation (75, 76), it is reasonable to assume that histamine released from activated basophils acts on keratinocytes, leading to the hyperplasia and thickening of epidermis that in turn hamper tick attachment or blood-feeding in the skin during the 2nd infestation (**Figure 4B**).

If the promotion of epidermal hyperplasia by basophil-derived histamine is indeed one of the mechanisms underlying ATR, it is intriguing to hypothesize that the length of tick mouthparts may be correlated with the degree of tick resistance (77). Some ticks such as *H. longicornis*, *D. andersoni*, and *B. microplus* have short mouthparts while others including *A. amricanum* and *Ixodes holocyclus* have longer mouthparts. So, it is plausible that the thickening of epidermis makes the former's but not the latter's mouthparts difficult to penetrate deep into the dermis in order to efficiently take a blood meal. In accordance with this assumption, the former tick species are highly responsive to histamine in terms of the induction of tick resistance whereas the latter are less responsive to histamine. Factors other than the length of mouthparts may also influence ATR. For example, the amounts of histamine-binding proteins injected by ticks into host animals may be correlated with differential responsiveness of ticks to histamine in the induction of tick resistance.

WHY AND HOW DO NATIVE HOST ANIMALS SHOW NO OR WEAK ATR IN CONTRAST TO NON-NATIVE HOSTS?

Many studies on ATR have examined tick feeding on laboratory animals that the particular tick species could not encounter naturally. It is generally thought that when ticks feed on their natural or reservoir host animals, animals show no or weak ATR. In contrast, non-reservoir host animals display strong ATR when repeatedly infested with ticks. For example, *Peromyscus leucopus* (white-footed mouse), the reservoir host for *I. scapularis*, does not show ATR upon repeated infestation with *I. scapularis* nymphal ticks even though they show a strong inflammatory response, including leukocyte accumulation, in the tick-feeding skin lesion (13). This is also the case in laboratory mice (*Mus musculus*) analyzed as surrogates of reservoir host animals. BALB/c and C3H/HeN mice could not develop ATR to nymphal *I. scapularis* or *I. ricinus* ticks upon repeated infestation (78–80). In contrast, these laboratory mice can show strong ATR when repeatedly infested with other tick species, such as *D. variabilis* and *H. longicornis*, that are not native ticks for mice. This suggests that *I. scapularis* has co-evolved with its natural host *Peromyscus leucopus*, and therefore the *I. scapularis-P. leucopus* interactions might be optimized for successful tick feeding (81).

The exact mechanism underlying poor development of ATR in natural host animals remains to be determined. Notably, the histopathological comparison of skin lesions of re-infestation with *I. scapularis* nymphal ticks in natural hosts (mice) and non-natural hosts (guinea pigs) revealed that the architecture of the skin lesions was distinct between them even though there was increased inflammation in the dermis of both hosts (13). The tick-feeding site in the non-natural hosts was characterized by a prominent scab-like epidermal hyperplasia and hyperkeratosis whereas the skin structure was not substantially disturbed in the natural hosts. This suggests that a certain step toward ATR development, including the production of tick-specific IgE, the generation of skin-resident, memory CD4 T cells, basophil recruitment, histamine release and epidermal hyperplasia (**Figures 3** and **4**), may not be operative in natural hosts, perhaps due to the modulation of host immune system by tick-derived molecules. Transcriptome and proteome analyses of tick salivary glands demonstrated that ticks of the same species differentially express tick saliva proteins, depending on host animals they feed (80, 82). This difference in the composition of saliva proteins might contribute, in part, to differences in host immune responses. This needs to be taken into account when anti-tick vaccine target antigens are selected. The findings obtained using laboratory animals may not be applied to wildlife animals and humans.

ATR CAN REDUCE THE RISK OF PATHOGEN TRANSMISSION FROM INFECTED TICKS TO HOST ANIMALS

Francis and Little (23) reported that the transmission of *B. bigemina* and *Babesia argentina* to tick-resistant cattle is significantly lower than that to nonresistant hosts. Bell et al. (24) provided clear and convincing evidence that ATR can reduce the risk of pathogen transmission from infected ticks to host animals. Rabbits were infested twice with pathogen-free *D. andersoni*, and they displayed resistance to tick infestation during the 2nd exposure. When infested further with *Francisella tularensis*-infected ticks, only 36% of the tick-resistant rabbits died as a result of *F. tularensis* infection whereas 100% of control naive rabbits died. Nazario et al. (25) demonstrated that repeated infestation of guinea pigs with pathogen-free *I. scapularis* nymphal ticks induced tick resistance in association with reduced capacity of *B. burgdorferi*-infected *I. scapularis* to transmit borrelia infection to guinea pigs. Analysis of people living in Lyme disease-endemic regions demonstrated that residents who experienced itching associated with attached ticks had fewer episodes of Lyme disease than those who reported no such episodes (26). This suggests that acquired immunity to ticks may limit the transmission of *B. burgdorferi* in humans as well.

The exact mechanisms underlying host resistance to tick-borne pathogens in association with resistance to tick infestation remain ill-defined. The resistance to pathogen transmission might be simply ascribed to the decrease of tick feeding and salivation in tick-resistant host animals. However, this does not seem to be always the case. Dai et al. (83) demonstrated that antibodies raised against Salp15, a tick saliva protein, protected C3H/HeJ mice from the transmission of borrelia infection mediated by *B. burgdorferi*-infected *I. scapularis* nymphal ticks. Salp15 binds to the surface of *B. burgdorferi*, increasing the ability of *B. burgdorferi* to infect mice. Salp15 antibodies appear to interact with Salp 15 on the surface of *B. burgdorferi* and hence enhance clearance of spirochete by phagocytes. Salp 15 antibodies showed no apparent influence on the ability of ticks to normally engorge, suggesting that the effect of the antibodies

on pathogen transmission cannot be ascribed to the reduced tick feeding/salivation in this case. In accordance with this, an earlier work by Wikel et al. (79) showed that repeated infestation of BALB/c mice with pathogen-free *I. scapularis* nymphal ticks induced host resistance to transmission of *B. burgdorferi* even though mice displayed no apparent ATR. Therefore, the host resistance to tick infestation might not necessarily be a prerequisite for the host resistance to pathogen transmission, even though both types of resistance are often observed in parallel.

DEVELOPMENT OF ANTI-TICK VACCINES

A number of chemicals have been used for controlling ticks. However, the application of such acaricides has had limited efficacy in reducing tick infestation and often comes with serious side effects, including the selection of acaricide-resistant ticks and the contamination of the environment and animal products, such as milk and meats, with chemical residues. Therefore, alternative strategies for controlling ticks and preventing tick-borne diseases are desired, including vaccines against ticks or pathogens. Because ticks can transmit a variety of pathogens, the development of vaccines against ticks rather than individual pathogens appears to represent one of the most promising and economical alternatives (7, 84, 85). Trager (14) already investigated in 1939 the possibility of the artificial immunization with tick extracts to obtain tick resistance in guinea pigs. Since then, the artificial induction of significant levels of tick resistance has been achieved by immunizing guinea pigs with extracts of tick tissues including salivary glands (21, 86–88). These findings are the basis for the development of tick antigen-based vaccines to forestall tick infestation and tick-borne diseases.

It is important to identify tick salivary antigens that are natural targets of acquired tick immunity, including those critical for ticks to feed, reproduce or transmit pathogens. This helps define salivary protein candidates that can serve as vaccine targets to inhibit tick feeding, reproduction and pathogen transmission to animals and humans. Transcriptomic analyses suggest that ticks of a given species may secrete more than 500 different proteins and peptides in their saliva during blood feeding (89, 90). The composition of saliva appears to change during blood feeding, perhaps confronting the different host defense responses. Targeting salivary proteins expressed early during tick feeding could have the advantage of inhibiting tick feeding early and preventing the pathogen transmission. The functional genomics approach, including RNA interference technology, will help assess the function of each tick gene and identify key molecules that mediate tick-host-pathogen interactions and can serve as vaccine targets (84).

CONCLUSIONS

A series of studies on the cellular and molecular mechanisms underlying ATR suggest the following scenario. In the sensitization phase of ATR (during and after the 1st infestation), tick saliva antigens injected into the skin are taken up by dendritic cells and delivered to draining lymph nodes where tick antigen-specific B and CD4$^+$ T cells are activated and expand, leading to the production of tick antigen-specific IgE that in turn binds to the surface of blood-circulating basophils through FcεRI (**Figure 3**). Some of tick antigen-specific CD4$^+$ T cells are distributed to the skin all over the body and remain as skin-resident, memory T cells (**Figure 3**). In the effector phase of ATR (during the 2nd infestation), such memory T cells are activated in response to the stimulation with saliva antigens injected by ticks to produce IL-3 that in turn facilitates the recruitment of IgE-armed, blood-circulating basophils to the tick re-infestation site (**Figure 4A**). Skin-infiltrating basophils are stimulated with tick antigens to release histamine that acts on keratinocyte, leading to epidermal hyperplasia that interferes with tick attachment or blood feeding (**Figure 4B**). This is the simplest mode of action proposed for the induction and manifestation of ATR, mainly based on the findings in the models of tick infestation in guinea pigs and mice. Further studies on the tick-host-pathogen interactions are definitely needed to develop the sophisticated strategy to forestall tick infestation and tick-borne diseases.

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

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Amblyomma sculptum Salivary Protease Inhibitors as Potential Anti-Tick Vaccines

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Amblyomma sculptum is the main tick associated with human bites in Brazil and the main vector of *Rickettsia rickettsii*, the causative agent of the most severe form of Brazilian spotted fever. Molecules produced in the salivary glands are directly related to feeding success and vector competence. In the present study, we identified sequences of *A. sculptum* salivary proteins that may be involved in hematophagy and selected three proteins that underwent functional characterization and evaluation as vaccine antigens. Among the three proteins selected, one contained a Kunitz_bovine pancreatic trypsin inhibitor domain (named AsKunitz) and the other two belonged to the 8.9 kDa and basic tail families of tick salivary proteins (named As8.9kDa and AsBasicTail). Expression of the messenger RNA (mRNA) encoding all three proteins was detected in the larvae, nymphs, and females at basal levels in unfed ticks and the expression levels increased after the start of feeding. Recombinant proteins rAs8.9kDa and rAsBasicTail inhibited the enzymatic activity of factor Xa, thrombin, and trypsin, whereas rAsKunitz inhibited only thrombin activity. All three recombinant proteins inhibited the hemolysis of both the classical and alternative pathways; this is the first description of tick members of the Kunitz and 8.9kDa families being inhibitors of the classical complement pathway. Mice immunization with recombinant proteins caused efficacies against A. *sculptum* females from 59.4% with rAsBasicTail immunization to more than 85% by immunization with rAsKunitz and rAs8.9kDa. The mortality of nymphs fed on immunized mice reached 70–100%. Therefore, all three proteins are potential antigens with the possibility of becoming a new tool in the control of *A. sculptum*.

Keywords: ***Amblyomma sculptum*, saliva, antihemostatic, complement inhibitors, vaccine**

INTRODUCTION

Amblyomma sculptum is the major tick species of medical importance in Brazil. It displays frequent parasitism of humans, high distribution in green areas of populated regions in both rural and urban areas, and is the main vector of *Rickettsia rickettsii*, the bacterium that causes the most frequent and severe form of Brazilian spotted fever (1–4). In addition, *A. sculptum* causes several problems in livestock rearing (5).

The reduction of host and environmental infestations of *A. sculptum* is of the utmost importance to prevent tick bites and pathogen transmission. The main control methods applied are based on the use of acaricides that, despite good results when used correctly, have raised concerns about the risk of contamination of products of animal origin and the environment, as well as the possibility of activity loss from the selection of resistant populations (6). *Amblyomma sculptum* is a three host tick, with a seasonal life cycle and short periods of contact with hosts that vary from 5 to 10 days (7, 8). Therefore, the use of acaricides has to be undertaken more frequently as strategic treatments recommend up to 16 to 28 applications throughout the year at weekly intervals (9). Such a strategy was developed to control ticks on horses, which are the main domestic hosts of *A. sculptum*. However, this treatment becomes extremely costly and even impractical when it needs to be administered on wild animals with serious difficulties regarding capturing, restraining, and application. Much of this concern is directed at the capybaras, which are considered to be the main wild hosts of *A. sculptum*, and can maintain high environmental tick infestations in urban and rural areas and are directly involved in the epidemiology of Brazilian spotted fever (1–3).

Therefore, the development of new tools for the control of *A. sculptum*, which can be applied alone or combined with acaricides, is extremely important. Anti-tick vaccines have emerged as an attractive alternative because these could reduce the use of acaricides and the costs associated with the manual labor required for animal capture, restraint, and application (10).

The development of arthropod vaccines is based on the selection of antigens that can induce an immune response in the hosts to prevent blood ingestion or the development of ticks (10). An important organ in tick physiology with many potential targets of vaccine antigens is the salivary glands (11). Ixodid ticks are hematophagous ectoparasites that spend long periods on the host (12), thus requiring the production of a cocktail of bioactive molecules in the salivary glands with important roles that guarantee the success of blood ingestion. These molecules are injected into the host skin to counteract the immune, inflammatory, and hemostatic reactions triggered at the site of the bite (13, 14). The complement system and coagulation are two examples of important mechanisms of immune and hemostatic responses, respectively, which are exerted by vertebrate hosts in blood spoliation and opposed by active molecules produced by the ticks (15).

The main function of complement inhibitors secreted in the saliva is to avoid inflammation at the bite site, prevent opsonization of salivary molecules, and protect the cells of the digestive tract (16–18). Components produced along the complement cascade, such as C3a and C5a, are strong inflammation anaphylatoxins, whereas molecules opsonized by two or three molecules of C3d can be 1,000 to 10,000 more immunogenic, respectively (19, 20). In addition, activated complement components in the intestinal environment during or after hematophagy may lead to cell lysis and, hence, tissue damage (17, 21, 22).

Hematophagous arthropods also face the mechanisms involved in hemostasis activated in the host in response to bite and blood spoliation. The fluidity of the diet is important as the tick must ingest proper amounts of blood during the feeding process to facilitate digestion. Therefore, these organisms must produce anticoagulant molecules that can oppose the blood coagulation cascade (23, 24).

Over the past few years, the molecules produced by the salivary glands of *A. sculptum* have been described in different transcriptomes (11, 25, 26); however, few of those molecules have been subjected to functional characterization. Concerning the development of tick vaccines, various studies have focused on the tick *Rhipicephalus microplus* or other tick species and there is a lack of studies concerning the selection and testing of specific antigenic targets against *A. sculptum*. Therefore, in the present study, we searched for sequences related to *A. sculptum* salivary proteins that might be involved in the feeding process and selected three proteins that underwent functional characterization and evaluation as possible vaccine antigens.

MATERIALS AND METHODS

Experimental Ticks and Ethical Statements

Amblyomma sculptum ticks used in the present study were obtained from a colony maintained at the Department of Parasitology, UFMG. Specimens were kept inside incubators under semi-controlled conditions of temperature (28 ± 2°C) and humidity (90 ± 5% relative humidity). Ticks were fed on Swiss mice (*Mus musculus*) using feeding chambers according to the methodology described by Bouchard and Wikel (27). Ticks used in all experiments were 20 to 40 days after molt.

The tick colony maintenance and animal experimental procedures performed in the present study were reviewed and approved by the Ethics Committee for Animal Use at UFMG (CEUA-UFMG) under protocols 60/2020 and 103/2017.

Target Selection

The parameters used for target selection were: putative antihemostatic activity, the presence of signal peptide, and high abundance among the protein family. For such, three transcriptomes of the salivary glands were analyzed (11, 25, 26) and the classes of salivary secreted proteins with more transcripts among the ones with putative antihemostatic function were selected. The classes of proteins with Kunitz domains and proteins of the families basic tail and 8.9 kDa were selected. Then, the most abundant transcript in each of

those three classes was identified and used to search for homologous sequences in a transcriptome of the salivary glands of *A. sculptum* produced with the tick population used in the present work (not published). The transcriptome was sequenced using a methodology similar to that described by Araujo et al. (28). A pool of four salivary gland pairs were used in the transcriptome that were isolated from one unfed male and three females (one unfed, one partially fed, and one fully engorged). Three target sequences were selected and submitted to the experiments (**Supplementary Material**)

RNA Extraction and cDNA Synthesis

RNA was extracted from pools of five larvae or two nymph whole bodies, or one female salivary gland extracted in a 0.9% saline solution using a stereomicroscope and dissecting fine tip forceps. The salivary glands or whole bodies were transferred to 1.5 ml microtubes containing 50 µl of de TRIzol® Reagent (Life Technologies®), where the total mRNA was extracted following the manufacturer's protocol. Total RNA quality and quantity were determined using a Nanodrop™ (Thermo Scientific) and were used for cDNA synthesis using a M-MLV Reverse Transcriptase (Promega) following the manufacturer's instructions.

Relative Expression Analysis of Salivary Proteins

Quantitative polymerase chain reaction (qPCR) was performed in triplicate with 5 µl of Power SYBR Green PCR Master Mix Kit (Applied Biosystems), 1 µl of the cDNA previously produced as a template, 200 nM of specific primers, and Milli-Q water to achieve a final volume of 10 µl per reaction. The reactions were conducted in 96-well plates using a StepOnePlus™ System (Thermo Fisher Scientific) at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative quantification data were presented as $2^{-\Delta\Delta Ct}$ (29).

PCR primers for targets were designed using the Primer3 webtool (http://bioinfo.ut.ee/primer3-0.4.0/) and the sequences were as follows: AsKunitz (F - 5′ AGACGCCAACCTGCTTT CTA 3′/R - 5′ ATTGTCCCTCCTTCGCTTTT 3′), As8.9kDa (F - 5′ TCTGTACCCTCGTCGCTTTT 3′/R - 5′ AGCGTGTTACG CTCGAAGAT 3′), and AsBasicTail (F - 5′ TTTTGGGCGAAG GATAACAC 3′/R - 5′ TTTGGCTTCTTCGTGCTTTT 3′). Elongation factor 1-α (F - 5′CGTGCCCACAAAATCCTTAT 3′/R - 5′ GGAAGTCTCAAAAGCCGGTA 3′) was used as a reference gene (30).

Cloning, Expression, and Purification of Recombinant Salivary Proteins

The target sequences were amplified by PCR and cloned into the pET28a(+) 6xHis-TEV expression vector. All primers used in the reactions were designed using the Primer3 webtool and restriction sites for *XhoI* (CTCGAG) and *NheI* (GCTAGC) were inserted in the forward and reverse ends, respectively. The final primer sequences were as follows: AsKunitz (F - 5′ CTCGAGTATAAAC GGCCCAAATTTTGCT 3'/R - 5′ GCTAGCTCATGGGGCG TTGAGAATA 3′), As8.9kDa (F - 5′ CTCGAGGTCCAGGAAC ATGGCCACTC 3′/R - 5′ GCTAGCGTTGGTGCCATCG CAGACT 3′), and AsBasicTail (F - 5′ CTCGAGTATGATA TTGTCCGTGGTTGC 3′/R - 5′ GCTAGCTTCGCCCAGGAT TTTACCAT 3′). The reactions were performed using 10 µM of each primer, 1 µl of the previously synthesized cDNA, and Platinum™ Taq DNA polymerase (Invitrogen) following the manufacturer's instructions at a 20 µl final volume mix. A Veriti™ 96-Well Thermal Cycler (Thermo Fisher Scientific) was used and the conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 40 s, 60°C for 40 s, 72°C for 40 s, and a final step of 72°C for 7 s. The products were cloned into pET28a(+) 6xHis-TEV digested with *XhoI* and *NheI*. The vector was transformed following heat shock and multiplied in *Escherichia coli* DH5α cells and then purified with a Wizard® Plus SV Minipreps DNA Purification System (Promega) following the manufacturer's specifications. The purified vectors were sequenced to confirm identity (**Supplementary Material**) and then transformed following heat shock in *E. coli* BL21. The expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (0.4 mM) and shaking at 180 rpm at 37°C overnight. Purification of recombinant proteins was performed using ProBond™ Purification System (Thermo Fisher Scientific) columns with a nickel-chelating resin. Eluted fractions were analyzed with 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) stained with silver nitrate solution to confirm the presence and mass weight of the recombinant proteins. Fractions containing recombinant proteins received 10 mM of 1,4-dithio-D-threitol and were dialyzed in dialysis membranes (Sigma-Aldrich®) for 48 h, with the addition of crescent volumes of phosphate buffered saline (PBS) at pH 7.4, overnight at 4°C, until a total volume of 200× the sample volume. Purified recombinant proteins were quantified (31) and stored at −20°C.

Western Blotting of Recombinant Proteins

Western blotting was performed to confirm the expression and purification of the recombinant proteins. The proteins were separated using 12.5% SDS-PAGE and then transferred to nitrocellulose membranes at 100 V for 2 h. The membranes were blocked with 5% milk powder diluted in PBS/0.05% Tween 20 (Sigma) for 2 h and then washed three times with PBS/0.05% Tween 20. After washing, the samples were incubated with His-tag antibodies produced in mice (Sigma-Aldrich®) that had been diluted 2,000× in PBS/BSA 1% for 1.5 h. After three more washes, the membranes were incubated with peroxidase-conjugated rabbit anti-mice-IgG secondary antibodies (Sigma-Aldrich®) that had been diluted 4,000× in PBS/0.05% Tween 20 for 1.5 h. Detection was performed using a Peroxidase Substrate DAB kit (Vector Laboratories®).

Human Plasma Recalcification Time Assay

Citrated human plasma (30 µl) was incubated with 30 µl of each recombinant protein at 0.25 µM or 0.5 µM concentration that had been diluted in PBS (pH 7.4) at 37°C for 5 min in a 96-well plate. The coagulation cascade was triggered by the addition of 30 µl of $CaCl_2$ (25 mM) in each well and the absorbance was measured in a microplate reader (VersaMax™, Molecular Devices) at 650 nm with 10 s read intervals for 20 min at 37°C.

Inhibition of Serine Protease Activity

Different concentrations of each recombinant protein were incubated with three different serine proteases in Tris buffer (20 mM Tris-HCl, 150 mM NaCl, and 0.1% BSA; pH 7.4): factor Xa (FXa) (5×10^{-5} U), thrombin (2.5×10^{-3} U), or trypsin (9 U) (Sigma-Aldrich®) at 37°C for 15 min in 0.5 ml microtubes. Then, the tube contents were placed in 96-well plates and the enzymatic reactions were triggered in the presence of the specific substrates CH_3OCO-D-CHA-GlyArg-pNA-AcOH (Sigma-Aldrich®) for FXa, Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride (Sigma-Aldrich®) for trypsin, and N-(p-tosyl)-Gly-Pro-Arg p-nitroanilide acetate salt (Sigma-Aldrich®) for thrombin at 4 mM concentration and 100 µl final volume. Immediately after the addition of the substrate, the absorbance variation was assessed at 405 nm every 10 s with 2 s agitation between reads in a microplate reader (VersaMax™, Molecular Devices) for 30 min. The maximum reaction velocity (Vmax) values were obtained for reactions lacking inhibitors and were used as a reference for reactions in the presence of different concentrations of inhibitors.

Complement System Hemolytic Inhibition Assays

To evaluate the effect of the salivary recombinant proteins on the classical pathway-mediated complement activation, hemolytic assays were performed using antibody-coated sheep erythrocytes (32). Erythrocytes were opsonized with IgG anti-sheep erythrocytes produced in rabbits (Sigma-Aldrich®) and diluted in GHB^{+2} solution (5 mM HEPES, 145 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.1% gelatin; pH 7.4) at 2×10^8 cells/ml. A total of 25 µl of normal human sera (NHS) that had been diluted 30× in GHB^{2+} and 25 µl of each recombinant protein that had been diluted in PBS at different concentrations were combined and incubated with 25 µl of diluted erythrocytes at 37°C for 30 min. To assess the hemolysis rate, 250 µl of PBS was added to each tube and the supernatants were removed after the samples were centrifuged at 1,700 × *g* for 1 min. The samples were placed in 96-well plates and evaluated using a microplate reader at 414 nm (VersaMax™, Molecular Devices). Negative controls (no serum) were subtracted from the other samples and the inhibition rates were calculated by comparing the samples containing recombinant proteins to the positive controls (samples without inhibitors). To evaluate the effect of the same proteins on the alternative pathway-mediated complement activation (33), rabbit erythrocytes diluted in Mg-EGTA (HEPES 1 mM, NaCl 30 mM, EGTA 10 mM, $MgCl_2$ 7 mM, glucose 3%, and gelatin 0.02%; pH 7.4) at a concentration of 1×10^8 cells/ml and NHS diluted 20× were used in the reactions.

To assess the effect of the mouse antiserum on the recombinant activities, hemolytic assays were performed as described above; however, 1 µM of each protein was incubated with its respective antiserum (produced according to the immunization procedures below) at different concentrations, at a final volume of 25 µl, before being mixed with NHS and the erythrocytes (34). Assays using rAsKunitz and rAs8.9kDa were performed with the classical pathway-mediated complement activation, whereas those with rAsBasicTail were performed using alternative pathway activation methodology. Controls were prepared by incubating each recombinant related antiserum with the erythrocytes to check whether they could cause hemolysis.

Immunization Procedures

Swiss mice (4 to 8 weeks old) were injected subcutaneously three times at 2-week intervals with 5 µg of each recombinant protein plus 0.1 mg of aluminum hydroxide gel (Sigma-Aldrich®) as an adjuvant diluted in 100 µl of sterile PBS. Animals in the control group were inoculated only with adjuvant. Individual blood samples were collected by puncturing the tail vein of the mouse and the serum samples were obtained by blood centrifugation at 4,000 × *g* for 10 min. Samples were stored at −20°C until antibody titration assays.

Measurement of Antigen-Specific IgG Levels in the Serum of Immunized Mice

Antigen-specific IgG levels for each salivary recombinant protein were estimated by indirect ELISA. The 96-well ELISA plates (Nunc MaxiSorp™, Thermo Fisher Scientific) were coated with 0.5 µg/well of each recombinant protein overnight. The plates were then blocked with 5% skimmed milk powder diluted in PBS/0.05% Tween 20 for 2 h at 37°C and washed with PBS/0.05% Tween 20. Serum samples diluted 1:160 in PBS/0.05% Tween 20 were added to the plates and incubated for 1.5 h. The plates were then washed and incubated with peroxidase-conjugated rabbit anti-mice-IgG secondary antibodies (Sigma-Aldrich®) that had been diluted 4,000× in PBS/0.05% Tween 20 for 1.5 h. After three washes, 100 µl of 100 mM phosphate-citrate buffer (pH 5.0) containing 0.075% H_2O_2 and 2 mg/ml o-phenylenediamine dihydrochloride was added to each well and the plates were incubated in a dark container for 20 min at 37°C. After incubation, 100 µl of 2 M H_2SO_4 was added to stop the reaction and the IgG levels were estimated with a 492 nm read on a microplate reader (VersaMax™, Molecular Devices).

Tick Infestation and Vaccine Efficacy Calculations

Two weeks after the third injection of the immunization protocol, all mice were prepared with a feeding chamber attached to the dorsal region (27) and infested with one adult tick couple or 10 nymphs per mouse. The levels of protection were determined by measuring the following feeding parameters: mortality that occurred during blood feeding and before oviposition or molt, the engorged tick weight, and the feeding period for nymphs and females. To assess the reproductive parameters, measurements were taken of the egg mass weight and percentage of eggs hatching. Vaccine efficacy rates against female parasitism were calculated according to the following formula: (%E) = 100 [1−(CRT × CR0 × CRF)], which comprises the coefficient of reduction in the number of engorged female ticks in the vaccinated/control group (CRT), coefficient of reduction in oviposition in the vaccinated/control group (CR0), and coefficient of reduction in egg fertility in the vaccinated/control group (CRF) (35).

RESULTS

Selection and Expression Levels of the Tick Salivary Transcripts

A transcript coding for a protein of 8.7 kDa containing a Kunitz_bovine pancreatic trypsin inhibitor domain and that was 99% identical to the "putative tick Kunitz 78" (accession: JAC23688.1) was selected. This protein was named AsKunitz.

A transcript coding for a 9.8 kDa protein with a Von Willebrand factor (vWF) type c domain was also selected. The protein has eight conserved cysteine characteristics of the 8.9 kDa tick superfamily. Its mature sequence was identical to the "hypothetical protein (*Amblyomma cajennense*)" (accession: JAC23736.1). This protein was named As8.9kDa.

A 16.7 kDa protein was also selected. It has several lysine residues in the carboxy terminal region, three disulfide bonds, and a YY block, which are characteristics of the Basic Tail protein family and are exclusive to ticks. The protein was 86% identical to a sequence named "putative basic tail protein (*A. cajennense*)" (accession: JAC23973.1). This protein was named AsBasicTail.

Expression of the three selected transcripts was observed in the larvae, nymphs, and adults of *A. sculptum* at different levels during feeding. In general, transcripts were expressed at basal levels in the unfed ticks and their expression increased as feeding started (**Figure 1**). For immature tick instars, the highest expression was observed in larvae after feeding, with As8.9kDa expression significantly ($p < 0.05$) increasing by ~15-fold (**Figure 1B**). For nymph feeding, the expression of AsKunitz significantly increased ($p < 0.05$) by ~750-fold (**Figure 1D**).

In adult females, the profile of the mRNA expression in the salivary glands during feeding was similar between As8.9kDa and AsBasicTail, which had peaks of expression close to the end of the feeding (at day 5 of feeding) (**Figures 1H, I**). AsKunitz was upregulated at the beginning of blood feeding (**Figure 1G**). When the expression level was analyzed at the peak times, AsKunitz was the most abundant with mRNA levels that were almost 20 million times higher than those in the unfed ticks (**Figure 1G**). As8.9kDa and AsBasicTail also had a significant ($p < 0.05$) upregulation; however, their mRNA levels were 365- and 471-fold higher than those in the fasting ticks (**Figures 1H, I**).

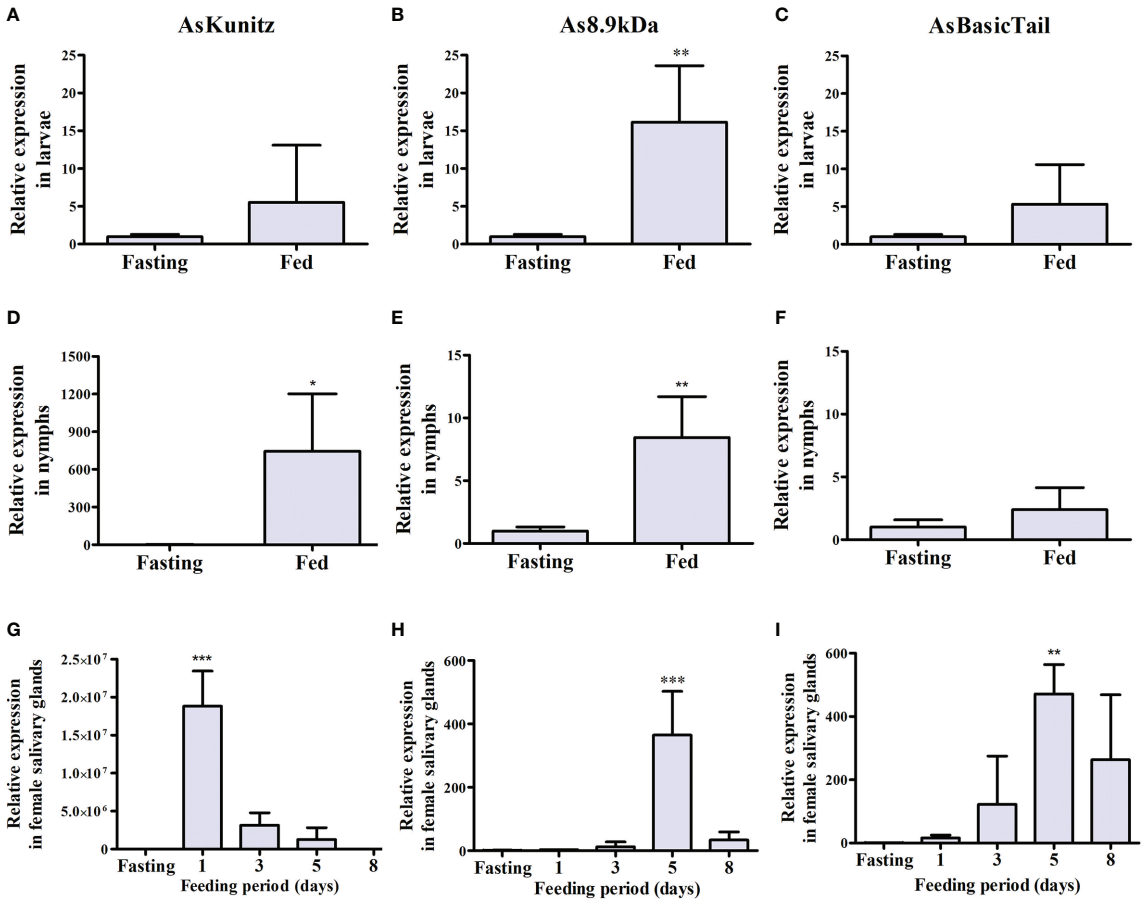

FIGURE 1 | Relative messenger RNA (mRNA) levels of AsKunitz, As8.9kDa, and AsBasicTail in *Amblyomma sculptum* instars during feeding. Measurements were performed using quantitative polymerase chain reaction in whole body for larvae **(A–C)** and nymphs **(D–F)** and in the salivary glands for females **(G–I)**. Feeding = 3 days of feeding. Statistical analysis: **(A–F)**—t-test, **(G–I)**—ANOVA–Dunnett, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate difference from the fasting group. Data is shown as mean ± standard deviation (SD).

Recombinant Expression and Functional Characterization of Selected Salivary Proteins

To assess the biological activities of these three proteins, they were expressed as recombinant proteins in *E. coli*. Recombinant proteins appeared on SDS-PAGE and western blotting with molecular weights approximately twice their estimated size (**Figure 2A**).

The three recombinant proteins prolonged the coagulation time, with higher activity for rAsBasicTail (**Figure 2B**). These results confirm that the recombinants were correctly folded and active. Coagulation was completely inhibited (no clots formed during the assay period) when 0.5 μM of each recombinant was used.

The coagulation cascade has several serine proteases; therefore, we checked the ability of the recombinant proteins to act on FXa, thrombin, and trypsin. rAsKunitz was specific for thrombin, where it had a significant inhibitory activity (IC_{50} = 0.65 μM) and had no effect on FXa and trypsin (**Figures 3A, D, G**). rAsBasicTail and rAs8.9kDa significantly affected ($p < 0.05$) the activity of all serine proteases tested, but with distinct levels of activity. Inhibition promoted by rAsBasicTail was stronger on FXa and trypsin (**Figures 3C, F, I**), whereas rAs8.9kDa showed higher inhibition of thrombin (**Figures 3B, E, H**).

Recombinant proteins also affected the human complement cascade. All three proteins inhibited the activation of the complement system in both the classical and alternative pathways. rAsKunitz and rAs8.9kDa were more efficient in inhibiting the classical pathway (**Figures 4A–C**), whereas rAsBasicTail was the best inhibitor of the alternative pathway (**Figures 4D–F**), achieving more than 80% hemolysis inhibition with 4 μM. To confirm the complement inhibition activity, the assays were repeated, but the recombinants were incubated with mice specific anti-sera before being added to the other components of the assay. Each recombinant was tested in a pathway that promoted more inhibition and the results showed that the antibodies could partially and significantly ($p < 0.05$) impair their inhibition of the complement system by all three recombinants (**Figures 4G–I**).

Effect of Recombinant Proteins as Vaccine Antigens Against Tick Infestation

To assess the potential of the proteins as vaccine antigens, the mice were immunized with each recombinant and used as a feeding source for nymphs and females of *A. sculptum*. Immunization induced two distinct profiles of specific IgG levels (**Figure 5**). Mice immunized with rAsKunitz and rAsBasicTail had an increase in specific IgG levels until the challenge followed by a gradual decrease (**Figures 5A, C**), whereas the specific anti-rAs8.9kDa IgG levels increased gradually until the end of the experiment (**Figure 5B**).

When females were fed on mice previously immunized with each recombinant protein, no significant ($p > 0.05$) effects were observed on the feeding period, engorged tick weight, and egg mass laid by females (**Figures 6A–C**). However, mortality and egg hatching were considerably affected in females from the groups fed on immunized mice. Mortality reached up to 58% when the nymphs were fed on mice that had been previously immunized with the recombinant proteins compared to a rate of 30% for the control group (**Table 1**) and egg viability was at least 50% lower than that of the control groups (**Figure 6D**). rAs8.9kDa induced higher female mortality by 94% (**Table 1**) and rAsKunitz and rAs8.9kDa both induced more than 80% fewer egg hatching than that of the control group (**Figure 6D**). The efficacy of the recombinant proteins as antigens showed that immunization with rAsKunitz and rAs8.9kDa had the highest effects (reduced infestation by at least 85%), whereas immunization with rAsBasicTail was only 59.4% lower than that of the control group (**Table 2**).

The vaccination experiments performed with the nymphs also had high efficacies, with mortality being the most affected parameter. The control group had 32% mortality, whereas all the nymphs died after feeding on mice immunized with rAs8.9kDa and rAsBasicTail, and 70% died when fed on rAsKunitz-immunized mice (**Table 1**). All the nymphs from the rAs8.9kDa and rAsBasicTail groups died while attached to the host feeding. Nymphs of the rAsKunitz group that survived required significantly ($p < 0.05$) more days to complete feeding (**Figure 7A**); however, they had final weights similar to those of the control group (**Figure 7B**).

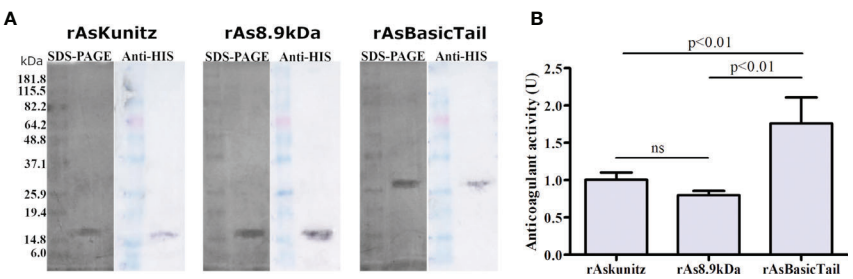

FIGURE 2 | Expression of rAsKunitz, rAs8.9kDa, and rAsBasicTail and their effect on the coagulation cascade. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (silver staining) and western blotting (developed using anti-His-tag antibodies) of each recombinant protein **(A)**. Effect of 0.25 μM of each recombinant on the human coagulation cascade **(B)**. One unit (U) of anticoagulant activity indicates that the coagulation time was doubled. Statistical analysis: ANOVA–Tukey, ns = not significant ($p>0.05$). Data is shown as mean ± SD.

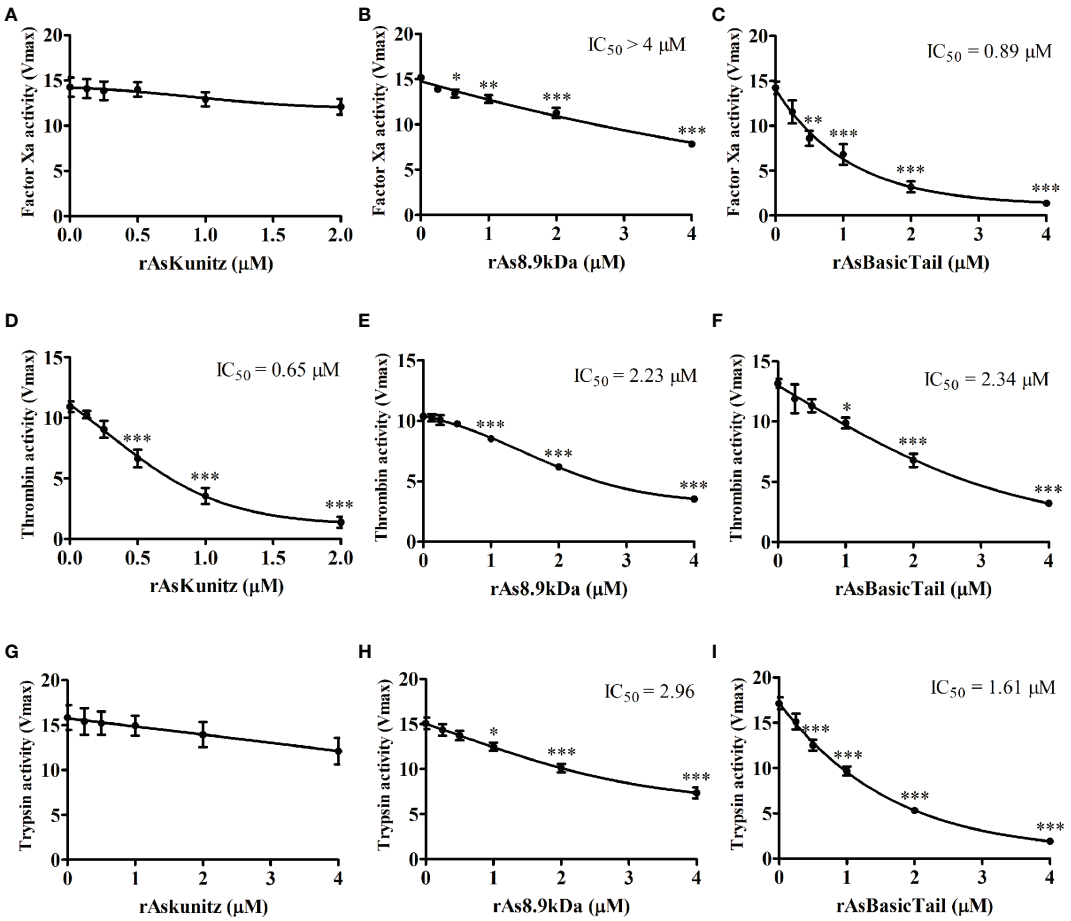

FIGURE 3 | Effect of the recombinant proteins on the activity of factor Xa **(A–C)**, thrombin **(D–F),** and trypsin **(G–I)**. Data is shown as mean ± standard error (SE). Statistical analysis: ANOVA–Dunnett, *p < 0.05, **p < 0.01, and ***p < 0.001 indicate difference from the sample without recombinants (0 μM).

DISCUSSION

The present study characterized three salivary molecules from *A. sculptum* and evaluated their potential as vaccine antigens for the development of novel tick control methods. All three proteins showed antihemostatic and anti-complement activities and promising anti-tick efficacies, which make them potential vaccine antigens against *A. sculptum*. The results also provide information on the biological activities of proteins belonging to three families of proteins. Despite being relatively common in tick salivary glands (13, 36), functional studies on members of the 8.9 kDa and Basic Tail families are scarce.

AsKunitz, As8.9kDa, and AsBasicTail were expressed in all tick instars and presented upregulation, with distinct levels, after feeding started. The larvae and nymphs had lower levels of upregulation that varied from 2.4- to 745-fold, whereas the variation in adults, at peak production, was considerably higher and ranged from 365- to almost 20 million-fold. The larvae and nymphs were analyzed at day 3 of feeding and, therefore, the peak production of each protein might not have been assessed.

In the female salivary glands, the expression profile showed that AsKunitz was more highly expressed at the beginning of feeding, whereas As8.9kDa and AsBasicTail were more important at the end of feeding. Several studies have shown that the levels of salivary secreted molecules may vary with tick feeding (11, 37–40); however, further studies are required to elucidate the meaning of such profiles. In the study by Esteves et al. (11), the expression of secreted proteins in the salivary glands of *A. sculptum* increased from 11 to 36% at 72 h after the beginning of hematophagy and several genes from the Kunitz, 8.9kDa, and basic tail families were among the most upregulated. These results are in agreeance with the results from our study and indicate that these proteins are important for blood feeding with an expression profile that might be useful in a multicomponent vaccine because tick salivary molecules will be attacked by the immune system of vaccinated hosts during most of the hematophagy period.

The recombinant expression produced proteins with higher molecular weights (MW) on SDS-PAGE than expected. Once their identity was checked by sequencing and anti-His

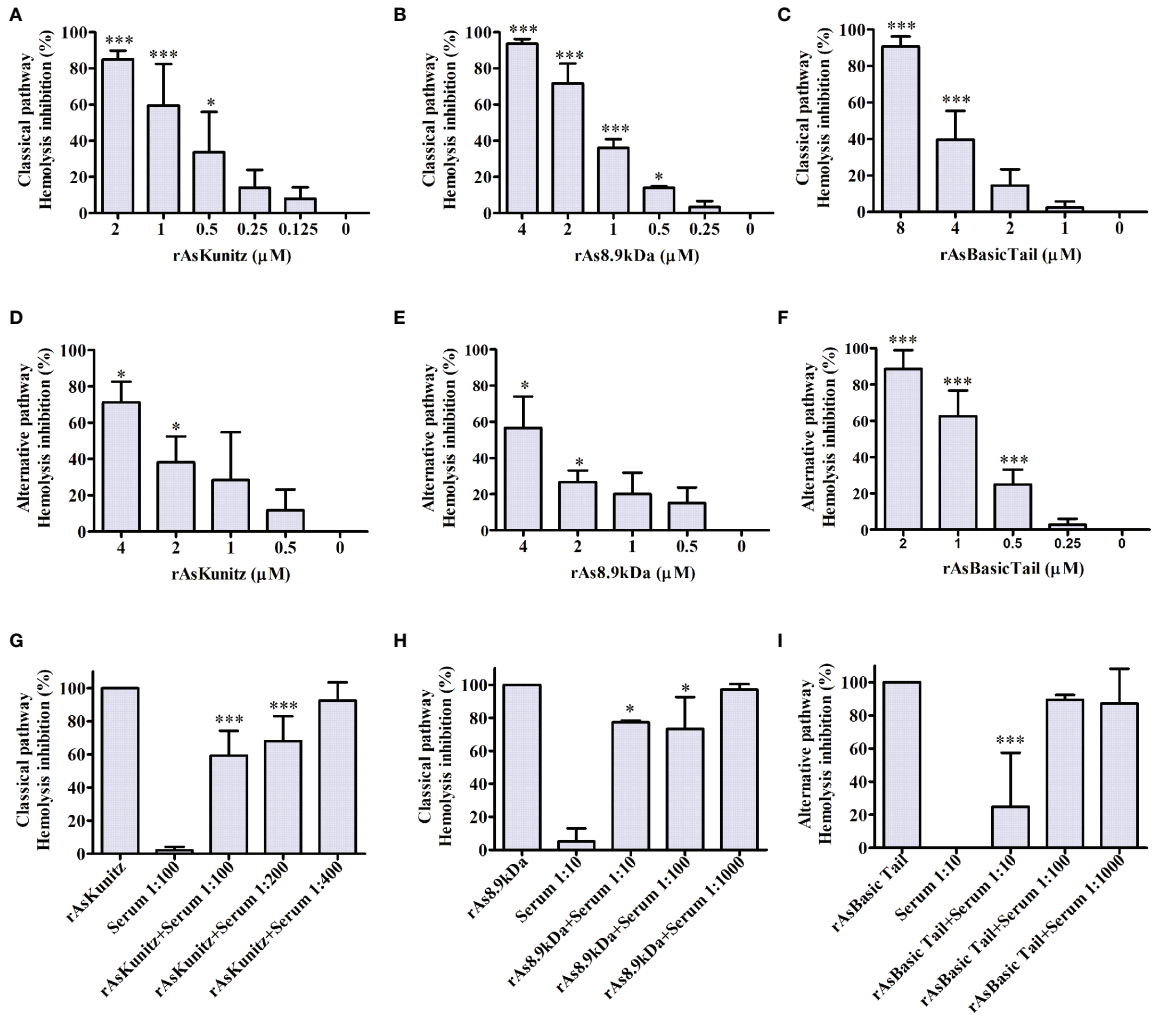

FIGURE 4 | Effect of rAsKunitz, rAs8.9kDa, and rAsBasicTail on the classical **(A–C)** and alternative **(D–F)** pathways of the human complement system. Recombinant proteins were incubated with the respective mice antiserum before being added to the classical **(G, H)** or the alternative **(I)** pathway assays. Data is shown as mean ± SD. Statistical analysis: ANOVA–Dunnett, *p < 0.05 and ***p < 0.001. In **(A–F)**, asterisks indicate statistical difference from controls, i.e., 0 μM in **(A–F)** and the sample without specific antiserum in **(G–I)**.

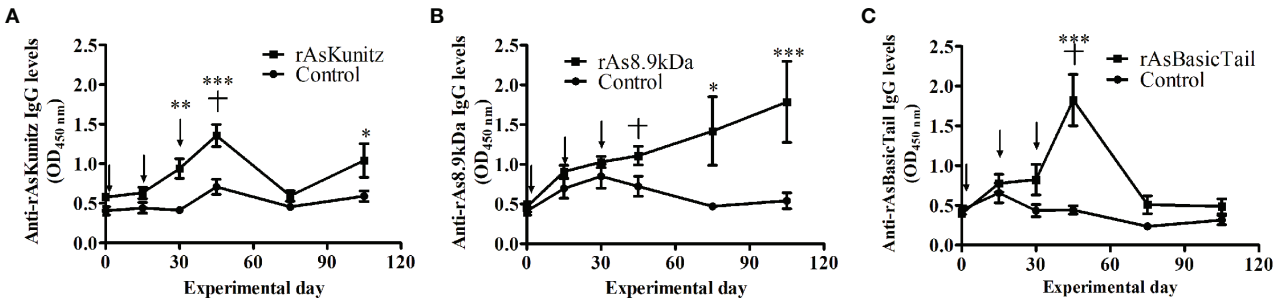

FIGURE 5 | Profile of specific serum IgG levels in mice vaccinated with rAsKunitz **(A)**, rAs8.9kDa **(B),** and rAsBasicTail **(C)**. Serum IgG levels against each of the recombinant were accessed by ELISA. Arrows indicate the three injections used for immunization and the cross indicates the challenge with ticks. Statistical analysis between control and vaccinated groups: Two-way ANOVA–Bonferroni, *p < 0.05; **P < 0.01; ***p < 0.001.

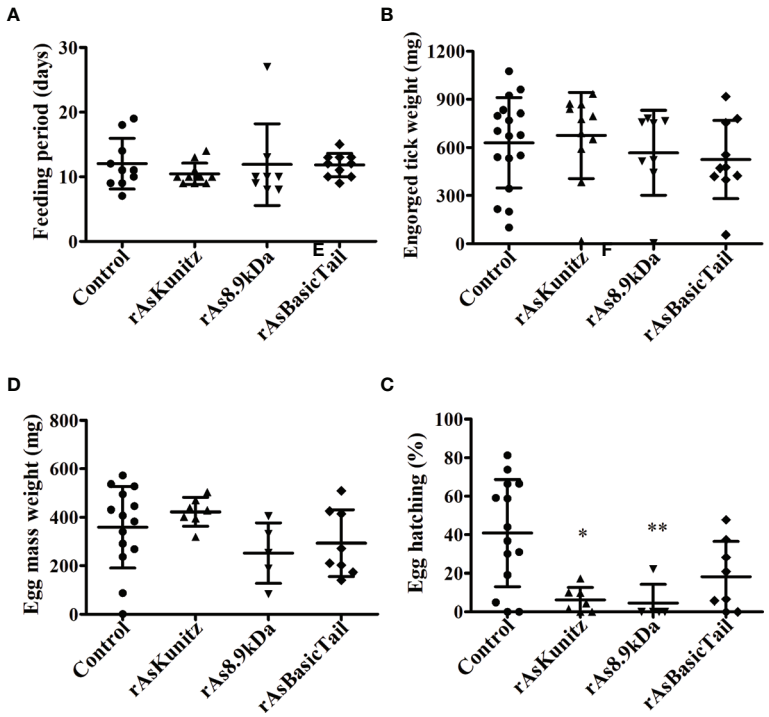

FIGURE 6 | Feeding **(A, B)** and reproductive **(C, D)** parameters of *Amblyomma sculptum* females fed on mice previously immunized with rAsKunitz, rAs8.9kDa, or rAsBasicTail. Data is shown as mean ± SD. Statistical analysis: Kruskal-Wallis–Dunn's, *p < 0.05 and **p < 0.01 indicate significant difference from controls.

TABLE 1 | Mortality of *Amblyomma sculptum* females and nymphs fed on mice previoulsly immunized with rAsKunitz, rAs8.9kDa, and rAsBasicTail.

Instar/Group	Control	rAsKunitz	rAs8.9kDa	rAsBasicTail
Female mortality*	30.0	41.6	58.3	33.3
Nymph mortality*	32.0	70.0	100.0^{+}	100.0^{+}

**Data show the percentage of ticks that died among the total specimens used in each group.*

$^{+}$All nymphs from the rAs8.9kDa and rAsBasicTail groups died while attached to the host feeding.

TABLE 2 | Efficacy of vaccination of mice with rAsKunitz, rAs8.9kDa, or rAsBasicTail against *Amblyomma sculptum* ticks.

	rAsKunitz	rAs8.9kDa	rAsBasicTail
CRT[a]	1.07	0.78	0.98
CRO[b]	0.90	0.53	0.79
CRF[c]	0.15	0.17	0.52
Efficacy (%)[d]	85.3	92.8	59.4

[a]*Coefficient of reduction in the number of engorged female ticks in the vaccinated/control group (CRT).*

[b]*Coefficient of reduction in oviposition in the vaccinated/control group (CR0).*

[c]*Coefficient of reduction in egg fertility in the vaccinated/control group (CRF).*

[d]*Vaccine protection against ticks considering the effects on CRT, CTO, and/or CRF. Percentage of efficacy (%E) = 100 [1–(CRT × CR0 × CRF)].*

western blotting, it was possible that the His-tag influenced the protein mobility in the SDS-PAGE or that recombinant proteins formed multimers, as discussed in previous studies (33, 41, 42).

The biological assays undertaken with the recombinant proteins revealed that they were all efficient in delaying the coagulation time of the blood plasma, suggesting that they are inhibitors of serine proteases. They could act on serine proteases, but with different activities and specificities. rAsKunitz was more specific with inhibition only on thrombin activity, whereas rAs8.9kDa and rAsBasicTail showed more general activity, acting on the three serine proteases tested.

Thrombin inhibitors with Kunitz-like domains have been previously identified and characterized in other species of ixodid ticks, such as the intestinal inhibitors boophilin, hemalin, and ixophilin, which have been described in *R. microplus*, *Haemaphysalis longicornis*, and *Ixodes scapularis*, respectively (43–45). The protein amblin also inhibits thrombin; however, it is present in the hemolymph of *Amblyomma hebraeum* (46). AsKunitz is the first Kunitz protein to show thrombin inhibitory activity from a tick salivary gland. Unlike the previously mentioned inhibitors, which have two Kunitz domains, AsKunitz has only one and has a considerably smaller MW than the others. Nevertheless, rAsKunitz showed efficiency in doubling the time needed for plasma coagulation similar to that of recombinant hemalin (43) and compatible with that observed for the native boophilin (44).

The AsKunitz mechanism of action remains unknown; however, it appears to be related to that of amblin (46) because both have a basic nature (amblin has a pI of 9.7) and the presence of two cysteine residues in the carboxy-terminal region, which may be responsible for the formation of another disulfide bond.

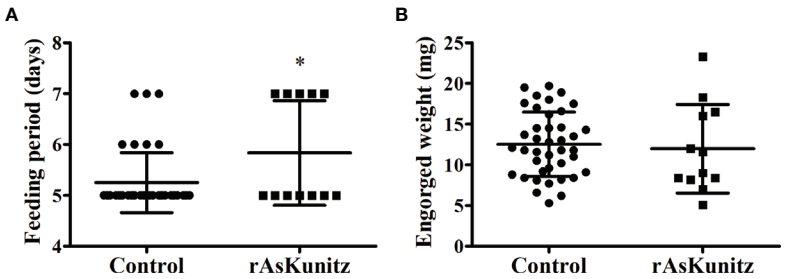

FIGURE 7 | Feeding parameters of *Amblyomma sculptum* nymphs fed on mice previously immunized with rAsKunitz. Duration of blood feeding **(A)** and tick weight after detachment **(B)**. Data is shown as mean ± SD. Statistical analysis: Mann-Whitney, *p < 0.05.

This, in turn, could interfere with the exosite I binding region of thrombin (47).

rAsKunitz as well as the other Kunitz proteins cited above could not inhibit FXa. However, other salivary FXa inhibitors of the Kunitz family have already been described as amblyomin-X from *A. sculptum* (48) and Ixolaris from *I. scapularis* (49). Ixolaris has two Kunitz domains and is a non-competitive inhibitor of FXa exosite. The tick anticoagulant protein (TAP), a salivary inhibitor of the argasid tick *Ornithodoros moubata* (50), has only one Kunitz domain similar to rAsKunitz and can inhibit FXa. However, the TAP mechanism of action is linked to the presence of a tripeptide (Tyr–Asn–Arg) in the amino terminal region, which is absent from rAsKunitz and acts by blocking the active site of the enzyme (47).

rAs8.9kDa was the recombinant with the lowest inhibition of the coagulation cascade. Although it could significantly inhibit the enzymatic activity of FXa, thrombin, and trypsin, but the IC_{50} achieved indicates that inhibition occurred with low efficiency. These findings indicate that As8.9kDa is not a specific inhibitor for any of the serine proteases tested and that it may play a role in *A. sculptum* hematophagy other than the inhibition of the coagulation cascade. Coagulation inhibitors have not yet been described in the 8.9 kDa family of proteins.

rAsBasicTail was the recombinant with the greatest effectiveness in inhibiting the catalytic activity of FXa. This was expected because the basic tail family has members previously characterized as FXa inhibitors, such as the 11.8 kDa salivary protein Salp14 from *I. scapularis* (51). *I. scapularis* Ixonnexin is also a basic tail protein that interacts with FXa (52). AsBasicTail has characteristics similar to those of Ixonnexin, such as a tail rich in lysine residues and the presence of three disulfide bonds responsible for maintaining its secondary structure. Although the AsBasicTail mechanism of action was not investigated, the interaction with FXa might be related to the presence of the basic carboxy-terminal region because proteins with structures similar to those of the tick salivary lectin pathway inhibitor and the recombinant Salp9, which do not have this structure, did not show anticoagulant activity (51, 53).

The hemolytic assays demonstrated that the salivary recombinants, tested separately, inhibited the activation of the human complement system in both the classical and alternative pathways. Although anti-complement activity has already been observed in the saliva of *A. sculptum* (32), anti-complement molecules have not been identified until the present study.

rAsKunitz and rAs8.9kDa blocked the activation of the classical pathway and are the first complement inhibitors described for ticks in their respective families. The complement factor inhibited by rAsKunitz still requires further studies to be elucidated; however, the different inhibition promoted in the classical and alternative pathways might suggest that more than one component of the cascade was affected. The anti-complement activity observed in rAs8.9kDa, in turn, may be related to the presence of the vWF domain in its sequence, as vWF acts as a cofactor in the regulation of the complement system in vertebrates via the cleavage of C3b (54). Thus, rAs8.9kDa could also be acting in the inactivation of C3b and, consequently, inhibiting the activation of the cascade.

AsBasicTail strongly opposed the activation of the complement by the alternative pathway. It is the first inhibitor of *A. sculptum* and the first molecule of the Basic Tail family, which is described as an inhibitor of the alternative pathway. However, it is not the first complement inhibitor characterized in the family because the tick salivary lectin pathway inhibitor is also a Basic Tail protein and can block the activation of the lectin pathway (53).

In addition to the hemolytic assays, the results showed that specific antibodies present in the serum of animals immunized with each of the recombinant salivary proteins could interfere with their anti-complement activity, probably due to opsonization by the antibodies. Similar results were observed in hemolytic assays with SALO and lufaxin, the salivary complement inhibitors of *Lutzomyia longipalpis* (33, 34). The importance of the complement in host resistance against ticks has been previously shown (21, 55, 56) and this effect is highly desirable if the proteins are intended to be used as vaccine antigens. Salivary molecules of hematophagous arthropods also act in the intestine (57); therefore, the suppression of anti-complement activity could contribute to the activation of the complement cascade in the intestinal lumen, culminating in lesions on the epithelial cells (17) and, consequently, loss of digestive and reproductive efficiency or even death of the parasite (58). Additionally, it has been observed that blocking the activity of salivary proteins with anti-complement function can impair the transmission of pathogens to vertebrate hosts (53, 59–61);

thus, the immunization of hosts with the salivary antigens of *A. sculptum* could also interfere with the transmission of *R. rickettsii*.

The simultaneous presence of anticoagulant and anti-complement activities performed by *A. sculptum* recombinant proteins is not uncommon in serine protease inhibitors produced by other invertebrates. Salivary proteins from *L. longipalpis* and *Amblyomma americanum* were previously shown to be able to delay plasma clotting time and block the activation of the complement system (34, 62, 63). This may be related to the non-specific inhibition of different serine protease members of the cascades. In natural circumstances, the effect may be even higher once it is known that both systems are intrinsically related. Components of the coagulation cascade, such as FXa and thrombin, can act in the cleavage of complement factors C3 and C5, contributing to their activation (64). Thus, we can infer that the salivary proteins addressed in the present study may be related to various functions in the parasite-host interaction, which encourages further studies of functional characterization.

Vaccine trials using recombinant proteins as antigens showed promising efficacy against *A. sculptum*, especially rAs8.9kDa and rAsKunitz. The tick mortality rate was the first parameter affected by the vaccine. It increased in groups of both females and nymphs fed on vaccinated mice; however, it was much greater for nymphs and reached 100% for two recombinant proteins. It is not clear why the mortality rate was higher in nymphs; however, the cause may be explained by the lower expression levels of the native proteins. Lower levels of the inhibitors could lead to a more expressive blocking of their activity by the antiserum of vaccinated mice.

The vaccinations did not decrease the oviposition of engorged females, which was similar to that found by Andreotti et al. (65). However, the great variation in the hatching rate of the eggs was the main parameter affected by the vaccine. One possible explanation would be the influence of feeding on immunized hosts on the tick microbiota. In this case, the ingested blood containing antibodies against anti-complement molecules could increase the complement system activation in the intestinal environment, thereby reducing the symbiotic microbiota of the arthropods, culminating in the reduction of reproductive fitness. Previous studies have shown the importance of the intestinal microbiota in tick fertility. Zhong et al. (66) demonstrated that the oviposition and fertility of eggs of engorged females of *A. americanum* treated with antibiotics were significantly decreased. Machado-Ferreira et al. (67) evaluated the bacteria present in the eggs of *A. sculptum* and showed that the microbiota can act as a chemical defense and would be beneficial for its development in the environment.

Vaccine trials against ticks of the *A. cajennense* complex are rare in the literature. The BM86 antigen, which is the base of the commercial vaccines against *R. microplus*, was ineffective against nymphs and adults of *A. cajennense* sensu lato when calves were used as hosts (68). In an experiment conducted with larvae on rabbits, the tick P0 peptide showed 54% efficacy against *Amblyomma mixtum*, which is another species of the *A. cajennense* complex (69).

Several research groups are looking for an efficient anti-tick vaccine. Studies have shown that results vary; however, some candidates have reached up to 97% efficacy (70, 71). The efficacies obtained here for rAs8.9kDa and rAsKunitz were superior to those described in studies with *A. cajennense* s.l. and other trials that tested antigens against other species of ticks (70, 71). However, mice were used in the present study, which are not natural hosts of *A. sculptum* and the results might be divergent if natural hosts were used in the vaccine trials. In studies that tested salivary antigens against ticks in their natural hosts, such as *R. microplus* on cattle, considerably lower efficacies were found, as seen for the 32% efficacy from a thrombin inhibitor of the Kunitz family (65), 73.2% for a multicomponent vaccine containing a serine protease inhibitor (72) and 60% for a salivary metalloprotease (73).

The activities of the proteins shown in the present study are also in agreeance with the host concern. If coagulation and complement inhibition are truly related to vaccine efficacy, it is important to check their activity when using plasma/sera from natural hosts (human material were used here) as activity may vary significantly when samples from different animal species are used (74, 75).

The data obtained here are encouraging for further studies to be performed with AsKunitz, As8.9kDa, and AsBasicTail, mainly for the evaluation of the efficacy against *A. sculptum* on other hosts and to verify a possible interference on the transmission of *R. rickettsii* by the vectors (76). In addition, tests with formulations containing antigenic combinations should be performed to increase the efficacy of the vaccine, which would be another step in the search for a commercial vaccine against *A. sculptum*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Comissão de Ética no Uso de Animais (CEUA) from the Federal University of Minas Gerais (UFMG).

AUTHOR CONTRIBUTIONS

GC and IR—performed the experiments, and analyzed and discussed the data. NG, MS, MP, GP, LK, and RA were involved in procedures related to colony maintenance, evaluation of relative mRNA levels, and production of recombinant proteins. FO, JV, DB, NG, and RA conceived and designed the procedures to select protein targets and characterize the activity of the recombinants. OJ, RG, RF, and RA conceived and designed the vaccination trials.

GC, IR, and RA wrote the paper. RNA supervised and coordinated the experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.611104/full#supplementary-material

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Identification of Tick *Ixodes ricinus* Midgut Genes Differentially Expressed During the Transmission of *Borrelia afzelii* Spirochetes Using a Transcriptomic Approach

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Lyme borreliosis is an emerging tick-borne disease caused by spirochetes *Borrelia burgdorferi* sensu lato. In Europe, Lyme borreliosis is predominantly caused by *Borrelia afzelii* and transmitted by *Ixodes ricinus*. Although *Borrelia* behavior throughout tick development is quite well documented, specific molecular interactions between *Borrelia* and the tick have not been satisfactorily examined. Here, we present the first transcriptomic study focused on the expression of tick midgut genes regulated by *Borrelia*. By using massive analysis of cDNA ends (MACE), we searched for tick transcripts expressed differentially in the midgut of unfed, 24h-fed, and fully fed *I. ricinus* nymphs infected with *B. afzelii*. In total, we identified 553 upregulated and 530 downregulated tick genes and demonstrated that *B. afzelii* interacts intensively with the tick. Technical and biological validations confirmed the accuracy of the transcriptome. The expression of five validated tick genes was silenced by RNA interference. Silencing of the uncharacterized protein (GXP_Contig_30818) delayed the infection progress and decreased infection prevalence in the target mice tissues. Silencing of other genes did not significantly affect tick feeding nor the transmission of *B. afzelii*, suggesting a possible role of these genes rather in *Borrelia* acquisition or persistence in ticks. Identification of genes and proteins exploited by *Borrelia* during transmission and establishment in a tick could help the development of novel preventive strategies for Lyme borreliosis.

Keywords: *Borrelia afzelii*, *Ixodes ricinus*, transcriptome, tick, midgut, RNAi, massive analysis of cDNA ends (MACE)

INTRODUCTION

Lyme borreliosis is an emerging human disease, occurring predominantly in temperate regions of the northern hemisphere (1, 2). It is caused by spirochetes *Borrelia burgdorferi* sensu lato and is spread by ticks from the genus *Ixodes*. In Europe, ~65,000 new cases are reported annually (3). However, the real prevalence is substantially higher due to under-reporting (4). In North America,

the transmission cycle primarily involves the spirochete *B. burgdorferi* sensu stricto and the tick *Ixodes scapularis*. In Europe, the disease is caused by several *Borrelia* species and is transmitted by related tick species, *Ixodes ricinus* and *Ixodes persulcatus*. The early disease typically manifests itself with a bulls-eye rash on the skin, called *erythema migrans*. The spirochetes then disseminate throughout the body to diverse tissues and are associated with arthritis, neurological symptoms, and dermatitis (5). Prompt antibiotic treatment usually cures the disease and symptoms. Despite several promising trials (6–9), a vaccine against human Lyme borreliosis is not currently available and prevention mainly depends on avoiding tick bites (10).

Ixodes ricinus is the most common tick in Europe and is typically found in humid sheltered environments and forests, mainly from early spring until late fall. It is a three-host tick, where all developmental stages (larva, nymph, and adult female) must feed on the host blood to undergo molting into the next instar. *B. afzelii* is the dominant spirochete in Europe (11). *Borrelia* enter the tick gut when the larvae feed on an infected mouse. The spirochetes then multiply and are transstadially maintained in the tick through the molts (12). The nymph's ability to survive without feeding for years contributes to stabilization of *Borrelia* prevalence in the reservoir host population. Because of their small size, the tick nymphs are considered to be the most critical tick stage for human infections (13). During engorgement, which typically lasts for two to four days, the spirochetes continuously migrate from the tick into the host. An interval between 24 and 48 h after tick attachment is considered the most critical time for transition of *B. afzelii*. Although *Borrelia* can already be detected in the skin on the first day of feeding, this early spirochetal population cannot initiate a systemic infection (12). Unlike *B. burgdorferi* s.s. in *I. scapularis* (14), which migrate through the hemolymph and salivary glands into the host, *B. afzelii* probably infect the host directly from the midgut of *I. ricinus* (12).

The segmented tick midgut is well adapted to accommodate an enormous volume of host blood. Unlike other blood-feeding arthropods, digestion in ticks occurs intracellularly (15), so extracellular pathogens are not directly exposed to the harsh effects of secreted proteases. Despite this, the tick midgut is still a relatively sterile environment (16), maintained presumably by combining active components of the blood and tick immune molecules. Adaptations of *Borrelia* spirochetes inhabiting the tick midgut are still not satisfactorily explained. However, it has been documented that during tick colonization, *Borrelia* change expression of their genes (17). For instance, the main surface protein outer surface protein A (OspA) is preferentially expressed within the tick midgut and is downregulated during transmission of the spirochete to the vertebrate host (18). The tick receptor for OspA (TROSPA), is a midgut protein identified in *Ixodes scapularis*, ensuring adherence of *B. burgdorferi* to the midgut surface. Expression of *trospa* is significantly upregulated in *Borrelia*-infected nymphs. Moreover, the silencing of *trospa* expression reduces colonization and transmission of the pathogen (19). Another example of this co-adaptation is *Borrelia*-induced overexpression of the tick salivary protein 15 (Salp15) necessary for *Borrelia* survival in the host (20). *Borrelia*-infected nymphs have also been shown to accumulate significantly more fat reserves (21) to better survive unfavorable temperatures and humidities (22). These examples point to the existence of delicate gene interactions between *Borrelia* spirochetes and the tick.

Here we show that midgut cells of infected nymphs before, during, and after feeding on the vertebrate host react to *B. afzelii*. By employing the MACE transcriptomic method, we were able to identify, in total, 1,083 *Borrelia*-responding tick midgut genes. Silencing of tick uncharacterized protein (GXP_Contig_30818) by RNA interference reduced transmission of *Borrelia* spirochetes from the tick to the host, whereas silencing of several other candidate tick genes had no effect. This suggests that these genes may have a role in processes associated with acquisition rather than transmission of *Borrelia*, and persistence in the vector.

MATERIAL AND METHODS

Biological Material

Adult females of *I. ricinus* were collected by flagging in a forest near Ceske Budejovice and kept at 95% humidity, 24°C, and 15/9 daylight settings. The adult ticks were fed on a single guinea pig. The laid eggs were preserved to hatch separately to form individual populations, each coming from a single female. For the transcriptomics purposes, the larvae from three populations were mixed together to scale up the number of ticks and then divided into two groups to prepare for infected and uninfected nymphs (**Figure 1**). Prior to feeding, a half of 6–8 week old C3H/HeN mice (Charles River Laboratories, GER) were infected with *B. afzeli* CB43 (23) by subcutaneous injection of 0.2 ml of culture (approximately 10^6 spirochetes). Mouse infection was checked by PCR on ear punctures taken 3 weeks after injection. The *Borrelia*-infected nymphs were obtained by feeding the larvae on *Borrelia*-infected mice. Uninfected nymphs were obtained by feeding the larvae on uninfected C3H/HeN mice. The resulted nymphs, molted 4–6 weeks after repletion, were rested for 2 weeks and used in these experiments. The prevalence of *Borrelia* infection in nymphs was checked by PCR and reached >90%. All experiments were carried out according to the animal protection law of the Czech Republic (§17, Act No. 246/1992 Sb) with the approval of CAS (approval no. 79/2013). The experiments with *Borrelia* were performed in BSL2 conditions.

Tick Dissection and RNA Extraction

The *Borrelia*-infected nymphs were divided into three groups (MACE 1,3,5), as well as the uninfected nymphs, which were also divided into three groups (MACE 2,4,6). The nymphs of MACE groups 1 and 2 remained unfed. The nymphs of MACE groups 3 and 4 were forcibly removed from the naïve 6–8 weeks old C3H/HeN mice at 24 h after attachment. The nymphs of MACE groups 5 and 6 were allowed to feed on the naïve 6–8 weeks old C3H mice until repletion (around 72 h). All tick were surface-sterilized by washing in 3% H_2O_2, 70% ethanol, and distilled

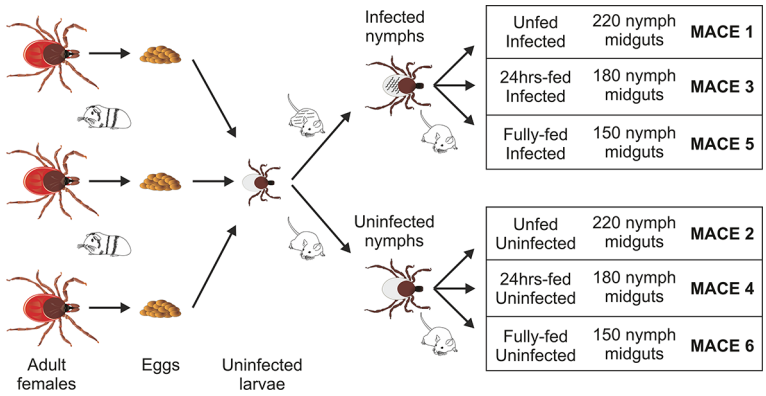


FIGURE 1 | Scheme of sample preparation for massive analysis of cDNA ends (MACE) analysis. The uninfected larvae, originating from three individual females fed on a single guinea pig, were mixed and fed on *B. afzelii*-infected or uninfected mice. The nymphs then were fed on uninfected mice and dissected for midguts (150–220 nymphs for each group) at the three indicated time points. The MACE analyses were performed on six different RNA pools (MACE 1-6).

water (30 seconds each wash). The nymphs were dissected for midguts [pools of: 220 unfed nymphs (MACE 1,2), 180 24 h-fed nymphs (MACE 3,4), and 150 fully fed nymphs (MACE 5,6)] under the stereomicroscope (Olympus) on wax dishes with diethyl pyrocarbonate (DEPC)-treated cold phosphate buffered saline (PBS) and then transferred in RA1 buffer (NucleoSpin miRNA Kit, Macherey-Nagel, GER) supplemented with β-mercaptoethanol (Sigma-Aldrich). Before extraction, the midguts were homogenized in an insulin syringe. Total RNA (including miRNA) was extracted using the above extraction kit by following the manufacturer's protocol ("small+large" protocol). The concentration of RNA was measured by NanoDrop ND-1000 (Thermo Fisher Scientific), its consistency was checked on an agarose gel, and stored at −80°C until further use.

MACE Analysis

The massive analysis of cDNA ends (MACE) was performed as previously described (24) using the GenXPro MACE Kit (GenXPro) according to the manufacturer's protocol (www.genxpro.net). The isolated RNA was subjected to an additional DNAse I treatment and its quality was assessed on an Agilent 2100 Bioanalyzer. First and second-strand cDNA synthesis was then performed, initiated from biotinylated oligo dT primers. The cDNA was fragmented randomly by ultrasonication, resulting in fragments with an average size of 300bp as determined by an Agilent 2100 Bioanalyzer. The biotinylated 3′ cDNA ends were bound to a streptavidin matrix, while the remaining fragments were eliminated through the washing step. Then, the p5 "TrueQuant" sequencing adapter was ligated to the unbound end of the fragments using tailed Illumina p5 and p7 oligonucleotides as primers. The quality of the final library was determined using an Agilent 2100 Bioanalyzer. The next-generation single-end sequencing of the 5' cDNA fragments was performed on an Illumina HiSeq2000 sequencer. To remove the PCR bias, all duplicate reads detected by the GenXPro in-house TrueQuant technology were removed from the raw datasets. In addition, low-quality sequence nucleotides and poly(A)-tails were clipped off using Cutadapt (25). Overlapping sequencing reads were then assembled into contigs. The reads were aligned to different reference sequences using NovoAlign (www.novocraft.com/products/novoalign/), resulting in "GXP_Contigs" (sequences derived from our previously published nymphal RefSeq database (Bioproject PRJNA657487), "Contigs" (*I. ricinus* sequences were derived from NCBI nuccore and the BioProjects 177622, 230499, 34667, and 183509), and "noHITAssemblies" (assemblies of MACE sequences that could not be mapped to sequences from the existing BioProjects or our own RefSeq database). The contigs of the assemblies were annotated further by BLASTX to either the SwissProt or Trembl database (www.uniprot.org). Contigs that did not match to one of these databases were annotated by BLASTN to all "*Ixodes*" mRNA sequences available in the NCBI database, against the "nt" (nucleotide collection from GenBank, RefSeq, TPA, and PDB) of NCBI, or the *I. scapularis* genome (NW_002505054). Only uniquely mapped reads were accepted for the quantification of the MACE tags. Finally, gene expression was normalized per million reads and tested for differential gene expression between the different conditions using the DEGSeq R/Bioconductor package (26) (R package version 1.16.0.). The final table was produced as an Excel file (**Supplemental Table 1**).

In Silico Analysis

The selection of *Borrelia*-upregulated and downregulated genes at different time points was performed using the MACE Excel file according to these selection criteria: 1) the transcript was annotated (e-value ≤ $10E^{-6}$) in the *I. ricinus* genome PRJNA270959, the *I. scapularis* genome PRJNA314100, or in all *Ixodes* sequences available in NCBI; 2) "noHitAssemblies" contigs were removed from the analysis because of no homologies with tick sequences (no hits, host contaminants, and short-length sequences); 3) to select upregulated genes: fold change upregulation of expression in the infected *vs.* uninfected

group was set to ≥ 5 and expression in the infected group to ≥ 5 transcripts per million; selection of downregulated genes was done *vice versa* (expression in the uninfected *vs*. infected group was set to ≥ 5 and expression in the uninfected group to ≥ 5 transcripts per million). The selected candidate sequences were translated into proteins (DNASTAR) and screened for the presence of a signal sequence by SignalP 5.0 (www.cbs.dtu.dk/services/SignalP/) and for cellular localization by DeepLoc-1.0 (www.cbs.dtu.dk/services/DeepLoc/).

Technical and Biological Validation of the MACE Analysis

An aliquot of RNA from each MACE analysis was used for the technical validation of MACE results. For biological validation, we prepared 10 genetically distinct larval populations of *I. ricinus* ticks coming from wild-captured adult females fed on a guinea pig (**Supplemental Figure 1**). Each of the batches of larvae was divided in half and fed on *B. afzelii* CB43-infected or uninfected 6–8 weeks old C3H/HeN mice (Charles River Laboratories, GER) mice. The resulting infected and uninfected nymphs were then fed on naïve mice for 0h, 24h, and until replete (fully fed), midguts were dissected (for each group and time point pools of: 50 unfed nymphs, 20 24h-fed nymphs, and 10 fully fed nymphs (equal number of females and males), and RNA was extracted following the methods and time points used for the MACE analysis. Then, the RNA was reverse transcribed into cDNA (0.5µg RNA per 20µl reaction; random hexamers) using the Transcriptor High-Fidelity cDNA Synthesis Kit (Roche) and diluted 20-times in sterile water. Gene-specific qRT-PCR primers were designed in Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) and verified by PCR using cDNA prepared from a mix of infected nymphs at different time points. Gene expression in technical and biological replicates was measured by quantitative real-time PCR (qRT-PCR) using a LightCycler 480 (Roche) and SYBR green chemistry, as described previously (27) and primers listed in **Supplemental Table 2**. Relative expression was normalized to *I. ricinus* elongation factor (GU074769) and ferritin 1 (AF068224, data not shown) using the mathematical model of Pfaffl (28).

RNA Interference and Its Effect on Nymph Feeding and Development

To prepare the gene-specific dsRNA, 200–600bp long gene fragments were amplified from *I. ricinus* cDNA and cloned into the pll10 vector with two T7 promoters in reverse orientations (29), using primers listed in **Supplemental Table 2** and containing additional restriction sites ApaI and XbaI. The dsRNA was synthesized as described previously (30). The dsRNA (3 µg/µl) was injected through the coxa of the third pair of legs into the hemocoel of nymphs (32 nl) using Nanoinject II (Drummond). After 3 days of rest in a humid chamber at room temperature, the nymphs (20 nymphs per mouse, 3 mice per group) were fed on BALB/c mice (Velaz, CR). The level of gene silencing was checked by qRT-PCR in a mix of five fully fed nymphs and compared to the dsGFP control group. For each group, we recorded feeding success, length of feeding, the weight of individual nymphs after feeding, and molting into adults (took approximately 2 months; recorded every 2 weeks until molting in the dsGFP control group reached 80%).

Borrelia-Transmission Experiments

Borrelia afzelii CB43-infected nymphs were prepared as described previously (31). The infected nymphs were injected with 32nl of gene-specific dsRNA or dsGFP (control), rested for 3 days, and fed on the uninfected 6-weeks old C3H/HeN mice (five nymphs per mouse, 5–8 mice per group) in plastic cylinders attached to the murine back. Detached engorged nymphs were weighed. The level of *Borrelia* infection in each mouse was measured the second week after tick detachment by qRT-PCR using DNA isolated from an ear biopsy and normalized to the number of mouse genomes (actin). Three weeks after tick detachment, mice were sacrificed and the numbers of *Borrelia* in the ear, urinary bladder, and heart tissue were determined by qRT-PCR as reported previously (12).

Statistical Analysis

For biological validations, feeding experiments, and transmission experiments, statistical significance of differences were analyzed using GraphPad Prism 8.0 (GraphPad Software, CA) employing the One-way ANOVA Kruskal-Wallis test or the non-parametric Mann-Whitney test and $P < 0.05$ (∗), $P < 0.01$ (∗∗), or $P < 0.001$ (∗∗∗) were considered as significant. If not further specified, all results were expressed as the mean ± standard error (SEM).

RESULTS

MACE Analysis

Initially, we measured differences in gene expression of *Borrelia*-infected ticks by employing the MACE technology, where high throughput sequencing of cDNA fragments provides a high resolution of gene expression and can reveal expression of low-abundance transcripts, compared to standard RNA sequencing (24, 32). We pooled more than 150 nymph midguts from each stage of tick feeding to minimize variations in gene expression. Being aware of intra-species genetic variation of wild-captured ticks, we limited the transcriptomes to the mixed population of nymphs originating from only three tick females (**Figure 1**). During the preparation of ticks for the transcriptomes and biological validations, we did not observe any adverse effects of the *Borrelia* infection on tick survival, fitness, or feeding, as demonstrated by body weights of fully fed infected nymphs compared with uninfected controls (**Supplemental Figure 2**). As a result, we obtained a total of 38,199,641 raw reads from the six cDNA MACE libraries. By mapping these sequences to our previously sequenced RefSeq library [containing 32,897 high-quality GXP contigs; Bioproject PRJNA657487 (33)] and the public *Ixodes* genomic and transcriptomic databases, we identified in each MACE library, on average, 17,257 GXP contigs and 1,302 additional tick genome/transcriptome contigs (gi|contigs absent from the RefSeq database) (**Supplemental Table 3**). Overall, in the midgut transcripts, we

observed a total of 24,276 tick genes. This number is in line with the 26,179 transcripts identified in our previous MACE transcriptomic project of the nymph *I. ricinus* salivary glands (33) and lower than the total number of genes described in the tick *I. scapularis* genome project (32,572 protein-coding genes) (34).

Identification of the Differentially Expressed Genes

To sort the database for genes upregulated or downregulated in the presence of *B. afzelii*, we defined a transcript as differentially expressed when the fold change was ≥ 5 (log2fold change ≤ −2.3 or ≥ 2.3) and the p-value ≤ 0.05. This primary selection led to the identification of 553 upregulated and 530 downregulated unique genes (**Figure 2**). Interestingly, in the group of fully fed nymphs (**Figure 2C**), we identified the largest number of *Borrelia*-upregulated genes (fold change > 1) and the highest ratio between upregulated and downregulated transcripts. Then, to produce a slimmed list of the differentially regulated genes, potentially confirmable by qRT-PCR in technical and biological validations, we selected transcripts with a fold change ≥ 5 and expression ≥ 5 transcripts per million in the infected (for upregulated genes) or uninfected (for downregulated genes) groups. By applying these criteria, we obtained a list of 118 upregulated and 96 downregulated genes (**Figure 3A**), of which 34, 49, and 55 genes were upregulated by infection at unfed (UF), 24h-fed (24-h), and fully fed (FF) stages, respectively. Conversely, 38, 33, and 30 genes were downregulated. Interestingly, five genes were upregulated, and one gene downregulated in all three time points (**Supplemental Tables 4**–**7**). The genes encode potentially secreted proteins (SignalP) containing a signal sequence [labeled as "SP(Sec/SPI)"] or intracellular proteins (labeled as "OTHER"). We did not observe any pattern in the prediction of subcellular localization (DeepLoc). The full list contained extracellular proteins, as well as proteins localized to the cytoplasm, mitochondrion, nucleus, or lysosome. Most of the proteins were predicted to be soluble, although the list also contained several membrane proteins (e.g., receptors, channels, glycoproteins). In summary, we identified 214 tick genes with various functions and localizations, highly differentially expressed in the presence of *B. afzelii*, suggesting a significant interaction of the tick midgut tissue with the spirochetes.

Technical and Biological Validations of MACE

We confirmed the expression of several differentially regulated genes arising from the MACE analysis by technical and biological validations. We focused only on genes from our upregulated candidate list as only these could be later silenced by RNA interference and tested in our *B. afzelii*-transmission model. We selected 46 candidates (from various time points) with homologous sequences present in the genomic databases of *I. ricinus* and *I. scapularis* (for the selection criteria see *Methods*). For 33 of these genes, we were able to design gene-specific PCR primers and for 22 of these genes, these primers worked well in a standard PCR assay. Their expression was then validated in technical and biological validations by qRT-PCR. All 22 candidate genes passed the technical validation and were proven to be upregulated at specific time points (**Figure 3**). Gene expression levels in 10 genetically distinct *I. ricinus* populations of nymphs were then determined to validate these candidate genes biologically. Through this strict validation level, seven genes passed, representing 32% of the 22 pre-selected genes. Of these, four candidates were shown to be overexpressed at the same time point compared to MACE, while the other three genes were overexpressed at other time points. The seven gene sequences represented: 1) cytosolic iron-sulfur protein assembly protein CIAO1 homolog (GXP_Contig_7059), 2) uncharacterized protein (GXP_Contig_30818), 3) BTB domain-containing protein (GXP_Contig_6657), 4) cytochrome p450 cyp2 subfamily protein (GXP_Contig_26946), 5) solute carrier organic anion transporter family member (GXP_Contig_29696), 6) cyclin-D-binding Myb-like transcription factor 1 (GXP_Contig_16121), and 7) Kolobok-5 tv protein (GXP_Contig_1931). All transcripts encoded intracellular proteins without predicted signal sequences (SignalP) and were predicted for various cellular localizations (DeepLoc).

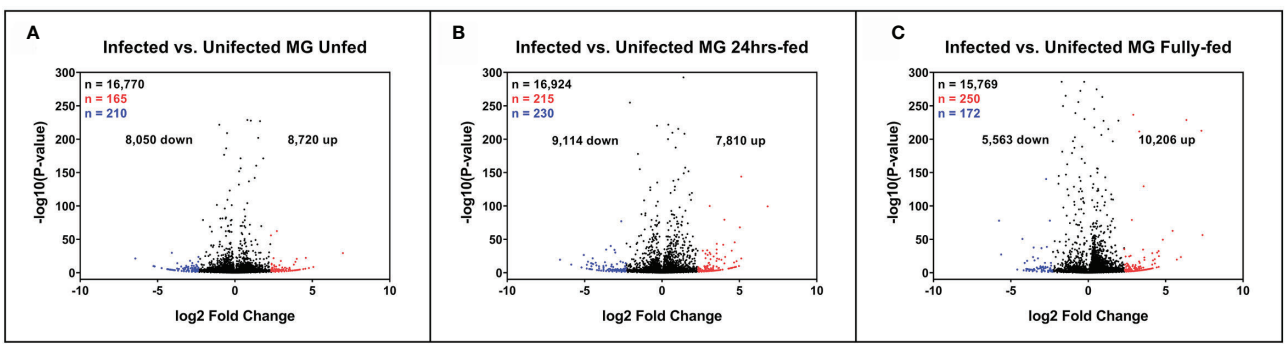
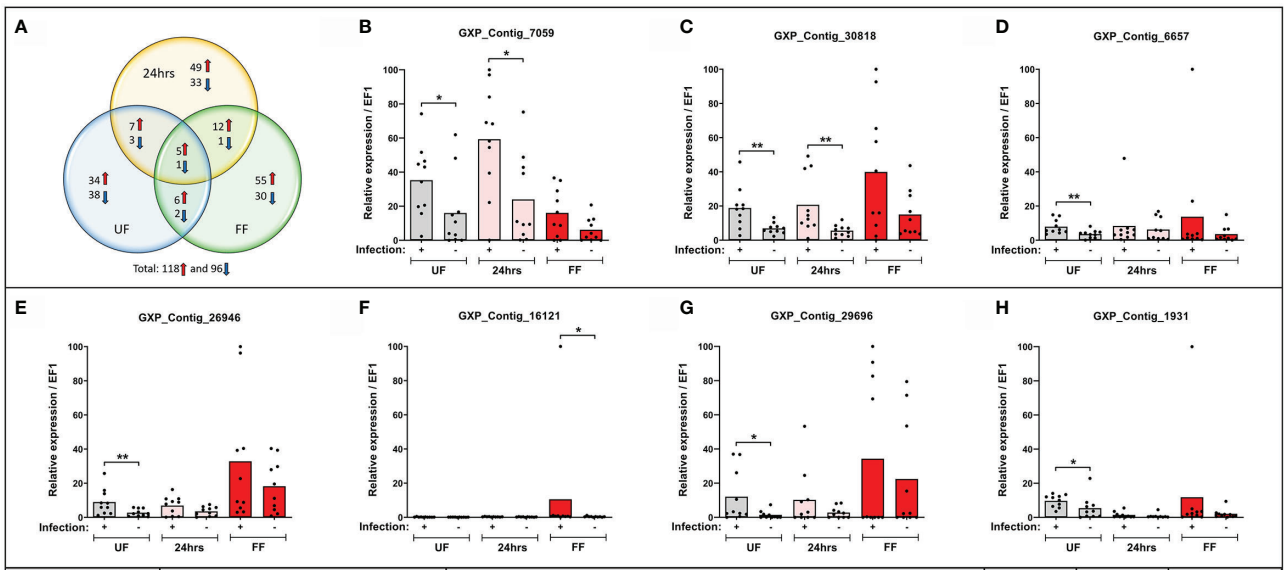


FIGURE 2 | Expression of tick midgut genes is altered in the presence of *Borrelia afzelii*. Volcano plots showing differentially expressed tick transcripts analyzed by MACE at individual time points. **(A)** Unfed nymphs **(B)** Nymphs fed for 24 h **(C)** Fully fed nymphs. n = number of differentially expressed transcripts. Total differentially expressed transcripts (black), upregulated transcripts (red; p-value ≤ 0.05 and log2 fold change ≥ 2.3), and downregulated transcripts (blue; p-value ≤ 0.05 and log2 fold change ≤ −2.3). up = total upregulated transcripts, down = total downregulated transcripts, MG, midgut.

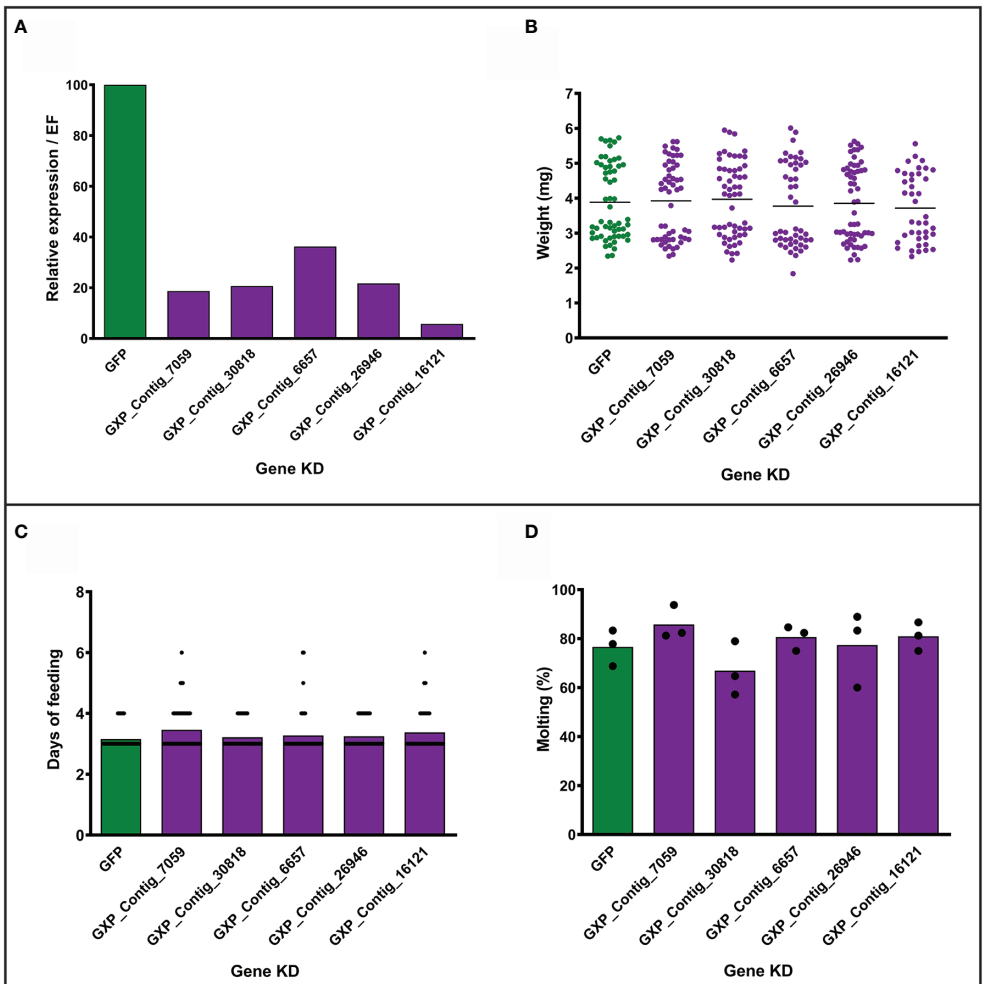


Database of upregulated transcript	Contig	Putative description	Technical validation	Biological validation	Timepoint of upregulation
UF	GXP_Contig_7059	Cytosolic iron-sulfur protein assembly protein CIAO1 homolog	YES	YES	UF, 24hrs
UF	GXP_Contig_31514	Hypothetical protein	YES	NO	
UF	GXP_Contig_30818	Uncharacterized protein	YES	YES	UF, 24hrs
UF	GXP_Contig_5839	Glycine-rich protein	YES	NO	
24hrs	gi|241123951|ref|XM_002403994.1|	Hypothetical protein	YES	NO	
24hrs	GXP_Contig_8911	Glycine-rich protein	YES	NO	
24hrs	GXP_Contig_6131	Pla2 bee venom like a sub-family of phospholipase a2	YES	NO	
24hrs	GXP_Contig_2889	Salivary lipocalin	YES	NO	
24hrs	GXP_Contig_28121	WC protein	YES	NO	
24hrs	GXP_Contig_7109	Enoyl-coa hydratase/isomerase	YES	NO	
24hrs	GXP_Contig_30557	Diacylglycerol acyltransferase dgat	YES	NO	
FF	GXP_Contig_16607	Putative DNA-bridging protein baf	YES	NO	
FF	GXP_Contig_6657	BTB domain-containing protein	YES	YES	UF
FF	GXP_Contig_25185	Uncharacterized protein	YES	NO	
FF	GXP_Contig_26946	Cytochrome p450 cyp2 subfamily protein	YES	YES	UF
FF	GXP_Contig_476	RNA-directed dna polymerase from mobile element jockey-like protein	YES	NO	
FF	GXP_Contig_29696	Solute carrier organic anion transporter family member	YES	YES	UF
FF	GXP_Contig_29976	Solute carrier organic anion transporter family member	YES	NO	
FF	GXP_Contig_16121	Cyclin-D-binding Myb-like transcription factor 1	YES	YES	FF
UF+24hrs+FF	GXP_Contig_21561	Secreted salivary gland peptide	YES	NO	
UF+24hrs	GXP_Contig_1931	Kolobok-5 tv	YES	YES	UF
24hrs+FF	GXP_Contig_1305	Salivary secreted peptide	YES	NO	

FIGURE 3 | Expression of selected transcripts can be verified by technical and biological qRT-PCR validations. (Upper) **(A)** Venn diagram of the top-score differentially expressed *B. afzelii*-infected nymph midgut transcripts (fold change ≥ 5 fold and expression ≥ 5 transcripts per million). The upregulated transcripts are marked by a red arrow, downregulated by a blue arrow. **(B–H)** qRT-PCR profiles (relative expression) of seven biologically validated transcripts were significantly upregulated by *B. afzelii* infection (Mann-Whitney test). The biological validations were carried out on 10 individual tick populations. Each dot represents expression in a single nymph population. In each graph, cDNA with the highest expression was set as 100. The tick *elongation factor* was used as a housekeeping gene. (Lower) Summary table of the validated transcripts. In total, 22 transcripts from different time points of feeding (see *Results* for the selection criteria) were analyzed by the technical and biological validations. UF, unfed; 24hrs, fed for 24hrs; FF, fully fed. $P < 0.05$ (*), $P < 0.01$ (**).

RNA Interference and *Borrelia*-Transmission

To assess the role of the stimulated genes in transmission of *Borrelia*, we employed the method of RNA interference and injected nymphal ticks individually with five different gene-specific dsRNAs designed against the previously biologically validated transcripts. Before the transmission experiments with infected nymphs, we tested the effect of silencing in uninfected nymphs. The genes were successfully silenced in the fully fed nymphs to expression levels ranging from 6 to 36% comparing to the dsGFP control (**Figure 4A**). We did not observe any significant impact on feeding success, duration of feeding, tick weight after feeding, or molting of nymphs to adults (**Figures 4B, D**). We then performed the silencing in infected nymphs. Initially, we tested



Gene	Ear week 2	Ear week 3	Heart week 3	Urinary bladder week 3	Cumulative
GFP control	9 / 13	12 / 13	12 / 13	12 / 13	12 / 13
GXP_Contig_7059	5 / 5	5 / 5	5 / 5	5 / 5	5 / 5
GXP_Contig_30818	**2 / 13**	10 / 13	10 / 13	**7 / 13**	10 / 13
GXP_Contig_6657	8 / 13	11 / 13	12 / 13	11 / 13	12 / 13
GXP_Contig_26946	4 / 5	5 / 5	5 / 5	5 / 5	5 / 5
GXP_Contig_16121	4 / 5	5 / 5	5 / 5	5 / 5	5 / 5

FIGURE 4 | Effect of gene silencing by RNA interference on nymph feeding and *Borrelia afzelii* transmission. (Upper) Silencing of five tick genes in uninfected nymphs. **(A)** Evaluation of the silencing level by qRT-PCR (each group represents a mix of five fully fed nymphs). **(B)** Weights of individual fully fed nymphs. Each dot represents a single tick. **(C)** Duration of nymph feeding. **(D)** Molting success of fully fed nymphs into adults (percentage of molted nymphs fed on each mouse; biological triplicates). (Lower) Summary table of two transmission experiments with the gene-silenced *B. afzelii*-infected nymphs. Numbers indicate total qRT-PCR positive/total mouse tissues during the infection (ear week 2) and after mice scarification (week 3). dsGFP was used as a negative control. A decrease of positivity by >25% is highlighted in red.

transmission with five mice per group. Silenced genes associated with the blocking of transmission of *B. afzelii* in at least one mouse, were further tested with an additional eight mice per group. Similarly, as observed with the uninfected nymphs, gene silencing did not affect tick feeding (**Supplemental Figures 3A, B**). The transmission of *B. afzelii* from the tick to the mouse was not noticeably blocked after the silencing of GXP_Contigs_7059, _6657, _26946, and _16121 (**Figure 4**). The number of spirochetes in deeper mouse tissues, as measured by qRT-PCR, was also not significantly altered (**Supplemental Figures 3C–F**). Interestingly, in the group with silenced uncharacterized protein (GXP_Contig_30818), the progress of infection in mice was delayed (only 15% of ears were *Borrelia*-positive by the second week compared to 70% in control), which was then reflected in a

reduction of *Borrelia* prevalence in the ear (3rd week), heart, and urinary bladder by 23, 23, and 46%, respectively (**Figure 4**).

DISCUSSION

The hypothesis that parasites actively modify the physiology and behavior of their hosts to enhance transmission is an intriguing and well-documented phenomenon in many species of living organisms (35). However, evidence of manipulation of ticks by *Borrelia* spirochetes is still mostly unknown. In this study, we have revealed differential gene expression in the midgut of *I. ricinus* nymphs infected with the Lyme borreliosis spirochete *B. afzelii* before, during, and after blood-feeding. This study represents the first transcriptome produced from ticks focusing on midgut genes stimulated by *Borrelia*. Previous transcriptomic studies described differential gene expression in salivary glands (33, 36), or used alternative approaches for such gene identifications (19, 20, 37–42).

The motile *Borrelia* enters the tick when larvae or nymph feed on an infected reservoir host. The spirochetes are attracted to the feeding site by the tick proteins secreted into the saliva (43). During the acquisition phase, the ingested spirochetes change their gene expression and multiply in the tick midgut contents (12, 44) to successfully infect the vector. After tick molting, the midgut appears empty. The midgut walls are localized close to each other, and the peritrophic matrix, a layer consisting of glycoproteins bound to the chitin network, is absent. In these harsh conditions of limited nutrients, which can last for months or years, the spirochetes switch into their "sleeping mode" and can be found attached to the midgut cell wall. OspA, a membrane lipoprotein produced by the *Borrelia*, was shown to bind the tick TROSPA protein present on the surface of *I. scapularis* midgut cells (19). *Trospa* was the first tick gene recognized as upregulated by the presence of *Borrelia* in the unfed nymph. Surprisingly, we were not able to identify *trospa* in our RefSeq database nor the recent TSA databases of *I. ricinus* available at NCBI. However, this gene has previously been sequenced from *I. ricinus* (NCBI: EU034646) (45), indicating that in *I. ricinus*, *trospa* was probably expressed to a limited level.

It is unknown how *Borrelia* spirochetes change expression of the tick midgut genes and how these modifications help *Borrelia* multiply and persist in the gut lumen. Using the MACE method on unfed midguts we have identified 210 downregulated and 165 upregulated tick genes as a result of infection (p-value ≤ 0.05 and log2 fold change ≤ −2.3 or ≥ 2.3). We found that *mitochondrial carboxypeptidase* (V5HK70) and *cytochrome C oxidase subunit VIa* (a component of the respiratory complex IV, XM_002435666) were downregulated > 8 fold in expression, indicating possible suppression of energy metabolism in unfed infected ticks. Among the highly upregulated genes, we identified several peritrophins and chitinases, constituents of the peritrophic matrix. However, the peritrophic matrix is formed in *I. ricinus* > 18h after the beginning of feeding (46), meaning that mRNAs of these genes could be pre-synthesized to accelerate the formation of the peritrophic matrix after the initiation of feeding. Alternatively, these proteins could be involved in establishing and maintaining other chitin structures such as tracheae, which supplement the midgut tissue with oxygen.

Ticks do not receive any nutrients from the environment, and the blood-feeding represents a significant milestone in their life cycles. The *Borrelia* spirochetes residing in the tick midgut become activated by a mechanism that is not completely clear [probably by nutrients in the blood, temperature, pH (47), osmolarity (48)] and thereby accelerate the expression of genes necessary for their transmission and survival in the vertebrate host. In the case of *B. burgdorferi* sensu stricto, the number of spirochetes multiplies from several hundred in an unfed nymph to a hundred thousand in a fully fed nymph (49). Next, *B. burgdorferi* migrate to the basolateral surface of the midgut epithelium, cross the basal membrane, and enter the hemocoel and salivary glands to infect the host through the secretion of saliva (14). However, *B. afzelii* appears to behave differently. These spirochetes do not multiply during feeding, but their numbers reduce continuously, possibly by direct traversal of the spirochetes from the midgut into the host (12). Importantly, spirochetes of *B. afzelii* have not been found to infect the salivary glands. In addition, and in contrast to *B. burgdorferi*, the number of *B. afzelii* spirochetes dramatically decreases over the next few months after molting (50). Bontemps-Gallo et al. previously showed that physicochemical parameters such as the level of oxygen, osmolality, and oxidative stress, affect growth and motility differently in these two genetically distinct bacterial species (51). Consistent with this, from 42 previously identified tick *Borrelia*-responsive genes (19, 20, 37–42) (including *tre31*, *isdlp*, *pixr*, *stat*, etc.), in our databases we found only *duox* (52) and *alcohol dehydrogenase* (42) being upregulated more than two-fold, further supporting the behavioral differences between *B. burgdorferi* and *B. afzelii*.

The primary purpose of this study was to identify tick proteins suitable for developing new anti-tick therapies. Ideally, such candidates should be abundantly expressed during feeding and targeted to the tick midgut wall or secreted from the cells into the midgut content in order to be accessible to antibodies or drugs present in the host blood. It was demonstrated that *B. afzelii* enters the host skin within 24h of attachment, but this population of spirochetes is not infectious. This means that the *Borrelia* need >24h for activation in the tick midgut to become infectious. We identified several tick genes altered in expression at the 24h time point. We do not know if this response was evoked explicitly by the *Borrelia* to gain an advantage during transmission, reflecting ongoing host modifications, or was induced by the tick as a reaction (immune) against the spirochetes.

We observed that at the fully fed time point, the number of upregulated genes were almost doubled when compared to the downregulated genes. We hypothesized that this overexpression was evoked by *Borrelia* during feeding to alter the tick physiology in order to transmit the spirochetes from the tick midgut into the host. To test the necessity of this upregulation, we silenced five previously biologically validated tick genes by RNA interference and tested the ability of nymphs to transmit *Borrelia*. All these candidates were predicted to be intracellular proteins, and many of them were transcription factors, so we tested whether silencing of these genes could block the expression of their downstream-regulated genes. We observed that the silencing of GXP_Contig_30818 caused the absence of *Borrelia* infection in the ear the second week after tick detachment (the beginning of infection) in 85% of mice (11 of 13).

This delay in onset of disease probably triggered a further decrease in *Borrelia* prevalence in the ear (week 3) and destination tissues, heart, and urinary bladder. This transcript encodes yet uncharacterized protein with predicted nuclear localization. The expression of genes possibly regulated by this protein deserves further attention. The silencing of other genes did not affect *Borrelia* transmission. Therefore, we propose that upregulation of these genes is necessary for processes other than transmission, possibly for the acquisition and persistence of *Borrelia*. Additionally, in the transcripts upregulated during feeding, and similar to the unfed stage, we more often identified genes connected with synthesis and reconstruction of the peritrophic matrix (e.g., peritrophins and chitinases), whose expression has been previously shown to influence spirochete colonization of ticks (53).

We believe that this work will enable further identification and characterization of the tick midgut proteins necessary for acquisition, persistence, and transmission of *B. afzelii* from *I. ricinus*. In our MACE transcriptomic database, we found, in total, 55 *Borrelia*-stimulated, well expressed, and secreted or cell membrane-associated midgut proteins. We assume that some of these candidate proteins are necessary for *Borrelia* activation and transmission and that blocking of these proteins by a specific vaccine or a drug will contribute to the development of novel therapies against Lyme borreliosis.

DATA AVAILABILITY STATEMENT

The dataset presented in this study is deposited as **Supplementary Table 1** in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the animal protection law of the Czech Republic (§17, Act No. 246/1992 Sb) with the approval of CAS (approval no. 79/2013).

AUTHOR CONTRIBUTIONS

RS, JT, PK, JH, and OH designed the experiments. SM, RS, VU, and OH prepared the infected nymphs, performed the dissections, isolated RNA, and accomplished the feeding and transmission experiments. NK and PW did the MACE analysis and annotations. SM analyzed the transcriptomic data and performed the technical and biological validations. SM and OH wrote the manuscript with an input from all co-authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.612412/full#supplementary-material

Supplementary Figure 1 | Scheme of sample preparation for biological validations. Ten populations of uninfected larvae, each originating from a single female fed on a guinea pig, were fed on *B. afzelii*-infected or uninfected mice. The nymphs then were fed on uninfected mice and dissected for midguts (10-50 nymphs for each group) at the three indicated time points.

Supplementary Figure 2 | *Borrelia* infection does not affect tick feeding or final weights of the fully-fed nymphs. Each group of nymphs was comprised of females (higher weights) and males (lower weights). Each dot represents a single nymph. The data in each group contain a collection of 20 individual feedings (in total 360 infected and 339 uninfected nymphs). INF = infected nymphs, UNINF = uninfected nymphs. The horizontal bar indicates a mean. n.s. = not significant (Mann-Whitney test).

Supplementary Figure 3 | Effect of gene silencing by RNA interference on nymph feeding and *B. afzelii* transmission.**(A)** Weights of individual fully-fed nymphs. Each dot represents a single tick. **(B)** Duration of nymph feeding. **(C–F)** The absolute number of *B. afzelii* in individual mouse tissues measured by qRTPCR. Two genes with no detectable *B. afzelii* in the heart tissue from the silencing Experiment 1 (left) were once more tested in the silencing experiment 2 (right). dsGFP was used as a negative control.

Supplementary Table 1 | A list and expression of all Ixodes ricinus nymph midgut genes identified in individual MACE transcriptomes. UF = unfed, 24hrs = fed for 24 hours, FF = fully-fed, INF = B. afzelii-infected nymphs, UNINF = uninfected nymphs.

Supplementary Table 2 | List of primers. Restriction sites for ApaI/XbaI are underlined.

Supplementary Table 3 | Raw reads and mapped contigs obtained after the sequencing of MACE libraries. Raw reads mapped to our previously sequenced RefSeq library (Bioproject PRJNA657487) were labeled as GXP sequences. Sequences absent from the RefSeq library, but present in other Ixodes tick genomes and transcriptomes, were labeled as gi|. UF = unfed, 24hrs = fed for 24 hours, FF = fully-fed, INF = infected nymphs, UNINF = uninfected nymphs.

Supplementary Table 4 | A list of Ixodes ricinus nymph midgut genes upregulated in the presence of Borrelia afzelii at three different timepoins of feeding. n.c. = not calculated.

Supplementary Table 5 | A list of Ixodes ricinus nymph midgut genes co-upregulated by Borrelia afzelii at different timepoints. n.c. = not calculated.

Supplementary Table 6 | A list of Ixodes ricinus nymph midgut genes downregulated in the presence of Borrelia afzelii at three different timepoins of feeding. n.c. = not calculated.

Supplementary Table 7 | A list of Ixodes ricinus nymph midgut genes co-downregulated by Borrelia afzelii at different timepoints. n.c. = not calculated.

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REVIEW
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Induced Transient Immune Tolerance in Ticks and Vertebrate Host: A Keystone of Tick-Borne Diseases?

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Ticks and tick transmitted infectious agents are increasing global public health threats due to increasing abundance, expanding geographic ranges of vectors and pathogens, and emerging tick-borne infectious agents. Greater understanding of tick, host, and pathogen interactions will contribute to development of novel tick control and disease prevention strategies. Tick-borne pathogens adapt in multiple ways to very different tick and vertebrate host environments and defenses. Ticks effectively pharmacomodulate by its saliva host innate and adaptive immune defenses. In this review, we examine the idea that successful synergy between tick and tick-borne pathogen results in host immune tolerance that facilitates successful tick infection and feeding, creates a favorable site for pathogen introduction, modulates cutaneous and systemic immune defenses to establish infection, and contributes to successful long-term infection. Tick, host, and pathogen elements examined here include interaction of tick innate immunity and microbiome with tick-borne pathogens; tick modulation of host cutaneous defenses prior to pathogen transmission; how tick and pathogen target vertebrate host defenses that lead to different modes of interaction and host infection status (reservoir, incompetent, resistant, clinically ill); tick saliva bioactive molecules as important factors in determining those pathogens for which the tick is a competent vector; and, the need for translational studies to advance this field of study. Gaps in our understanding of these relationships are identified, that if successfully addressed, can advance the development of strategies to successfully disrupt both tick feeding and pathogen transmission.

Keywords: tick, skin immunity and microbiome, immune tolerance, tick-borne diseases, innate immunity, adaptive immunity

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INTRODUCTION

Tick-borne diseases initially viewed as a triad of vector-pathogen-host, have evolved toward a very complex network of interactions. A fourth actor has appeared, the microbiome, present within the tick (1, 2), but also at the skin interface of the vertebrate host (3) (**Figure 1**). More recently, a fourth factor has emerged as an important cellular regulator, the non-coding RNAs (4).

Tick-borne pathogens should be viewed as danger signals, a concept developed by Polly Matzinger in 1994 (5, 6) and later refined by Medzhitov and Janeway (7). How do these pathogens manipulate the tick and the vertebrate host immunity to not be eliminated? Up and down

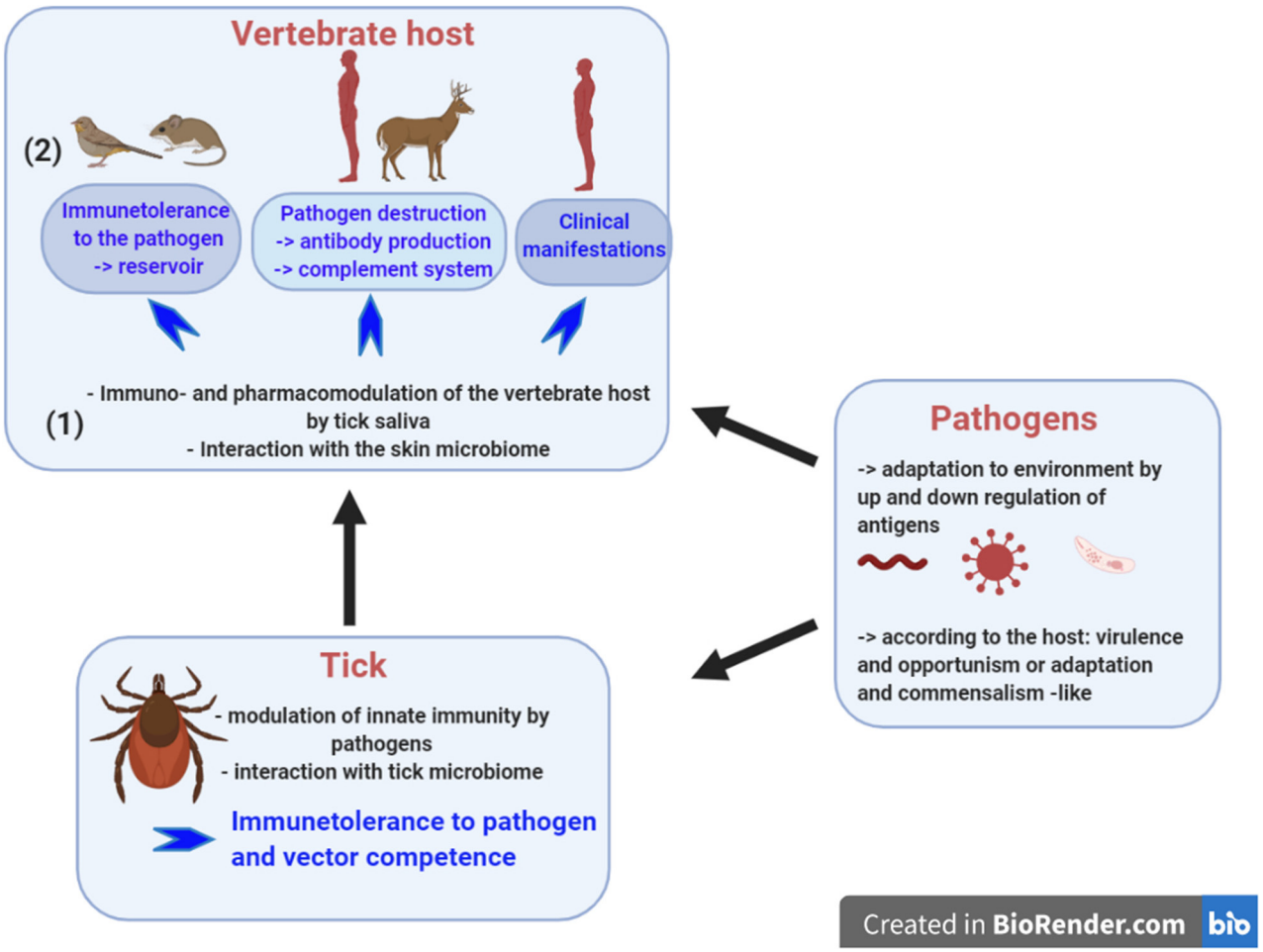

FIGURE 1 | Tick-borne diseases rely on interplays between the tick, the pathogen and the vertebrate host. To be a competent vector, the tick must control the pathogen population by its innate immunity and the tick microbiome seems to contribute to this control. During pathogen inoculation into the skin, tick saliva modulates the pharmacology and the immunology of the vertebrate host. Skin immunity plays a major role in tolerance of tick-borne pathogens. It is likely that the skin microbiome participates in this immunomodulation. Once inoculated, the infection outcome varies: (1) in animal reservoir like rodents, where no clinical manifestations develop and the pathogens survive for months allowing their persistence in the environment; (2) the vertebrate host has a sufficient immune system to neutralize the pathogens and antibody presence provides evidence of contact with the pathogens; and, (3) the vertebrate host does not trigger a sufficient and protective immune response and as a consequence develops clinical disease. Created with BioRender.com.

regulation of antigens helps the pathogens to adapt to its environment. Significantly, the tick itself must also be considered as a danger signal for the vertebrate host during the bite process, however its saliva makes it tolerant for the immune system of the vertebrate host. Tick modulation complements the contribution of tick-borne pathogen manipulation of the host environment (**Figure 1**). Tick saliva prepares the site of inoculation and makes it tolerant for inoculated pathogens, except for viruses that are inoculated within a few minutes of starting the blood meal. A delay of 12–24 h or more in pathogen inoculation is observed for bacteria and parasites, transmitted by hard ticks (8, 9). The final outcome of this tripartite relationship is determined by the interplay of the immune responses of the host and tick vector on the pathogen; modulation of vertebrate host defenses by the tick and pathogen; and the largely unknown manipulation of tick innate immunity by the tick transmitted pathogen.

Major advances in immunology will help to understand the different levels of interactions and tolerance which occur in tick-borne diseases. What are the role of the different T cell populations such as the Treg or the T_{RM} (T resident memory cells) (10) and Innate Lymphoid Cells (11) in the control of infection at the skin interface? Skin immunity should be particularly investigated since the skin represents a site of pathogen inoculation, and for some tick-borne pathogens a site of multiplication and persistence. For example, why does *Borrelia burgdorferi* sensu lato (sl), the bacteria responsible for Lyme borreliosis, multiply so intensively in the skin early after its inoculation (12)? Does it take advantage of the immunologically permissive environment created by tick modulation of host defenses? Is it to induce an immune tolerance and facilitate *Borrelia* persistence in the skin for months (13)? Additional factors might help successful tick-borne multiplication and

persistence. While the role of adipocytes and hair follicle has been shown for *Plasmodium* in malaria infection (14, 15) and for *Trypanosoma* in sleeping sickness (15, 16), for tick-borne diseases these relationships are yet to be defined.

New technologies should help to answer some of these questions. They have greatly evolved from early proteomics and transcriptomics to more powerful functional genomic, deep sequencing and bioinformatics analyses (17). With single cell technology, we might expect to unravel the complex interactions of host-pathogen-tick interaction (18). In this review, we will present the gaps existing presently to understand the different interactions taking place during the complex travel of tick-borne pathogens through the vector and the vertebrate host. We will also highlight some recent advances in skin immunity and its microbiome that we should explore.

TICK

Ticks are an ancient group of organisms that transmit a large array of pathogens, more than other haematophagous arthropods. This is likely explained by their life cycle, spending their free life in leaf litter and humus rich in microorganisms and then as an ectoparasite on vertebrate host skin rich in other types of microorganisms, microbiota (3), that can be potentially acquired during the course of their long blood meal. To adapt to these different environments, ticks developed innate immunity (19). Some of these tick associated microorganisms are endosymbionts and others evolved to become tick-transmitted pathogens that are responsible for tick-borne diseases (2). Tick-borne pathogens possibly circumvent or actively modulate tick innate immune defenses, resulting in tolerance to their presence within the tick vector.

Tick Innate Immunity

To defend itself from microbial insults and injury, ticks rely solely on innate immunity. Microbial insults can be generated through their blood meal or in response to physical damage to the cuticle. Tick immune system comprises central tissues like fat body, the equivalent of vertebrate liver and adipose tissue, and different types of hemocytes. In the periphery, the epithelium of different organs secretes effector molecules to protect ticks (20). This innate immune system can be particularly challenged during the blood meal. Ticks are strictly hematophagous, and all events occurring during the blood meal can induce the immune system, especially if the tick feeds on an infected vertebrate host.

The innate immunity of the tick relies on different structures. Mesodermic **fat body** is present in all tick stages. It is located beneath the epidermis and around organs, particularly the trachea. It is mainly a source of vitellogenin, but also a source of antimicrobial molecules secreted into the hemolymph (21). In the **hemolymph**, tick innate immunity relies on cellular immunity including active phagocytosis, nodulation and encapsulation orchestrated by **hemocytes** circulating in an open circulatory system. In ixodid ticks, three types of hemocytes have been described: prohemocytes, granulocytes and plasmatocytes that participate in phagocytosis, clotting system, and encapsulation of microbes (22). More recently, humoral immunity has been investigated in ticks, building on research on *Drosophila melanogaster* (23). The discovery of cecropin, the first antimicrobial peptide (AMP) in primitive insect, *Hyalophora cecropia* (24), open the avenues to the discovery of innate immunity in *Drosophila*. The two main pathways, Toll activated by Gram-positive bacteria or fungi, and Imd activated by Gram-negative bacteria, were discovered (25), leading to the identification by homology to the cloning of Toll (TLR—toll-like receptors) in human (26). In addition to these specific immune organs, some barriers protect the tick. While feeding, a **peritrophic membrane** (PM) is formed by secretion from the midgut epithelium, at least in some species of Ixodidae (27). This chitin-rich matrix formation was first described in the three life stages of *I. ricinus*. It appears 18 h after the beginning of blood digestion and remains intact for several days (28). It surrounds the blood meal and protects the midgut epithelium. Then, the blood digestion occurs intracellularly *via* phagocytosis into the midgut cells (29). The epithelium is the next component of the **gut barrier** that operates upon uptake of the blood meal and movement of cells and fluid across the gut to the hemolymph. The innate immunity of the epithelium has been well-investigated in insects, particularly in *Drosophila* (30) and in the *Anopheles* mosquito as insect vector (30). This topic deserves greater examination in tick-pathogen interactions (31).

Tick innate immunity is regulated by different pathways and molecules. Hemocytes, midgut epithelium, and salivary glands produce defensive, anti-microbial molecules (31). This generally happens upon recognition of the pathogen-associated molecular patterns (PAMPs) of microorganisms by specific receptors. In *Drosophila*, some of these receptors include the peptidoglycan recognition proteins (PGRPs) and the Gram-negative binding proteins (GNBPs). In tick genome, all the components of these two cascades have not been identified so far (32, 33). Interestingly in *Ixodes* ticks, lipid moieties (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol or POPG and 1-palmitoyl-2-oleoyl diacylglycerol or PODAG) of certain pathogens elicit IMD (Immune deficiency) pathway (34). In hemolymph, activation of different proteolytic cascades is triggered similar to a complement cascade and clotting cascade, but no phenoloxidase cascade has been identified in tick (19, 35). This activation leads to release of soluble factors and antimicrobial molecules (lysozyme, defensin and hemoglobin peptides) (31). In addition to the well-known defensins, some molecules have been identified in ticks: microplusin, ixodidin and hebraein (36). Microplusin for example has a bacteriostatic effect by sequestering copper used by bacteria (19). Other soluble factors in tick humoral immunity include antimicrobial proteins such as lectins, lysozyme, proteases and inhibitors of proteases like alpha2-macroglobulin. This molecule belongs to the thioester-containing proteins with similarity to the C3-components of the complement system and insect TEPs that inhibit pathogen proteases.

Regulation of the tick innate immune response relies on three pathways: IMD, JAK-STAT and Toll pathways. Activation occurs through different mechanisms of pathogen recognition and leads to secretion of effector molecules that further neutralize the pathogens. The IMD pathway has been the most investigated.

PAMP recognition leads to the interaction of XIAP (ubiquitin ligase: X-linked inhibitor of apoptosis) with its substrate, the protein p47. The silencing of this protein *in vitro* and *in vivo* affects pathogen control and enhances *A. phagocytophilum* intracellular burden in ISE6 hemocytes and *B. burgdorferi* infection of *I. scapularis* nymphs (34, 37). This pathway also protects *Dermacentor andersoni* against *A. marginale* (34). In the **gut epithelium** of *I. scapularis*, the mRNA of a defensin-like peptide was identified; the amino-acid sequence having 79 % similarity with a *D. variabilis* defensin (38). In *Haemaphysalis longicornis*, an antimicrobial peptide, longicin, was also expressed in the gut and had antimicrobial activity on different microbes (bacteria, fungi, and *Babesia*) (39). In addition, the JAK/STAT pathway is a key signaling pathway in gut immunity in *I. scapularis,* as was shown in *Drosophila* (32). In tick hemolymph, defensins were identified as effector molecules as well as lectins and TEP (thioester-containing proteins) (19). Similarly, in salivary glands, transcriptomic studies revealed the presence of AMPs in different tick genera (19). By comparative genomics, an RNA interference (RNAi) pathway has also been described (40), that is, mainly involved during tick-virus interactions. This is a gene silencing process triggered upon interaction with double-stranded RNA. Most viruses infecting ticks are RNA viruses (36). In *Ixodes scapularis* tick, genome sequencing identified several genes participating in these different pathways, but characterizations of some components are still missing (19, 41). The use of tick hemocyte cell lines has been proposed to investigate the molecular mechanism involved in tick immune response. The interaction of ISE6 hemocytes with *A. phagocytophilum* has been particularly explored (34, 37, 42). More precisely, using metabolomics, transcriptomics and proteomics, it has been shown that the intracellular bacterium affects the protein processing in the endoplasmic reticulum and decreases the glucose metabolism. The bacterium also limits the tick immune response within the hemocytes and inhibits apoptosis facilitating its survival and possibly its further transmission to the vertebrate host (42). Through its co-evolution of more than 300 million years (43), it seems that pathogens establish with the tick innate immune system an intimate equilibrium at different levels, where both need to survive. The tick is not killed by the tick-borne pathogen and the pathogen population is controlled by the tick immunity limiting damage to the tick.

During the blood meal, some molecules of the vertebrate host can interact with the tick immune system. Recently, the JAK-STAT pathway has been shown to be controlled by cross species signaling between mice and ticks (44). Indeed, **mouse INF-gamma** acquired during the blood meal on a *Borrelia*-infected mouse activated *Ixodes* STAT leading to the secretion of the antimicrobial peptide (AMP) Dae2 that in turn controls the *Borrelia* population in the gut of the infected tick. Similarly, this pathway was shown to regulate the *A. phagocytophilum* population in ticks (45). Although different components of the Toll pathway were identified in ticks, its direct involvement in pathogen control was not yet demonstrated. Host **hemoglobin** also participates in the control of infection against Gram (+) bacteria and fungi. In the tick midgut, hemoglobin is cleaved in large peptides (hemocidins) with antimicrobial activity. A seminal observation was that hemoglobin passed across the gut from the blood meal of the insect, *Rhodinus prolixus*, into hemolymph and subsequently was incorporated into salivary glands (46). This phenomenon has been observed in both soft and hard ticks (19). Host-derived **plasminogen** also helps some pathogens to escape tick immunity and facilitates their migration through the gut epithelium, as shown for *Borrelia* (47).The outcome of **host immunoglobulin** in the tick has been particularly investigated. Immunoglobulins consumed in the tick blood meal passed serologically intact across tick gut into hemolymph and subsequently were detected in salivary gland extract (48, 49). Immunofluorescent microscopy confirmed that rabbit antibodies raised against tick ovaries and salivary glands, when consumed in a blood meal, retained tissue antigen binding specificity in *D. variabilis* hemolymph (48). Likewise, hemolysins raised in rabbits to sheep erythrocytes retained their antigen specificity in the hemolymph of female *I. ricinus*. Ticks fed upon re-infested rabbits had higher titers of hemolysin in their hemolymph than observed during an initial infestation (50). Intact rabbit immunoglobulin G was also present, post-blood meal, in hemolymph of the argasid tick, *Ornithodoros moubata* (51). Argasid ticks consume a much smaller blood meal in 2 h, while ixodid larvae and nymphs may complete a blood meal in 4 days and adults may require more than a week (52, 53).

What are the quantitative aspects of host immunoglobulin in hemolymph of argasid and ixodid species? Concentration of host immunoglobulin G was found in a comparative study of seven species to be highest in *Hyalomma excavatum* with 30% intact (54). Immunoglobulin concentration in *O. moubata* was comparatively very low; however, 100% of the molecules were intact (54). Blood meal immunoglobulin G did not pass into hemolymph of the argasid species, *Argas persicus* and *Ornithodoros tholozani* (54). Hemolymph immunoglobulin G antibody specific activity was 35 to 42% for *Rhipicephalus appendiculatus* females that fed upon guinea pigs immunized with killed *Escherichia coli* (49). Immunoglobulin binding proteins present in both hemolymph and salivary glands of *R. appendiculatus* were hypothesized to be involved in removing foreign proteins from the tick (49). Functional bovine antibodies persisted in *Rhipicephalus (Boophilus) microplus* hemolymph for at least 48 h post-engorgement (55). Host immunoglobulin G entered *Amblyomma americanum* adult hemolymph at 6.5% of the concentration in the capillary tube feeding solution after 6 h with no evidence of binding to cells (56). The Fc piece was identified as the immunoglobulin G molecule region essential for specific uptake across the *A. americanum* midgut into hemolymph with receptor mediated endocytosis speculated to be the mechanism for preferential transport of immunoglobin G from midgut to hemolymph (57).

Why is host immunoglobulin taken up from the blood meal, moved to the hemolymph, and then found in the salivary glands of a feeding tick? This process may be a means of removing large proteins in the blood meal. Could antibody molecules recycled back into the host bite site down regulate host immune defenses? Host species can be immunized with tick internal tissue molecules essential for normal physiological function that results

in antibodies taken up in the blood meal moving from tick midgut into the hemolymph that bathes internal tissues where host antibodies can bind and disrupt tick physiological pathways and cell function.

Tick Microbiome and Virome

In addition to pathogenic microorganisms, the tick also harbors symbiotic microorganisms. A few years ago, two metagenomics studies were performed to analyze the bacterial diversity of the cattle tick, *R. microplus* (58) and *I. ricinus* (59). In both studies, more than a 100 bacterial genera were identified in the different tick stages. Variations were found according to geography and environment. Among all genospecies of hard ticks, the most studied group, bacteria of the phylum *Proteobacteria* are predominant followed by *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*. These endosymbionts likely either evolved toward virulent vertebrate pathogens or colonized ticks to become endosymbionts of specific tick tissues (1). Mechanisms that govern this evolution toward virulent microorganisms remain to be elucidated. Within the tick, these symbionts are well-known for their beneficial effects, notably their role on arthropod nutrition as a provider of B vitamin. The nutritional role of symbionts has been particularly well-investigated in the model tsetse-fly-*Trypanosoma* for a potential use in paratransgenesis (60, 61). In ticks, it has also drawn attention for the biocontrol of ticks with a first study in 1998 on *Ixodes scapularis* microbiome (62). Then, with the development of metagenomics, additional tick genera have been investigated for their microbiome and the list of identified microorganisms has been implemented (58, 59). In *Ixodes*, among the different bacterial families characterized, the Enterobacteriaceae have been shown as essential bacteria in the tick microbiome (63). An assay of vaccination with one of these bacteria, *Escherichia coli*, has been tempted in C57BL/6 mice deficient alpha-1,3-galactosyltransferase. Indeed, this sugar residue is broadly distributed in bacteria, fungi and mammals except humans and old word monkeys (63). *Ixodes* nymphs that fed on these immunized mice were hampered in their blood meal and showed high mortality. BalB/c and C57BL/6 mice were not affected, pointing out the role of the genetic background in the response to this sugar residue (63).

Three bacteria genera, *Coxiella*, *Francisella*, and *Rickettsia* are particularly interesting in ticks since within each of these genera, some evolved as true pathogens while others are endosymbionts (2). Some symbionts seem also to directly compete with TBP as described within the *Rickettsia* genus in *Dermacentor*. An interesting relationship operates in *Dermacentor andersoni* between non-pathogenic, *Rickettsia peacockii,* and pathogenic rickettiae, *Rickettsia rickettsii,* in tick vectors. This relationship goes back to the Bitterroot Valley of Montana with the non-pathogenic "east side agent" and highly pathogenic *Rickettsia rickettsii* on the west side of the valley (64). Interestingly, when both *Rickettsiae* are present within the tick, *R. rickettsii* has a reduced prevalence and the incidence of Rocky Mountain Spotted Fever is reduced. Genome sequence analysis of the two *Rickettsia* species revealed that the virulence could be mainly associated with an ankyrin repeat containing protein (65).

The roles of both microbiome and virome in development of tick innate immunity and immune tolerance to microbial agents within the vector are interesting and important topics to address.

Tick Microbiome at the Skin Interface?

The co-transmission of vector microbiome and Vector-borne pathogen (VBP) has been suspected in insect. During regurgitation process as present in the transmission of *Yersinia pestis* by the flea or *Leishmania* by the sandfly, the presence of gut microbiome of the insect in the vertebrate host skin is likely. Stercoral transmission of *Trypanosoma cruzi* during the reduvid bite likely also involves gut microbiome deposit at the skin interface of the vertebrate host (66). Recent data on *Leishmania donovani* transmission confirmed this hypothesis. These parasites are co-inoculated with sandfly microbiota leading to inflammasome activation and secretion of IL-1 beta (67). In transmission of tick-borne pathogens, very few studies have been performed to elucidate the potential transmission of gut microbiota during the process of the tick bite *via* exosomes (68). Interestingly, some of these tick symbionts have been shown to be transmitted during the tick bite process, due to their presence in salivary glands. It has been shown for *Coxiella*-like endosymbiont, found in a human skin biopsy and inducing a human infection in Europe (69). Similarly, *Midichloria mitochondrii*, an intra-mitochondrial symbiont of hard tick has been detected in tick salivary glands and transmitted to vertebrate hosts as evidence by the presence of antibodies against the bacteria in humans (70) and in rabbits (71). Application of next generation sequencing and advanced bioinformatics tools at the site of the vector bite should help to build upon these preliminary data and identify tick microbiota present in the host. However, detailed studies are needed to investigate whether the inoculated microbiota play a role in initiating the immune response of the vertebrate host (66). A recent work (68) demonstrated that tick saliva of *Amblyomma maculatum* and *I. scapularis* contain exosomes. In an *in vitro* system using a keratinocyte cell line (HaCaT cells), the authors demonstrated that these exosomes delayed the wound healing process by down regulating CXCL12 and upregulating IL-8.

Tick-Borne Pathogen Interaction With Tick Gut and Salivary Glands

The midgut is the largest organ with several diverticuli in the body cavity of the tick. Its size greatly enlarges during the blood meal. The digestion of blood occurs intracellularly and the midgut cells serve as a storage cells for the blood nutrient, enabling ticks to survive extended period (72). Pathogens entering the gut during the blood meal first have to overcome the acellular barrier constituted by the **peritrophic membrane** (PM). It protects the gut epithelium from injury potentially induced by ingested particulates or pathogens during the blood meal. The JAK-STAT pathway regulates its formation. Indeed, a decrease in the expression of the transcription factor STAT induces a lower expression of peritrophin, a glycoprotein of the PM (73). STAT expression is itself regulated by the gut microbiota as shown in dysbiosed larvae of *I. scapularis* (73). In this study, it is also demonstrated that the integrity of the PM is necessary for the

colonization of the gut by *B. burgdorferi*. The role of a protein present in the PM, a chitin deacetylase, has been investigated in relation to *Borrelia* infection within the gut, but no clear role of the protein has been established (74). For *Anaplasma*, the opposite effect of peritrophic membrane is observed (75). The symbiotic bacteria induce the tick to synthesize a glycoprotein, IAFGP (*Ixodes scapularis* anti-freeze glycoprotein), that modifies the formation of bacteria biofilm essential for the formation of the peritrophic membrane. *A. phagocytophilum* can then more easily invade the gut tick cells and migrate to the salivary glands (75). The role of PM has also been studied for the parasite *Babesia microti*. *Babesia* goes through the PM by help of a specific parasite structure, the arrowhead (28). Finally, the precise role of PM in the context of tick-borne pathogens remains to be investigated.

Then, microorganisms need to pass through the **gut epithelium**. Unlike in insects, microorganisms ingested during the tick blood meal do not face directly the digestive enzymes in the gut lumen. Some, like *Rickettsia*, are internalized and escape endosomes and digestion, to develop in tick cell cytoplasm (36). Using a yeast surface display library of tick gut proteins, four *B. burgdorferi*-interacting tick proteins have been identified. Two have been characterized. A fibronectin type III domain-containing tick gut protein (Ixofin3D) was shown to interact with *Borrelia* proteins (76). Similarly, a dystroglycan-like protein was identified on the surface of tick gut epithelium (77). RNAi silencing of these proteins demonstrated their essential role in the migration of *Borrelia* through the gut epithelium toward tick salivary glands and ultimate transmission to the host. Likewise, in a *D. variabilis* cell line infected by the intracellular bacterium, *A. marginale*, initial differential transcriptomic studies identified four tick genes involved in cell infection and *Anaplasma* trafficking through the tick. RNAi silencing on the whole infected tick revealed their role in the regulation of infection and transmission (78). Potential applications in anti-tick vaccines have been explored (78). Recently, one protein, subolesin was tested as a potential vaccine candidate (30). This tick protein is particularly interesting because it is well-conserved among tick species and it is an ortholog of akirin, known to function as a transcription factor for NK-kB gene expression and regulation of the innate immune response (79).

To move to the **salivary glands,** where pathogens will be inoculated into the host, pathogens need to pass through the hemolymph and face innate immune defenses. Tick-borne pathogens developed different strategies to escape the tick immune system and allow their transmission to the vertebrate host. Some have been particularly well-explored like *Borrelia* and *Anaplasma*, others like *Rickettsia* and *Babesia* deserve further investigation (43). The Imd pathway was activated upon interaction with *B. burgdorferi* and *A. phagocytophilum*, both transmitted by the *I. ricinus* complex, and it limits their proliferation (34). However, there are differences in the activation of this pathway between insects and ticks (34).

Transcriptomics and proteomics studies of uninfected and infected ticks revealed up and down regulation of molecules upon infective blood meal (17, 80, 81). Some specific tick molecules are used by the pathogens to facilitate their development and persistence within the tick. This topic has been particularly well-studied in *Borrelia*-infected ticks. First, within the tick gut, a bacterial protein, OspA, interacts specifically with a receptor, TROSPA (tick receptor for Outer surface protein A) (82). In addition, the presence of *Borrelia* upregulates some specific tick proteins to facilitate their survival within the salivary glands. A salivary gland protein, Salp25D, is a gluthathione peroxidase that helps *Borrelia* to establish within the gut (83, 84). Another tick protein, tre31 is induced in the gut and interacts with a *Borrelia* lipoprotein BBE31 (85). The use of RNAi demonstrated the essential role of these proteins for the colonization of the tick by *Borrelia*. The most studied tick saliva protein is likely Salp15, which was shown to interact with OspC and facilitate the transmission of *Borrelia* to the vertebrate host (86). This protein targets different immune cells of the vertebrate host: dendritic cells, T cells, keratinocytes, B cells (87). Another model particularly well-investigated is *I. scapularis* infected with *A. phagocytophilum*. As an intracellular bacterium, the interaction tick-bacteria has been first analyzed in the tick hemocyte cell line ISE6 (88). Then, a combination of transcriptomics and proteomics on nymphs and adult female midguts and salivary glands revealed a major impact of *Anaplasma* on the apoptosis process. The bacteria inhibit this pathway to facilitate their survival within the tick (89). In one hand, they increase histone modifying enzymes (90) and on the other hand inhibit gluconeogenesis and activate glycolysis (91). Similar "omics" approach has been undertaken to analyze the development of *Babesia* (19) in ticks. It also led to the identification of different tick molecules. Recent reviews describe the major role of tick saliva in the virulence and transmission of TBPs to the vertebrate host (92–94).

Duration of Tick Feeding to Pathogen Transmission

As examined for *I. ricinus*, tick-borne pathogen enzootic cycles are maintained through complex interactions of multiple factors that include abundance and diversity of hosts, larval tick density, likelihood of tick encounters with preferred hosts, pathogen effects on host and tick behavior, aggregation of ticks among hosts, pathogen transmission efficacy, success of larvae molting to nymphs, and success of nymph host seeking, feeding and pathogen transmission (95, 96). Pathogen transmission depends on the tick establishing successful blood feeding and avoiding host defenses of pain, itch induced grooming, hemostasis, and immune rejection at the host cutaneous interface (13, 97, 98).

Ixodid adult female tick blood feeding is divided into the initial slow phase of a week or more, during which weight increases ten-fold, followed by a rapid engorgement period of ∼12 to 24 h, during which the tick increases to 100 times or more the unfed weight (53). Cellular and molecular developmental and physiological interactions occur between the pathogen and the tick vector, including during the blood feeding phase (41, 99). Impacted by developmental events within the tick, an important parameter related to these phenomena is the duration of tick blood feeding prior to successful passage of an infectious agent into the bite site and establishment of infection (9, 100). These parameters have practical implications for disease prevention.

Reducing risk of tick-borne infections is predominantly an individual responsibility and relies significantly on use of repellents, protective clothing, and checking one's body for ticks (101, 102). Therefore, knowing how long a tick must feed prior to transmission of specific pathogens can help inform potential effectiveness of specific prevention measures, such as tick checks.

Argasid tick blood meals are much smaller than those of ixodid ticks, and they are completed in approximately 1 to 2 h, depending upon the life cycle stage (52). The argasid, *O. turicata* transmits relapsing fever, *Borrelia turicata*, spirochetes within 15 to 40 s of initiating feeding (103). Rapid transmission and host infection are attributed to preadaptation of *B. turicata* in tick salivary gland to the vertebrate host environment (103).

Variations occur in duration of ixodid tick feeding prior to transmission of a specific tick-borne pathogen as well as for different pathogens (100). *B. burgdorferi* transmission is well-studied in regard to development within the feeding tick and transmission to a vertebrate host by the North American vector, *I. scapularis* (100, 104, 105). Nymphs and adults transmit spirochetes, with nymphs transmitting the majority of infections (106, 107). Determinants of transmission include a six-fold increase in the number of spirochetes in tick gut from initiation to 48 h of blood feeding followed by a rise at 72 h of engorgement of salivary gland spirochetes by 21-fold (105). Using larval xenodiagnoses, spirochete transmission from infected nymphs occurred in one of 14 hamsters at 24 h of feeding, 5 of 14 at 48 h, and 13 of 14 after 72 h or longer of engorgement (104). However, due to different *Ixodes* vector and different pathogen species in Europe, the transmission delay can be shortened, especially for *B. afzelii* (108). The pathogen can already be transmitted after 24 h.

Anaplasma phagocytophilum was transmitted to 9% of experimentally infested mice by 24 h, 76% by 36 h, and 85% at 50 h of tick feeding (104). A study that examined two time points found *A. phagocytophilum* transmission did not occur by 40 h of tick attachment; however, 100% of mice were infected by 48 h (109). *Babesia microti* sporozoite transmission occurred in 9% of hamsters at 36 h and 50% after 54 h of infestation (110). A *B. burgdorferi* co-transmission study found that 71% of hosts were positive for *B. microti* infection at 54 h of tick attachment (104). *A. phagocytophilum* and *B. microti* can occur individually as co-infections with *Borrelia burgdorferi*, resulting from the bite of a tick infected with both microbes (111, 112).

B. burgdorferi, A. phagocytophilum and *B. microti* are transmitted by ticks of the nearly globally distributed *I. ricinus* species complex that includes *I. ricinus*, *I. persulcatus*, *I. scapularis*, *I. pacificus* and additional species (113). These tick species are also competent vectors for tick-borne encephalitis (TBE) virus (114). TBE virus exists as three geographically defined pathogenic subtypes in endemic foci from Japan across Eurasia to France (115, 116). Powassan virus is the reemerging North American representative of the TBE virus groups, and it occurs as two lineages (117, 118). Powassan virus was also isolated in Russia (119). *I. scapularis* nymphs transmitted Powassan virus to mice by 15 min of initiating feeding with maximum transmission efficiency at 180 min (120).

Borrelia miyamotoi is a relapsing fever spirochete also transmitted by members of the *I. ricinus* species complex and occurs over the same geographic regions (121, 122). Transmission risk increased with infestation by a single infected *I. scapularis* nymph from 10% after 24 h to 73% at 72 h (123). A single *I. scapularis* infected nymph transmitted *B. mayonii* with a 31% probability of infection at 72 h of engorgement with no evidence of transmission at 24 or 48 h (124).

Unclear is the process by which reactivation of spotted fever group rickettsial virulence occurs within the vector tick during the period from attachment to the host through blood feeding (125). Reactivation occurs during the 6 to 10 h infected ticks feed before rickettsiae are transmitted (126). In addition to blood feeding, virulence can also be restored by exposing unfed, infected ticks to 37°C for 24 to 72 h (127). Overall, bacteria and parasites need to migrate and undergo development within the ticks explaining a delay in pathogen transmission to the vertebrate host, while viruses are transmitted as soon as the tick blood meal is initiated (8, 9).

Salivary Glands: A Key Organ in Pathogen Transmission

The structure of Ixodid tick salivary glands is composed of three types of acini in females and four types in males (128–131). Type I acini occur in all ixodid life cycle stages; these acini lack secretory granules; and, they contribute to maintaining off host water balance by production of hygroscopic saliva (130). Type II and III acini both increase in size and granularity over the course of engorgement combined with release of granular contents (129, 130).

Number and diversity of salivary gland derived proteins were greatly increased by application of reverse genetics strategies that included sequencing of full length cDNA libraries in combination with increasingly powerful bioinformatic and proteomic analyses tools (17, 132). Next generation sequencing platforms combined with proteomics informed by transcriptomics revealed even greater salivary gland gene product complexity (133–135). Transcribed salivary gland gene analyses revealed differences between and within prostriate and metastriate species; gene transcription changes during infection with tick-borne pathogens; widely conserved multigenic families; pluripotency and redundancies in gene products that target specific host defenses; and, saliva composition changes occurring during the course of feeding, including members within a gene family (135–142). Analyses can now be performed on a single pair of salivary glands rather than on pooled glands, revealing individual tick specific properties and variations within a population (17, 143). Host species related specific salivary gland gene expression adaptations also occur (144). While these gene expression studies focused on proteins, salivary glands also produce non-protein compounds, purine nucleoside and prostaglandins, with important biological activities (145). More recently, non-proteinaceous molecules like **small RNAs** (miRNAs and small-interfering RNAs) were described as gene regulators. They are produced after cleavage by the DICER protein and they bind to complementary mRNA target leading to gene silencing. They have been studied in several tick species. MiRNAs can be involved in the regulation of tick development

(146) or blood feeding (147). MiRNAs have been detected in *I. ricinus* saliva and might be excreted in exosomes that could modulate the vertebrate host homeostasis at the skin interface (148). Interestingly, some of these miRNAs have been characterized in *I. scapularis* salivary glands during the transmission of Powassan virus to mouse model (149). They have been also detected in tick hemocyte ISE6 cell line infected by the bacterium, *A. phagocytophilum*. A specific miRNA, isc-mir-79, was particularly upregulated. It targets a transmembrane protein belonging to the Robo immunoglobulin family involved in inflammatory processes (150).

Transcriptomic and proteomic analyses also identified a multitude of salivary gland protein predicted biological activities, molecular targets, and functions that include modulators of host pain and itch, vasodilation, platelet aggregation, coagulation pathways, innate and adaptive immune effectors and regulators, and wound healing (92, 135, 151–153). Attention is increasingly focused on characterizing major groups of broadly bioactive molecules present in saliva cross multiple tick species, such as cystatins and Kunitz inhibitors (154). Histamine-binding lipocalins (155) and releasing factor (155) are examples of targeted differential effects of tick saliva on a host response mediator during different phases of blood feeding. Although the number of identified salivary gland genes and miRNAs continues to increase, the fundamental problem remains of linking individual molecules to specific biological activities at the tick-host-pathogen interface.

Differential production of bioactive molecules correlates with anatomical and histological changes occurring in tick salivary glands during the course of blood feeding; however, regulatory events controlling saliva production require continued study.

VERTEBRATE HOST

Immune tolerance to tick-borne pathogens differs whether the vertebrate is a reservoir (a host, source of infection to tick and not clinically ill) or a susceptible host (a host, that ticks feed on in nature) (156). This susceptible host can be either clinically ill or neutralizes the pathogens and is only serologically positive (157).

In this process of tolerance to the intruder (the tick and the potential pathogen), the skin plays a key role by its immunity (158) and its microbiome (159). In addition to its role as an inoculation site, it has been shown to be a site of persistence in some insect-borne diseases such as malaria parasite (14) and for trypanosomiasis (16, 160, 161). In TBDs, Lyme borreliosis has been the most investigated for this aspect (13). Additional studies on other TBDs deserve further investigations to conclude to a common role of the host skin in pathogen persistence.

Structure and Immunity of the Skin

The skin is the largest organ and more than just a physical barrier. It is structured into three major layers: the epidermis, the dermis, and the hypodermis (162). The **epidermis** is the outermost layer with a stratified epithelium, mainly constituted by tissue resident cells, the keratinocytes, which undergo sequential differentiation, and melanocytes. Keratinocytes are integral components of the skin innate immune system (158). They have been studied for their role in secretion of the defensins (163–165), cathelicidin (166, 167), and control of skin infection. It is well-documented that these antimicrobial peptides (AMPs) increase adaptive immune responses (165, 168). Langerhans cells reside mainly in the epidermis and represent 2–8% of the epidermal cell population (169). The **dermis,** which underlies the epidermis, is a connective tissue with fibroblasts as resident cells secreting extracellular matrix, collagen and proteoglycans (170), giving the dermis its toughness and resilience (158). Dermis is well-drained by both blood and lymphatic vessels, which facilitate circulation of immune cells. It is therefore rich in migrating immune cell populations: dendritic cells, mast cells, macrophages, T lymphocyte subsets (CD4 T cells and CD8 T cells), natural killer cells and innate lymphoid cells (ILCs) (171). All these cells possess a strong ability to recognize pathogens and to be activated (158, 172). Below these two layers, **adipose tissue** constituted of subcutis, **hypodermis** and dermal white adipose tissue (dWAT). The dWAT within the reticular dermis, is involved in thermoregulation, hair cycling, wound healing and most recently in immunity (173). Its main cells are adipocytes, secreting adipokines and AMPs, but also immune cells. Adipocytes display various pattern recognition receptors and then produce various cytokines and chemokines (173, 174). The dWAT also surrounds the hair follicle. A specific interplay exists between the hair follicle cycle and the intradermal adipocyte. PDGF (platelet-derived growth factor) secreted by immature adipocytes, activates the growth of the hair follicle (175). Recently, the hair follicle has been shown to harbor a complex microbial community due to its moist and less acidic environment compared to the epidermal surface. This community is regulated by specific AMPs and constitutes an immune-privileged site, potentially used by persistent pathogens (176) (see below).

Our improved knowledge of the structure and immunological function of the skin provides the framework for understanding tick and tick-borne pathogen induced immune tolerance. To protect from invaders the skin has developed a complex network of cellular interactions that ensure host defense and preserve homeostasis (158, 177). This network relies on (1) innate immunity with the resident skin cells of the epidermis and the dermis, and more specific immune cells like Langerhans cells, mast cells, dendritic cells, macrophages and innate lymphoid cells (ILCs), and (2) adaptive immunity which relies on various subpopulations of T cells (169). Within this structure different appendages like hair follicles, sebaceous glands and sweat glands participate in skin homeostasis and protection.

The role of macrophages, and more particularly of neutrophils, has been investigated in vector-borne diseases (178, 179). While macrophages and neutrophils are studied in the contexts of infectious diseases and tissue repair, the roles of lymphocytes are reevaluated at the skin interface. ILCs respond to epithelium-derived signals (cytokines, cell-surface receptors and lipid mediators) and therefore constitute an important actor of skin homeostasis. They divide into three subgroups according their cytokines profiles. The secreted cytokines modulate the immune response and ILC functions overlap and complement T cells (11, 180, 181). Then, acquired immunity relies on antigen-specific T cells. First, effectors T cells are generated upon acute

infection leading to a long lasting immunity in the skin, with the development of resident-memory T cells (T_{RM}) (182). In adult human skin, memory T cells are four times more important than in peripheral blood and four distinct populations of these T cells have been identified according to their surface receptors (183). These skin-homing T cells are produced in skin-draining lymph nodes, where they acquire specific chemokine receptors (CCR4, CCR8, and CCR10) and leukocyte integrins to come back to skin tissue (172). Regulatory T cells play a key role in homeostasis and inflammation in the skin, where they are particularly abundant. These cells are also part of the resident cell population and interact with fibroblasts and Langerhans cells (10). How this complex immune network control so efficiently tick-borne pathogens at the skin interface needs to be elucidated.

Tick Induction of Cutaneous Immune Tolerance

When ticks introduce their mouthparts into the skin tissues, they lacerate the epidermis and the dermis due to their telmophage bite that induces a blood pool in the dermis, where they inject saliva and consume blood. While the presence of chitin on mouthparts should trigger an immune response (184), it seems that the tick succeeds once again in escaping the host immune system. Tick saliva is responsible of this immune escape (185, 186). Indeed, it has been known for years that tick saliva exerts a potent local immunosuppression by secreting a large array of molecules that target multiple elements of the immune system (93, 153, 187, 188).

Salivary gland transcriptomes and proteomes have shown how tick saliva modulates vertebrate host innate and adaptive immune responses and wound healing (134, 189). Increased examination of mediators, cells, and crosstalk among these elements will greatly enhance or understanding of events occurring at the tick-host-pathogen interface. In addition to the receptor populations, cells, cytokines, chemokines, and interleukins that are well-studied in the context of tick induced modulation, emphasis can be placed upon less well-studied cells in the tick-host relationship, such as keratinocytes, melanocytes, fibroblasts, adipocytes, and innate lymphoid cells and mediators such as alarmins. Resident skin cells (keratinocytes, fibroblasts, and adipocytes) deserve further investigation based on the increasingly recognized roles of these cells in immunity that are emerging (15, 190, 191). Innate lymphoid two cells are also of interest relative to potential cytokine polarization to a Th2 profile at the tick bite site. These innate immune cells have not been studied so far in the context of TBDs. Due to their role in the regulation of the innate immune response at the skin interface (180, 181, 192), they must also play a role during tick feeding and inoculation of pathogens. It may be particularly relevant to analyze these ILC2 cells, since it is well-documented that Th2 lymphocyte response is induced during the introduction into the host of tick-borne pathogens such as *B. burgdorferi* (193, 194) and spotted fever group rickettsiae (195).

Finally, since tick saliva modulates pain and itch responses, the interactions of saliva with dermal peripheral nerve endings deserve investigation since the role of the nervous system and its connection with the immune system is unknown during the tick blood feeding that lasts for days (196).

Tick Attachment and Feeding Site: Role of Tick Saliva

Understanding tick-host-pathogen interactions requires characterizing and defining the biological activities of tick saliva molecules during the course of feeding and infectious agent transmission. Ixodid tick feeding presents unique challenges due to larvae and nymphs blood feeding for days while adult feeding may require more than a week (53). Host defense systems evolved to reduce or eliminate insults on homeostasis; however, ticks developed effective countermeasures to host pain and itch responses, hemostasis, innate and adaptive immunity, and wound healing (92–94, 97, 98, 135, 154).

Tick-borne infectious agents exploit tick saliva modulation of host defenses that create an immune tolerant bite site environment favorable for pathogen transmission and establishment (13, 92, 153, 193). Balance is not static between host immunity to tick feeding and tick modulation of host immune defenses, as occurs during repeated infestations (97, 98, 154). Acquired resistance to tick bite represents a tipping of that balance toward host immune dominance that results in impaired tick engorgement, blocked molting, and tick death (197–202). While tick modulation of host defenses can facilitate pathogen transmission, acquired resistance to tick bite significantly inhibited *Dermacentor andersoni* transmitted infection with *Francisella tularensis* type A (203). Development or absence of acquired resistance depends upon the tick species and host species infested (197, 200, 204).

The complexity of tick salivary gland derived molecules increased dramatically during the past five decades due to the emergence of transcriptomics, next generation sequencing, and quantitative proteomics (17, 135). Early studies relied on isolation and biochemical characterization of individual bioactive molecules from salivary glands of feeding ticks (132, 205–207). Valuable insights were obtained about saliva activities. Biochemical isolation and characterization combined with analysis of biological activity studies were generally labor intensive; required large amounts of starting material; and, depended upon activity identification assays at each fractionation step.

In addition to secreted saliva molecules into the host skin, attachment cement is a salivary gland secretion that serves as a holdfast structure and sealant of the bite site whose production starts within minutes of host attachment and, depending on the tick species, occurs in different patterns during the course of blood feeding (131). Attachment cement production is linked to distinct cell types within type II and III acini (128) along with a possible contribution from type I acini (130). Tick saliva can be trapped in attachment cement (208) along with tick-borne pathogens (209).

To conclude, tick saliva induces a transient potent immune tolerance at the bite site to avoid its rejection. Pathogens, when present in infected ticks, behave as opportunistic

microorganisms at the skin interface and take advantage of this immunosuppression (210).

Tick and Host Pharmacology

Concerning the pharmacomodulation of tick saliva at the skin interface, successful tick blood feeding depends upon inhibiting host hemostasis and wound healing that allows access to a continuous supply of blood. Ticks evolved salivary secretions that inhibit platelet aggregation and activation, act as vasodilators, and block the action of multiple components of the coagulation cascade (154, 188, 211). Hemostasis is also the first phase of the multi-step process of acute injury cutaneous wound healing (212), a process that ticks regulate primarily during hemostasis and inflammatory response phases (97, 98, 135). Argasid and ixodid ticks evolved multiple, redundant strategies to counteract platelet aggregation and activation of the different vertebrate host species from which individual tick species are capable of obtaining a blood meal by blocking platelet integrins, binding platelet activating molecules, or inhibiting protease activated receptors (154, 188, 211, 213, 214). Tick saliva contains numerous Kunitz domain serine protease inhibitors that disrupt platelet aggregation and coagulation cascade activation (154, 188, 213). Saliva Kunitz inhibitors act upon coagulation cascade factors Xa and thrombin due to their activation of platelets and multiple coagulation pathway enzymes (188, 215–218). Tick saliva contain few vasodilators (214).

Itch-induced grooming is a threat to ticks that are continuously attached and blood feeding for several days. The relationships among tick feeding, host acquired tick resistance, and the itch response were described for *Rhipicephalus (Boophilus) microplus* infestations of cattle either restricted from self-grooming or allowed to groom, lick, freely (219–222). Experimental infestations of cattle restricted from grooming resulted in an average yield of 33% engorged adults whilst the adult recovery from animals allowed to groom freely was 9% (219). Grooming-induced tick mortality was directed primarily toward larvae within the first 24 h of infestation (221) that resulted in larval losses of up to 54% (223).

Infested cattle developed acquired resistance to *R. (Boophilus) microplus* that upon reinfestation resulted in reduced tick feeding weight, yield of adults, and egg mass (220). Acquired resistance was linked to a cutaneous allergic hypersensitivity response characterized by an influx of eosinophils and development of serous exudates that trapped ticks (220). Significantly, highly tick resistant cattle blood histamine levels peaked at 48 h after applying larvae and persisted for 8 days, while little or no changes occurred in blood histamine concentrations for infested, non-resistant cattle (220). Histamine and its receptors are commonly associated with the temporary sensation of cutaneous itch (224, 225), and anti-histamines are well-recognized treatments for itch (226). Serotonin, 5-hydroxytryptamine, elicits both pain an itch responses independent of histamine with only an itch response stimulated at lower concentrations (227, 228). Recent reviews focus on molecular and neural mechanisms of itch that include peripheral initiation of the response, sensory neurons, mediators, receptor, central nervous system perception, scratching response to eliminate an acute stimulus, and shared features of itch and pain responses (224, 225, 229, 230).

Cutaneous injury, such as a tick bite, results in mast cell release of histamine, serotonin, cytokines, chemokines, and proteases that mediate vasodilation, inflammatory cell influx, and stimulation of itch receptors (225, 231). Platelet aggregation in response to injury also releases histamine and serotonin (232, 233).

Regulating the actions of histamine and serotonin are central to tick modulation of the itch response. Chinery and Ayitey-Smith (234) reported that *Rhipicephalus sanguineus* salivary gland extract contains a histamine blocker. Three histamine binding proteins were found in *R. appendiculatus* saliva and each had one high and one low affinity binding site for histamine (235, 236). Similar dual receptor binding affinity active sites occur on a histamine binding protein in *D. reticulatus* salivary glands that was demonstrated to have one high affinity histamine receptor and a low histamine affinity receptor that bound serotonin with high affinity (155). These two different receptors on one saliva protein bind two important mediators of acute itch to tick bite.

Histamine and serotonin directly impact tick feeding. Upon exposure to histamine and serotonin in a blood meal, *D. andersoni* female salivation and blood uptake were inhibited (237). Mechanisms mediating acquired tick resistance remain to be fully defined, elevated bite site histamine levels negatively impact tick feeding and induce host grooming (238, 239). Elevated histamine can also be linked to the basophil rich inflammatory cell influx at tick attachment sites and development of epidermal hyperplasia that disrupts tick feeding (240–242).

Basophils and Acquired Resistance to Ticks

Basophil responses at tick attachment sites are linked to the phenomena of acquired host resistance to tick infestation and the immunological basis underpinning the response. Two studies are foundational in linking host immune responses to tick bite. Jellison and Kohls (243) hypothesized that host immunity was responsible for poor tick feeding on rabbits repeatedly infested with adult *D. andersoni* and for development of crust-like lesions at tick attachment sites. In a foundational study, Trager (244) observed that guinea pigs developed resistance to infestation with *D. variabilis* larvae after one infestation, and that resistance was expressed during a second infestation as reduced tick engorgement, death of ticks, discolored feeding ticks, and small blisters at attachment sites. Histology of first exposure larval attachment sites was characterized by slight epidermal thickening with little cellular reaction, while second exposure bite sites contained large numbers of polymorphonuclear leukocytes, few eosinophils, and epidermal thickening extending below the inflammatory cell containing "mass" (244).

Allen (240) established that the cellular response in vesicles in hyperplastic epidermis beneath larval mouthparts on guinea pigs expressing acquired resistance to *D. andersoni* consisted of high concentrations of basophils attributed to a cutaneous basophil hypersensitivity response. During a repeated infestation in which acquired resistance was strongly expressed, histologic

changes at attachment sites consisted of epidermal acanthosis and acantholysis; dermal influx of eosinophils, lymphocytes, and macrophages with no increase in mast cells; and, numerous basophils accumulating in vesicles beneath mouthparts (245). Likewise, cattle expressing acquired resistance to *I. holocyclus* developed basophil rich inflammatory responses at tick attachment sites (245). Basophils also accumulated at tick bite sites in humans (246).

In an elegant and definitive study that maintained the functional integrity of mast cells, selective ablation of basophils established their non-redundant role in murine acquired resistance to infestation with *Haemaphysalis longicornis* larvae (247). Mast cell deficient mice developed resistance to *D. variabilis* larvae after repeated infestations; however, their mast cell sufficient counterparts developed a more marked resistance, suggesting a minor role for mast cells in this tick-host association (248). Basophils were detected by ultrastructural examination of *D. variabilis* larval attachment sites on mast cell deficient mice after three infestations (249). In contrast to *D. variabilis* larval infestations, mast cell deficient mice of the same strain failed to develop acquired resistance to *H. longicornis* larvae (250). Tabakawa et al. (251) subsequently established that histamine derived from basophils infiltrating the tick bite site, not mast cells, were responsible for expression of acquired resistance. Recruitment of basophils to the tick attachment site was linked to interleukin-3 produced by skin resident memory CD4+ T lymphocytes (252). The central role of basophils in acquired resistance to ticks was recently reviewed (241, 242).

Does acquired resistance to tick infestation alter pathogen transmission? Rabbits expressed acquired resistance after one infestation with uninfected *D. variabilis* adults that provided significant protection against transmission of the bacteria, *Francisella tularensis* type A, by an infestation with infected *D. variabilis* nymphs (253). The mechanism by which resistance to this highly virulent pathogen was expressed remains to be determined. One possibility is that the inflammatory reaction at the bite site creates a milieu that reduces infectivity or kills the bacteria.

Basophils and mast cells were recently reviewed in the regard to similarities and differences in their biology, roles in host defense and disease pathogenesis, and availability of specific molecular tools to distinguish the effector functions of these two important cell types (254–258). Roles of mast cells and basophils in cutaneous immunity and inflammation were reviewed in the context of Th2 responses, innate lymphoid cells, and eosinophils (259, 260). Basophil function as antigen presenting cells for Th2 responses remains a topic of ongoing study (242, 260).

Skin Microbiome

The skin microbiome is part of skin immunity (261). Its major role increased lately in studies on skin inflammation (166, 262). The vertebrate host and its microbiota are now considered as a holobiont or a hologenome (263, 264). The cutaneous surface, the largest organ of 1.8 m^2, is colonized by a diverse population of microbes, ranging from bacteria, mites, yeasts, and viruses (3). The composition of the human skin microbiota varies according to moist, dry or sebaceous microenvironments. These symbiotic microorganisms occupy the skin surface and specific niches, such as hair follicles and sebaceous glands. A precise 3D mapping by mass spectrometry and 16S rRNA sequencing revealed the impact of the skin surface environment on the composition and chemistry of human skin microbiome (265).

Various forms of interaction exist between these microorganisms, encompassing mutualism, parasitism and commensalism depending on the context (159). In addition, these microorganisms cooperate with the host immune system to maintain skin homeostasis. Immune tolerance to these commensal microbes is essential. It seems to take place in neonatal life with help of Treg cells as shown in a mouse model colonized specifically with a *Staphylococcus epidermidis* transformed to express a model 2W peptide coupled to a fluorescent protein (266). How skin Treg cells induce tolerance to commensal antigens remain to be investigated, but it seems to be different from the mechanisms operating in the intestine and then to be tissue specific. Scharschmidt et al. speculate that the hair follicles could be the site where Treg reside since both, the hair follicle morphogenesis and Treg production take place at the same time. A hair-follicle related chemokine would attract the Treg into these appendages (266). The skin microbiome also educates the innate immunity by shaping the expression of IL-1 alpha, complement system and AMPs (mainly cathelicidin and beta-defensin). Therefore, commensal microbiota is considered as an adjuvant to the immune system (267). For example, *S. epidermidis*, a major constituent of the skin bacteria, participates in innate immunity by secreting its own antimicrobial peptides to control pathogens at the skin interface, also creating a favorable environment for itself (261).

The skin microbiome is an interesting and important area for future investigation in the context of VBDs. While several studies have been performed on the interaction of mosquitoes and skin microbiome, they are mainly focused on the role of commensal bacteria on mosquito attractiveness (268–270). Early studies revealed the role of *Brevibacterium epidermidis* of human host on the attractiveness of *Anopheles*, the vector of malaria parasites (271, 272). Very few studies have investigated the role of skin microbiota on pathogen transmission at the skin interface. They concern *Leishmania* parasites (273, 274). Germ-free mice develop larger lesions, a higher parasite load and their macrophages are less efficient to kill intracellular parasites (274). It has also been shown that germ free mice have an impaired immune response against *Leishmania* parasites that can be partially rescued by inoculation of the commensal skin bacteria, *S. epidermidis* (273). *Leishmania* might induce dysbiosis; this rupture in skin homeostasis would lead to the recruitment of neutrophils and IL-1 beta secretion increasing the severity of the disease (275). Surprisingly, up to now no such studies have been performed on ticks and tick-borne pathogens to analyze the role of host microbiota in tick-attractiveness and pathogen transmission. This last aspect is particularly relevant in TBDs, since the tick lacerates the host skin, creates a feeding pool and remains for several days attached to the host skin (276). It is very likely that the microbiota penetrates from the surface of the skin, deeper in the dermis and might contribute to local immunomodulation during the bite and pathogen transmission.

The role of skin microbiota is definitely a research area that deserves further investigation.

TICK-BORNE PATHOGENS

A Worldwide Increase

Due to major climate change, modifications of the ecosystem and global trade, tick population and TBPs increase worldwide (277–280). Ticks transmit a diverse array of pathogens; therefore, the public health impact of established, resurging, and emerging tick-borne infectious agents is increasing (98). Changing geographic ranges of tick species is associated with movement of tick-borne infections as well with the potential for creation of new endemic areas for diseases (281–283). Some of TBP are considered as emerging pathogens however this increase is also likely due to better detection methods, awareness of health practitioners and patients and closer contacts between ticks and populations.

The high incidence of Lyme borreliosis in northern hemisphere (107) has likely hidden other TBDs in humans with lower incidence like anaplasmosis, relapsing fever associated with *B. miyamotoi* among others. Since the 1990s, molecular tools allowed the identification of a number of microorganisms such as *Neoerhlichia mikurensis*, *B. miyamotoi* and different *Rickettsia* species within ticks (157). Facing clinical pictures different from Lyme borreliosis in patients, biologists looked for these pathogens by direct diagnosis in blood and tissues by PCR. Consequently, the panel of TBDs in human significantly increased, especially in patients suffering from immunosuppression (284–287). The immune status of the patient is a key element in the outcome of disease. Due to concurrent medical procedures and conditions (e.g., cancer and grafts), the number of immune-compromised patients has increased. It explains why new TBPs are detected in these patients, improving indirectly the knowledge on tick-borne diseases and potential pathogen isolation as shown for *B. miyamotoi* (288, 289) or *N. mikurensis* (290). In parallel, serological surveys performed in tick endemic areas revealed that the number of exposed people to TBP is significantly higher, as shown by the seroprevalence against TBPs, increasing worldwide (157).

Molecular and Cellular Tools to Identify and Study Tick-Borne Pathogens

Molecular tools such as next generation sequencing and functional "omics" (genomics, transcriptomics, and proteomics) for identification of potential emerging tick-borne pathogens are essential to make a direct detection of pathogens in tick or in the vertebrate host (17). PCR can be developed rapidly and can be tested on different matrices (whole tick, blood, skin biopsy or biological fluids). Application of molecular techniques was the basis for the reversed discovery of tick associated microbes that were subsequently recognized as human pathogens: *B. miyamotoi* (291), *N. mikurensis* (292), *Rickettsia helvetica* (293), *R. monacensis* (294), and other *Rickettsia* species identified by genomic methods (295). High throughput sequencing of the microbiomes of *I. scapularis*, *D. variabilis*, and *A. americanum* from a single site in New York State resulted in identification of nine new viruses (296). This study design was expanded to multiple sites in Connecticut, New York, and Virginia with the detection of nine previously characterized viruses and 24 presumably novel viral species (297). New microbial species were detected in Western Europe when the *I. ricinus* microbiome was analyzed by next generation sequencing (298).

Single cell technologies, for example, flow cytometry to analyze cell surface markers or single-cell RNA sequencing (sc RNAseq) should also greatly help to better understand host-pathogen interactions and identify key molecules involved in cell-cell interaction. The complexity of the immune system network involves in these interactions requires complementary techniques and comprehensive analysis (18). Two photon intravital imaging visualized the interaction of different parasites in the skin: the persistence of *Plasmodium* parasites in the hair follicle (299), the role of neutrophils in *Leishmania* (179) and *Trypanosoma brucei* infections (178). Very few studies have been performed with TBDs (300). For example, laser microdissection coupled to scRNA seq (18) could be used in infected and control skin to localize the site of pathogen persistence and better appreciate the respective role of dermal adipocytes and hair follicle environment.

Tick-Borne Pathogen Immune Modulation of Vertebrate Host: Inoculation, Multiplication and Persistence

Tick-borne pathogen modulates host defenses in a manner very similar to, or complementary to, tick induced host immune modulation. The immune modulation is involved in different events of the vertebrate host infection: pathogen transmission and pathogen persistence.

Two tick-pathogen systems have been particularly explored: *Rickettsia-Dermacentor* et *Ixodes-Borrelia*. The **spotted fever group rickettsiae control host defenses, and its competent vector, *Dermacentor andersoni*,** also controls host immune defenses in a very similar manner to that of the rickettsiae. Basically, the tick vector and the pathogen complement each other in manipulating host defenses (125). Transmission, pathogenesis and evasion of host defenses by spotted fever group rickettsiae reviewed by Sahni et al. (125) noted that knowledge was incomplete relative to the influences exerted by the tick vector in transmission and establishment of infection by rickettsiae. *D. andersoni*, Rocky Mountain wood tick, is a competent vector of *R. rickettsii* (301). Ability of this tick to modulate host innate and adaptive immune defenses has been the subject of multiple studies (302–305). Immunomodulatory molecules contained in tick saliva are introduced into the host prior to transmission of rickettsiae, creating a cutaneous environment that is favorable for both blood feeding and pathogen transmission (305). The tick vector is attached to the host for 6 to 10 h prior to transmission of *R. rickettsii* (100).

Here, we examine potential synergies between host immune evasion induced by rickettsiae and that induced by *D. andersoni* feeding. Immune elements controlling rickettsial infection include: endothelial cells, macrophages, dendritic cells, NK cells, innate immune signaling pathways, proinflammatory cytokines,

chemokines, CD4+ T lymphocytes, CD8+ T lymphocytes, and antibodies (125). Macrophages and dendritic cells at tick feeding site are initial targets of infection with rickettsiae, and TNF-α and IFN-γ activated macrophages are effectors capable of clearing rickettsiae within these cells (125). Furthermore, endothelial cells activated by TNF-α and IFN-γ are induced to kill intracellular rickettsiae, and CD4+ T lymphocyte derived IFN-γ important in host protection against rickettsiae (125). These host defenses against rickettsiae are modulated by the tick vector. *D. andersoni* salivary gland extracts prepared throughout the course of engorgement suppressed macrophage production of TNF-α and IL-1 and T lymphocyte elaboration of IFN-γ and IL-2 (303).

Severity of disease correlated with whether rickettsiae can survive and proliferate in macrophage-like cells. During rickettsial infection, protective response consists of IL-1, IL-6, and IFN-γ accompanied by inflammatory infiltrates of neutrophils and macrophages (125). In addition to salivary gland extract suppression of macrophage IL-1 and T lymphocyte production of IFN-γ (303), *D. andersoni* infestation suppresses T lymphocyte expression of the integrins LFA-1 and VLA-4, important molecules in leukocyte adhesion to endothelium and movement to sites of inflammation (306) and significantly down regulated vascular endothelial cell expression of ICAM-1 (304). *D. andersoni* saliva proteome analysis identified cystatins and serpins that are putative inhibitors of inflammation (134).

D. andersoni nymph infestation induced host cutaneous responses were characterized using a combination of genome arrays and histopathology (134). During an initial nymphal infestation, there occurred a decrease in the number of up regulated host cutaneous genes between 12 and 48 h' post-infestation. This early primary infestation inhibition of transcription and RNA processing was consistent with an observed inhibition of inflammation. Histologic examination revealed that the number of inflammatory cells infiltrating the tick bite site did not increase from 12 to 48 h of a primary infestation. These changes were consistent with inhibition of inflammation during the period when rickettsiae would be transmitted from tick and infection established (134).

A second very well-analyzed tick-host-pathogen relationship is ***I. scapularis* and *I. ricinus* modulation of host defenses in the context of *B. burgdorferi*** sl transmission and infection. One *Ixodes* protein, Salp15 (Saliva protein 15 kDa) has been particularly explored since it targets different aspects of the host immunity. First, this tick protein is specifically upregulated within the *Ixodes* tick (307) where it binds to OspC (Outer surface protein C), a surface lipoprotein involved in the early transmission of *Borrelia* (308). Then, salp15 targets the different arms of the immune system: innate immunity (complement system and TLR2 receptor) (191, 309) and cellular immunity (the dendritic cells and the T cells) (310, 311).

Once the TBP has been inoculated, two scenarios occur. The vertebrate host possesses a potent immune system and neutralize the pathogens and antibodies are generated in absence of clinical manifestations. This explains the seroprevalence of people or animals regularly exposed to infected tick bites and who do not develop clinical manifestations (312, 313). In some vertebrate hosts, clinical manifestations appear few days or few weeks after the tick bite (107). Interestingly, in the mouse model of Lyme borreliosis, a peak of *Borrelia* multiplication appears 7 to 10 days after the inoculation of bacteria whatever the *Borrelia* species (12). It would be interesting to investigate the potential signification of this peak, perhaps related to generation of immune tolerance in the host skin. In animal models, induction of immune tolerance to pathogens seems to develop and pathogens persist in the skin in absence of antibiotics (314). This has been particularly well-documented for *Borrelia* in the mouse model. The pathogen is alive since it can be reactivated by application of topical corticosteroid that induces a local immunosuppression and local multiplication of *Borrelia* (314, 315). In experimental inoculation of luciferase positive-*Borrelia*, the bacteria can be visualized moving extracellularly in mouse skin for several months (M. Wooten—personal communication).

Since adipocytes have been described as a haven for *Plasmodium* (malaria), *Trypanosoma cruzi* (Chagas disease) and *T. brucei* (sleeping sickness) (15), it would be interesting to investigate whether TBP escape the immune control of the vertebrate host in adipose tissue. More interestingly, the hair follicle, which interacts with the adipose tissue, would be an immune privileged site to explore. It has been clearly shown that the hair follicle constitutes a site of persistence for *Plasmodium* (14). Induced immune tolerance is a result of both tick and pathogen manipulation of the host environment and results in successful establishment of infection. This would be another interesting topic to stimulate future research.

CONCLUSIONS

Vector-borne diseases have evolved toward a very complex, multifaceted network (**Figure 2**) (316). Initially described as a simple triad, vector-pathogen-vertebrate host, additional factors appear to regulate the network like the microbiome (2, 3) and non-coding RNAs (4, 317).

The vector by its innate immune system regulates the pathogen population to allow vector survival (19, 73). It also differentiates its microbiome from tick-borne pathogens (2). How this operates is still not clear. **The vertebrate host** can tolerate the tick-borne pathogen and becomes a reservoir (156). This specific relationship between the host and the pathogen is essential for enzoonotic cycle maintenance. Accidental host is more or less susceptible to the pathogen. He can be an immune tolerant host who develops an immune response with a positive serology or a host who presents clinical manifestations. These manifestations are particularly important in case of immunocompromised patients. The skin might play a key role in the process of tolerance as the first interface met by the tick and the pathogen (13, 190). By modulating the host immune system, the ticks prepares the skin to the pathogen inoculation by various sophisticated mechanisms targeting all the skin cells (97, 185) The numerous molecules contained in tick saliva facilitate the process (93, 97). Although in certain circumstances as repeated infestations (318), the tick can be rejected, most of the time it remains attached to the skin. **The pathogen** uses the tick to facilitate its transmission and then multiply and persist in the

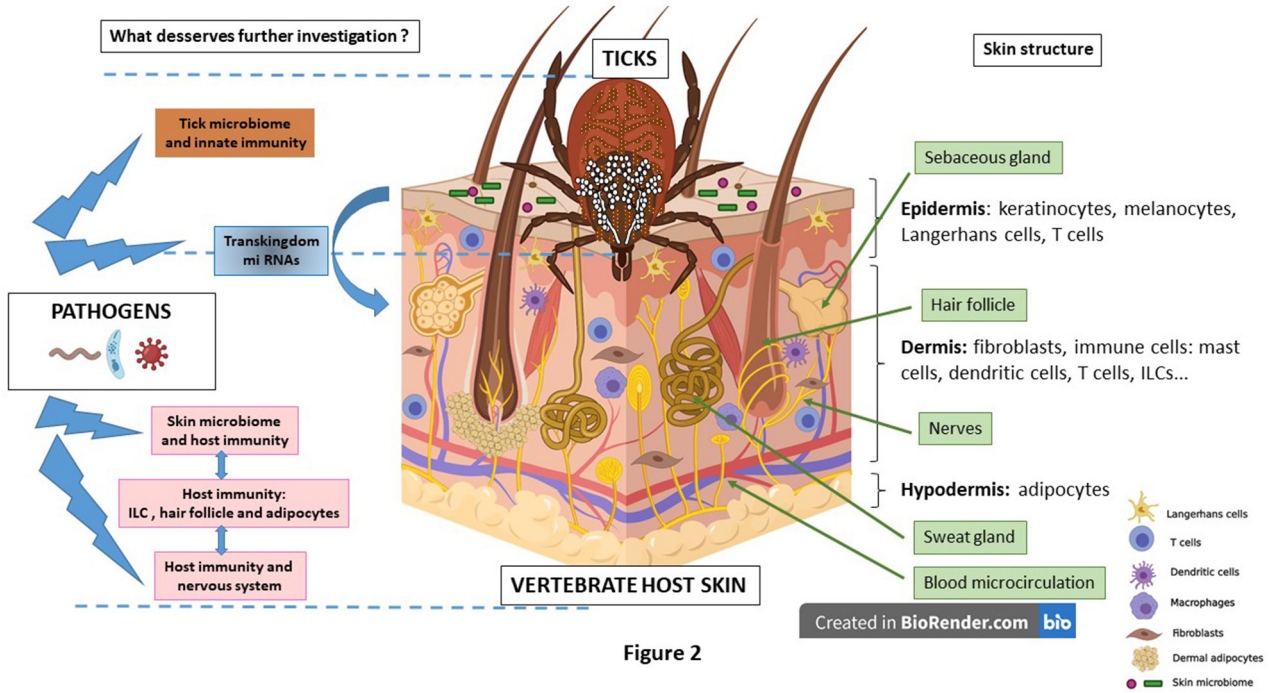

Figure 2

FIGURE 2 | The skin is structured into three interconnected layers. The epidermis, the uppermost layer, mainly composed of keratinocytes accompanied by immune cells such as Langerhans cells and T cells. On its surface, the skin microbiome (bacteria, yeasts and virus) plays a key role in skin homeostasis. The dermis is responsible for resistance and elasticity of the skin. Dermal resident cells are fibroblasts secreting extracellular matrix with numerous immune cells such as dendritic cells, T cells, mast cells, Innate Lymphoid cells (ILCs) and others. Different appendages are present: hair follicles surrounded by dermal adipocytes, sweat glands, microcirculation blood vessels, nerves and sebaceous glands. Then beneath the dermis is the hypodermis that contains numerous adipocytes. During the process of an ixodid, hard tick bite, a feeding pool develops around the tick mouthparts. Saliva is introduced into the bite site and modulates the local host immune response with the goal of avoiding tick rejection. The tick microbiome can be secreted into the skin as exosomes. Likely, transkingdom miRNAs participate in the regulation of the infection. The role of skin immunity and skin and tick microbiomes deserves to be better investigated during the process of pathogen inoculation, multiplication and persistence. Some immune privileged sites like the hair follicle and the adipocytes might help pathogens to better survive within the skin. Recently, the potential role of nerves in the skin immune regulation has been evoked. Created with BioRender.com.

skin. This has been particularly well-studied in Lyme disease (13), in mouse (314, 315) and in dog model (319). It will be very interesting to perform similar analyses in human. The exact process of tolerance at the skin interface is only starting to be defined. In VBDs, certain skin structures and cells seem to be involved in this process like adipocytes (15, 173) and the hair follicle (175).

To orchestrate these different interactions, the **microbiome** developed a sophisticated tuning. First, within the tick gut, it is the interaction of tick innate immunity with the tick microbiome, which contributes to determining the pathogenicity of the microorganisms. What makes one rickettsia a pathogen and the other one a symbiont? These practical questions remain to be answered. At the skin interface, the microbiome also contributes to regulation of inflammation and likely the host response to the tick bite. Due to the long blood feeding of a hard tick and the formation of a feeding pool, the skin microbiome of the vertebrate host enters the dermis. What is the role of the microbiota in the case of pathogens co-inoculated with tick saliva? Some preliminary data exists for certain VBDs like malaria (320) and leishmaniasis (275), but none for TBDs. In malaria, the skin microbiome is clearly involved in attractiveness of mosquito and the intestinal microbiome of the vertebrate host seems also to influence the outcome of the disease, at least in mouse model. Depletion of the bacteria from the phylum Firmicutes, mainly Gram (+) bacteria, is correlated with more severe disease (320).

Recently, **non-coding RNAs** (long non-coding RNAs and small non-coding RNAs: siRNAs, miRNAs, and piRNAs) have drawn much attention due to their diversity of function (4). It appears that small RNAs are particularly interesting because of their role in different regulation systems like innate immunity but also in the communication between host and pathogens, for example, from humans to malaria parasite and from *Escherichia coli* bacteria to *Caenorhabditis elegans* nematode (317). This phenomenon is referred to trans-kingdom silencing (317). We can then question at which level would they play a role in the different interactions occurring in TBDs and then whether they participate in the induction of tolerance. In tick saliva, several micro RNAs have been identified *in silico* that could target host genes, especially those related to inflammation (4). They also seem to be involved in the regulation of tick infection (150) and during the process of arbovirus transmission (149).

Our understanding of **skin immunity** has also made tremendous progress. The innate immunity with the engagement

of PAMs with PRRs leads to secretion of cytokines, chemokines and AMPs. They chemoattract different cells to the site of tick feeding and they also activate ILCs which in turn secrete cytokines and express cell receptors that activate T cells (180). A focus for future research is the connection of the skin immune system and the nervous system (196).

To explore this complex network, molecular techniques have been very helpful. Next generation sequencing of 16S ribosomal RNA gene allowed a better identification of microbiomes (3). We need now to validate many of these observations *in situ*, in animal models or even better in patients. We require new tools in addition to molecular techniques and traditional proteomics, like targeted quantitative proteomics (321–323). It has been tested successfully on skin biopsies of mouse and human infected by *B. burgdorferi* sl to detect markers of infection (315, 324). It might be used to identify tick saliva proteins inoculated into the host skin. Targeted proteomics will be also very useful to identify vaccine candidates (325). To develop effective vaccines against VBDs, after identification of good vaccine candidates, efficient delivery system will be necessary in the future to take into consideration the skin microbiome and the skin immunity (326).

Due to the complexity of the system, involving different expertise (immunology, entomology, ecology, human and veterinary medicines, etc.), there is a real need for multidisciplinary team to answer these different scientific questions (327, 328) and find new tools to control expanding TBDs (8, 98, 329, 330).

AUTHOR CONTRIBUTIONS

Both authors searched, read the literature, edited the manuscript, and wrote the manuscript.

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Arthropods Under Pressure: Stress Responses and Immunity at the Pathogen-Vector Interface

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Understanding what influences the ability of some arthropods to harbor and transmit pathogens may be key for controlling the spread of vector-borne diseases. Arthropod immunity has a central role in dictating vector competence for pathogen acquisition and transmission. Microbial infection elicits immune responses and imparts stress on the host by causing physical damage and nutrient deprivation, which triggers evolutionarily conserved stress response pathways aimed at restoring cellular homeostasis. Recent studies increasingly recognize that eukaryotic stress responses and innate immunity are closely intertwined. Herein, we describe two well-characterized and evolutionarily conserved mechanisms, the Unfolded Protein Response (UPR) and the Integrated Stress Response (ISR), and examine evidence that these stress responses impact immune signaling. We then describe how multiple pathogens, including vector-borne microbes, interface with stress responses in mammals. Owing to the well-conserved nature of the UPR and ISR, we speculate that similar mechanisms may be occurring in arthropod vectors and ultimately impacting vector competence. We conclude this Perspective by positing that novel insights into vector competence will emerge when considering that stress-signaling pathways may be influencing the arthropod immune network.

Keywords: vector-borne diseases, vector competence, vector-borne pathogens, arthropod immunity, eukaryotic stress response, integrated stress response, unfolded protein response

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INTRODUCTION

Among arthropods, the adaptation to blood-feeding is a life history trait that evolved independently at least 20 times (1). From the arthropod's perspective, a hematophageous lifestyle has both benefits and drawbacks. Blood is a good source of proteins and lipids, which are necessary for development and egg production (2–4). However, blood-feeding comes with a variety of risks and stressors (5) including long periods between nutrient supplementation (6–13), thermal stress associated with the influx of a hot blood meal (5, 14–16), heme toxicity (17–30) and excess amounts of ions and water (31). Cells respond to acute environmental changes by activating stress responses that temporarily increase tolerance limits in adverse conditions and/or eliminate stressful stimuli. Being able to

respond to stressful stimuli is an evolutionary advantage, which explains the highly conserved nature of cellular stress responses across eukaryotes (32–40).

For arthropods that transmit disease, another stressor is the presence of pathogens with incoming blood meals (41–44). Although vector-borne pathogens do not typically kill their arthropod vectors (45), infection does impart stress on the host by parasitizing nutrients, secreting toxic by-products and/or causing physical damage (46). For this reason, arthropod immune processes responding to infection are a key factor influencing vector competence (47–53). From mammals, it is now recognized that innate immunity is tightly intertwined with cellular stress responses and may represent an ancient mode of host defense against infection (32–34, 54–60). Whether stress-responses also intersect with arthropod immunity and how this may influence vector competence of blood-feeding arthropods is not known. Herein we briefly outline current knowledge of two well-characterized cellular stress response mechanisms, the Unfolded Protein Response (UPR) and the Integrated Stress Response (ISR) and discuss evidence that stress signaling impacts immunity. We then cite examples of cellular stress responses mediating outcomes at the host-pathogen interface in mammals and conclude that, given the well-conserved nature of the UPR and the ISR, similar crosstalk may be occurring in arthropods that would fundamentally impact vector competence.

ARTHROPOD INNATE IMMUNE SIGNALING

Arthropod innate immune pathways are best characterized in the model organism *Drosophila* which are briefly summarized owing to space constraints. The Toll pathway is generally characterized as responding to Gram-positive bacteria and fungi, resulting in activation of Rel-family transcription factors Dorsal and Dif (Dorsal-related immunity factor) (61–64). The IMD (immune deficiency) pathway is analogous to the tumor necrosis factor receptor (TNFR) pathway in mammals (65) and is initiated by Gram-negative bacteria, although exceptions outside of *Drosophila* have been observed (66–71). The Janus Kinase (JAK)-signal transducers and activators of transcription (STAT) pathway, first described in mammals, is activated in the presence of bacterial or protozoan pathogens as well as more than 35 ligands, including interferons (IFN) and interleukins (IL) (72, 73). In arthropods, the JAK-STAT pathway is induced by the endogenous cytokine Unpaired (Upd) (74). Owing to space constraints, we refer readers to excellent reviews that comprehensively cover arthropod Toll, IMD and JAK-STAT signaling (65, 75, 76).

THE UNFOLDED PROTEIN RESPONSE

The UPR is a cellular stress response mechanism that is highly conserved across species, from single-celled eukaryotes to mammals (**Figure 1**). The UPR is triggered when the endoplasmic reticulum (ER) is under stress, which can result from a variety of stimuli such as oxidative stress, changes in temperature or pH, lack of nutrients or infection (32–34, 77–84). Such conditions can impart stress when protein-folding requirements exceed the processing capacity of the ER, causing an accumulation of unfolded proteins in the ER lumen. The UPR is activated through any combination of 3 transmembrane receptors: PERK (PKR-like ER kinase), ATF6 (activating transcription factor 6) or IRE1α (inositol-requiring enzyme 1α). In a non-stressed state, the negative regulator, BiP (binding immunoglobulin protein; also known as GRP78), keeps all three receptors in an inactive state by binding to them. Upon activation, BiP disassociates from the receptors, thereby activating signaling. Disassociation allows IRE1α and PERK to oligomerize and autophophorylate (85–87), whereas ATF6 is released for migration to the Golgi (88, 89). If homeostasis cannot be restored, the UPR will switch from pro-survival to proapoptotic outcomes (90).

PERK is a type I transmembrane protein kinase that has dual roles in the UPR as well as the ISR (91). When activated, PERK dimerizes, autophosphorylates and then also phosphorylates the regulatory molecule, eIF2α (eukaryotic translation initiation factor 2α). Phosphorylated eIF2α promotes cell survival by temporarily inhibiting protein translation, which decreases the amount of proteins entering the ER and alleviates the demand for protein folding. While inhibiting the translation of most mRNAs, eIF2α selectively induces the expression of some proteins including ATF4 (activating transcription factor 4). ATF4 can activate transcription of the growth arrest and DNA damage-inducible protein, GADD34, which negatively regulates eIF2α phosphorylation, or CHOP (C/EBP homologous protein), which is a proapoptotic factor (85, 92, 93).

ATF6 is a type II transmembrane protein that contains a bZIP transcription factor within the cytosolic domain. Once BiP disassociates from ATF6, it is transported to the Golgi compartment by COPII-containing vesicles (33, 94, 95). ATF6 is proteolytically processed by the Golgi-resident proteases S1P and S2P (site-1/2 proteases), which cleave the amino-terminal portion and allow the bZIP transcription factor to translocate to the nucleus (81, 94, 96). ATF6 upregulates the expression of GRP94 (endoplasmin) to increase the ER's folding capacity and p58IPK to induce the ER associated degradation pathway (ERAD). ATF6 also induces the expression of other UPR-associated proteins including BiP and XBP1 (X-box binding protein) (32, 60, 97, 98).

IRE1α is a type I transmembrane protein with a cytosolic serine/threonine kinase domain and an RNase (ribonuclease) domain. When the ER is stressed, IRE1α autophosphorylates and splices the inactive mRNA $xbp1^U$ into $xbp1^S$, which is then translated into a protein (33, 81, 99, 100). XBP1 translocates to the nucleus where it induces genes that are involved in lipid biosynthesis, ERAD and chaperone production (100, 101). IRE1α signaling also limits the amount of new proteins entering the ER through regulated IRE1α-dependent decay (RIDD), which degrades mRNA (102). With high levels of ER stress, IRE1α recruits the adaptor protein TRAF2 (TNF receptor-

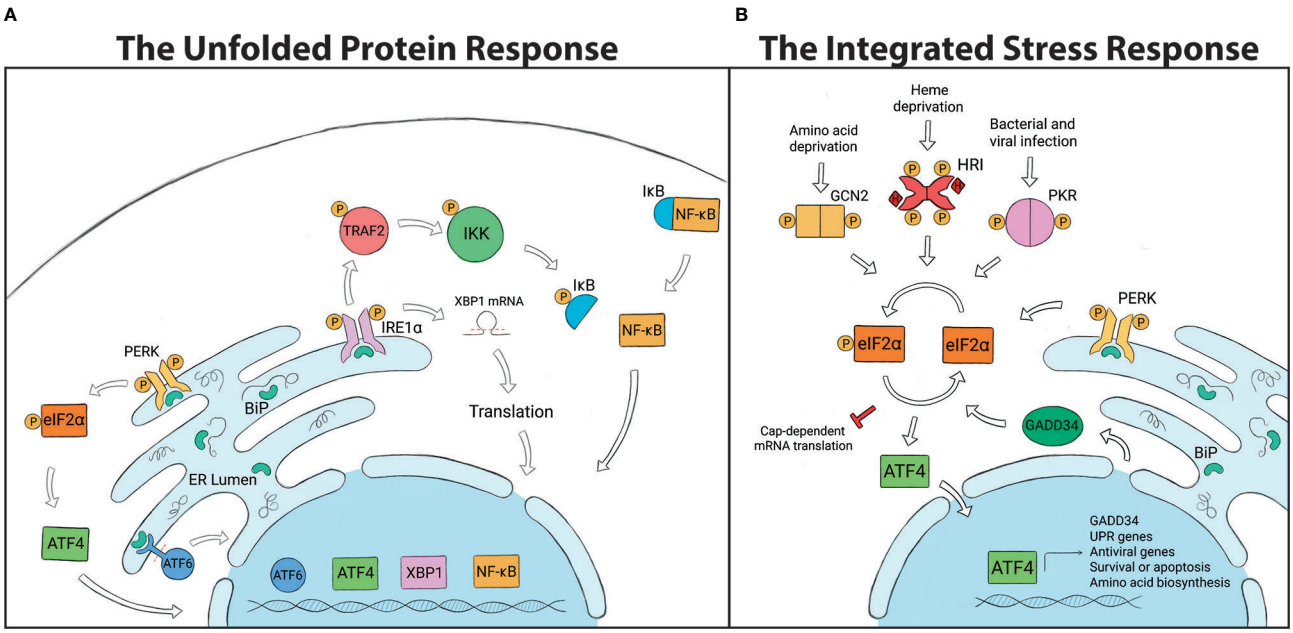

FIGURE 1 | **(A)** The Unfolded Protein Response (UPR) is regulated by three transmembrane endoplasmic reticulum (ER) receptors: PERK, ATF6 and IRE1α. BiP binds to and holds the receptors in an inactive state under non-stressed conditions. The accumulation of unfolded proteins in the ER leads to the disassociation of BiP from the receptors and UPR activation. PERK phosphorylates eIF2α and induces ATF4, which controls transcription of *chop* and *gadd34*. ATF6 is cleaved and translocated to the nucleus to regulate expression of GRP94, p58IPK, and UPR-associated proteins. IRE1α splices *xbp1*, which is translated into a protein that controls genes involved in lipid biosynthesis, ER associated degradation pathway (ERAD) and chaperone production. IRE1α can also recruit TRAF2, which leads to NF-κB, ASK1 and JNK signaling. **(B)** The Integrated Stress Response (ISR) is initiated by stress-specific stimuli that activate kinases GCN2, HRI, PKR and PERK, which all converge on the regulatory molecule eIF2α. Phosphorylation of eIF2α halts global protein translation and upregulates the transcription factor ATF4, which determines cell fate. To terminate the ISR, ATF4 induces GADD34 expression which dephosphorylates eIF2α and allows global protein translation to resume.

associated factor 2) a component of the TNFR pathway. IRE1α-TRAF2 association recruits the IKK complex, leading to NF-κB activation and proinflammatory responses (33, 103, 104). Alternatively, IRE1α-mediated signaling through TRAF2 can also lead to ASK1 (apoptosis signal regulating kinase 1; also known as mitogen-activated protein kinase 5, MAP3K5) activation and downstream JNK (JUN N-terminal kinase) signaling to induce apoptotic outcomes (33, 100, 104, 105). Less is known about UPR mechanisms in arthropods, but genome comparisons indicate that UPR-encoding genes are well-conserved between species, which may suggest similar mechanisms of action (**Table 1**).

THE INTEGRATED STRESS RESPONSE

The ISR is responsible for alleviating cellular stress and restoring homeostasis in eukaryotes (93, 106–109) (**Figure 1**). In mammalian cells, the ISR can be activated by one of four stress-sensing kinases: PKR (protein kinase double-stranded RNA-dependent), GCN2 (general control nonderepressible 2), HRI (heme-regulated inhibitor) and PERK (110, 111). These serine-threonine kinases are stimulated by pathological and physiological changes in the cellular environment. GCN2, a highly conserved cytoplasmic kinase, is stimulated by UV irradiation and nutrient deprivation (e.g. amino acid, glucose) (107, 108, 112). PERK is stimulated when misfolded proteins accumulate in the ER, causing ER stress (91, 113, 114). PKR is activated primarily in response to viral infections, as well as bacterial infections and oxidative stress (109, 115–117). Unlike the other kinases, HRI is mostly expressed in erythrocytes and acts as a heme sensor that is activated by iron deficiency (118).

All four stress-sensing kinases converge on a common regulatory factor: the phosphorylation of eIF2α (106, 110). Under non-stressed conditions, protein translation is initiated when the eIF2 complex (consisting of eIF2α, eIF2β and eIF2γ) binds with GTP and Met-tRNA (initiator methionyl tRNA). The ternary complex then associates with the 40S ribosome subunit to form the 43S pre-initiation complex that binds to the 5' end of mRNA and scans for start codons. Upon recognition, eIF2-GTP (active state) is hydrolyzed to eIF2-GDP (inactive state) and causes a conformational change to the pre-initiation complex, halting the mRNA scanning process and allowing protein translation to begin (119–123). eIF2B, a guanine nucleotide exchange factor, is essential for recycling GDP to GTP for new rounds of protein translation. Phosphorylated eIF2α attenuates protein synthesis owing to its increased affinity for eIF2B that prevents eIF2-GDP to eIF2-GTP exchange (110, 121–123).

As previously discussed in reference to PERK signaling, ATF4 is activated downstream of eIF2α phosphorylation and can act as

TABLE 1 | Distribution of Unfolded Protein Response (UPR) and Integrated Stress Response (ISR) genes across arthropod vectors.

Common Name	Genus	UPR and ISR genes									
		BiP	ATF6	IRE1α	XBP1	ATF4	eIF2α	PERK	GCN2	PKR	HRI
Fruit Flies	*Drosophila*	+	+	+	+	+	+	+	+	–	–
Mosquitoes	*Culex*	+	+	+	+	+	+	+	+	–	+
	Aedes	+	+	+	+	+	+	+	+	–	+
	Anopheles	+	+	+	+	+	+	+	+	–	+
Fleas	*Xenopsylla**	+	+	+	+	+	+	+	+	–	–
	Ctenocephalides	+	+	+	+	+	+	+	+	–	+
Lice	*Pediculus*	+	+	+	+	–	+	+	+	–	+
Triatome bugs	*Triatoma**	+	+	+	+	+	+	+	+	–	+
Ticks	*Ixodes*	+	+	+	+	+	+	+	+	–	+
	*Dermacentor**	+	+	+	+	–	+	+	+	–	+
	*Ornithodoros**	+	+	+	+	+	+	+	+	–	+
Mites	*Leptotrombidium*	+	–	+	+	–	+	+	+	–	+

NCBI's Basic Local Alignment Search Tool (BLAST) was used with query sequences from Homo sapiens to identify putative homologs. (+) homologs identified (–), homologous gene targets not found.

**homologs found in vector transcriptomes.*

both a transcriptional activator and repressor of genes important for determining cell fate, including *chop* and *gadd34* (124–126). In response to prolonged ER-stress, ATF4 interacts with CHOP to generate reactive oxygen species (ROS), a key signal for mediating cell death (125). Once cellular stress is resolved, ATF4 induces GADD34 which interacts with protein phosphatase 1 (PP1) to dephosphorylate eIF2α and terminate the ISR (126).

The ISR has known roles in regulating host immunity. In response to ISR activation, cells will form cytoplasmic aggregates of untranslated mRNA and proteins termed "stress granules" that influence immune signaling. Phosphorylated eIF2α induces stress granules, which become cell signaling hubs that can intercept molecules from other pathways to modulate processes such as immunity (35, 127, 128). For example, TRAF2 is sequestered into stress granules, which ultimately suppresses NF-κB-mediated inflammation (127). The ISR can also act as an antiviral defense mechanism (129–131). A stress granule-nucleating protein G3BP1 (Ras-GTPase-activating protein SH3 domain binding protein 1) recruits and activates PKR to suppress viral protein synthesis. It also activates innate immune responses through NF-κB and JNK pathways and promotes the expression of proinflammatory cytokines such as IL-17 (117). The arthropod ISR is less-studied when compared to mammals, but comparative genomic analyses demonstrate that many ISR genes are well-conserved in arthropod vectors (**Table 1**).

INTERSPECIES INTERACTIONS

UPR-Pathogen Interface

The UPR is increasingly implicated in host defense against infection (132–137). For example, mammalian macrophages limit methicillin resistant *Staphylococcus aureus* (MRSA) through IRE1α with sustained ROS production and concentrated delivery of ROS to bacteria-containing phagosomes (138). The foodborne pathogen *Camplyobacter jejuni* activates eIF2α and CHOP, while decreasing expression of *perk*, *ire1α* and *atf6* in human intestinal epithelial cells. Pharmacologically inducing the UPR prior to infection resulted in decreased *C. jejuni* invasion (139), highlighting the UPR as a host defense strategy.

Some pathogens manipulate or selectively induce the UPR to promote growth and survival. *Legionella pneumophila*, causative agent of Legionnaires' disease, inhibits UPR-induced apoptosis with secreted effector proteins Lgt1 and Lgt2 that block IRE1α-mediated $xbp1^{U}$ splicing (140, 141). The intracellular pathogen *Brucella* manipulates the UPR in mammalian cells with secreted effectors TcpB and VceC (142–144), which induce IRE1α signaling and NF-κB to potentiate pro-inflammatory responses (81, 144–147). This effector-mediated manipulation appears to benefit the bacterium, with VceC providing an advantage for long-term *in vivo* colonization (144).

Vector-borne pathogens are also reported to cause ER stress and UPR activation in mammalian hosts. *Francisella tularensis*, causative agent of Tularemia vectored by ticks (*Dermacentor* spp. and *Amblyomma americanum*) and biting insects (148–151), alters the expression of *bip*, increases IRE1α phosphorylation and ATF6 activation, but decreases PERK phosphorylation and CHOP (152). IRE1α-XBP1 signaling is reported to limit *F. tularensis in vivo* with increased pathogen burdens in the liver, spleen and lungs of $xbp1^{-/-}$ mice (153). *Orientia tsutsugamushi*, the causative agent of Scrub Typhus transmitted by trombiculid mites (chiggers), induces the UPR and ERAD in HeLa cells to benefit bacterial growth, owing to the increase in available amino acids (154). The *Ixodes scapularis*-transmitted bacterium *Anaplasma phagocytophilum* induces all the three UPR branches in THP-1 cells (155).

Due to their very nature, viral replication requires host cells for protein production, which often engages the UPR. For example, herpesviruses activate one or more of the UPR receptors, but limit downstream signaling to ensure global protein translation, including viral proteins, is not halted (156–158). Many arthropod-transmitted viruses activate one or more UPR sensors in mammalian cells as well. Bluetongue virus, transmitted by *Culicoides* spp. (biting midges), induces autophagy to benefit viral replication by activating PERK-eIF2α signaling of the UPR (159). Mosquito-transmitted viruses Chikungunya (CHIKV), Dengue (DENV) and West Nile (WNV) all activate one or more UPR sensors in

mammalian cells. CHIKV activates BiP and the ATF6 and IRE1α branches of the UPR, but blocks PERK signaling by suppressing eIF2α phosphorylation through the nonstructural viral protein nsP4 (160, 161). The flaviviruses DENV and WNV induce $xbp1^{U}$ splicing, ATF6 proteolysis, and eIF2α phosphorylation to benefit viral propagation in mammalian cells and, in the case of WNV, to inhibit type I IFN signaling (161–164). Tick-borne encephalitis virus (TBEV) and Langat virus (LGTV) are Ixodid-transmitted flaviviruses. TBEV infection activates the IRE1α and ATF6 pathways to facilitate viral replication (165). In contrast, while LGTV infection activates the UPR, PERK signaling restricts viral load (166), highlighting the importance of the UPR as a host defense mechanism.

Much less is known about the UPR-pathogen interface in arthropod vectors. Recent work shows differential regulation of ER-resident proteins involved in ERAD in *Borrelia burgdorferi*-infected adult *I. scapularis* (167). There is also evidence that arthropod-transmitted plant pathogens manipulate the UPR in their arthropod vectors. *Candidatus* Liberibacter asiaticus (CLas), causative agent of Asian citrus greening disease, is vectored by the Asian citrus psyllid *Diaphorina citri* Kuwayama (*D. citri*). Infection of *D. citri* with CLas upregulates expression of ERAD and UPR components. A related bacterium, *Candidatus* Liberibacter solanacerarum, also upregulates *ire1α* and multiple genes involved in ERAD in its arthropod vector, the potato psyllid (*Bactericera cockerelli*) (168). Although more work is required to understand what role the UPR and ERAD-mediated protein degradation have during arthropod infection, these studies demonstrate that vector-borne pathogens are interfacing with the UPR in arthropods.

ISR-Pathogen Interface

The ISR broadly responds to a variety of stress-inducing stimuli, including invasion and damage caused by infection. *Listeria monocytogenes*-infected mammalian cells activate PERK, ATF4, eIF2α, PKR and induce the expression of CHOP. *L. monocytogenes* secretes listeriolysin O (LLO), which is a pore-forming hemolysin required for phagosomal escape and bacterial survival. Cells treated with *L. monocytogenes* LLO resulted in a similar ISR activation phenotype as infection, indicating that this effector is partially responsible for inducing the ISR. Downstream production of type I IFN activates the ISR kinase PKR, increasing its expression and activation, and further stimulating eIF2α signaling (169). Epithelial cell infection with *Shigella flexneri* disrupts host cell membranes, causing GCN2-mediated eIF2α phosphorylation. This halts global protein translation, leading to stress granule formation and autophagy that eliminates bacteria (170).

These examples reflect the role of the ISR as a host-defense strategy against infection. In response, many pathogens have evolved methods to counteract ISR-mediated defenses. For example, the causative agent of Q fever, *Coxiella burnetii*, increases eIF2α phosphorylation in a Type IV Secretion System-dependent manner and induces ATF4 and CHOP in human macrophages. However, nuclear translocation of CHOP is blocked by *C. burnetii* to prevent ER stress-induced apoptosis (171). Coronavirus protein AcP10 and picornavirus protein AiVL promote viral protein synthesis by acting as competitive inhibitors for phosphorylated-eIF2 and eIF2B interactions (172). Excellent reviews summarizing ISR-mediated antiviral responses, including stress granules, and concurrent viral evasion strategies have been published in the past several years which readers are referred to (129, 173).

Interactions between vector-borne pathogens and ISR mechanisms have been reported, with several focusing on insect-borne viruses. In mammalian cells, the sandfly fever Sicilian phlebovirus evades PKR defense mechanisms by expressing a nonstructural protein that binds to eIF2B, blocking translation inhibition and promoting viral replication (174). Rift valley fever virus, transmitted by mosquitoes and sandflies, degrades host PKR and inhibits IFN induction (175–177). WNV inhibits PKR activation and downstream phosphorylation of eIF2α and stress granule formation (178). Zika virus likewise has evolved to evade stress granule formation in host cells by repurposing host proteins, including G3BP1, to facilitate viral replication and repress normal stress granule assembly (179, 180). Other flaviviruses such as WNV, DENV and Japanese encephalitis virus hijack or further inhibit stress granule machinery to benefit replication (181–184). These studies illustrate that vectored pathogens evade ISR signaling to facilitate replication and survival.

CONCLUDING REMARKS

Vector-borne pathogens selectively interface with the UPR and the ISR to promote survival and infection in mammals. Given the well-conserved nature of both the UPR and the ISR between evolutionarily distant species (**Table 1**), it is reasonable to speculate that vectored microbes may also be modulating the stress responses in their arthropod vectors. Moreover, this type of manipulation may be a common survival strategy used by vector-borne pathogens to suppress host defenses and create replicative niches.

Cellular stress responses are increasingly recognized as being closely intertwined with innate immunity (32–34, 54–60). For example, there are multiple reports that mammalian Toll-like receptors (TLRs) influence the UPR and the ISR. TLR2 and TLR4 in mammalian macrophages activate the IRE1α-XBP1 axis, which leads to proinflammatory TNFα and IL-6 cytokine production (153). MRSA induces $xbp1^{U}$ splicing in wildtype bone marrow-derived macrophages, but not in $TLR2/4/9^{-/-}$ or $myd88^{-/-}$ mutant cells, indicating that TLR signaling is required for IRE1α activation (138). The ISR kinase PKR is activated downstream from TLR3 and TLR4, which induces type I IFN (185, 186). Whether a similar phenomenon occurs in arthropods is not known, but considering the well-conserved nature of the Toll pathway between arthropods and mammals (70, 187) it is possible that this type of crosstalk is occurring across species.

Beyond TLR signaling, overlap between the UPR and ISR with other innate immunity components has also been noted. During infection, the mammalian UPR is capable of initiating an immune response by crosstalking with the TNFR pathway (33, 56–58, 188–190). IRE1α can produce proinflammatory

responses by signaling through TRAF2, a component of the TNFR pathway, recruiting the IKK complex for phosphorylation and releasing NF-κB for nuclear translocation (32–34). Arthropod immunity may similarly be influenced by the UPR, as the arthropod IMD pathway is analogous to the mammalian TNFR network. PERK was shown to interact with JAK-STAT signaling in mammalian glial cells (191) and several examples of IFN production being influenced by the ISR and UPR have been reported, which could potentially influence JAK-STAT signaling in an indirect manner (169, 175–177, 185, 186, 192). Similar crosstalk between JAK-STAT and cellular stress responses may also be occurring in arthropod vectors.

Since one of the major factors determining vector competence is arthropod immunity (47–53), it is feasible that cellular stress responses may be influencing vector competence. With this in mind, understanding how stress responses may interface with arthropod immunity and conversely how vector-borne pathogens may be inducing or manipulating cellular stress responses could be important for opening new avenues in vector-borne disease control. This knowledge could be leveraged for the future design of disease transmission-blocking strategies to reduce the global burden of vector-borne diseases.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found on NCBI.

AUTHOR CONTRIBUTIONS

KR, LS-L, JH, and DS wrote this perspective. EF designed and constructed **Figure 1** and contributed to intellectual discussions. All authors contributed to the article and approved the submitted version.

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Tick-Tattoo: DNA Vaccination Against *B. burgdorferi* or *Ixodes scapularis* Tick Proteins

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Introduction: *Borrelia burgdorferi* sensu lato (sl) is the causative agent of Lyme borreliosis. Currently there is no human vaccine against Lyme borreliosis, and most research focuses on recombinant protein vaccines. DNA tattoo vaccination with *B. afzelii* strain PKo OspC in mice has proven to be fully protective against *B. afzelii* syringe challenge and induces a favorable humoral immunity compared to recombinant protein vaccination. Alternatively, several recombinant protein vaccines based on tick proteins have shown promising effect in tick-bite infection models. In this study, we evaluated the efficacy of DNA vaccines against *Borrelia* OspC or tick antigens in a tick-bite infection model.

Method: We vaccinated C3H/HeN mice with OspC using a codon-optimized DNA vaccine or with recombinant protein. We challenged these mice with *B. burgdorferi* sensu stricto (ss)-infected *Ixodes scapularis* nymphs. Subsequently, we vaccinated C3H/HeN mice with DNA vaccines coding for tick proteins for which recombinant protein vaccines have previously resulted in interference with tick feeding and/or *Borrelia* transmission: Salp15, tHRF, TSLPI, and Tix-5. These mice were also challenged with *B. burgdorferi* ss infected *Ixodes scapularis* nymphs.

Results: DNA tattoo and recombinant OspC vaccination both induced total IgG responses. *Borrelia* cultures and DNA loads of skin and bladder remained negative in the mice vaccinated with OspC DNA vaccination, except for one culture. DNA vaccines against tick antigens Salp15 and Tix-5 induced IgG responses, while those against tHRF and TSLPI barely induced any IgG response. In addition, *Borrelia* cultures, and DNA loads from mice tattooed with DNA vaccines against tick proteins TSLPI, Salp15, tHRF, and Tix-5 were all positive.

Conclusion: A DNA tattoo vaccine against OspC induced high specific IgG titers and provided near total protection against *B. burgdorferi* ss infection by tick challenge. In contrast, DNA tattoo vaccines against tick proteins TSLPI, Salp15, tHRF, and Tix-5 induced low to moderate IgG titers and did not provide protection. Therefore, DNA tattoo

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vaccination does not seem a suitable vaccine strategy to identify, or screen for, tick antigens for anti-tick vaccines. However, DNA tattoo vaccination is a straightforward and effective vaccination platform to assess novel *B. burgdorferi* sl antigen candidates in a relevant tick challenge model.

Keywords: lyme disease, *borrelia*, DNA tattoo, DNA vaccination, OspC, tick proteins

INTRODUCTION

Lyme borreliosis is the most common vector-borne disease in the Northern hemisphere and is caused by the spirochete *Borrelia burgdorferi* sl, which is transmitted by *Ixodes* ticks. Vaccination would be an effective way to prevent Lyme disease. Currently there is no human vaccine available. Vaccines to prevent *Borrelia burgdorferi* sl infection could work in two ways: killing the pathogen to stop infection or targeting the vector to prevent successful transmission. Research therefore focuses on either protective antigens derived from the pathogen, *B. burgdorferi* sl, or from the vector, *Ixodes* ticks (1). When focusing on possible protective antigens from *Borrelia*, the most promising candidates in human vaccine studies are the outer surface proteins. Especially OspA, which is primarily expressed by *Borrelia* in unfed ticks, has been widely studied and was the primary component of the withdrawn human LYMErix™ vaccine (2–6). During transmission from tick to host, the *Borrelia* spirochete downregulates OspA and upregulates outer surface protein C, which is necessary to facilitate migration to the tick salivary glands and also plays a role in spirochete infection of the mammalian host. OspC was also shown to be an effective vaccine target, but has a high heterogenicity among different *B. burgdorferi* sl species and strains (7, 8).

In an alternative approach where the tick vector is targeted, tick saliva could play a pivotal role. Tick saliva contains several proteins that facilitate transmission and survival of tick-borne pathogens by using anti-inflammatory, anti-coagulant and immunosuppressive abilities (9, 10). *Borrelia burgdorferi* sl exploits tick salivary gland proteins to facilitate their transmission from tick to host, and vice versa to increase their chances of survival within the tick (11, 12). For example, OspC binds to *Ixodes scapularis* salivary protein Salp15 which protects the spirochete from antibody-mediated killing (12–14). In addition, Salp15 also has immunosuppressive properties in inhibiting $CD4^+$ T cell and dendritic cell activation (15, 16). Interestingly, a vaccine directed against Salp15 has been shown to partially block *B. burgdorferi* ss infection (14, 17). Dai et al. also characterized tick histamine release factor, present in tick saliva and important to tick feeding (18). They showed significantly impaired tick feeding on mice when tHRF was silenced by RNA interference. Tick feeding and transmission of *B. burgdorferi* ss was also significantly diminished in tHRF immunized mice (18). Schuijt et al. identified Tick Salivary Lectin Pathway Inhibitor (TSLPI), an *I. scapularis* salivary protein, which was shown to impair complement-mediated killing of *B. burgdorferi*. *B. burgdorferi* transmission was impaired in mice that were injected with TSLPI rabbit antiserum (19). TIX-5 (tick inhibitor of factor Xa toward factor V) is another tick protein, with anticoagulant activity, that has been investigated in vaccination studies (20, 21). Adult *I. scapularis* tick engorgement weights from rTIX-5–immune rabbits were dramatically reduced compared to control rabbits. The effect on *B. burgdorferi* ss transmission has not been assessed. Thus, multiple promising tick salivary gland proteins have been identified and investigated as vaccine candidates to prevent tick feeding and/or transmission of *B. burgdorferi* sl from tick to host. It has been described in literature that these salivary gland antigens are expressed in infected ticks; Salp15 and TSLPI are even upregulated in infected ticks (13, 19). In addition, transcriptomic data from *B. afzelii* infected *I. ricinus* nymphs show that all these antigens are also expressed in infected *I. ricinus* salivary glands, indicating that the expression of these specific antigens appears particularly conserved even cross-species (22).

In addition to *B. burgdorferi* sl, *Ixodes* ticks also transmit other tick-borne diseases that can cause human infection such as Babesiosis and Anaplasmosis and several Flaviviruses. An anti-tick vaccine that would prevent the tick from feeding on a host could have the advantage of being able to provide protection against multiple tick-borne diseases (23).

Most research on new Lyme vaccines focuses on recombinant proteins, but DNA vaccination constitutes an alternative vaccination platform (24). DNA vaccines are easy to produce, highly stable and induce both humoral and cellular immune responses (25). While no human genomic vaccines targeting infectious diseases are currently on the market, the COVID pandemic might establish its mainstream acceptance in infectious diseases, as several genomic vaccines are currently being developed (26–28). A previous study by Wagemakers et al. has shown that DNA vaccination by tattoo with OspC from *B. afzelii* strain PKo as a model antigen was fully protective against *B. afzelii* syringe challenge in mice and induced favorable humoral immune responses compared to recombinant protein vaccination (29). In the current study, we have used OspC from *B. burgdorferi* ss strain N40, both as recombinant as well as DNA vaccine to evaluate whether DNA vaccination can also protect against *Borrelia* infection through tick challenge, more closely resembling the natural situation. The other goal of this study was to assess DNA vaccination as a modality to induce protective immune responses against tick antigens. We assessed tick salivary gland proteins TSLPI, Salp15, tHRF, and TIX-5 to test DNA vaccination as an easy screening vaccination platform for novel future candidates as an anti-tick vaccine. These described tick antigens were selected since they are known to be able to

interfere with tick feeding and/or *B. burgdorferi* transmission when investigated in conventional vaccination approaches.

MATERIALS AND METHODS

Ethics Statement

All experiments were reviewed and approved by the Animal Research Ethics Committee of the Academic Medical Center, Amsterdam, The Netherlands (protocol 208AI). Experiments have been conducted according to European and national guidelines.

Recombinant OspC Protein Generation

The OspC gene was amplified from genomic DNA from *Borrelia burgdorferi* ss strain N40 DNA and was cloned into pET-21b (Invitrogen), produced in *E. coli* and purified using Ni-NTA as detailed elsewhere (12).

Purity was checked using SDS-PAGE, and protein concentrations were measured using a Bradford assay.

Generation of DNA Vaccines

The DNA vaccines were designed as described before in Wagemakers et al. (29). From *the B. burgdorferi* N40 OspC gene sequence (NCBI reference DQ437463.1 and the respective tick salivary gland genes Salp15 (NCBI reference AAK97817.1), tHRF (NCBI reference DQ066335), TSLPI (NCBI reference AEE89466.1), and TIX-5 (NCBI reference AEE89467). The signal peptide (predicted by SignalP 4.0 web-based software, CBS, Lyngby, Denmark) was replaced with the human tissue plasminogen activator (hTPA) signal sequence (genbank AAA61213.1) (30). The resulting sequence was codon-optimized to mouse tRNA usage with Java Codon Adaptation tool (Braunschweig, Germany) (31). At the 5′ end a BamH1 and a Kozak sequence were added, and at the 3′ end a sequence encoding a double stop codon and a Xho1 were added. The insert was synthesized (BaseClear, Leiden, The Netherlands) and ligated into a BamH1/Xho1 restricted empty pVAX vector (Invitrogen, Carlsbad, CA, USA). The plasmid was amplified using a Nucleobond Xtra EF kit (Macherey-Nagel, Düren, Germany) and resuspended in DNase free water.

Generation of I. scapularis Nymphs with *B. burgdorferi* Strain N40

Low passage *B. burgdorferi* ss strain N40 spirochetes were cultured in MKP medium and counted by using a Petroff-Hausser counting chamber and dark-field microscopy. $1x10^6$ spirochetes in 200 µl was injected subcutaneously between the shoulders of four six-to-eight-weeks-old female C3H/HeN mice, purchased from Charles River. Mice were checked for *Borrelia* infection positivity by qPCR after 14 days. Once infection was confirmed, approximately 500 *I. scapularis* larvae (kindly provided by Center for Disease Control and Prevention, BEI Resources, NIAID, NIH: *Ixodes scapularis* (Live), NR-44116) were placed on each mouse. In the following 6 days, the fully fed larvae that had fallen off the mice were collected and were allowed to mold into the nymphal stage during the next 6–8 weeks. Ticks were housed in an incubator (Panasonic) at room temperature and at a constant relative humidity of 90%. Once molted, nymphal infection rates were assessed by qPCR. To establish tick infection rate, DNA was extracted from 10 ticks using the Qiagen Blood and Tissue kit (Qiagen, Venlo, The Netherlands). Quantitative (q)PCR was used to quantify *B. burgdorferi* ss DNA in mouse tissues and was performed according to previously described protocol (29) and also in the section below "Borrelia detection and quantification by culture and qPCR". Infection rate was > 90%.

Vaccination Experiments

Six-to-eight-weeks-old female C3H/HeN mice were purchased from Charles River. The vaccination experiment was carried out as previously described (29). Eight mice per experimental group were vaccinated at t=0, t=14, and t=28 days and sera were collected at each time point. For the recombinant OspC vaccine 10 µg protein was emulsified with complete Freund's adjuvant at t=0 and 5 µg in incomplete Freund's adjuvant at t=14 and t=28 days. All vaccinations were administered subcutaneously. For the DNA vaccines and the negative control, hair was removed from the mouse abdomen using hair removal cream. Using a Cheyenne Hawk tattoo machine carrying a Cheyenne 13-magnum tattoo needle (both MT.DERM, Berlin, Germany) 20 µg of the DNA vaccines was tattooed 0.5–1mm into the abdominal skin of the mice for 45 s at 100 Hz under isofluorane anesthesia. Two weeks after the third vaccination, at t=42, all mice were challenged with seven *Ixodes scapularis* nymphs, infected with *B. burgdorferi ss* strain N40. To determine tick attachment time and tick weights the *B. burgdorferi* ss strain N40-infected *I. scapularis* nymphs were placed in capsules on the vaccinated mice and allowed to feed to repletion. The nymphs were checked daily for attachment, collected and weighed when they had fallen off. Additional sera were collected at t=42 (pre-challenge) and at t=63 days mice were sacrificed and ear, skin, ankle, heart, bladder, and tissue was collected for analysis.

ELISA

To measure antigen-specific IgG, ELISAs were performed, as described previously (29). High-binding 96-well ELISA plates (Greiner Bio-one, Kremsmünster, Austria) were coated overnight at 4°C with 1 µg/ml recombinant protein (produced as described elsewhere (12, 18, 19, 21)), washed with PBS–Tween (phosphate-buffered saline–0.05% Tween) and incubated with blocking buffer (1% BSA in PBS, pH 6.5) for 2 h at room temperature. Mouse sera (collected at day 42 before tick challenge) were diluted in blocking buffer, added to the wells and incubated for 1 h at room temperature. Plates were washed and incubated for 1 h with horseradish peroxidase (HRP)-linked anti-mouse IgG (Cell Signaling, Beverly, MA, USA) diluted 1:1,000 in blocking buffer. The plates were washed again and developed using TMB substrate [50 µl TMB chromogene in 5 ml TMB substrate buffer (8,2 gr NaAc and 21 gr citric acid monohydrate dissolved in 1 liter H_2O + 10 µl 3% H_2O_2, pH

5)] and optical density was measured in a Biotek (Winooski, VT, USA) ELISA plate reader at 450–655 nm.

Borrelia Detection and Quantification by Culture and qPCR

Cultures were carried out as described elsewhere (12). Murine bladder and skin samples were cultured in modified Kelly Pettenkofer (MKP) medium with rifampicin, 50 µg/ml and phosphomycin, 100 µg/ml) at 33°C. The cultures were checked weekly (for a total of 8 weeks) for the presence of motile spirochetes with dark field microscopy as described before (12). For all samples DNA was extracted using Qiagen Blood and Tissue kit (Qiagen, Venlo, The Netherlands). Quantitative (q)PCR was used to quantify *B. burgdorferi* ss DNA in mouse tissues and was performed according to previously described protocol (29). OspA primers were used for quantification; forward 5'-AAAAATATTTATTGGGAATAGGTCT-3' and reverse 5'-CACCAGGCAAATCTACTGAA-3', mouse Beta-actin forward 5'-AGCGGGAAATCGTGCGTG-3' and reverse primer 5'-CAGGGTACATGGTGGTGCC-3' were used for normalization. The qPCRs were performed on the LightCycler480 (Roche, Nutley, NJ, USA) using SYBR green dye (Roche) using the following PCR protocol: 95°C 6 min, and 60 cycles of 95°C 10 s, 60°C 20 s, and 72°C 20 s. Reactions were performed in triplicate. Results were analyzed using LinRegPCR software (Amsterdam, The Netherlands) (32). Negative and positive controls were included in each qPCR run.

Statistical Methods

Differences between experimental groups between *B. burgdorferi* ss loads in qPCR were statistically tested by two-sided nonparametric tests (Mann-Whitney, GraphPad Prism software version 5.0, San Diego, CA, USA). Differences between experimental groups in tick weight were statistically tested by one-way ANOVA.

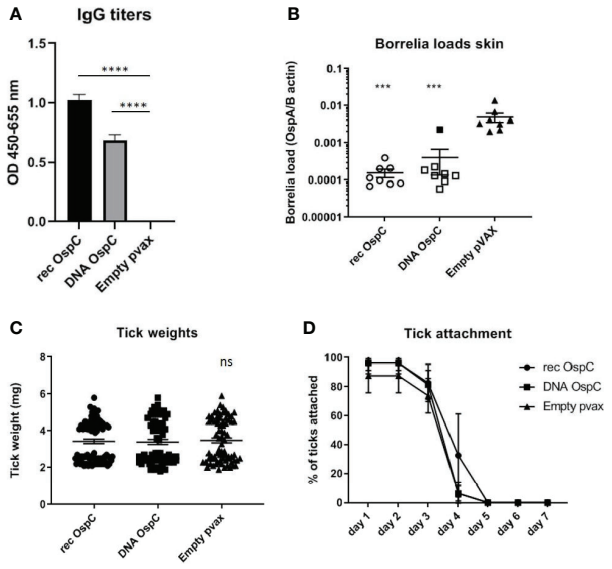

FIGURE 1 | **(A–D)** rOspC vaccination versus vaccination with OspC DNA vaccine in a 0-14-28 day immunization protocol. **(A)** Specific total IgG titers were measured in an Enzyme-linked immunosorbent assay (ELISA). Plates were coated with recombinant protein and incubated with mouse sera collected at timepoint 42 days pre-challenge. Sera was diluted in steps of 3 starting with 100 times and ending with 218,700 times dilution Presented are the total IgG titers incubated with mouse sera 1:8,100 diluted for OspC. Statistical significance was calculated for each experimental group compared to the control, Empty pvax, using a one-way ANOVA statistical test ($p< 0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$). **(B)** Borrelia loads in skin samples of the tick attachment site as determined by qPCR. Closed dots depict positive Borrelia loads, open dots depict PCR negative samples for which the OspA detection limit was divided by the sample's mouse beta actin load. The Borrelia loads were compared to the negative control Empty pvax using a two-sided non parametric test (Mann-Whitney $^{*}P < 0.05$, $^{**}P<0.01$, $^{***}P<0.001$). **(C)** Tick weights in mg. Immunized mice were challenged with *B. burgdorferi* N40-infected I. scapularis nymphs. Nymphs were placed in a collection capsule on the back of each mice. The capsules were checked daily and the ticks were allowed to feed to repletion. Once they had fallen off their weight was measured. **(D)** Tick attachment is presented as percentage of ticks that are still attached per day. ns, not significant.

RESULTS

Osp C Vaccination

Vaccination with recombinant OspC as a positive control was compared to vaccination with OspC as a DNA vaccine to determine the efficacy of the DNA vaccine strategy (**Figure 1**). Both the rOspC and OspC DNA vaccine were able to induce robust IgG responses, although the titer of rOspC was significantly higher (**Figure 1A**). As expected from vaccination with a *Borrelia* protein, the OspC vaccinated groups did not show decreased tick weight or duration of attachment (**Figures 1C, D**).

We also assessed whether the conventional and DNA OspC vaccines were able to provide protection against infection with *B. burgdorferi ss* strain N40 transmitted by *I. scapularis* nymphs. For this purpose we performed qPCR and culture of several tissues we obtained by sacrificing the mice 21 days after the challenge. *B. burgdorferi* ss DNA loads in the skin challenge site were negative in all of the rOspC vaccinated mice and in seven out of eight of OspC DNA vaccinated mice (**Figure 1B**). *B. burgdorferi* ss culture data of the skin challenge site and bladder, corresponded with the qPCR data; seven out of eight mice were *Borrelia* negative (**Table 1**).

Tick Salivary Gland DNA Vaccines

DNA vaccines against TSLPI, Salp15, tHRF, and Tix-5 did not induce robust IgG responses (**Figure 2A**). Although moderate

TABLE 1 | Culture positivity 8 weeks after challenge for OspC vaccination.

	Skin	Bladder
Rec OspC	0/8	0/8
DNA OspC	1/8	1/8
Empty pvax	8/8	8/8

Cultures of skin and bladder were checked weekly for growth of Borrelia.

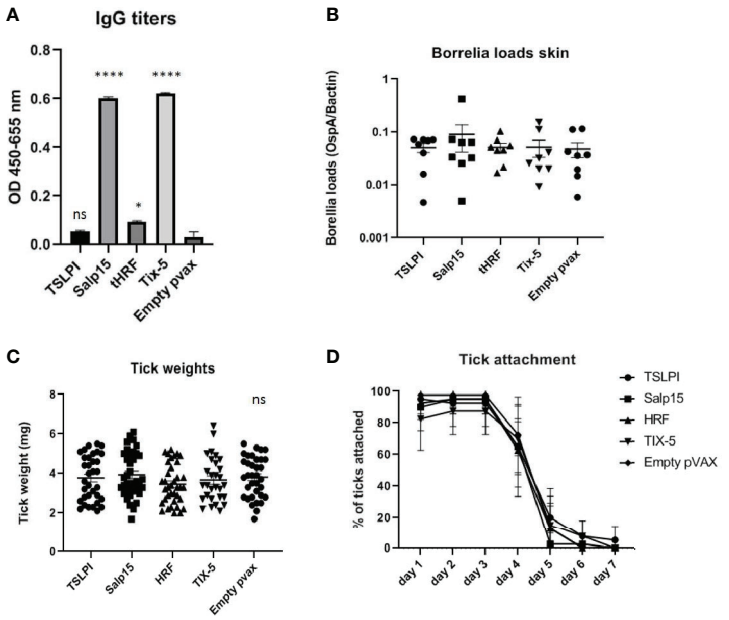

FIGURE 2 | **(A–D)** DNA vaccination with tick salivary gland genes TSLPI, tHRF, Salp15, and Tix-5 in a 0-14-28 day immunization protocol. **(A)** Specific total IgG titers were measured in an Enzyme-linked immunosorbent assay (ELISA). Plates were coated with recombinant protein and incubated with mouse sera collected at timepoint 42 days pre-challenge. Sera was diluted in steps of 3 starting with 100 times and ending with 218,700 times dilution. Presented are the total IgG titers incubated with mouse sera 1:300 diluted for the tick salivary gland gene DNA vaccines. Statistical significance was calculated for each experimental group compared to the control, Empty pvax, using a one-way ANOVA statistical test ($p< 0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$). **(B)** Borrelia loads in skin samples of the tick attachment site as determined by qPCR. Closed dots depict positive Borrelia loads, open dots depict PCR negative samples for which the OspA detection limit was divided by the sample's mouse beta actin load. The Borrelia loads were compared to the negative control Empty pvax using a two-sided non parametric test (Mann-Whitney $^{*}P < 0.05$, $^{**}P<0.01$, $^{***}P<0.001$). **(C)** Tick weights in mg. Immunized mice were challenged with *B. burgdorferi* N40-infected I. scapularis nymphs. Nymphs were placed in a collection capsule on the back of each mice. The capsules were checked daily and the ticks were allowed to feed to repletion. Once they had fallen off their weight was measured. **(D)** Tick attachment is presented as percentage of ticks that are still attached per day. ns, not significant.

antibody response against Salp 15 and Tix-5 compared to Empty pvax could be observed, TSLPI and tHRF did not elicit any noteworthy IgG response. When assessing tick attachment time and tick weight, the DNA vaccines against tick antigens also did not demonstrate a difference compared to the Empty pvax control (**Figures 2C, D**). As described above, OspC DNA vaccination protected all but one mouse against *Borrelia* infection. In stark contrast, *B. burgdorferi* ss DNA loads in TSLPI, Salp15, tHRF, and Tix-5 skin samples, were all positive (**Figure 2B**). Also, *B. burgdorferi* ss cultures from mice tattooed with DNA vaccines against tick proteins TSLPI, Salp15, tHRF, and TIX-5 were all positive and almost all within 2 weeks time (**Table 2**).

TABLE 2 | Culture positivity 8 weeks after challenge for tick salivary gland DNA vaccination.

	Skin	Bladder
TSLPI	8/8	8/8
Salp15	8/8	8/8
tHRF	8/8	8/8
Tix-5	8/8	8/8
Empty pvax	8/8	8/8

Cultures of skin and bladder were checked weekly for growth of Borrelia.

DISCUSSION

In this study, we assessed the utility of DNA vaccination against Lyme borreliosis using a known *Borrelia* antigen - OspC - that has been shown to elicit protection when used as a recombinant protein vaccine. Both rOspC and OspC DNA vaccination resulted in robust antibody production in mice and subsequently protected against *B. burgdorferi* ss transmission by tick challenge. DNA vaccination therefore embodies a promising vaccination strategy, as it allows for rapid vaccination schedules, they are easy to produce, and besides humoral immunity, are also capable of inducing cellular immunity. Especially humoral immunity is described to be very important for clearance of *Borrelia* (33, 34). Interestingly, as the OspC DNA vaccine was able to induce moderate to high antibody titers and provided near total protection against *B. burgdorferi* ss infection by tick challenge. Therefore, immunization with plasmid DNA for this particular *Borrelia* antigen is, as described previously, an alternative platform for vaccination, as opposed to recombinant protein vaccination (35). It also shows that DNA vaccination against *B. burgdorferi* ss antigens in itself is effective in protecting against infection in a tick challenge model. Given the heterogeneity of OspC protein

sequences among different *B. burgdorferi* sl species and strains, DNA vaccination could be an interesting platform, as multiple OspC sequence for a multivalent vaccine can be relatively easily combined (36). Future studies should focus on the effectiveness of a DNA vaccine targeting various OspC variants on protection against a range of *B. burgdorferi* sl isolates.

Secondly, we examined several anti-tick vaccine candidates as DNA vaccines. The ability of *Borrelia* spirochetes to establish an infection in mammals is partly dependent on tick (salivary gland) proteins, which makes these proteins interesting candidates for an anti-tick vaccine. Our goal was to determine the protective abilities of four tick salivary gland proteins used as DNA vaccines. In contrast to their recombinant protein vaccines described in literature, the tick salivary gland antigen DNA vaccines induced low (Salp15 and Tix-5) or hardly any (TSLPI and tHRF) IgG titers and did not provide protection against *B. burgdorferi* ss infection by tick challenge. It could therefore be speculated that the low or absent IgG titers are responsible for the fact that there was no protection observed and an adequate humoral immune response is essential to neutralize the function of these tick proteins and preventing transmission of *B. burgdorferi* ss. The low expression of these antigens by murine cells could be one reason for the low immunogenicity. Perhaps this could be circumvented by stronger adjuvants. Indeed, in addition to our strategy - i.e., addition of a hTPA signal and Kozak sequence and codon-optimization - adjuvant modifications can be made to DNA vaccines, such as genetic adjuvant strategies to improve the immune response induced by DNA vaccines (37). Regardless, both Salp15 and TIX5 were able to elicit a moderate IgG response, yet no protection against *B. burgdorferi* infection was observed. It should be mentioned that, although it has been established that an anti-tick vaccine based on TIX-5 impairs tick-feeding the effect of such a vaccine on *Borrelia* transmission has never been investigated (21). In contrast, for Salp15, for which an adjuvanted recombinant protein vaccine was able to interfere with *B. burgdorferi* transmission, the observed low IgG titers induced by our DNA vaccine are likely to cause for the lack of protection.

In this study, we have assessed DNA vaccination as a tool for two different vaccination approaches to protect against *B. burgdorferi* ss infection: targeting the spirochete with OspC or targeting the tick vector using Salp15, Tix-5, TSLPI, or tHRF. Although IgG levels are important for both approaches, the mechanism that leads to protection as a result of these antibodies differ greatly. Antibodies bound to OspC not only neutralize the ability of OspC to interact with Salp15 and shield against complement-mediated killing, they facilitate complement-mediated killing and phagocytosis of the spirochete. Antibodies against tick salivary gland proteins that are not directly bound to *Borrelia* can only neutralize the function of these proteins. As such high IgG levels against tick salivary gland proteins might possibly even be more important in protection compared to *Borrelia* antigens. Regardless, and as discussed above, it is clear that IgG levels induced by DNA vaccination against these tick proteins are insufficient (14).

In conclusion, we have shown that a successful vaccine against *Borrelia* is not restricted to conventional recombinant vaccination strategies and also works against tick-mediated transmission. This implies that DNA tattoo vaccination can be used to as a rapid and relatively easily screening tool to assess immunogenicity and efficacy of future novel *B. burgdorferi* sl vaccine candidates. In contrast, DNA vaccination appears not to be a suitable method to induce adequate immune responses against tick antigens and subsequent protection against *B. burgdorferi* ss; at least not for the selected tick salivary gland antigens Salp15, tHRF, TSLPI, and TIX-5.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article could be made available upon reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Research Ethics Committee of the Academic Medical Center, Amsterdam, The Netherlands.

AUTHOR CONTRIBUTIONS

MK, JT, and AW designed the study. JE, JT, and MK performed the study procedures. AB and AW provided information about the DNA vaccine design. JH supervised the study progress. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.615011/full#supplementary-material

Supplementary Figure 1 | Specific total IgG titers were measured in an Enzyme-linked immunosorbent assay (ELISA). Plates were coated with recombinant protein OspC 1µg/ml and incubated with specific mouse sera collected at timepoint 42 days pre-challenge. Sera were diluted to 218700 times.

Supplementary Figure 2 | Specific total IgG titers were measured in an Enzyme-linked immunosorbent assay (ELISA). Plates were coated with recombinant protein (TSLPI, Salp15, tHRF, Tix-5 1µg/ml) and incubated with mouse sera collected at timepoint 42 days pre-challenge. Sera were diluted to 218700 times.

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Iripin-3, a New Salivary Protein Isolated From *Ixodes ricinus* Ticks, Displays Immunomodulatory and Anti-Hemostatic Properties *In Vitro*

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Tick saliva is a rich source of pharmacologically and immunologically active molecules. These salivary components are indispensable for successful blood feeding on vertebrate hosts and are believed to facilitate the transmission of tick-borne pathogens. Here we present the functional and structural characterization of Iripin-3, a protein expressed in the salivary glands of the tick *Ixodes ricinus*, a European vector of tick-borne encephalitis and Lyme disease. Belonging to the serpin superfamily of protease inhibitors, Iripin-3 strongly inhibited the proteolytic activity of serine proteases kallikrein and matriptase. In an *in vitro* setup, Iripin-3 was capable of modulating the adaptive immune response as evidenced by reduced survival of mouse splenocytes, impaired proliferation of $CD4^+$ T lymphocytes, suppression of the T helper type 1 immune response, and induction of regulatory T cell differentiation. Apart from altering acquired immunity, Iripin-3 also inhibited the extrinsic blood coagulation pathway and reduced the production of pro-inflammatory cytokine interleukin-6 by lipopolysaccharide-stimulated bone marrow-derived macrophages. In addition to its functional characterization, we present the crystal structure of cleaved Iripin-3 at 1.95 Å resolution. Iripin-3 proved to be a pluripotent salivary serpin with immunomodulatory and anti-hemostatic properties that could facilitate tick feeding via the suppression of host anti-tick defenses. Physiological relevance of Iripin-3 activities observed *in vitro* needs to be supported by appropriate *in vivo* experiments.

Keywords: tick, serpin, X-ray crystallography, blood coagulation, inflammation, adaptive immunity, *Ixodes ricinus*, saliva

INTRODUCTION

The European tick *Ixodes ricinus* (Acari: Ixodidae) is an obligate blood-sucking ectoparasite that transmits several medically important pathogens such as Lyme disease spirochetes from the *Borrelia burgdorferi* sensu lato complex and tick-borne encephalitis virus (1). The insertion of the tick hypostome and two chelicerae into host skin disrupts the surrounding tissue and capillaries, to

which the host responds by activating a series of physiological defense processes including hemostasis and innate and adaptive immune responses (2–5). Cutaneous tissue injury and tick antigens are sensed by cells in the vicinity of the tick attachment site, such as keratinocytes, fibroblasts endothelial cells, mast cells, macrophages and dendritic cells (3). These cells release pro-inflammatory and chemotactic molecules that stimulate the recruitment of neutrophils and other immune cells to the area of tick feeding (3, 4, 6). Moreover, Langerhans cells and macrophages trap tick antigens and present them to T cells, which triggers T cell proliferation and ultimately results in the development of the acquired immune response (7). If unopposed, the host defense reaction rejects the tick via detrimental effects on tick viability and reproduction (8). Therefore, ticks surpass the host response by secreting hundreds of bioactive molecules via their saliva into the wound (9–11). Since these salivary molecules can target hemostasis and almost every branch of the immune response, they might be useful in the development of novel pharmaceuticals for the treatment of immune-mediated inflammatory diseases, hypercoagulable states, diseases associated with excessive complement activation, or even cancer (11–14). Moreover, tick salivary proteins represent potential targets for the development of anti-tick and/or transmission blocking vaccines (15).

Protease inhibitors form the largest functional group of tick salivary proteins (16). Based on their specificity, tick protease inhibitors can be divided into inhibitors of cysteine proteases (e.g., cystatins) and inhibitors of serine proteases (e.g., Kunitz domain-containing proteins and serpins) (17). Serpins (serine protease inhibitors) are mid-sized proteins consisting of about 330–500 amino acids (18, 19) with a conserved serpin domain and an exposed region near the carboxyl-terminal end referred to as the reactive center loop (RCL) (20). Cleavage of the scissile P1-P1′ bond in the RCL by a target serine protease results in the formation of a covalent serpin-protease complex and permanent inactivation of both the serpin and the protease (18, 20).

Serpins have been identified in many species of hard-bodied ticks of medical and veterinary importance such as *Amblyomma americanum* (21), *Haemaphysalis longicornis* (22), *I. ricinus* (23), *I. scapularis* (24), *Rhipicephalus appendiculatus* (25), and *Rhipicephalus microplus* (26, 27). Some of the functionally characterized tick serpins have been shown to suppress the enzymatic activity of blood clotting factors (mainly thrombin and factor Xa) and consequently inhibit the intrinsic and common coagulation pathways (28–31). Tick serpins that inhibit thrombin and cathepsin G can block platelet aggregation triggered by these two serine proteases (30–33). In addition to anti-hemostatic activities, many of the functionally characterized tick serpins interfere with the host innate immunity, since they inhibit the enzymatic activity of mast cell and neutrophil serine proteases, reduce vascular permeability and paw edema formation, suppress neutrophil migration *in vivo* and attenuate the production of pro-inflammatory cytokines by activated innate immune cells, such as macrophages and dendritic cells (32, 34–37). Last but not least, tick serpins can modify the host adaptive immune response via suppression of T lymphocyte proliferation and inhibition of Th1 and Th17 cell differentiation (35, 37–40). A number of RNA interference and vaccination experiments have demonstrated the important role of tick serpins in successful completion of a blood meal by prolonging the feeding period, reducing engorgement weight, or resulting in higher mortality rates or impaired oviposition (41–45).

To date, only two serpins from the tick *I. ricinus* have been assigned functions: Iris (*I. ricinus* immunosuppressor) (38) and IRS-2 (*I. ricinus* serpin-2) (32). Due to possible confusion arising from the previously used abbreviation IRS for *I. ricinus* serpins (32) (with insulin receptor substrates), we decided to name *I. ricinus* serpins Iripins (*Ixodes ricinus* serpins). Here we present the structural and functional characterization of Iripin-3 (*I. ricinus* serpin-3). Iripin-3 primarily inhibited two trypsin-like serine proteases, kallikrein and matriptase. When tested in various *in vitro* assays, Iripin-3 displayed several distinct functions: it inhibited the extrinsic blood coagulation pathway, attenuated interleukin-6 (IL-6) production by LPS-activated bone marrow-derived macrophages (BMDMs), impaired the survival and proliferation of $CD4^+$ T cells, and suppressed the Th1 immune response. The presence of Iripin-3 protein in tick saliva suggests that this serpin could play a role at the tick-host interface by suppressing various aspects of the host defense to *I. ricinus* feeding. Further *in vivo* studies, however, are necessary to confirm herein presented results. Finally, we determined the crystal structure of cleaved Iripin-3 at 1.95 Å resolution.

MATERIALS AND METHODS

Animals

C57BL/6N mice were purchased from Velaz, Ltd (Praha-Lysolaje, Czechia). C3H/HeN mice and OT-II transgenic mice were obtained from Charles River Laboratories (Wilmington, MA). Mice were maintained under standard, pathogen-free conditions in the animal house facility of the Department of Medical Biology, Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic. Guinea pigs utilized for *I. ricinus* feeding and a rabbit used for the production of anti-Iripin-3 antibodies were bred and maintained at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences (IP BC CAS), Czech Republic. All animal experiments were performed in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb. (ethics approval No. 34/2018) and protocols approved by the Ministry of Education, Youth and Sports of the Czech Republic (protocol No. 19085/2015-3) and the responsible committee of the IP BC CAS. Pathogen-free *I. ricinus* ticks were obtained from the tick colony maintained at the IP BC CAS.

Bioinformatics Analyses

The molecular weight and isoelectric point of Iripin-3 were computed by ProtParam (46). The presence of a signal peptide was predicted using the SignalP 4.1 server (47). The ScanProsite tool (48) was utilized to identify the serpin signature motif

PS00284 as well as two other consensus amino acid motifs N-[AT]-[VIM]-[YLH]-F-[KRT]-[GS] and [DERQ]-[VL]-[NDS]-E-[EVDKQ]-G (26, 49). The reactive central loop together with the amino acid residue at the P1 site were determined based on the eight-residue pattern p17[E]-p16[E/K/R]-p15[G]-p14[T/S]-p13[X]-p12-9[AGS]-p8-1[X]-p1′-4′ [X] (26, 49). NetNGlyc 1.0 (Gupta et al., unpublished) and NetOGlyc 4.0 (50) servers were used to predict potential N-glycosylation and O-glycosylation sites, respectively. To compare Iripin-3 with other known serpins, the Iripin-3 protein sequence was tested against the GenBank database of non-redundant protein sequences using BLASTP (51). Alignment of IRS-2 and Iripin-3 amino acid sequences was conducted with ClustalW (52). Visualization of the alignment and addition of secondary structure elements were performed using ESPript 3.0 (53).

Crystal Structure Determination

The production of recombinant Iripin-3 in an *Escherichia coli* expression system is detailed in the **Supplementary Materials**. Crystallization experiments were conducted using the sitting-drop vapor diffusion technique, and the obtained crystals were used to collect X-ray diffraction data on the beamline BL14.1 at the BESSY II electron storage ring operated by the Helmholtz-Zentrum Berlin (54). The structure of Iripin-3 was solved by the molecular replacement method, in which the known structure of IRS-2 (Protein Data Bank (PDB) code 3NDA) (32) was used as a search model. The whole procedure of Iripin-3 structure determination, starting with crystallization and ending with structure refinement and validation, is described in detail in the **Supplementary Materials**. Complete data processing and refinement statistics are summarized in **Supplementary Table 1**. Atomic coordinates were deposited in the PDB under accession code 7AHP.

Phylogenetic Analysis

For the purpose of phylogenetic analysis, the amino acid sequences of 27 tick serpins and one human serpin were retrieved from GenBank. Accession numbers of these sequences are provided in **Supplementary Table 2**. Retrieved sequences were aligned and edited manually using BioEdit 7.2.5 (55). Evolutionary history was deduced from the protein sequences without a signal peptide by using the maximum likelihood method and Jones-Taylor-Thornton (JTT) matrix-based model (56). Initial trees for the heuristic search were obtained automatically by applying the neighbor-joining (57) and BIONJ (58) algorithms to a matrix of pairwise distances estimated using the JTT model, and then the topology with a superior log likelihood value was selected. The reliability of individual branches was determined by bootstrapping. Bootstrap values were calculated for 1000 replicates. Evolutionary analyses were conducted in MEGA X (59).

Iripin-3 Expression in Ticks

I. ricinus nymphs were fed on C3H/HeN mice for 1 day, 2 days, and until full engorgement (3–4 days). *I. ricinus* adult females were fed on guinea pigs for 1, 2, 3, 4, 6, and 8 days. Tick removal from host animals at given time points was followed by the dissection of nymphs and adult female salivary glands, midguts, and ovaries under RNase-free conditions. RNA was isolated from tick tissues using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH), and 1 µg of total RNA was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. Five-fold diluted cDNA mixed with FastStart Universal SYBR Green Master (Roche Applied Science) and gene-specific primers were used for the analysis of *iripin-3* expression in the Rotor-Gene 6000 thermal cycler (Corbett Research, Saffron Walden, UK). Cycling conditions were 95°C for 10 min followed by 45 cycles of 95°C for 15 s, 60°C for 10 s and 72°C for 30 s. The relative quantification of *iripin-3* transcripts in tick tissues was performed using the ΔΔCt method (60). The *I. ricinus* gene encoding ribosomal protein S4 (*rps4*, GenBank accession number MN728897.1) was utilized as a reference gene for the calculation of relative expression ratios (61, 62). Nucleotide sequences of forward and reverse primers as well as amplicon lengths are provided in **Supplementary Table 3**.

Presence of Iripin-3 in Tick Saliva

Polyclonal antibodies against Iripin-3 were produced in a rabbit injected subcutaneously with 100 µg of purified Iripin-3 in 500 µl of complete Freund's adjuvant. The first immunization was followed by another two injections of Iripin-3 in 500 µl of incomplete Freund's adjuvant at 14-day intervals. On day 14 after the last injection, the rabbit was sacrificed, and its blood was collected. Prepared rabbit antiserum to Iripin-3 was subsequently utilized for the detection of Iripin-3 in tick saliva by indirect ELISA and western blotting. The saliva was collected from *I. ricinus* ticks feeding for 6–7 days on guinea pigs as described previously (63). ELISA and western blot analyses are detailed in the **Supplementary Materials**.

Inhibition of Serine Proteases

Preliminary screening of Iripin-3 inhibitory activity against a set of 17 serine proteases was performed as described previously (32), with the exception of factor VIIa (FVIIa). Human FVIIa (Haematologic Technologies, Inc., Essex Junction, VT) at 20 nM concentration was pre-incubated for 10 min at 30°C with 400 nM Iripin-3 before the addition of 250 µM fluorogenic substrate Boc-QAR-AMC. The assay buffer used consisted of 20 mM Tris, 150 mM NaCl, 0.01% Triton X-100, 5 mM $CaCl_2$, and 0.1% polyethylene glycol 6000, pH 8.0. After the determination of the substrate hydrolysis rate, the six most strongly inhibited proteases were chosen for more detailed analysis. The assessment of covalent complex formation between Iripin-3 and selected serine proteases and the determination of second-order rate constants of protease inhibition are detailed in the **Supplementary Materials**.

Blood Coagulation

The effect of Iripin-3 on blood coagulation was tested by prothrombin time (PT), activated partial thromboplastin time (aPTT), and thrombin time (TT) assays. All chemicals were purchased from Technoclone (Vienna, Austria). Citrated human plasma (Coagulation Control N) was mixed either with 6 µM

Iripin-3 or with five different Iripin-3 concentrations and then incubated for 10 min at room temperature. To perform the PT test, 100 µl of plasma with added Iripin-3 was incubated for 1 min at 37°C before the addition of 200 µl of Technoplastin HIS pre-warmed to 37°C. Plasma clotting time was measured on the Ceveron four coagulometer (Technoclone). In the aPTT test, the incubation of 100 µl of plasma mixed with Iripin-3 at 37°C for 1 min was followed by the addition of 100 µl of Dapttin TC. After incubating the mixture of plasma and Dapttin at 37°C for 2 min, 100 µl of 25 mM $CaCl_2$ was added to initiate the coagulation cascade. Plasma clotting time was determined as described above. To perform the TT test, 200 µl of plasma mixed with Iripin-3 was incubated at 37°C for 1 min. At the end of incubation, 200 µl of thrombin reagent was added, and plasma clotting time was measured as in the PT and aPTT assays.

Pro-Inflammatory Cytokine Production by BMDMs

Bone marrow cells were isolated from femurs and tibias of C57BL/6N mice. Both ends of the bones were cut with scissors, and bone marrow was flushed with complete medium. The complete medium was prepared by supplementation of RPMI 1640 medium containing glutamine (Biosera) with 10% heat-inactivated fetal bovine serum (FBS, Biosera), 50 µM 2-mercaptoethanol (Sigma Aldrich, St Louis, MO), 100 U/ml penicillin G (Biosera, Kansas City, MO) and 100 µg/ml streptomycin (Biosera). After erythrocyte lysis in RBC lysis buffer (eBioscience, San Diego, CA), bone marrow cells resuspended in complete medium were seeded into 10 cm Petri dishes and incubated in the presence of 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF, Sigma Aldrich) at 37°C and 5% CO_2 for 10 days. On days 4 and 7, non-adherent cells were removed and the medium was replaced with fresh complete medium containing 10 ng/ml GM-CSF. On day 10, adherent cells (macrophages) were collected, resuspended in RPMI 1640 medium supplemented only with 0.5% bovine serum albumin (BSA, Biosera), and seeded into 24-well culture plates (2×10^5 cells in 500 µl of culture medium per well). After 5 h incubation at 37°C and 5% CO_2, the medium was replaced with fresh RPMI 1640 medium containing 0.5% BSA, and BMDMs were pre-incubated for 40 min with 3 µM or 6 µM Iripin-3. Finally, 100 ng/ml of LPS (Sigma Aldrich; *E. coli* serotype O111:B4) was added, and macrophages were incubated in the presence of Iripin-3 and LPS for another 24 h. At the end of incubation, cells and cell-free supernatants were collected for RNA isolation and protein quantification, respectively. Relative expression of *Tnf*, *Il6*, and *Il1b* in macrophages was determined by RT-qPCR and concentrations of tumor necrosis factor (TNF), IL-6, and interleukin-1β (IL-1β) cytokines in collected supernatants were measured by DuoSet ELISA Development Kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions with only minor modifications. The RT-qPCR analysis is described in detail in the **Supplementary Materials**.

Splenocyte Isolation and Culture in the Presence of Iripin-3

Spleens harvested from OT-II mice were forced through a Corning 70 µm cell strainer to obtain a single cell suspension. Red blood cells (RBCs) were removed from the suspension by the addition of 1× RBC lysis buffer (eBioscience), and the erythrocyte-free spleen cells were resuspended in RPMI 1640 medium with stable glutamine (Biosera) supplemented with 10% heat-inactivated FBS (Biosera), 50 µM 2-mercaptoethanol (Sigma Aldrich), 100 U/ml penicillin G (Biosera), and 100 µg/ml streptomycin (Biosera). Splenocytes were then seeded into 24-well or 96-well culture plates and pre-incubated with 3 µM or 6 µM Iripin-3 for 2 h. Pre-incubation with Iripin-3 was followed by the addition of ovalbumin (OVA) peptide 323–339 (Sigma Aldrich) at a concentration of 100 ng/ml. Splenocytes were incubated in the presence of Iripin-3 and OVA peptide at 37°C and 5% CO_2 for either 20 h (assessment of cell survival) or 72 h (analysis of cell proliferation and transcription factor expression).

Survival of B and T Cells

Mouse splenocytes were seeded into 96-well culture plates (5×10^5 cells in 200 µl of complete medium per well), pre-incubated with Iripin-3, and stimulated with OVA peptide. After 20 h incubation at 37°C and 5% CO_2, cells were harvested for flow cytometry analysis. First, splenocytes were stained with fixable viability dye eFluor 780 (eBioscience). Subsequently, Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience, clone 93), and surface antigen staining was performed with following monoclonal antibodies purchased from eBioscience: anti-CD45-PerCP-Cyanine5.5 (clone 30-F11), anti-CD19-PE (clone eBio1D3(1D3)), and anti-CD3e-APC (clone 145-2C11). Finally, the active form of caspase 3 in splenocytes was labeled using the FITC Active Caspase-3 Apoptosis Kit (BD Biosciences). The percentage of live $CD19^+$ and $CD3e^+$ splenocytes as well as the level of active caspase 3 were analyzed on the BD FACSCanto II flow cytometer using BD FACSDiva software version 6.1.3 (BD Biosciences).

Proliferation of $CD4^+$ T Cells

Erythrocyte-free splenocytes were stained with red fluorescent dye eFluor 670 (eBioscience), which allows monitoring of individual cell divisions. The stained splenocytes were seeded into 96-well culture plates (5×10^5 cells in 200 µl of complete medium per well), pre-incubated with Iripin-3, and stimulated with OVA peptide. Cells were allowed to proliferate for 72 h and then were harvested for flow cytometry analysis. Collected cells were stained with FITC-labelled anti-CD4 monoclonal antibody (clone GK1.5, eBioscience) and propidium iodide (eBioscience), and the percentage of proliferating live $CD4^+$ splenocytes was measured on the BD FACSCanto II flow cytometer using BD FACSDiva software version 6.1.3 (BD Biosciences).

Transcription Factor Expression in $CD4^+$ T Cells (RT-qPCR)

Splenocytes were seeded into 24-well culture plates (4.5×10^6 cells in 500 µl of complete medium per well), pre-incubated with Iripin-3, and stimulated with OVA peptide. At the end of 72 h incubation, non-adherent cells were collected, stained with FITC-labeled anti-CD4 monoclonal antibody (clone GK1.5, eBioscience), and $CD4^+$ splenocytes were separated from the rest of the cell population using

the S3e Cell Sorter (Bio-Rad Laboratories, Hercules, CA). RNA was extracted from $CD4^+$ cells with the help of NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany), and 1 µg of total RNA was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). RT-qPCR was performed in the CFX384 Touch thermal cycler (Bio-Rad) by utilizing five-fold diluted cDNA, SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), and gene-specific primers. The PCR cycling conditions were 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. The relative quantification of *Tbx21* (*Tbet*), *Gata3*, *Rorc*, and *Foxp3* transcripts in $CD4^+$ splenocytes was performed using Pfaffl's mathematical model (64). Based on the results of geNorm analysis (65), *Actb* and *Gapdh* were utilized as reference genes for the calculation of relative expression ratios. Nucleotide sequences of forward and reverse primers as well as amplicon lengths are given in **Supplementary Table 3**.

Transcription Factor Expression in $CD4^+$ T Cells (Flow Cytometry)

Splenocytes were seeded into 24-well culture plates (2 x 10^6 cells in 500 µl of complete medium per well), pre-incubated with Iripin-3, and stimulated with OVA peptide. After 68 h incubation at 37°C and 5% CO_2, 20 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich) together with 1 µM ionomycin (Sigma Aldrich) were added to re-stimulate the cells. Brefeldin A (eBioscience) at a concentration of 3 µg/ml was added 1 h later, and splenocytes were incubated in the presence of PMA, ionomycin, and brefeldin A for another 4 h. At the end of incubation, non-adherent cells were collected and stained with fixable viability dyes eFluor 520 and eFluor 780 (eBioscience). Subsequently, Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience, clone 93), and surface antigen staining was performed with anti-CD4-Alexa Fluor 700 (BD Biosciences, clone RM4-5) and anti-CD25-PerCP-Cyanine5.5 (eBioscience, clone PC61.5) monoclonal antibodies. Surface antigen staining was followed by intracellular staining of transcription factors and cytokine IFN-γ, for which the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) was used in conjunction with following monoclonal antibodies: anti-T-bet-APC (clone eBio4B10 (4B10)), anti-GATA-3-PE (clone TWAJ), anti-RORγt-PE-CF594 (clone Q31-378), anti-Foxp3-PE-Cyanine7 (clone FJK-16s), and anti-IFN-γ-PE (clone XMG1.2). All antibodies were purchased from eBioscience except for the anti-RORγt antibody, which was obtained from BD Biosciences. Analysis was performed on the BD FACSCanto II flow cytometer using BD FACSDiva software version 6.1.3 (BD Biosciences).

Statistical Analyses

Data are presented in all graphs as mean ± the standard error of the mean (SEM). Differences between the mean values of two groups were analyzed by the unpaired two-tailed *t*-test. Differences between the mean values of three or more groups were analyzed by one-way ANOVA or randomized block ANOVA, which involved two variables: a fixed effect factor (treatment) and a random effect factor/block (an experimental run) (66). In the case of a statistically significant result ($p < 0.05$), Dunnett's *post hoc* test was performed to compare the mean of a control group with the means of experimental groups. All statistical tests were conducted using the software package STATISTICA 12 (StatSoft, Inc.). Statistically significant differences between groups are marked with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

RESULTS

Iripin-3 Belongs to the Serpin Superfamily

A full-length nucleotide sequence of Iripin-3 was obtained during a salivary gland transcriptome project (16) and was submitted to GenBank under accession number GADI01004776.1. This sequence, consisting of 1182 base pairs, encodes a 377-amino acid (AA) protein with predicted molecular weight of approximately 42 kDa and with theoretical isoelectric point (pI) 5.23. The SignalP 4.1 server found a 16-AA signal peptide at the N terminus of the protein sequence (**Figure 1A**), which indicates that Iripin-3 is a potentially secreted protein. Using ScanProsite, the serpin signature motif PS00284 was identified at AA positions 366-376 (**Figure 1A**). Moreover, two other serpin consensus AA motifs N-[AT]-[VIM]-[YLH]-F-[KRT]-[GS] and [DERQ]-[VL]-[NDS]-E-[EVDKQ]-G were recognized: NAMYFKG at AA positions 183-189 and EVNEEG at AA positions 338-343 (**Figure 1A**), suggesting that Iripin-3 belongs to the serpin superfamily. The hinge region of the Iripin-3 RCL has glycine at the P15 position, threonine at the P14 position, and residues with short side chains (alanine and valine) at positions P12-P9 (**Figure 1A**), which correspond to the RCLs of inhibitory serpins (68). The P1 site is occupied with the basic amino acid residue arginine (**Figure 1A**), suggesting Iripin-3 might target trypsin-like rather than chymotrypsin-like or elastase-like serine proteases (69). Using NetNGlyc 1.0 and NetOGlyc 4.0 servers, the Iripin-3 AA sequence was predicted to contain two potential N-glycosylation sites (N-X-[S/T]) and one putative O-glycosylation site (**Figure 1A**).

Iripin-3 Adopts a Typical Serpin Fold

Employing X-ray crystallography, we determined the 3D structure of Iripin-3 at 1.95 Å resolution. The crystal used exhibited symmetry of the $P6_222$ space group and contained one molecule in the asymmetric unit with a solvent content of 42.68%. The tertiary structure of Iripin-3 matched the 3D structures of other serpins, including the tick serpin IRS-2 (**Figure 1B**), with which it had the highest sequence similarity of all the serpin structures currently deposited in the PDB. More specifically, the Iripin-3 tertiary structure was composed of ten α-helices and three β-sheets, which were sequentially arranged in the order α1-β1-α2-α3-α4-α5-β2-α6-β3-α7-β4-β5-β6-β7-β8-α8-α9-β9-β10-α10-β11-β12-β13-β14-β15 (**Figures 1A**, **2**). The sheet A consisted of six β-strands (β2, β3, β4, β10, β11, β12), sheet B of five β-strands (β1, β7, β8, β14, β15), and sheet C of four β-strands (β5, β6, β9, β13) (**Figure 2**). Iripin-3 in the crystal adopted a conformation known as the relaxed (R) state, since its RCL was probably cleaved by some contaminating

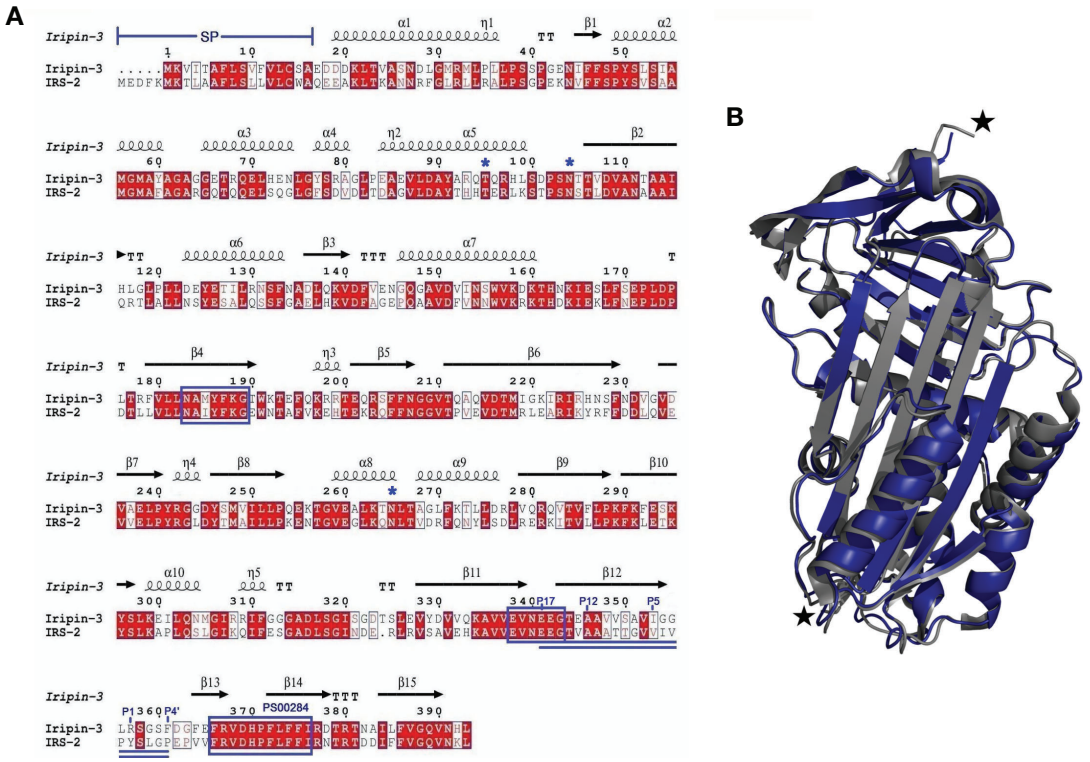

FIGURE 1 | A comparison of the primary, secondary and tertiary structures of Iripin-3 and IRS-2. **(A)** Structure-based sequence alignment of Iripin-3 and IRS-2. Secondary structure elements, which are shown above the aligned sequences, are depicted as spirals (α-helices, 3_{10}-helices) and arrows (β-sheets). Both Iripin-3 and IRS-2 possess a signal peptide (SP) at the N terminus of their sequences. Conserved AA motifs PS00284, N-[AT]-[VIM]-[YLH]-F-[KRT]-[GS], and [DERQ]-[VL]-[NDS]-E-[EVDKQ]-G are boxed in blue. The RCLs of both serpins are double underlined. Numbering of amino acid residues in the RCL is based on the standard nomenclature developed by Schechter and Berger (67). Putative N-glycosylation and O-glycosylation sites are marked with blue asterisks. **(B)** Superposition of the cleaved Iripin-3 structure (blue) on the structure of cleaved IRS-2 (gray). Cleavage sites are marked with black stars.

proteases before or during the crystallization experiment. A protein sample can contain traces of contaminating cysteine and serine proteases, as demonstrated previously (70). The cleavage of the RCL led to the insertion of the RCL hinge region into the β-sheet A as an additional β-strand S4 (**Figure 2**). The 3D structure of Iripin-3 contained 367 amino acid residues. The first 19 residues, which basically corresponded to the signal peptide of the protein, were missing. Moreover, the region $_{356}$LRSGSFD$_{362}$, in which the cleavage occurred, could not be modelled in the Iripin-3 structure due to its absence in the electron-density map. To compare the tertiary structure of Iripin-3 with that of IRS-2, the molecular structure of Iripin-3 was superposed with Cα atoms of IRS-2 with root-mean-square deviation of 0.8085 Å. The secondary structure elements were well conserved in both serpins, but there was a certain degree of divergence in disordered loop regions (**Figure 1B**).

Iripin-3 Is Most Closely Related to Serpins From *I. scapularis*

The BLASTP search of the GenBank non-redundant protein sequences identified three *I. scapularis* serpins (accession numbers XP_029826754.1, EEC19555.1, and AAV80788.1) whose sequences were highly similar to the Iripin-3 sequence (percentage identities 95.4%, 94.9%, and 93.6%, respectively). These homologs have not been functionally characterized. The phylogenetic relationship of Iripin-3 with 26 tick serpins, whose function was deciphered either by using recombinant protein or at least by gene knockdown via RNA interference in ticks, was determined by using the maximum likelihood method and JTT matrix-based model. The resulting phylogenetic tree, with human alpha-1-antitrypsin as an outgroup, showed two distinct groups of tick serpins (**Figure 3A**). The first group at the bottom of the tree included eight serpins without a signal peptide with presumably intracellular function (**Figure 3A**). Notably, these serpins usually contained one or more cysteines and methionines in their RCL (**Figure 3B**). The second, larger group at the top of the tree comprised 19 serpins with a signal peptide, including Iripin-3 (**Figure 3A**). Iripin-3 formed a small branch with one serpin from *I. scapularis* (IxscS-1E1) and one serpin from *I. ricinus* (IRS-2) (**Figure 3A**). In addition to the construction of the phylogenetic tree, we aligned the RCLs of the serpins used in the phylogenetic analysis (**Figure 3B**). Serpins that clustered together usually had similar RCLs, and the RCL of Iripin-3 resembled that of IxscS-1E1 (**Figure 3B**).

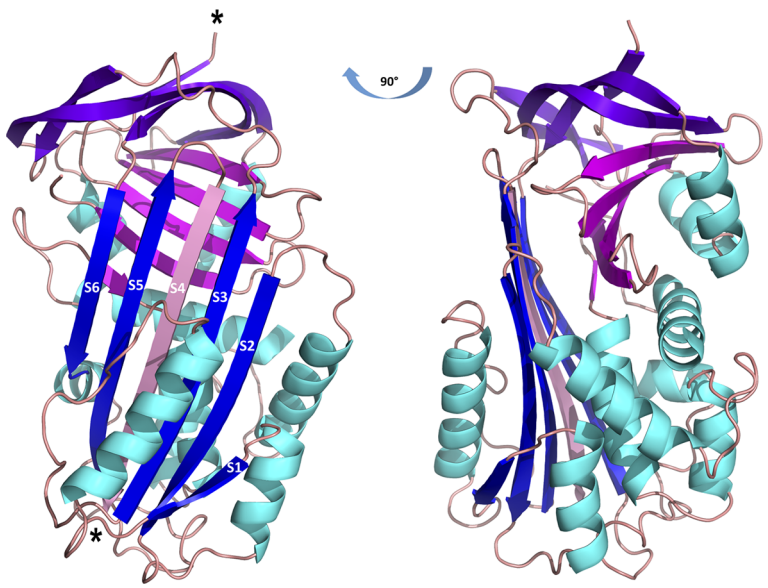

FIGURE 2 | Cartoon representation of the structure of cleaved Iripin-3. α-helices are colored cyan, β-sheet A is blue, β-sheet B is magenta, β-sheet C is purple, and loops are colored wheat. The insertion of the RCL hinge region between β-strands S3 and S5 (depicted in blue) resulted in the formation of an additional β-strand S4 (depicted in pink). Cleavage sites are marked with asterisks.

Iripin-3 Is Expressed in Feeding Ticks and Is Secreted Into Tick Saliva

In order to see how *iripin-3* expression changes during blood feeding, nymphal and adult ticks were allowed to feed on blood from host animals for various periods of time, and the amount of *iripin-3* transcript in tick tissues was subsequently determined by RT-qPCR. Overall, *iripin-3* expression was significantly induced in response to blood feeding in nymphs as well as in the salivary glands and ovaries of adult females (**Figure 4A**). In adults, the highest levels of *iripin-3* mRNA were detected in the salivary glands (**Figure 4A**). To prove the presence of Iripin-3 protein in tick saliva, we collected saliva from ticks that were feeding for 6 to 7 days on guinea pigs. By ELISAs, markedly higher optical density values were obtained after exposure of tick saliva to anti-Iripin-3 serum than to pre-immune serum (**Figure 4B**), suggesting that Iripin-3 is a salivary protein. This result was further confirmed by western blotting. Rabbit pre-immune serum did not recognize recombinant Iripin-3, and there was no band of appropriate size (around 42 kDa) in tick saliva (**Figure 4C**). Conversely, the use of anti-Iripin-3 serum led to the recognition of recombinant Iripin-3 and appearance of an approximately 45 kDa band in tick saliva, which might represent native Iripin-3 (**Figure 4D**). The difference in the sizes of native and recombinant Iripin-3 was probably caused by the fact that native Iripin-3 is glycosylated, whereas recombinant Iripin-3 was prepared in the *E. coli* expression system and therefore lacks glycosylation. The other bands with sizes greater or less than 45 kDa that appeared in the lanes with tick saliva after exposure of membranes to either pre-immune serum or anti-Iripin-3 serum are most likely a result of non-specific binding of antibodies to some components of tick saliva (**Figures 4C, D**).

Iripin-3 Primarily Inhibits Kallikrein and Matriptase

An initial screen for Iripin-3 inhibitory activity was carried out against 17 different serine proteases. Statistically significant reductions in enzymatic activity were observed for ten proteases, but only six of these, namely kallikrein, matriptase, trypsin, plasmin, thrombin, and FVIIa, had their proteolytic activity reduced by >20% (**Figure 5A**). Iripin-3 formed covalent complexes, typical for the serpin "suicide" mechanism of inhibition (71), with kallikrein, matriptase, thrombin, and trypsin, as shown by SDS-PAGE (**Figure 5B**). There was no visible complex between Iripin-3 and plasmin on the gel (**Figure 5B**). It is possible that the complex was hidden within an approximately 70 kDa protein band, which was also present in the lane with plasmin only (**Figure 5B**). Moreover, no SDS- and heat-stable complex was formed between Iripin-3 and FVIIa in the absence or presence of tissue factor under given conditions (**Supplementary Figure 1**), suggesting Iripin-3 probably does not reduce the proteolytic activity of FVIIa through the classic serpin inhibitory mechanism. Finally, the second-order rate constants k_2 for the interactions between Iripin-3 and kallikrein, matriptase, thrombin, and trypsin were measured by a discontinuous method under pseudo first-order conditions. Iripin-3 most potently inhibited kallikrein with $k_2 = 8.46 \pm 0.51 \times 10^4\ M^{-1}\ s^{-1}$ (**Figure 5C**). The k_2 for the interactions between Iripin-3 and matriptase and trypsin were determined as $5.93 \pm 0.39 \times 10^4\ M^{-1}\ s^{-1}$ and $4.65 \pm 0.32 \times 10^4\ M^{-1}\ s^{-1}$, respectively (**Figures 5D, F**). Thrombin was inhibited by Iripin-3 with the lowest potency ($k_2 = 1.37 \pm 0.21 \times 10^3\ M^{-1}\ s^{-1}$) (**Figure 5E**). Interface analysis between the active sites of matriptase, thrombin, kallikrein and trypsin and the P4-P4′ part of Iripin-3 RCL revealed possible polar interactions that could indicate the binding selectivity of

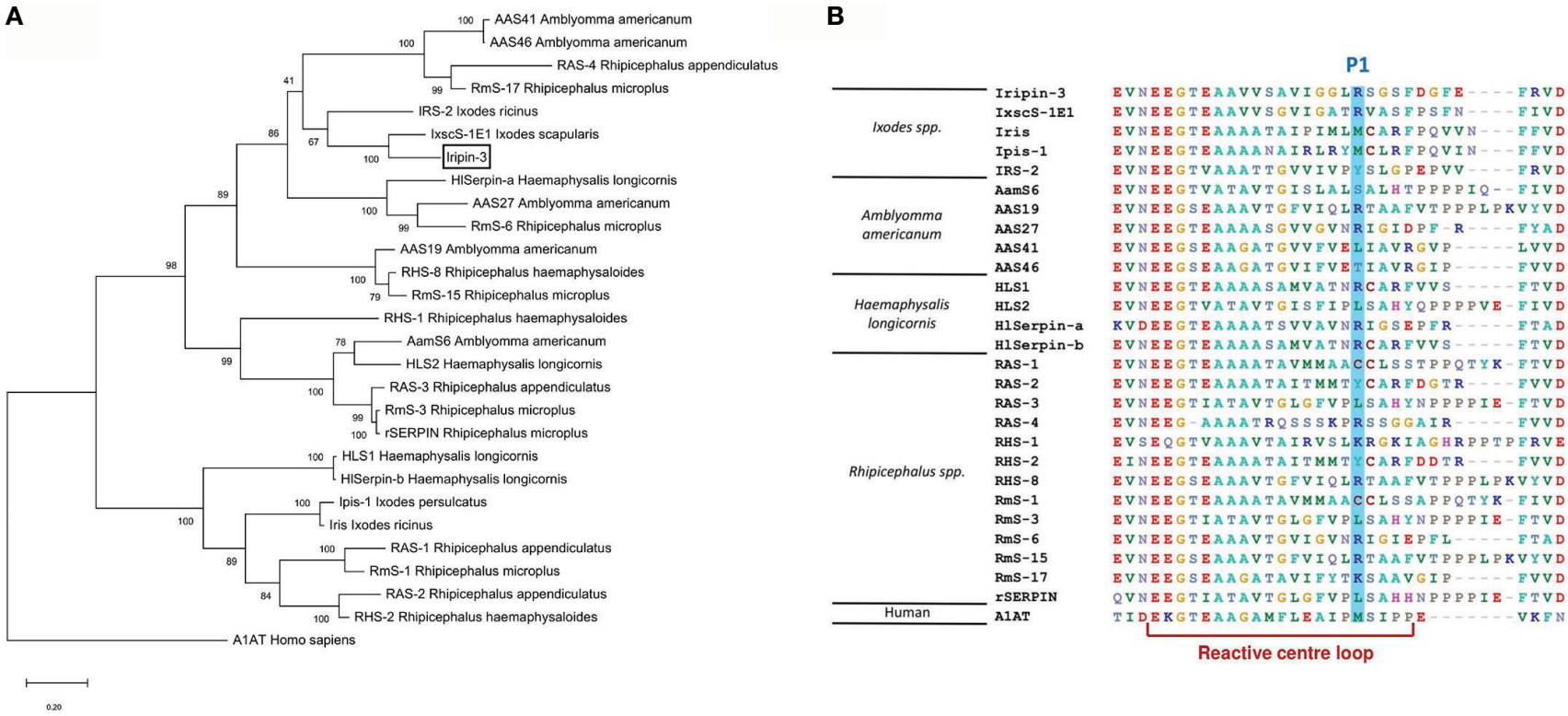

FIGURE 3 | Phylogenetic analysis of selected tick serpins. Protein sequences of previously characterized tick serpins were aligned and analyzed to determine phylogenetic relationships. **(A)** A phylogenetic tree was built using the maximum likelihood method and JTT matrix-based model. Alpha-1-antitrypsin (A1AT) was utilized as an outgroup to root the tree. The branch length represents the number of substitutions per site. The reliability of individual branches, assessed by bootstrapping, is expressed as a percentage of trees in which a given topology was present out of 1,000 replications. Iripin-3 is boxed. **(B)** Alignment of reactive center loop (RCL) regions of 27 tick serpins and one human serpin was performed using BioEdit. RCLs were determined based on the eight-residue pattern p17[E]-p16[E/K/R]-p15[G]-p14[T/S]-p13[X]-p12-9[AGS]-p8-1[X]-p1′-4′ [X] typical for inhibitory serpins (68). Amino acid residues at the predicted P1 site are highlighted in blue.

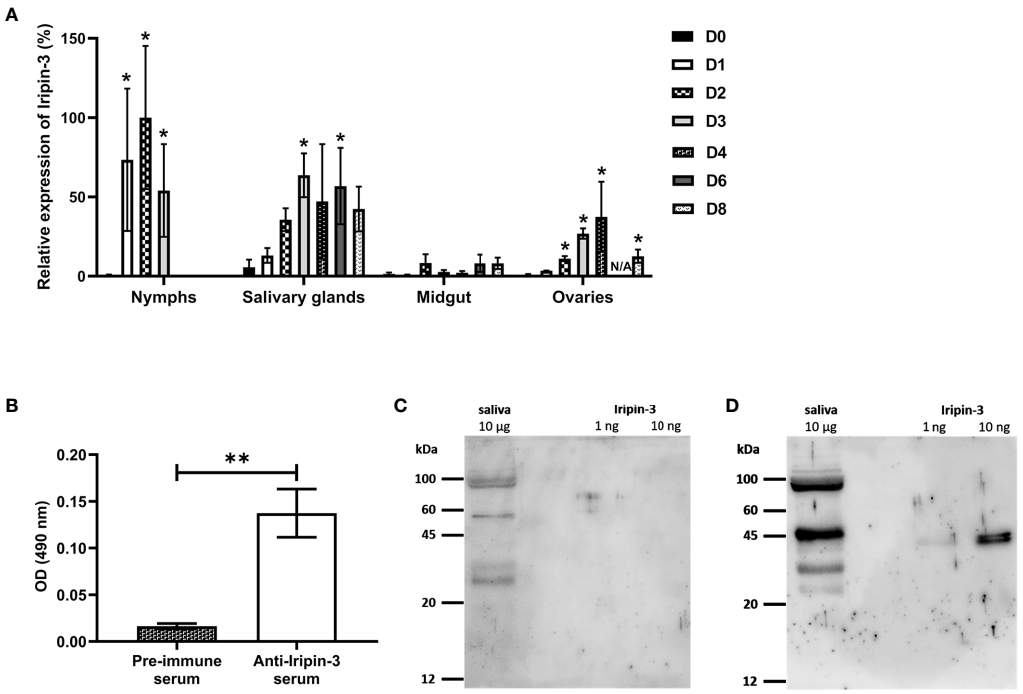

FIGURE 4 | *Iripin-3* transcription in *I. ricinus* ticks is increased in response to blood feeding, and Iripin-3 protein is present in the saliva of feeding ticks. **(A)** *Iripin-3* mRNA expression in nymphs and in the salivary glands, midguts and ovaries of adult females feeding for 1 (D1), 2 (D2), 3 (D3), 4 (D4), 6 (D6), and 8 (D8) days or not feeding at all (D0). In nymphs, the last column represents fully engorged ticks that completed their blood meal in 3 or 4 days. N/A – data not available. Relative expression values were calculated using the ΔΔCt (Livak) method (60), with *rps4* serving as a reference gene. A group with the highest *iripin-3* expression (nymphs feeding for 2 days) was utilized as a calibrator during calculations, and its expression value was set to 100%. Data are presented as mean of three biological replicates ± SEM. Statistically significant induction ($p < 0.05$) of *iripin-3* expression as compared to unfed ticks is marked with an asterisk. **(B)** ELISA results expressed as optical density (OD) values measured after exposure of tick saliva to either rabbit pre-immune serum or rabbit antiserum to Iripin-3. Data are presented as mean ± SEM of three values (**$p < 0.01$). **(C, D)** Tick saliva (10 µg) and Iripin-3 (1 ng or 10 ng) were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with rabbit pre-immune serum **(C)** or rabbit antiserum to Iripin-3 **(D)**.

Iripin-3 for target proteases (**Supplementary Figure 2**). The strongest interaction with the catalytic triad was calculated for matriptase, followed by trypsin, kallikrein and thrombin (data not shown). According to this analysis, thrombin and kallikrein should be inhibited by Iripin-3 with similar potency. This, however, was not supported by enzyme-substrate kinetic analyses (**Figures 5C–F**), in which kallikrein displayed 60 times higher k_2 value than thrombin. Therefore, the specificity of Iripin-3 is probably dependent on more factors. As shown in **Supplementary Figure 3**, matriptase and trypsin have open and shallow active sites, easily accessible to various substrates, including Iripin-3 RCL. Thrombin and kallikrein, on the other hand, possess narrower and deeper cavities with the catalytic triad (**Supplementary Figure 3**). It is possible that some subtle differences in spatial arrangement hinder the access of Iripin-3 RCL to the thrombin's active site, while facilitating its access to the kallikrein's active site cleft.

Iripin-3 Prolongs Plasma Clotting Time in the Prothrombin Time Assay

Since tick serpins commonly inhibit the host coagulation system (72), we tested the effect of Iripin-3 on the extrinsic coagulation pathway, intrinsic coagulation pathway, and common coagulation pathway by using prothrombin time (PT), activated partial thromboplastin time (aPTT), and thrombin time (TT) tests, respectively (73). Iripin-3 at 6 µM final concentration did not significantly prolong plasma clotting time in the aPTT and TT assays (data not shown). However, there was a statistically significant delay in blood clot formation in the PT test when plasma was treated with 1.5, 3, and 6 µM Iripin-3 (**Figure 6**). The highest Iripin-3 concentration prolonged the prothrombin time by 8.8 s when compared to control plasma (**Figure 6**). These results therefore indicate that Iripin-3 slightly inhibits the extrinsic pathway while not affecting the intrinsic and common pathways of blood coagulation.

Iripin-3 Decreases Production of IL-6 by BMDMs

Serpins secreted in tick saliva can facilitate blood meal uptake not only by inhibiting coagulation but also by suppressing host inflammatory responses (37, 72, 74). Therefore, we next investigated whether Iripin-3 attenuates pro-inflammatory cytokine production by LPS-stimulated BMDMs. The production of TNF, IL-6, and IL-1β was assessed at the mRNA level by RT-qPCR as well as at the protein level by ELISA. Iripin-

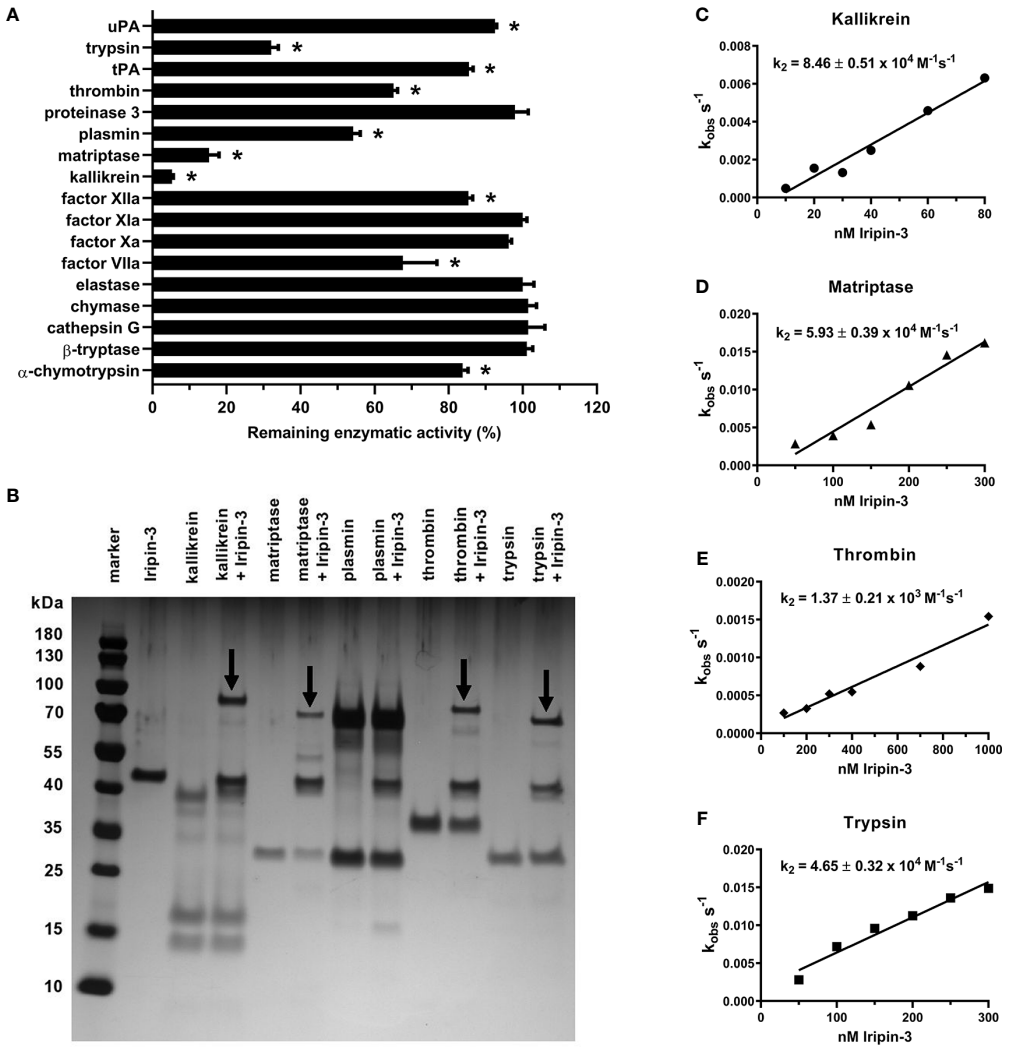

FIGURE 5 | Iripin-3 suppresses the enzymatic activities of kallikrein, matriptase, thrombin, and trypsin through the classic serpin inhibitory mechanism. **(A)** The residual enzymatic activities of 17 selected serine proteases in the presence of 400 nM Iripin-3. The experiment was performed in triplicate, and data are expressed as mean ± SEM. The enzymatic activities of individual proteases in the absence of Iripin-3 (control groups) were considered as 100%, and differences between control groups and Iripin-3-treated groups were analyzed by the unpaired two-tailed *t*-test. Enzymes labelled with an asterisk were inhibited with statistical significance ($p < 0.05$). **(B)** Formation of SDS- and heat-stable complexes between Iripin-3 and kallikrein, matriptase, plasmin, thrombin, and trypsin. Proteins were resolved on 4 to 12% NuPAGE Bis-Tris gels and visualized by silver staining. Covalent complexes between Iripin-3 and target proteases are marked with black arrows. **(C–F)** The apparent first-order rate constant k_{obs} was plotted against Iripin-3 concentration, and linear regression was performed to obtain the line of best fit. The slope of the line represents the second-order rate constant k_2 for the inhibition of kallikrein **(C)**, matriptase **(D)**, thrombin **(E)**, and trypsin **(F)** by Iripin-3. For each determination, the standard error of the slope is given.

3 caused a dose-dependent and statistically significant reduction in the transcription of all three genes (**Figures 7A–C**). However, decreases in the transcription of *Tnf* and *Il1b* did not result in corresponding changes in the concentrations of these two pro-inflammatory cytokines at the protein level (**Figures 7D, F**). Conversely, Iripin-3 was an efficient inhibitor of both IL-6 synthesis and secretion (**Figure 7E**).

Iripin-3 Impairs B and T Cell Viability *In Vitro*

In addition to inhibiting innate immune mechanisms, tick serpins can alter the host adaptive immune response (35, 37, 72). First, we tested whether Iripin-3 had an effect on B and T lymphocyte viability. Incubation of splenocytes derived from OT-II mice for 20 h in the presence of two different concentrations of Iripin-3 (3 μM and 6 μM) resulted in a pronounced dose-dependent reduction in the viability of both B cells ($CD45^+$ $CD19^+$ splenocytes) and T cells ($CD45^+$ $CD3e^+$ splenocytes), with B cell survival more negatively affected by the serpin presence than T cell survival (**Figures 8A–D**). B and T cell viability was impaired irrespective of whether the splenocytes were left unstimulated or were stimulated with OVA peptide (**Figures 8C, D**). Conversely, Iripin-3 did not reduce the viability of BMDMs or dendritic cells (**Supplementary Figures 4A, B**),

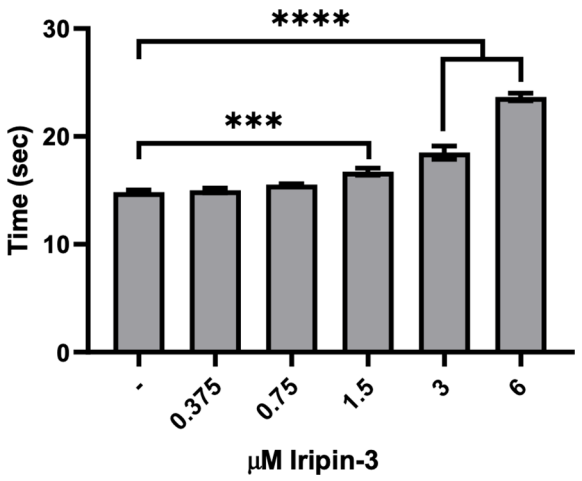

FIGURE 6 | Iripin-3 inhibits the extrinsic pathway of blood coagulation. Human plasma was treated with no Iripin-3 or with 0.375, 0.75, 1.5, 3, and 6 μM Iripin-3 and the time required for blood clot formation in the prothrombin time assay was subsequently determined on a coagulometer. Data are presented as mean ± SEM of three independent experiments (***p < 0.001, ****p < 0.0001).

and the viability of LPS-activated neutrophils was impaired only in the presence of the highest (6 μM) concentration of Iripin-3 (**Supplementary Figure 4C**). Therefore, Iripin-3 might selectively induce B and T cell death. To investigate the possibility that Iripin-3 triggers lymphocyte apoptosis, we measured active caspase-3 levels in both unstimulated and OVA peptide-stimulated splenocytes. Treatment of splenocytes with Iripin-3 did not lead to a statistically significant increase in the level of active caspase-3 (**Figures 8E, F**). Therefore, Iripin-3 probably does not induce B and T cell death through activation of a caspase-3-dependent pathway.

Iripin-3 Inhibits *In Vitro* CD4$^+$ T Cell Proliferation

Since Iripin-3 reduced T cell viability, we tested whether it also affected the survival and proliferation of CD4$^+$ helper T cells. OT-II splenocytes were pre-incubated with 3 μM or 6 μM Iripin-3 for 2 h before being stimulated with OVA peptide for 72 h. Propidium iodide staining in combination with the application of anti-CD4 antibody revealed a lower percentage of live CD4$^+$ cells in Iripin-3-treated groups than in the control group (**Figure 9A**), suggesting Iripin-3 has a negative effect on CD4$^+$ T cell viability. After the exclusion of dead cells, we assessed the

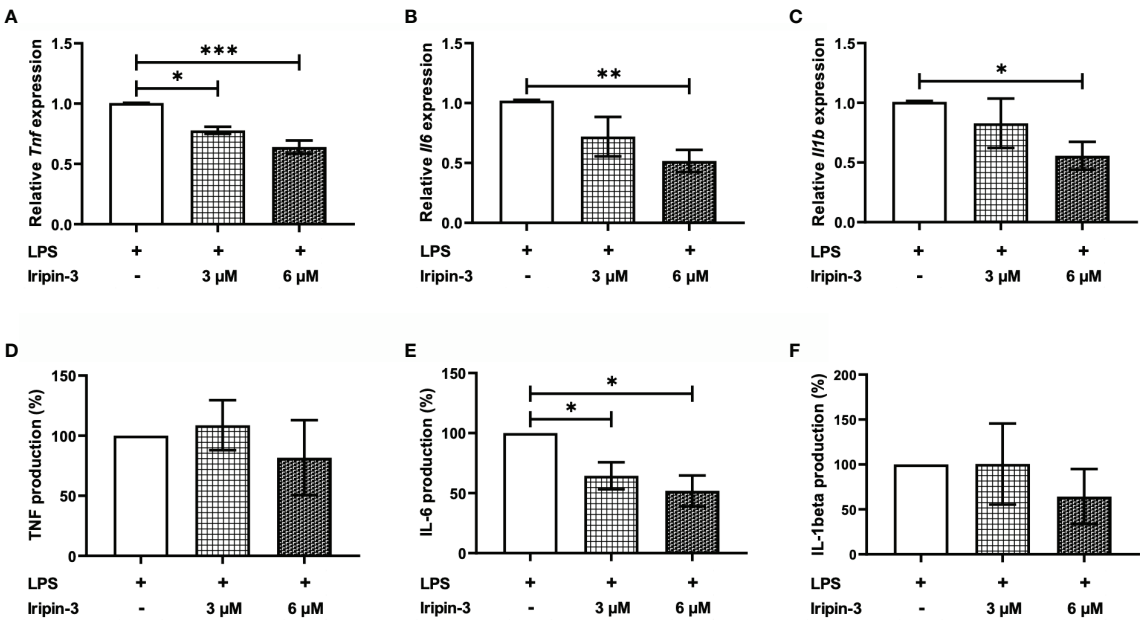

FIGURE 7 | Iripin-3 inhibits the expression of pro-inflammatory cytokines in LPS-stimulated BMDMs. Macrophages derived from bone marrow cells isolated from C57BL/6N mice were pre-incubated with 3 μM or 6 μM Iripin-3 for 40 min and were then stimulated with LPS (100 ng/ml) for 24 h. **(A–C)** At the end of 24 h incubation, cells were harvested for RNA extraction and the expression of *Tnf* **(A)**, *Il6* **(B)**, and *Il1b* **(C)** was determined by RT-qPCR. Relative expression values were calculated using the delta-delta Ct (Livak) method (60), with *Gapdh* serving as a reference gene. Cells incubated only in the presence of LPS were utilized as a calibrator during calculations. Data are presented as mean ± SEM of four independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001). **(D–F)** Supernatants were collected, and TNF, IL-6, and IL-1β concentrations in these supernatants were measured by sandwich ELISA. TNF **(D)**, IL-6 **(E)**, and IL-1β **(F)** production by Iripin-3-treated BMDMs is expressed as the percentage of the cytokine production by control macrophages, since there were large differences in the concentrations of the same cytokine between three independent repeats of the experiment. Data are expressed as mean ± SEM, and statistically significant differences (p < 0.05) are marked with an asterisk.

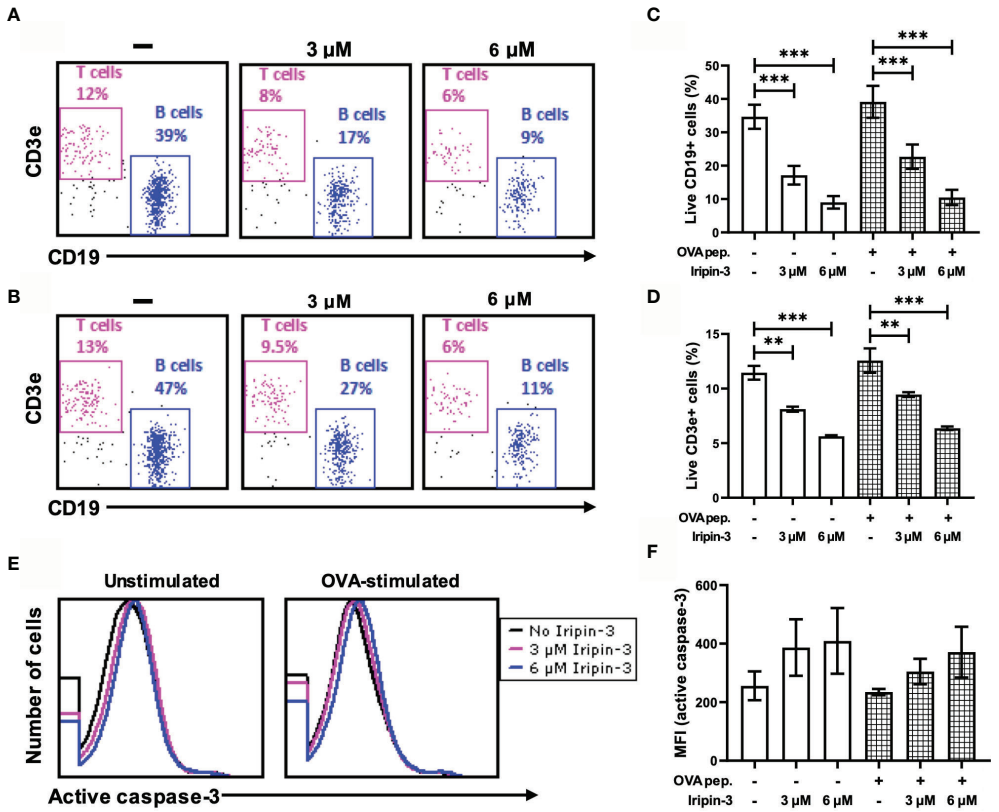

FIGURE 8 | Iripin-3 reduces B and T cell viability and does not significantly alter active caspase-3 levels. **(A, B)** Dot plots depicting the percentage of live $CD45^+CD19^+$ cells (B cells) and live $CD45^+CD3e^+$ cells (T cells) in unstimulated splenocytes **(A)** or OVA peptide-stimulated splenocytes **(B)**. Splenocytes were not treated with Iripin-3 (left) or were treated with 3 µM (middle) or 6 µM (right) Iripin-3. **(C, D, F)** The percentage of live B cells **(C)**, live T cells **(D)**, and median fluorescence intensity (MFI) corresponding to the level of active caspase-3 **(F)** after incubating the splenocytes for 20 h in the absence of Iripin-3 or in the presence of 3 µM and 6 µM Iripin-3. The cells were left either unstimulated or were stimulated with 100 ng/ml of OVA peptide. Data are presented as mean ± SEM of three independent experiments (**$p < 0.01$, ***$p < 0.001$). **(E)** Histograms showing the level of active caspase-3 in either unstimulated splenocytes (left) or splenocytes stimulated with OVA peptide (right). Splenocytes were incubated for 20 h without Iripin-3 or were treated with 3 µM or 6 µM Iripin-3.

proliferation of $CD4^+$ T cells. Unstimulated $CD4^+$ cells did not proliferate at all (**Figure 9C**), whereas addition of OVA peptide triggered proliferation in approximately 95% of cells (**Figures 9B, D**). Treatment with Iripin-3 caused a dose-dependent decrease in $CD4^+$ splenocyte proliferation (**Figure 9B**). While about 84% of cells proliferated in the presence of 3 µM Iripin-3 (**Figures 9B, E**), only 35% of cells were capable of proliferation after addition of 6 µM Iripin-3 (**Figures 9B, F**). Therefore, Iripin-3 impairs both the viability and proliferation of $CD4^+$ T cells.

Iripin-3 Inhibits a Th1 Immune Response and Promotes Differentiation of Regulatory T Cells (Tregs) *In Vitro*

To examine whether Iripin-3 alters the differentiation of naïve $CD4^+$ T cells into Th1, Th2, Th17, or Treg subpopulations, we evaluated the expression of transcription factors T-bet, GATA-3, RORγt, and Foxp3 in OVA peptide-stimulated $CD4^+$ splenocytes by RT-qPCR and flow cytometry. T-bet, GATA-3, RORγt, and Foxp3 are considered lineage-specifying transcription factors that govern Th1, Th2, Th17, and Treg differentiation, respectively (75–79). Iripin-3 markedly and dose-dependently inhibited the expression of T-bet in $CD4^+$ T cells at both the mRNA and protein levels (**Figures 10A–C**). Since T-bet controls *Ifng* transcription (76), we also tested the ability of Iripin-3 to inhibit the production of this hallmark Th1 cytokine. As with T-bet, Iripin-3 induced a pronounced and dose-dependent reduction in the percentage of $CD4^+$ T cells producing IFN-γ (**Figures 10D, E**). Despite the inhibition of the Th1 immune response, we did not observe significant changes in the differentiation of T cells into Th2 or Th17 subpopulations (**Figures 10F–K**). GATA-3 expression was slightly increased only in $CD4^+$ T cells treated with 3 µM Iripin-3 (**Figures 10G, H**). Similarly, both Iripin-3 concentrations induced only a small and non-significant increase in the percentage of $CD4^+$ T cells expressing RORγt (**Figures 10J, K**). Finally, Iripin-3 moderately stimulated the expression of Foxp3 at both the mRNA and protein levels (**Figures 10L–N**). Therefore, Iripin-3 might

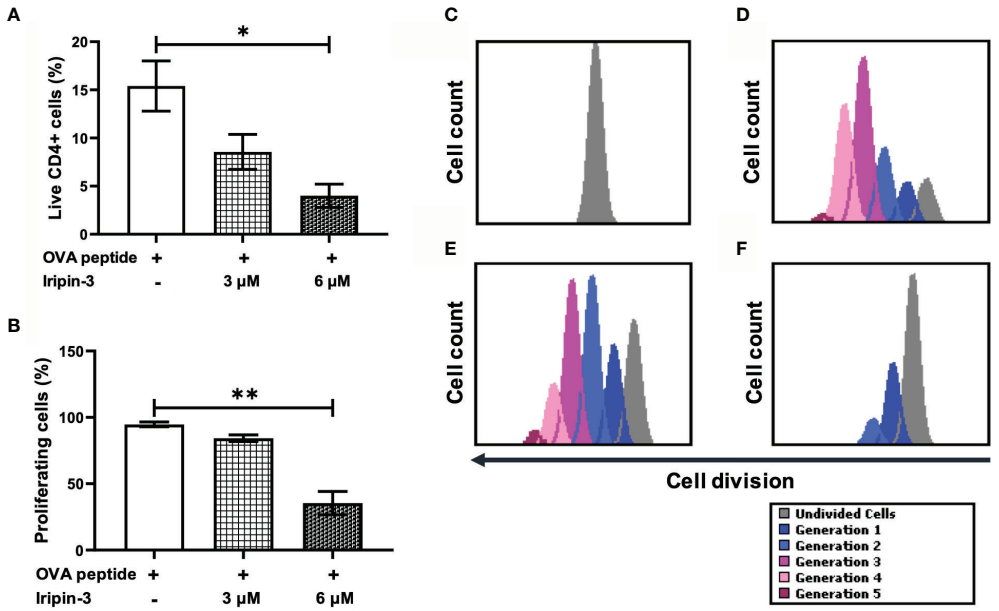

FIGURE 9 | Iripin-3 impairs the survival and proliferation of CD4$^+$ splenocytes. **(A, B)** The percentage of live CD4$^+$ cells **(A)** and the percentage of proliferating live CD4$^+$ cells **(B)** after exposure to 3 µM or 6 µM Iripin-3. Cells not treated with Iripin-3 were used as control. After 2 h pre-incubation with Iripin-3, cells were cultured in the presence of OVA peptide (100 ng/ml) for 72 h. Data are presented as mean ± SEM of three independent experiments (*$p < 0.05$, **$p < 0.01$). **(C–F)** Histograms showing the number of live CD4$^+$ cells that managed to divide once (blue), twice (light blue), 3 times (pink), 4 times (rose), 5 times (plum), or did not divide at all (gray) within the 72 h culture period. Cells were incubated in the absence of Iripin-3 and OVA peptide **(C)**, in the presence of OVA peptide only **(D),** or were treated with the combination of 3 µM Iripin-3 and OVA peptide **(E)** or 6 µM Iripin-3 and OVA peptide **(F)**.

induce the differentiation of Tregs in addition to inhibiting Th1 cell development.

Iripin-3 Is Not Essential for Feeding Success of *I. ricinus* Nymphs

Since *iripin-3* expression is induced in nymphs in response to blood feeding, we decided to assess the role of this serpin in the blood-feeding process by silencing *iripin-3* expression in nymphs via RNA interference. *Iripin-3* expression in *iripin-3* dsRNA-treated ticks was 34% when compared to *gfp* dsRNA-treated ticks (data not shown), suggesting that the knockdown of the target gene was successful. Despite diminished *iripin-3* expression, the time course of blood feeding and overall feeding success (i.e. the number of nymphs that reached full engorgement) did not significantly differ between control ticks and *iripin-3* dsRNA-treated ticks (**Supplementary Table 4**). The weight of fully engorged nymphs was not significantly affected by *iripin-3* silencing as well (**Supplementary Table 4**). Therefore, we can conclude that the deficiency of Iripin-3 alone is not sufficient to impair the blood meal acquisition and processing by nymphal *I. ricinus* ticks.

DISCUSSION

Tick saliva contains hundreds to thousands of proteins from diverse protein families (80). These salivary proteins are differentially expressed over the course of blood feeding and enable ticks to feed to repletion by maintaining blood fluidity and suppressing host defense responses (80). Serpins form one of four serine protease inhibitor families that have been discovered in ticks (72). Serpins are particularly intriguing to study, not only due to their unique trapping inhibitory mechanism but also because they regulate a variety of physiological processes in many organisms. The functional diversity of the serpin superfamily is exemplified by the widely studied human serpins, which have been shown to regulate blood pressure, transport hormones, and control blood coagulation, fibrinolysis, angiogenesis, programmed cell death, inflammation, or complement activation (81–84). We presume that ticks employ some of their serpins to modulate host defenses, as evidenced by several tick serpins with anti-platelet, anti-coagulant, anti-inflammatory, and/or immunomodulatory properties that have been shown to be secreted via saliva into the host (34–37, 72).

Here we determined the structure and partially deciphered the function of *Ixodes ricinus* serpin Iripin-3 by using several *in vitro* models. The size (377 amino acids), molecular weight (42 kDa), and 3D structure of Iripin-3, consisting of three β-sheets, ten α-helices, and a cleaved RCL, correspond to the structural parameters of typical serpins (18, 20, 71). *Iripin-3* expression was induced by blood feeding in both nymphs and adult females, suggesting Iripin-3 contributes to feeding success in both developmental stages. Of the three organs of adult ticks, the highest levels of *iripin-3* transcript were detected in the

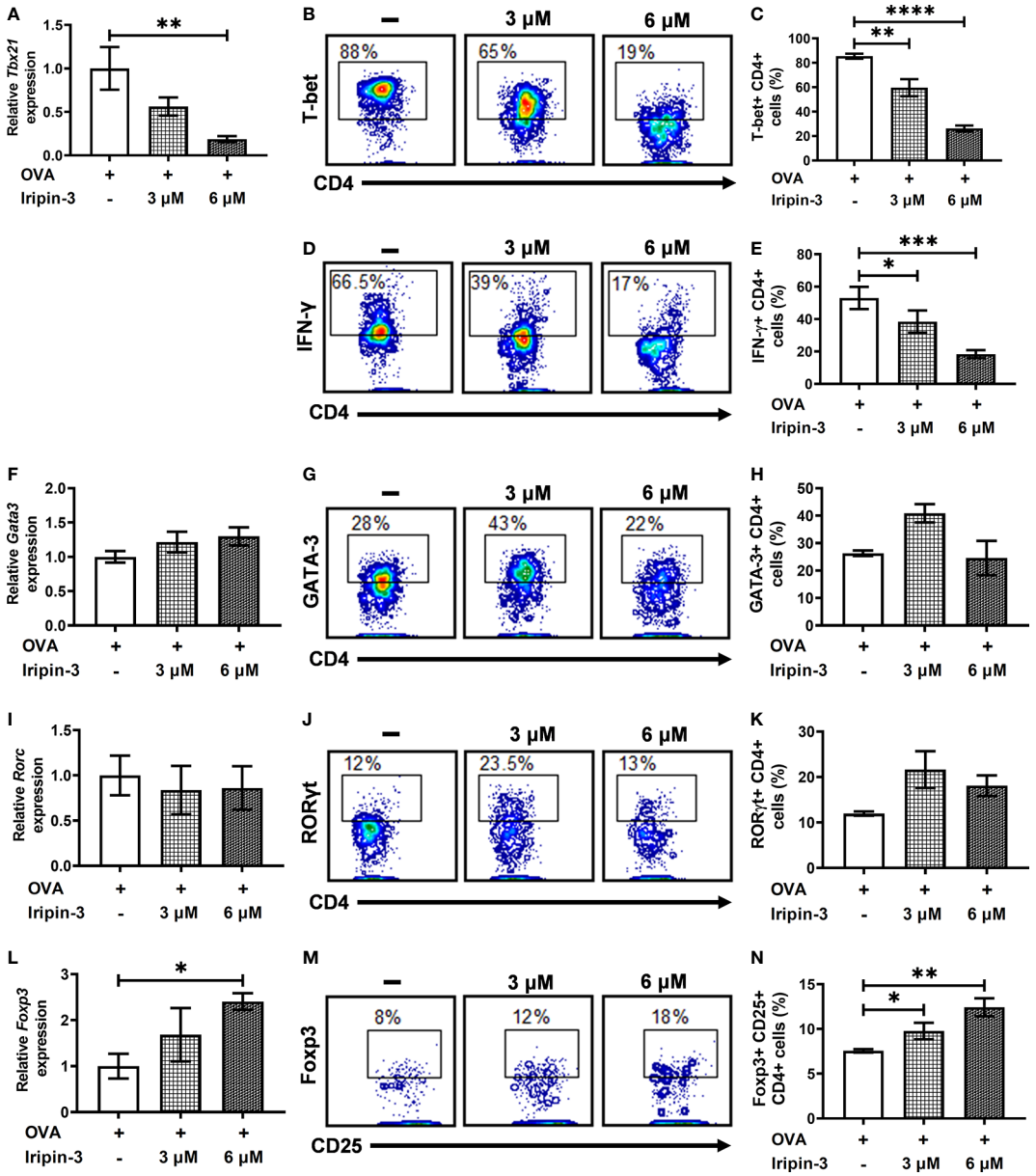

FIGURE 10 | Iripin-3 alters the expression of CD4$^+$ T cell transcription factors at both the mRNA and protein levels. **(A, F, I, L)** Expression of *Tbx21* **(A)**, *Gata3* **(F)**, *Rorc* **(I)**, and *Foxp3* **(L)** in CD4$^+$ cells stimulated with OVA peptide for 72 h. Cells were untreated with Iripin-3 or were treated with 3 µM or 6 µM Iripin-3. Cells incubated only in the presence of OVA peptide were utilized as a calibrator during calculations of relative expression values. Data are presented as mean ± SEM of four independent experiments (* $p < 0.05$, ** $p < 0.01$). **(B, D, G, J, M)** Representative contour plots showing the proportion of OVA peptide-stimulated CD4$^+$ splenocytes expressing T-bet **(B)**, IFN-γ **(D)**, GATA-3 **(G)**, RORγt **(J)** and the combination of CD25 and Foxp3 **(M)**. The cells were incubated in the absence of Iripin-3 (left) or in the presence of two different Iripin-3 concentrations: 3 µM (middle) and 6 µM (right). **(C, E, H, K, N)** The percentage of CD4$^+$ T cells producing the cytokine IFN-γ **(E)** and expressing transcription factors T-bet **(C)**, GATA-3 **(H)**, RORγt **(K)**, and Foxp3 together with CD25 **(N)**. Cells were cultured in the presence of Iripin-3 (3 µM or 6 µM) and OVA peptide for 72 h. Cells incubated without Iripin-3 were used as control. Data are presented as mean ± SEM of three or four independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

salivary glands. The presence of Iripin-3 protein in the saliva of partially engorged adults was confirmed by immunodetection. Thus, we can assume that Iripin-3 is secreted via saliva into the tick attachment site where it interferes with host anti-tick defenses. Statistically significant increase of *iripin-3* expression in response to blood feeding occurred not only in the salivary glands but also in the ovaries of adult ticks, which indicates that Iripin-3 might be somehow involved in the reproductive process.

The role of serpins in tick reproduction has been evidenced recently by *Rhipicephalus haemaphysaloides* serpin RHS-8, the knockdown of which impaired oocyte maturation due to the inability of oocytes to uptake adequate amount of vitellogenin (45).

The presence of the basic amino acid residue arginine at the P1 site of the Iripin-3 RCL indicates that Iripin-3 might inhibit trypsin-like rather than chymotrypsin-like or elastase-like serine proteases (69, 85). Indeed, out of 17 selected serine proteases, Iripin-3 most potently inhibited trypsin-like serine proteases kallikrein and matriptase and exhibited weaker inhibitory activity against trypsin, thrombin, plasmin, and factor VIIa. Kallikrein participates in the activation of the intrinsic blood coagulation pathway, promotes fibrinolysis, and is also responsible for the release of the potent inflammatory mediator bradykinin, which further induces vasodilation, increases vascular permeability, and evokes pain and itch (86, 87). Matriptase is a type II transmembrane serine protease that is primarily expressed in epithelial cells and is essential for the maintenance of skin barrier function (88). Moreover, matriptase seems to be involved in cutaneous wound healing (89, 90) and might contribute to the amplification and perpetuation of the inflammatory response through the activation of protease-activated receptor-2 (PAR-2) (91). Therefore, we speculate that Iripin-3-mediated inhibition of kallikrein and matriptase contributes to tick feeding success by suppressing the inflammatory response and consequent itch and pain and by impairing wound healing.

A phylogenetic analysis of 27 functionally characterized tick serpins revealed a close phylogenetic relationship between Iripin-3 and *I. scapularis* serpin IxscS-1E1. Both serpins possess arginine at the P1 site and inhibit trypsin and thrombin (30). However, while IxscS-1E1 prolonged plasma clotting time in aPTT and TT assays and had no effect on blood clot formation in the PT assay (30), Iripin-3 inhibited only the extrinsic coagulation pathway. This indicates that the Iripin-3-mediated inhibition of kallikrein and thrombin was not sufficient to significantly impair the intrinsic and common coagulation pathways. Other blood clotting factors (XIIa, XIa, Xa) involved in the intrinsic and common pathways were not markedly inhibited by Iripin-3. Several tick serpins are capable of inhibiting the common (and perhaps intrinsic) pathway of blood coagulation (28–31, 41, 92); however, none have shown any effect on the extrinsic coagulation pathway. The extrinsic coagulation pathway is initiated by damage to a blood vessel and subsequent formation of a FVIIa/tissue factor (TF) complex, which further activates factor X (93). In view of the fact that Iripin-3 exhibited weak inhibitory activity only in the PT test and not in the aPTT test or TT test, we hypothesized that it might target either FVIIa or TF, since these two proteins are the only unique components of the extrinsic pathway. FVIIa seemed to be a more likely target for Iripin-3 given that it is a serine protease (94), and some human serpins, such as antithrombin III or protein C inhibitor, have been shown to inhibit the proteolytic activity of FVIIa (95–97). In our hands, Iripin-3 did not form a covalent complex with FVIIa either in the absence or in the presence of TF. However, the proteolytic activity of FVIIa was reduced by approximately 30% in the presence of 400 nM Iripin-3 in the kinetic enzyme-substrate assay. Therefore, the prolongation of blood clot formation in the PT assay might be caused by the non-canonical inhibition of FVIIa by Iripin-3. Alternatively, a possible interaction between Iripin-3 and TF could also prevent FVIIa/TF complex formation, leading to a lower rate of FXa generation and inhibition of blood coagulation.

In addition to the inhibition of blood coagulation, Iripin-3 displayed anti-inflammatory activity *in vitro*, since it significantly and dose-dependently attenuated the production of pro-inflammatory cytokine IL-6 by LPS-stimulated bone marrow-derived macrophages. The decreased IL-6 production was probably caused by the inhibition of *Il6* transcription and not by reduced viability of macrophages, since the metabolic activity of macrophages remained unchanged in the presence of Iripin-3. Several tick serpins have been shown to inhibit IL-6 transcription and secretion (37–39, 74, 98), which can occur as a result of serpin-mediated inhibition of proteases such as cathepsin G and cathepsin B (37). However, the inhibition of pro-inflammatory cytokine production does not have to be dependent on serpin anti-protease activity because some serpins, like Iris and α-1-antitrypsin, can alter pro-inflammatory cytokine production by binding to immune cells via exosites (98, 99). An inflammatory environment with reduced IL-6 might favor differentiation of Tregs (100–102). Splenocytes, incubated in the presence of Iripin-3 for 72 h, increased the expression of Treg-specific transcription factor Foxp3 (77, 78), suggesting that Iripin-3 indeed induces the differentiation of naïve $CD4^+$ T cells into anti-inflammatory Tregs. Tregs would facilitate the suppression of the host immune response (103), which would be beneficial for feeding ticks. There is scarce evidence that tick saliva induces Treg differentiation (104, 105). The results of our *in vitro* assay indicate that salivary serpins could contribute to this particular activity of tick saliva.

Besides the reduction in IL-6 production and increase in Foxp3 expression, Iripin-3 caused a pronounced, dose-dependent decrease in B and T cell viability *in vitro*. This effect appears to be B and T cell-specific since macrophage and dendritic cell survival was not affected by Iripin-3 and the viability of LPS-stimulated neutrophils was slightly impaired only at the highest (6 μM) concentration of Iripin-3. Serpins usually protect cells from dying by reducing the proteolytic activity of enzymes (such as granzymes and caspases) involved in programmed cell death (106). However, certain serpins, e.g., kallikrein-binding protein, pigment epithelium-derived factor, or maspin, induce apoptosis of endothelial cells and some cancer cells through distinct mechanisms such as the activation of the Fas/FasL/caspase-8 signaling pathway or the permeabilization of the outer mitochondrial membrane followed by a loss of transmembrane potential (107–111). Active caspase-3 levels were only slightly and non-significantly increased in Iripin-3-treated splenocytes. Therefore, the induction of caspase-dependent apoptosis was not the main cause of impaired splenocyte viability. Various forms of caspase-independent cell death have been described such as autophagy, paraptosis, necroptosis, or necrosis (112, 113). Elucidation of the exact

mechanism behind the extensive splenocyte death in the presence of Iripin-3 is, however, beyond the scope of this paper.

I. ricinus saliva and salivary gland extracts inhibit T cell proliferation and suppress Th1 cell differentiation while simultaneously augmenting the Th2 immune response (114–117). Iripin-3 might contribute to this immunomodulatory effect of saliva, since in our *in vitro* assays it inhibited $CD4^+$ T lymphocyte proliferation and impaired the differentiation of naïve $CD4^+$ T cells into Th1 cells. Impaired Th1 cell generation was evidenced by decreased expression of the Th1 lineage-specifying transcription factor T-bet and a reduced percentage of $CD4^+$ T cells producing the hallmark Th1 cytokine IFN-γ. Several studies have reported inhibition of splenocyte and peripheral blood mononuclear cell proliferation in the presence of tick serpins (35, 37, 38, 40). Interestingly, the inhibition of mitosis observed in these studies was usually accompanied by decreased IFN-γ production (35, 38, 40), which might indicate, among other things, the suppression of Th1 cell differentiation. The causative mechanism of reduced cell proliferation and impaired Th1 cell differentiation in the presence of tick serpins remains unknown, but it could be associated with decreased production of certain cytokines such as IL-2, IL-12, and IFN-γ. In the case of Iripin-3, there might be a connection between the inhibition of cell proliferation and impaired viability of splenocytes, i.e., the mechanism behind B and T cell death could be also responsible for the suppression of $CD4^+$ T cell division. Iripin-3-mediated differentiation of naïve $CD4^+$ T cells into Tregs might also contribute to the reduction in $CD4^+$ T cell proliferation, since Tregs can inhibit cell multiplication by various mechanisms including the production of immunosuppressive cytokines TGF-β and IL-35, consumption of IL-2, and conversion of ATP to adenosine (103, 118).

It is worth mentioning that the Iripin-3 concentrations used in *in vitro* experiments (3 μM and 6 μM) are probably higher than the amount of Iripin-3 at the tick feeding site. This fact, however, does not make the anticoagulant, ant-inflammatory and immunomodulatory activities of Iripin-3 observed *in vitro* physiologically irrelevant. Tick saliva is a complex mixture of proteins from the same or different protein families, and some of these salivary proteins can share the same function (119). Therefore, even a low concentration of one tick protein may be sufficient to achieve a desired effect at the tick attachment site if this protein acts in concert with other tick proteins (119). For instance, the ability of *I. ricinus* saliva to inhibit $CD4^+$ T cell proliferation is probably a result of combined action of more proteins with anti-proliferative properties, such as the serpins Iripin-3 and Iris, the cystatin Iristatin and the Kunitz domain-containing protein IrSPI (38, 120, 121). That *I. ricinus* saliva may contain other proteins possessing Iripin-3-like activities was demonstrated by the RNA interference experiment. *Iripin-3* knockdown did not significantly affect the overall feeding success, time course of blood feeding and weight of fully engorged nymphs, which indicates that other similarly acting salivary proteins might compensate for the loss of *iripin-3* expression.

It is also important to note that native Iripin-3 is most likely glycosylated. However, recombinant Iripin-3 was prepared in an *E. coli* expression system, and therefore it lacks glycosylation. Glycosylation has been shown to reduce the propensity of serpins for polymerization (122) and increase the stability and half-life of circulating serpins by conferring resistance to proteolytic degradation (123, 124). The impact of glycosylation on the biological function of serpins is less clear. Recombinant Iripin-3 inhibited the proteolytic activity of some serine proteases, suggesting that its functions dependent on anti-protease activity (like anticoagulant properties) may not be affected by missing glycosylation. However, the absence of glycosylation might have an impact on anti-inflammatory and immunomodulatory activities of Iripin-3 mediated by its binding to cell surfaces and soluble immune mediators. For example, only glycosylated, but not non-glycosylated, α-1-antitrypsin was capable of binding IL-8, thus inhibiting IL-8-CXCR1 interaction (125).

CONCLUSION

To conclude, Iripin-3 is a pluripotent salivary protein secreted by *I. ricinus* ticks via saliva into the feeding site, where it might suppress various aspects of host anti-tick defenses. The attenuation of IL-6 production, suppression of $CD4^+$ T cell proliferation, and inhibition of Th1 immune responses have also been observed with other tick serpins and are consistent with the previously reported immunomodulatory effects of *I. ricinus* saliva and salivary gland extracts (114–117). On the other hand, our study is the first to describe the inhibition of the extrinsic pathway of blood coagulation, impaired B and T cell survival, and the induction of Treg differentiation by a tick serpin. The pluripotency and redundancy in Iripin-3 functions are consistent with the theory about the importance of these protein features for successful tick feeding (119). Although several distinct *in vitro* activities of Iripin-3 were observed in this study, their physiological relevance, mechanisms behind them and potential of Iripin-3 to be a candidate for drug or vaccine development remain to be determined. Therefore, further *in vivo* experiments and mechanistic studies are needed to validate and elucidate the Iripin-3 functions described in this work.

DATA AVAILABILITY STATEMENT

The data sets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

All animal experiments were performed in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb. (ethics approval no. 34/2018) and protocols approved by the Ministry of Education, Youth and Sports of the Czech Republic (protocol no. 19085/2015-3) and the responsible committee of

the IP BC CAS. Pathogen-free *I. ricinus* ticks were obtained from the tick colony maintained at the IP BC CAS.

AUTHOR CONTRIBUTIONS

AC designed and performed experiments, analyzed data, and wrote the manuscript. JK, ZB, BK, LAM, HL, TP, ME, and IKS designed and performed experiments and analyzed data. MK edited the manuscript. JC directed the study, designed experiments, analyzed data, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.626200/full#supplementary-material

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REVIEW
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Tick Immune System: What Is Known, the Interconnections, the Gaps, and the Challenges

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Ticks are ectoparasitic arthropods that necessarily feed on the blood of their vertebrate hosts. The success of blood acquisition depends on the pharmacological properties of tick saliva, which is injected into the host during tick feeding. Saliva is also used as a vehicle by several types of pathogens to be transmitted to the host, making ticks versatile vectors of several diseases for humans and other animals. When a tick feeds on an infected host, the pathogen reaches the gut of the tick and must migrate to its salivary glands *via* hemolymph to be successfully transmitted to a subsequent host during the next stage of feeding. In addition, some pathogens can colonize the ovaries of the tick and be transovarially transmitted to progeny. The tick immune system, as well as the immune system of other invertebrates, is more rudimentary than the immune system of vertebrates, presenting only innate immune responses. Although simpler, the large number of tick species evidences the efficiency of their immune system. The factors of their immune system act in each tick organ that interacts with pathogens; therefore, these factors are potential targets for the development of new strategies for the control of ticks and tick-borne diseases. The objective of this review is to present the prevailing knowledge on the tick immune system and to discuss the challenges of studying tick immunity, especially regarding the gaps and interconnections. To this end, we use a comparative approach of the tick immune system with the immune system of other invertebrates, focusing on various components of humoral and cellular immunity, such as signaling pathways, antimicrobial peptides, redox metabolism, complement-like molecules and regulated cell death. In addition, the role of tick microbiota in vector competence is also discussed.

Keywords: cell-mediated immunity, immune signaling pathway, immune system, microbiota, tick-borne pathogen

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INTRODUCTION

Ticks (Acari: Ixodida) are ectoparasitic arthropods that obligatorily feed on the blood of a diverse list of vertebrate hosts, including mammals, birds, reptiles, and even amphibians. More than 950 tick species have been described to date, which, according to morphological and physiological characteristics, are divided into two main families, Ixodidae (hard ticks), comprising more than

75% of tick species, and Argasidae (soft ticks); a third family, known as Nuttalliellidae, is monospecific (1, 2). As a result of blood spoliation [a single ixodid adult female can ingest more than ~1 mL of blood (3)], the host can suffer from anemia, which negatively impacts the productivity of livestock and causes a huge economic burden worldwide. For example, the estimated annual losses due to reductions in weight gain and milk production caused by the cattle tick *Rhipicephalus microplus* are approximately 3.24 billion dollars in Brazil alone (4).

In addition to ingesting blood, ticks also secrete saliva into the host during feeding. Tick saliva, produced by their salivary glands, returns excess water and ions to the host, thereby concentrating the blood meal (5). Tick saliva contains an arsenal of bioactive molecules that modulate host hemostasis and immune reactions, thus enabling blood acquisition (6, 7). The antihemostatic and immunomodulatory properties of saliva can also facilitate the infection of pathogens that use saliva as a vehicle to be transmitted to the host during tick blood feeding (6, 8). Indeed, ticks are versatile vectors of viruses, bacteria, protozoans and nematodes, which cause life-threatening diseases to humans as well as to other animals, including livestock, pets, and wildlife (9). Among human diseases, we highlight Lyme disease, the most common tick-borne zoonosis, which is caused by spirochetes from the *Borrelia burgdorferi* sensu lato complex. After transmission by the bite of an infected tick, the typical clinical sign of Lyme disease is erythema migrans, but infection can spread and affect joints, heart, and the nervous system (10).

The first organ that a pathogen acquired within the blood meal interacts with is the tick gut (**Figure 1**). Then, the pathogen must colonize the gut epithelial cells and/or cross the gut epithelium to enter the hemocoel, an open body cavity filled with hemolymph, the fluid that irrigates all the tissues and organs in the tick. The pathogen must then reach the salivary glands. In each of these organs, the pathogen must counteract tick immune factors to be successfully transmitted through saliva to the vertebrate host in a subsequent blood-feeding (11). Some pathogens also have the ability to invade tick ovaries and can therefore be transovarially transmitted to progeny (**Figure 1**). Thus, elucidation of the immune factors involved in the interactions between ticks and tick-borne pathogens (TBPs) in each of these steps is essential to understand the biology of tick-transmitted diseases and may help to identify targets for the development of new strategies to block pathogen transmission. In this review, we present an update on humoral and cellular tick immunity components (**Figure 1**), including signaling pathways, antimicrobial peptides (AMPs), redox metabolism, complement-like proteins, and regulated cell death. Using a comparative approach with the immune system of other invertebrates, we highlight the challenges of studying tick immunity, the gaps, such as prophenoloxidase (PPO) and coagulation cascades, and the interconnections, such as immune system signaling pathway crosstalk. In addition, the role of tick microbiota in vector competence is also discussed.

A BRIEF HISTORY OF STUDIES ON THE IMMUNE SYSTEM OF ARTHROPODS

The first records of studies on arthropod disease date to the 19th century, when Louis Pasteur investigated the cause of brown dots on the cuticle of larvae of *Bombyx mori* that predestined larvae to death and affected silk production in France (12). In the 1980s, the isolation of several immune factors from the hemolymph of arthropods that have a large volume of hemolymph, such as larvae of dipteran and lepidopteran insects, horseshoe crabs and crayfish, was achieved. Indeed, the first animal AMP to be characterized was cecropin, isolated from the hemolymph of the moth *Hyalophora cecropia* (13). After that, AMPs were identified as important effectors of mammalian immunity (14, 15). In addition to AMPs, components of the PPO cascade from the hemolymph of *H. cecropia* (16), *B. mori* (17), and the crayfish *Pacifastacus leniusculus* (18) were also elucidated. Some years later, the components of the coagulation cascade, another important arthropod immune reaction, were characterized in *P. leniusculus* (19) and horseshoe crabs (20).

In the 1990s, relevant studies on the immune pathways that regulate AMP production were conducted using the fruit fly *Drosophila melanogaster* (hereafter referred to as *Drosophila*) as a model (21–24). Among them, we highlight the identification of a kappa B (κB)-binding region in the promotor region of certain insect AMP genes (25) and the identification of Toll receptors, posteriorly identified to be homologous to interleukin-1 receptor of mammals (26). Some years later, with the improvement of molecular techniques and funding by major support agencies, such as the MacArthur Foundation, the World Health Organization, and the National Institutes of Health (USA), studies on the arthropod immune system were redirected to vectors of human diseases, principally mosquitoes (27). In this period, Sanger-based technology was largely used to elucidate genomes and generate datasets of expressed sequence tags (ESTs). After the development of next-generation sequencing (NGS) technologies, additional information on arthropod genomes and transcriptomes was added to public databases (28). Indeed, currently, more than 40 arthropod genomes are available in the VectorBase database (https://www.vectorbase.org/organisms).

Knowledge of vector genomes and ESTs allowed *in silico* comparisons of immune factors among species [for example, see (29–33)]. Moreover, studies with diverse approaches, such as transcriptomics, proteomics, and metabolomics analyses, to assess the arthropod response to different microbial stimuli were significantly expanded in the postgenomic era (28). The development and application of RNA interference (RNAi) and CRISPR-Cas9 technologies to arthropods [(34, 35), respectively] were also important to determine the role played by immune factors in the interaction between vectors and vector-borne pathogens.

Despite the importance of ticks as disease vectors, studies on their genomes and the molecular factors involved in their interactions with pathogens are scarce compared to studies on other arthropod vectors. The large size of tick genomes and the

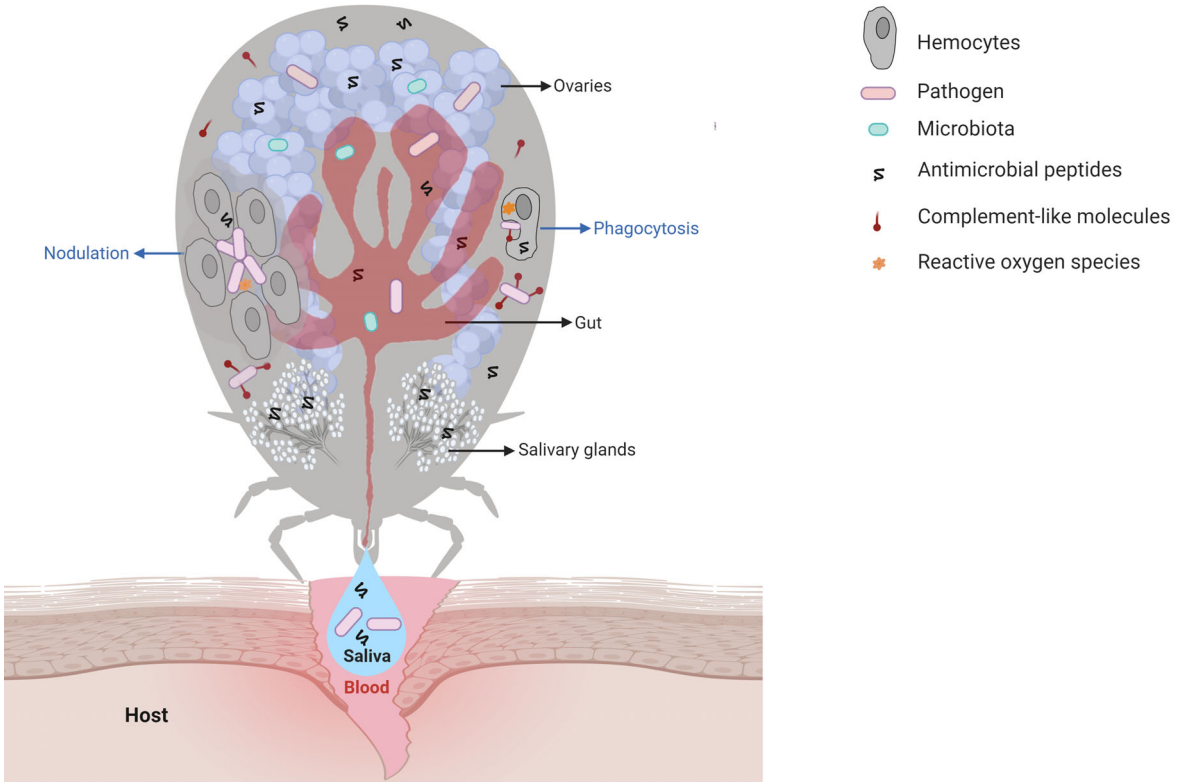

FIGURE 1 | Main interactions among tick immune system components, microbiota, and pathogens. Pathogens ingested within the blood meal initially reach the tick gut, where they interact with components of the gut microbiota and with cytotoxic molecules, such as AMPs (hemocidins and endogenous AMPs) and possibly with factors of redox metabolism, despite not being fully comprised. Pathogens must colonize and/or cross the gut epithelium to reach the hemocoel, which is filled with hemolymph. In hemolymph, complement-like molecules attach to pathogens that can be engulfed or trapped by hemocyte-mediated processes named phagocytosis and nodulation, respectively. Invaders can also be killed by several types of effector molecules, including AMPs, complement-like molecules, and factors of redox metabolism. The tick salivary glands return excess water and ions from the blood meal to the host through saliva, which also contains antihemostatic and immunomodulatory molecules. Pathogens use tick saliva as a vehicle to be transmitted to the host, in which infection can be facilitated by saliva properties. Some pathogens can also colonize the tick ovaries and are transmitted to progeny. In the tick salivary glands and ovaries, as in the gut, pathogens must deal with the members of resident microbiota as well as tick immune reactions. Additional studies are required to elucidate the molecules responsible for hemolymph clotting and melanization in ticks.

high contents of repetitive regions make genome assembly difficult. Indeed, the size of tick genomes is approximately 1.3 Gbp in argasids and 2.6 Gbp in ixodids (36). However, the genome of the cattle tick *R. microplus* is even larger and has been estimated to be approximately 7.1 Gpb, which is more than twice the size of the human genome (37). In addition, approximately 70% of the tick genome includes repetitive regions (37, 38). For this reason, until very recently, only the genome of the tick *Ixodes scapularis* had been annotated (38). Additional genomes were recently assembled by the use of NGS (37, 39). The scarcity of studies on the molecular factors involved in ticks and TBPs is in part due to the need for sophisticated structures to raise vertebrate animals to feed ticks, which is laborious and involves ethical concerns. In the last few years, artificial feeding systems have been successfully used to maintain laboratory tick colonies; however, an animal blood source is still required (40). Finally, the development of continuous cell lines derived from tick embryos, despite representing a mixture of different cell types, has also contributed considerably to studies on tick biology and their interactions with TBPs (41).

TICK IMMUNE SIGNALING PATHWAYS

Blood feeding represents a challenge for hematophagous arthropods due to the large diversity of pathogens to which these animals are exposed. In contrast to other arthropods, hard ticks are strictly hematophagous, feeding on the blood of their host for several days. In addition, some species feed on a different host in each developmental stage (larvae, nymphs, and adults), thereby increasing the chance of either acquiring or transmitting pathogens. Therefore, ticks are important vectors of a large list of disease-causing pathogens (42). In addition to host pathogens, ticks are in close contact with the microbiota of the host skin, which may also be acquired within the blood meal (43). Ticks are also exposed to microorganisms in the environment during the

nonparasitic phases of their life cycle. Hence, the immune system of ticks must be activated continuously to protect them from harmful infections.

Most of our knowledge on arthropod immune responses has come from studies on dipteran insects, especially *Drosophila* and the mosquitoes *Aedes* spp. and *Anopheles* spp. In *Drosophila*, invading microorganisms are mainly recognized by the Toll, immune deficiency (IMD), Jun-N-terminal kinase (JNK), Janus kinase/signal transducer and activator of transcription (JAK/STAT) and/or RNAi pathways (44). Nonetheless, the hypothesis that the level of conservation of arthropod immune responses might be high has been rejected by several studies on ticks, mites, lice, hemipterans, and others, and it is now recognized that the immune system displays remarkable diversification across the Arthropoda phylum (30). Tick immunity is, however, still greatly neglected and unexplored (45). Hence, we review the prevailing knowledge on tick immune signaling pathways alongside the connections between them and other equally important factors, such as AMPs, redox metabolism, complement-like proteins, and regulated cell death.

Nuclear Factor-Kappa B Signaling Pathways: Molecular Regulators for Pathogen Recognition

The Unexplored Toll Pathway

The Toll signaling pathway is well studied in *Drosophila*, in which it is preferentially activated in the presence of bacterial [by recognition of lysine-type peptidoglycan (PGN) from the cell

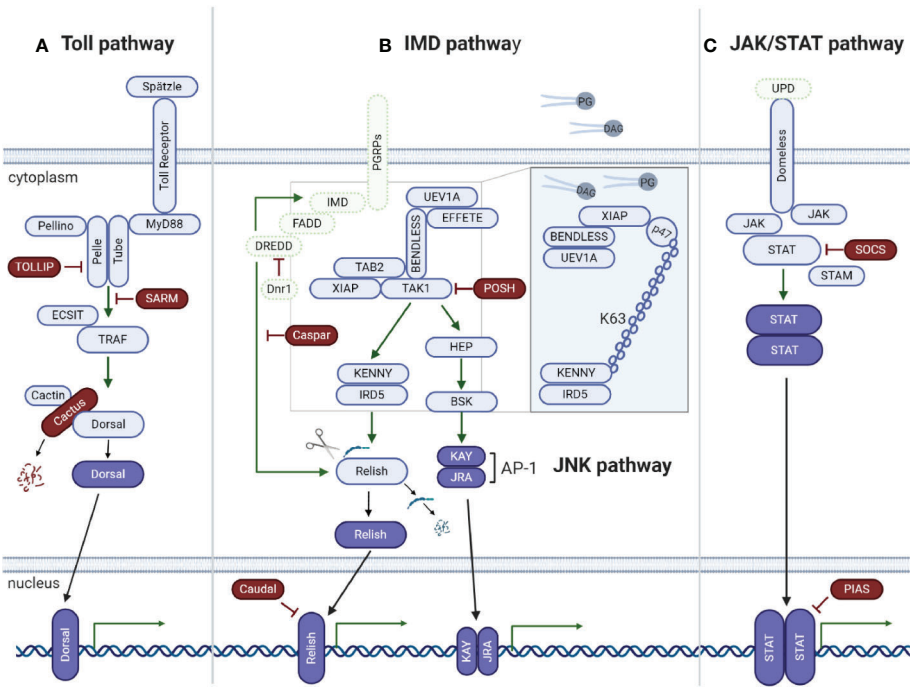

FIGURE 2 | Tick signaling-related genes in the three main immune signaling pathways of arthropods: **(A)** Toll, **(B)** IMD, and **(C)** JAK/STAT. **(A)** A previous *in silico* study (31) showed that components of the Toll signaling pathway of arthropods are conserved in ticks: extracellular cytokine Spatzle (Spz), transmembrane cytokine receptor Toll, Toll-interacting protein (TOLLIP), adaptor protein MyD88, kinases Tube (interleukin-1 receptor-associated kinase 4 or IRAK4), Pelle (interleukin-1 receptor-associated kinase 1 or IRAK1), Pelle-interacting protein Pellino, TNF receptor associated factors (TRAFs), evolutionarily conserved signaling intermediate in toll pathway (ECSIT), sterile alpha- and armadillo-motif-containing protein (SARM), Rel/NF-kappa B transcription factor Dorsal, Dorsal inhibitor protein IkappaB Cactus (IkB), and interacting protein Cactin of the IkB. **(B)** Regarding the IMD pathway, genes encoding downstream members of both the NF-kB/Relish and Jun N-terminal kinase (JNK) branches were identified: peptidoglycan recognition proteins (PGRPs), enzymes involved in ubiquitination (UEV1a, Effete/Ubc13 and Bendless/Ubc5), X-linked inhibitor apoptosis protein (XIAP), negative regulators Caspar (Fas-associating factor 1) and POSH (E3 ligase Plenty of SH3), transforming growth factor-beta activated kinase 1 (TAK1), TAK1-binding protein 2 (TAB2), IRD5 and Kenny/NEMO (IKKγ), and Relish-like Rel/NF-kB transcription factor. The adaptor protein IMD (immune deficiency), its associated molecule FAAD (Fas associated protein with death domain), the caspase DREDD (death related ced-3/Nedd2-like) and Dnr1 (defense repressor 1) have not yet been described in ticks. Components of the JNK branch of the tick IMD pathway include mitogen-activated protein (MAP) kinase hemipterous (HEP), Jun-kinase basket (BSK), activator protein 1 (AP-1) transcription factors JRA (Jun-related antigen) and KAY (Fos-related antigen, Kayak). Some IMD pathway components were functionally characterized by (48) (Insert). The authors showed that the IMD pathway is activated by PODAG (1-palmitoyl-2-oleoyl diacylglycerol) or POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol). Once activated, XIAP interacts with the heterodimer Bendless : UEV1a, leading to the ubiquitination of p47 in a K63-dependent manner. Ubiquitylated p47 connects to Kenny (also named NEMO) and induces the phosphorylation of IRD5 and Relish. **(C)** Components of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway are also conserved in ticks: the transmembrane cytokine receptor Domeless, tyrosine kinase JAK (Hopscotch), transcription factor STAT, signal transducing adaptor molecule (STAM) and the inhibitor proteins PIAS (protein inhibitor of activated STAT) and SOCS (suppressor of cytokine signaling). The ligand of the Domeless receptor (UPD gene) was not identified in ticks **(C)**. Activated transcription factors are represented in dark blue; the immune signaling pathway components not yet described in ticks are represented in green.

wall of Gram-positive bacteria) and fungal (by recognition of (1,3)-glucan polymers of D-glucose from the cell wall] pathogen-associated molecular patterns (PAMPs) (44, 46). *In silico* and genomic analyses have shown that ticks encode most Toll pathway components (31, 33, 38, 47) (**Figure 2A**), including the NF-κB Dorsal, indicating that conserved mechanisms of Toll pathway activation may exist. Indeed, the NF-κB transcription factor dorsal-related immunity factor (DIF) is the only component of the Toll pathway not yet reported in any tick species.

How the tick Toll pathway operates is largely unclear. Rosa and collaborators showed that the Toll pathway components are differentially expressed in the tick cell line BME26, which is derived from the tick *R. microplus*, in response to live *Anaplasma marginale* and *Rickettsia rickettsii* (two obligate intracellular bacteria) and heat-killed *Saccharomyces cerevisiae* (yeast), *Enterobacter cloacae* (Gram-negative bacterium) and *Micrococcus luteus* (Gram-positive bacterium) (31). Interestingly, heat-killed microorganisms upregulated the gene expression of the majority of the Toll pathway components, *R. rickettsii* upregulated some Toll pathway components and downregulated others, and infection with *A. marginale* (a pathogen naturally transmitted by *R. microplus*) downregulated most of the Toll pathway components. These results suggest that *A. marginale* may downregulate Toll pathway components in an attempt to favor vector colonization, which might correspond to coevolutionary adaptation. Of note, similar results were found for the IMD, JNK, and JAK/STAT signaling pathways (31). However, studies on the mechanisms used by this pathogen to overcome tick immune responses are warranted to confirm the authors' hypothesis. In adult *R. microplus*, only Dorsal was downregulated in both the gut and salivary glands of *A. marginale*-infected ticks, while Relish and STAT remained unmodulated (49). Moreover, Dorsal silencing promoted an increase in the *A. marginale* burden as well as knockdown of Relish and STAT. However, while Relish dsRNA (dsRelish) specifically silenced Relish, this transcription factor was also downregulated in both the dsDorsal and dsSTAT groups, which might explain the increase in the *A. marginale* load in these two groups as well. To determine the pathway responsible for infection control, the gene expression of specific effectors of each immune signaling pathway, which are currently unknown, is warranted. As Dorsal-, Relish-, and STAT-encoding genes do not exhibit significant sequence similarity, the authors suggested the existence of putative crosstalk among the Toll, IMD, and JAK/STAT signaling pathways (49) (**Figure 3C**). Nonetheless, an off-target effect cannot be ruled out. It is also possible that the

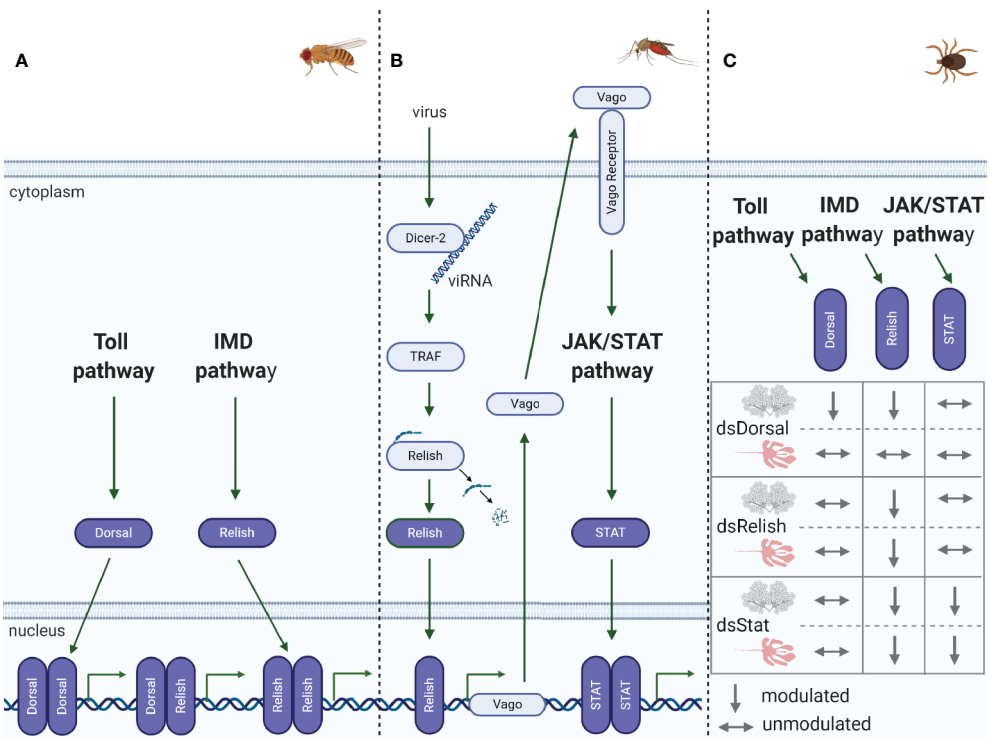

FIGURE 3 | Immune pathway crosstalk. **(A)** In *Drosophila*, the DIF-Relish heterodimer activates the expression of both Toll and IMD pathway effectors, resulting in a stronger response against infection (50). **(B)** In *Culex*, after recognition of West Nile virus (WNV) dsRNA by Dcr-2, TNF receptor-associated factor (TRAF) stimulates Relish, upregulating Vago expression (51, 52). Vago is then secreted by the infected cell and activates the JAK/STAT pathway in adjacent cells, upregulating the expression of antiviral genes. **(C)** In *R. microplus*, knockdown of Dorsal downregulates both Dorsal and Relish expression in salivary glands, while the levels of all the transcription factors remain unaltered in the gut. Relish is also downregulated in the gut and salivary glands of STAT-deficient ticks. Conversely, knockdown of Relish results in the specific silencing of its target gene in both the gut and salivary glands (49).

knockdown of immune signaling transcription factors exerts an effect on the gut microbiota, which, in turn, may modulate their gene expression. In contrast to the results obtained with *R. microplus*, gene silencing of Toll (ISCW018193) did not exhibit any effect on the *Anaplasma phagocytophilum* burden in the salivary glands of *I. scapularis* nymphs (53). However, gut colonization was not evaluated; therefore, it is not possible to guarantee that the Toll pathway is not involved in controlling *A. phagocytophilum* infection in this tick species. In a study carried out with *I. ricinus* cells (IRE/CTVM20), it was shown that the expression of a *Toll* gene (homologous to the *Toll* ISCW022740 of *I. scapularis*) is upregulated after 72 and 120 h of infection with flaviviruses [tick-borne encephalitis virus (TBEV) and louping ill virus (LIV)] but remained unmodulated in response to *A. phagocytophilum* (54). Conversely, infection with these flaviviruses downregulated the expression of three other Toll transcripts (homologous to ISCW017724; ISCW007727; ISCW007724 of *I. scapularis*), while Toll ISCW00727 expression was downregulated by *A. phagocytophilum* (54). The expression of another component of the Toll pathway, MyD88, was also downregulated by infection with these three pathogens, suggesting that they might suppress this pathway to promote vector colonization. To confirm this hypothesis, it is necessary to functionally characterize the role played by Toll components in pathogen proliferation.

The Unconventional Immune Deficiency Pathway

In *Drosophila*, bacterial infections caused by Gram-negative bacteria and certain Gram-positive bacteria, such as *Bacillus* and *Listeria* species, are mainly controlled by the IMD pathway through the recognition of diaminopimelic acid (DAP)-type PGN, which is present in the bacterial cell wall, by PGN-recognition proteins (PGRPs) (44, 55). Genomic and *in silico* studies have shown that ticks lack orthologs of many key elements of the IMD pathway, including the transmembrane PGRP, the Fas-associated protein with death domain (FADD), the adaptor molecule IMD, and the death-related ced-3/Nedd2-like protein (DREDD) (31, 33, 38, 48) (**Figure 2B**). Losses of IMD pathway components are not exclusive to ticks since they have also been described in other arachnids and hemipterans (30, 56). Nevertheless, it is important to highlight that some arthropods have unusual gene architectures, resulting in inaccurate annotation due to the use of software based on standard gene structures, as reported for the kissing bug *Rhodnius prolixus* (57). Gathering data from the genome and transcriptome associated with reciprocal BLAST (Basic Local Alignment Search Tool) and hidden Markov model profile searches, the authors showed that most of the missing IMD pathway components are present in this hemipteran. Therefore, it is possible that the missing IMD pathway components might be a consequence of incorrect annotations due to structural divergences. Indeed, assays showed that the IMD cascade is functional in the insect fat body and is predominantly responsive against Gram-negative bacterial infection (57).

Despite missing several elements, the tick IMD pathway is functional and responsive to distinct pathogens (48, 49, 58). RNAi silencing of several IMD pathway components, including Bendless, ubiquitin E2 variant 1A (UEV1a), Relish, and Caspar (**Figure 2B**, insert), showed that this cascade controls *A. phagocytophilum* and *B. burgdorferi* burden in *I. scapularis* nymphs (48). In contrast to the classical *Drosophila* model of DAP-PGN recognition by PGRPs (44, 55), glycerophospholipids from bacterial membranes, including 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoglycerol (POPG) and 1-palmitoyl-2-oleoyl diacylglycerol (PODAG), were reported to act as PAMPs for IMD pathway activation in ticks (48) (**Figure 2B**, insert). However, the mechanisms of POPG and PODAG recognition remain unclear, but it is hypothesized that they are sensed by a yet uncharacterized pattern-recognition receptor. X-linked inhibitor of apoptosis protein (XIAP) is an upstream signaling component of the IMD pathway and, when activated, specifically and directly interacts with the heterodimer E2 conjugating enzyme complex Bendless : UEV1a (48). Upon microbial activation, XIAP, together with Bendless : UEV1a, binds and ubiquitylates its p47 substrate in a K63-dependent manner. Ubiquitylated p47 connects to Kenny (also named NEMO) and induces, by a yet unknown mechanism, phosphorylation of the inhibitor of NF-κB kinase (IKK) β (also known as IRD5) and Relish, the IMD transcription factor. Consequently, Relish is cleaved and translocated to the nucleus (58) (**Figure 2B**, insert). On the other hand, RNAi knockdown of two other components of the IMD pathway, transforming growth factor-β activated kinase 1 (TAK1) and TAK1 adaptor protein 1 (TAB1) (**Figure 2B**), presented no effect on the *A. phagocytophilum* burden in the salivary glands of *I. scapularis* nymphs (53). Therefore, studies carried out by Dr. Pedra's group (48, 58) showed how the IMD pathway is activated in ticks, which is highly relevant since there is a lack of components in this pathway, different from the classic *Drosophila* model (44). However, the effector molecule(s) regulated by the IMD pathway that control(s) infections by pathogens such as *A. phagocytophilum* and *B. burgdorferi* still need to be identified.

In adult *R. microplus*, RNAi silencing of immune signaling of the Toll, IMD, and JAK/STAT pathway transcription factors identified the IMD pathway as the main controller of *A. marginale* infection in the tick gut and salivary glands (49). The expression of the genes encoding the AMPs microplusin, defensin, ixodidin, and lysozyme was analyzed in the gut and salivary glands of *R. microplus* after knockdown of Relish and infection with *A. marginale*. Interestingly, only the microplusin transcript levels were downregulated in dsRelish ticks, implicating this AMP as an effector of the IMD signaling pathway, which may act against *A. marginale* (49). However, although microplusin appears to be under IMD pathway regulation, possible coregulation by the JAK/STAT pathway cannot be discarded (49).

The other branch that constitutes the IMD pathway is JNK signaling (**Figure 2B**). In *Drosophila*, JNK has been shown to be involved in a wide range of biological processes, including cellular immune and stress responses, but it seems to not be required to induce AMP gene expression (59). Although activation of both the JNK and Relish branches of the IMD

pathway occurs *via* TAK1 in *Drosophila* (59), additional studies are warranted to determine the activation of JNK pathways in ticks (48, 53, 58).

JAK/STAT Pathway: Just a Support Molecular Circuit?

In *Drosophila*, the JAK/STAT signaling pathway only plays an indirect role in controlling bacterial and fungal infection. Therefore, this pathway is considered a support circuit to the Toll and IMD pathways; however, it is especially sensitive to viral infections (60). Beyond its effects on the immune response, the JAK/STAT signaling pathway also regulates multiple biological processes, including repair and renewal of the gut epithelial layer (61), a function that was also reported to occur in ticks (62).

Although still poorly understood, the tick JAK/STAT pathway **(Figure 2C)** was reported to be functional, playing an important role in the control of pathogens (53, 62, 63). However, it is not clear how ticks activate the JAK/STAT signaling pathway, as the unpaired (Upd) encoding gene, a cytokine-like signaling molecule ligand of the transmembrane receptor Domeless, is missing. In *I. scapularis*, knockdown of the transcription factor STAT and JAK yielded evidence that this pathway is key to the control of *A. phagocytophilum* infection (53). The results also showed that the 5.3-kDa AMP is an effector regulated by the JAK/STAT pathway, which is essential to restrict *A. phagocytophilum* proliferation in tick salivary glands and hemolymph but not in the gut, indicating that additional effectors under JAK/STAT pathway regulation are required in this organ (53). Interestingly, it was reported that *I. scapularis* employs a sophisticated immune strategy that uses a vertebrate host-derived cytokine to stimulate its own JAK/STAT immune pathway (63). During feeding, the interferon-gamma (INFγ) acquired within the infected bloodmeal activates STAT by a yet unknown receptor and, through mediation of a Rho-like GTPase, leads to the synthesis of the AMP domesticated amidase effector 2 (Dae2), limiting the level of *B. burgdorferi*. Other evidence that indicates that the JAK/STAT pathway is associated with the regulation of AMPs was reported by Capelli-Peixoto and collaborators in adult *R. microplus* (49). The authors observed the downregulation of the AMPs ixodidin and lysozyme in the salivary glands and defensin in the gut and salivary glands of STAT-deficient ticks.

Effectors from signaling pathways, such as JAK/STAT, can act as either positive or negative regulators of infection. As presented above, Dae2 (63) and the 5.3-kDa AMP (53) are negative regulators, as they control pathogen proliferation. In contrast, peritrophin-1, another effector from the tick JAK/STAT pathway, was reported to increase *B. burgdorferi* survival in the gut of *I. scapularis* nymphs (62). Knockdown of STAT had a direct impact on the gut epithelium, affecting its mitotic activity as well as decreasing peritrophin-1 expression, which consequently disrupted the structural integrity of the peritrophic matrix (62). Therefore, peritrophin-1, which is a component of the peritrophic matrix, favors *B. burgdorferi* establishment (62). Interestingly, peritrophin-1 exhibits the opposite effect on *A. phagocytophilum*, another pathogen naturally transmitted by *I. scapularis* (64). Infection with *A. phagocytophilum* upregulates a tick antifreeze glycoprotein, which, in turn, alters bacterial biofilm formation and, consequently, disturbs the natural gut microbiota. This microbiota alteration affects the integrity of the peritrophic matrix, favoring pathogen colonization (64). Knockdown of peritrophin-1 and, therefore, the reduction in the thickness of the peritrophic matrix increases the *A. phagocytophilum* load in the tick gut (64).

RNAi as a Tick Innate Immunity Component

RNAi is a biological process that plays an important role in the defense of arthropods against viruses and transposable elements. Four main RNAi-related pathways have been described based on the origin of the activating small RNAs. The origin of three of these small RNAs is endogenous [microRNA (miRNA), small interfering RNA (endo-siRNA), and piwi-interacting RNA (piRNA)], while the origin of the fourth is exogenous (siRNA) (65). The exogenous siRNA pathway is especially important and has been proposed to be the main antiviral response in *Drosophila* and mosquitoes (66). In general, after infection, long viral dsRNA is recognized and cleaved by Dicer-2 (Dcr-2) into 21 nucleotide (nt) siRNAs, known as viRNAs (65, 66). These viRNAs are then transferred to Argonaute-2 (Ago2), which couples to other members of the RNA-induced silencing complex (RISC). Only one strand of the viRNA remains coupled to RISC and guides the degradation of complementary viral RNA (65, 66). miRNAs use a similar mechanism, although involving Dcr-1 and Ago-1 (67).

The genome of *I. scapularis* exhibits significant gene expansion in RNAi elements, including five *Ago* homologous genes: *Ago-78*, homologous to insect *Ago-1*, and *Ago-96*, *-68*, *-16*, and-*30*, homologous to insect *Ago-2* (68). Additionally, two *Dcr* genes, *Dcr-89* and *-90*, were clustered with *Drosophila Dcr-2* and *-1*, respectively. Similar gene expansion was identified in *Hyalomma asiaticum* RNAi components (viz., two copies of *Dcr-2* and five copies of *Ago-2*) (69). Infection of *I. scapularis* IDE8 cells with Langat virus (LGTV) showed that Ago-16 and Ago-30 neutralized both LGTV and its replicon, as well as Dcr-90, despite the clustering of the last element with insect Dcr-1, which is involved in miRNA processing but not in siRNA (68). Shortly thereafter, knockdown of Ago-30 and Dcr-90 confirmed their antiviral role upon LGTV infection in *I. scapularis* IDE8 and *I. ricinus* IRE/CTVM19 cell lines (70).

Interestingly, viral or endogenous siRNAs were shown to be mostly 22 nt in length depending on the tick (68), in contrast with *Drosophila* and mosquito viRNAs and endo-siRNAs, which contain 21 nt (66). Moreover, these viRNAs mapped at the highest frequency around the 5' and 3' UTRs of the viral genome and antigenome (68). The 3' UTRs of LGTV and TBEV express subgenomic flavivirus RNAs (sfRNAs), which are a counterdefense against the tick RNAi system, assuring vectorial competence (68). Of note, sfRNAs are expressed by almost all Flaviviridae members as an evolved balance between arthropods and viruses (67).

Grubaugh and collaborators (71) validated the *in vitro* data previously obtained by Schnettler et al. (68), showing that most viRNAs are, indeed, 22 nt in length and originate from the UTR of the viral genome and antigenome of *I. scapularis* in its life stages (larvae, nymphs, and adults) naturally infected with Powassan virus (POWV) (71). Moreover, the viral genetic diversity in ticks is lower than that in mice, suggesting that ticks exert stronger viral control than their vertebrate hosts. Therefore, POWV evolution seems to depend on RNAi-mediated diversification and selective constraints (71).

Regarding endogenous miRNAs, recent studies have shown that pathogens, such as viruses (72) and bacteria (73), modulate tick miRNA profiles, with a potential role in controlling pathogen replication within the vector (72, 73). On the other hand, the piRNA response to infection is still unknown in ticks. Nonetheless, the piRNA response has been implicated in the response of mosquitoes to viral infections (74, 75). Moreover, Hess and colleagues (76) suggested that the mosquito piRNA response precedes the RNAi-Dcr-2-dependent (siRNA) response during viral infection. In contrast with siRNAs, piRNA activation seems to be mediated by single-stranded RNAs that are Dcr1- and Dcr2-independent and possibly mediated by the endonuclease activity of Piwi proteins, resulting in 24–30 nt small RNAs, as found in *Drosophila*. In addition to antiviral activity, piRNAs seem to have important roles in controlling the activity of transposable elements in the genome and in the development of reproductive tissues (65). Considering the knowledge of the role played by RNAi in the defense of insects against infections, the tick RNAi system represents a wide and still unexplored field awaiting investigation.

Independent Immune Pathways or Dynamic and Indispensable Crosstalk?

Although the term crosstalk is commonly applied to the arthropod immunity literature, its definition remains conflicting, and in many cases, the mechanism by which it occurs remains unknown. Here, we consider crosstalk to occur when (i) the same effector is regulated by more than one immune signaling pathway (50, 56, 77) and (ii) the components of a specific immune signaling pathway modulate the components of other pathways (49, 51, 52, 78–80).

The regulation of AMP expression by Toll and IMD pathways was initially established in *Drosophila*, as well documented in the historical review by Imler (24). Originally, it was accepted that AMPs were regulated by a specific immune pathway; however, subsequent studies carried out by different research groups showed that this regulation was more complex than initially known, and crosstalk among immune pathways could occur, as described in the examples below. Although AMPs are mostly regulated by either Toll or IMD pathways in *Drosophila*, it has been reported that some AMP-encoding genes can be activated synergistically by both immune pathways (50) (**Figure 3A**). It was shown that the NF-κB transcription factors Dorsal, DIF, and Relish can dimerize as homo- or heterodimers with varying degrees of efficiency. The DIF-Relish heterodimer mediates the crosstalk between the Toll and IMD pathways, resulting in the activation of effectors from both pathways and, consequently, targeting a broader spectrum of infectious microorganisms (50).

Another example of a certain effector being regulated by more than one immune pathway occurs in the hemipteran stinkbug *Plautia stali* (56). As shown by Nishide and collaborators, knockdown of IMD, as well as Toll pathway components, modulates effectors of both pathways. Interruption of both pathways at the same time had a more conspicuous effect on AMP production, strengthening crosstalk (56). The authors proposed an intriguing hypothesis that the redundancy between these two immune signaling pathways may have predisposed them to and facilitated the loss of some IMD-related genes in *P. stali*.

The crosstalk between RNAi and immune signaling pathways has been shown in recent publications (51, 52, 78, 79). In *Culex* mosquitoes, Dcr-2, a central component of the siRNA pathway, recognizes West-Nile virus (WNV) dsRNA and activates a signaling cascade to stimulate Relish *via* tumor necrosis factor (TNF) receptor-associated factor (TRAF) to increase Vago expression **(Figure 3B)** (51, 52). Following this transcriptional upregulation, Vago is secreted from infected cells and acts as a vertebrate cytokine functional homolog, binding to a still unknown cellular receptor in surrounding cells and triggering the JAK/STAT pathway. Activation of the JAK/STAT pathway ultimately results in an appropriate antiviral response in uninfected cells, such as upregulation of *vir-1* and other antiviral genes. These studies thereby revealed a paracrine signaling response mediated by a complex network of crosstalk, opening up several intriguing lines of investigation for future studies on arthropod immunity. Other studies have shown crosstalk between RNAi and the Toll pathway in *Ae aegypti* Aag2 cells (78) and *Drosophila* (79). In the first study, the miRNA aae-miR-375 upregulated Cactus, inhibiting the activation of the NF-κB transcription factor and reducing AMP synthesis, consequently enhancing dengue virus (DENV) infection (78). In *Drosophila*, on the other hand, four distinct members of the miR-310 family directly regulate drosomycin expression, a Toll-derived AMP (79). In addition to the connection between RNAi and signaling pathways, the redundancy of distinct miRNAs cotargeting the same transcript highlights the tight regulation imposed by miRNAs on the innate response.

It was also shown that the transcription factors activator protein 1 (AP-1; from the JNK pathway) and STAT neutralize Relish-mediated activation during the innate immune response in *Drosophila*, which is necessary for a proper and balanced immune response. The mechanism for controlling Relish-mediated transcriptional activation is through the formation of a complex composed of AP-1 and STAT with the dorsal switch protein (Dsp1), which recruits a histone deacetylase to prevent Relish transcription (80).

In ticks, to the best of our knowledge, there is only one study reporting putative crosstalk among the immune signaling pathways, which was reported by Capelli-Peixoto and collaborators (49). The authors showed that knockdown of the transcription factors Dorsal, Relish, or STAT downregulates

Relish expression (**Figure 3C**), with a consequent increase in the *A. marginale* load in *R. microplus* salivary glands. In contrast, Dorsal-deficient ticks presented no effects on Relish expression in the gut, where, intriguingly, ticks exhibited only modest silencing of Dorsal itself. Relish levels were also diminished in STAT-deficient guts. Only treatment with dsRelish resulted in specific silencing of its target gene in both the gut and salivary glands (**Figure 3C**). Nonetheless, the *A. marginale* burden was higher in the gut of ticks from all groups (dsDorsal, dsRelish, and dsSTAT) than in the control (49). As similarities among *Dorsal*, *Relish*, and *STAT* gene sequences were insignificant, the authors hypothesized that crosstalk of the immune pathways in ticks might occur to enhance the immune response. However, an off-target effect cannot be completely disregarded. Although the regulation of AMPs by the IMD and JAK/STAT pathways has been established, as already described above, it is still necessary to silence AMP-encoding genes to assign their role in *A. marginale* control. Therefore, the tick immune system, as shown in some insects, is also integrated, versatile, and possibly capable of making a network of connections among innate signaling pathways, giving rise to effective antimicrobial responses.

ANTIMICROBIAL PEPTIDES: MAY THE "SOURCE" BE WITH YOU!

AMPs are important effectors of the immune systems of both invertebrates and vertebrates, having a broad spectrum of activity against microorganisms (81). In ticks, the main sites of AMP expression are hemocytes, fat body, gut, ovaries, and salivary glands, where they can be modulated in response to either blood feeding or microbial challenge (82). Several reviews of tick AMPs addressing their characterization, as well as their interaction with microorganisms, have been published in the last decade (11, 33, 83, 84).

Interestingly, ticks use host hemoglobin, one of the most abundant proteins within the blood meal, as a source for the production of antimicrobial-derived fragments (85–88). Hemoglobin-derived AMPs, referred to as hemocidins (89), are produced by the proteolytic activity of aspartic and cysteine (catepsin-L like) proteinases from the tick gut (90). Structural studies with the synthetic amidated hemocidin Hb33-61a of *R. microplus* showed that its α-helical C-terminus is responsible for the permeabilization of the microbial membrane (91). However, it is still unknown whether hemocidins act intracellularly or if they are released to the tick gut lumen, where they can fight against microorganisms.

In addition to hemocidins, ticks also produce endogenous (ribosomally synthesized) AMPs (11, 83). Among the several tick AMPs identified to date, microplusins (also known as hebraeins) are among the most well characterized. Microplusin is a cysteine- and histidine-rich AMP that was first isolated from the hemolymph of adult *R. microplus* (92) and *Amblyomma hebraeum* (93). Microplusin was also identified in the ovaries and eggs of *R. microplus* (94), suggesting that in addition to protecting adults, it may also play a role in the protection of embryos before and after oviposition. Microplusin exhibits an α-helical globular domain and chelates metal ions (95). The bacteriostatic activity of microplusin against the Gram-positive bacterium *M. luteus* was reversed by the addition of copper II but not iron II. Indeed, microplusin interferes with the respiration (a copper-dependent process) of both *M. luteus* (95) and the fungus *Cryptococcus neoformans* (96). Microplusin was also reported to affect melanization and capsule formation, which are important virulence factors of *C. neoformans* (96). Interestingly, knockdown of microplusin increased the load of *R. rickettsii* in *Amblyomma aureolatum* (97). On the other hand, this AMP had no effect on either rickettsial transmission or tick fitness. Defensins compose another class of AMPs that have been described in several tick species, displaying activity against different types of microorganisms [for review, see (11, 83)]. For example, defensin-2 of *Dermacentor variabilis* was shown to protect against another bacterium of the genus *Rickettsia*, *R. montanensis*, as its neutralization with antidefensin-2 IgG increased the rickettisal load in the tick gut (98). Defensin-2 causes permeabilization of the bacterial membrane with consequent leakage of cytoplasmic proteins (98).

Dae2 is an AMP of *I. scapularis* that was acquired by horizontal bacterial gene transfer and has become an important effector to control *B. burgdorferi* infection (99), although it does not exhibit direct action on this pathogen (63). Indeed, it was recently shown that Dae2 is physically unable to overcome the outer membrane structure of the outer membrane of Gram-negative bacteria; thus, it does not present lytic activity against *B. burgdorferi*, suggesting the need for other factors, such as membrane-permeabilizing agents (100). As Dae2 is delivered to the vertebrate host bite site *via* saliva and exhibits strong activity against bacteria usually encountered in the host skin, this AMP may protect ticks from the acquisition and proliferation of host skin microbes (100).

Serine proteinase inhibitors have also been reported to play a role in the arthropod immune system. For instance, serine proteinase inhibitors mediate both coagulation and melanization processes of hemolymph and the production of AMPs (101). In addition, serine proteinases may also exert antimicrobial activity, possibly inhibiting proteinases that microorganisms use to colonize host tissues and evade the immune system (102). The first report of a tick serine proteinase inhibitor with antimicrobial properties was the ixodidin of *R. microplus* (103), which presents the key features of trypsin inhibitor-like domain proteins (104). Interestingly, one Kunitz inhibitor was reported to control *R. montanensis* infection in the gut of *D. variabilis* (105). In contrast to defensin, *D. variabilis* Kunitz-type inhibitors present a bacteriostatic effect on *R. montanensis* (106). Therefore, serine proteinase inhibitors are also used by ticks as powerful antimicrobial molecules.

Despite the diverse nature of molecules used by ticks as antimicrobials, little information on their synthesis regulation is available, as discussed above. Therefore, additional studies on the regulation of tick AMPs by immune signaling pathways are required to better understand their role in the control of distinct pathogens.

REDOX METABOLISM AS AN IMPORTANT PLAYER IN THE INFECTION CONTROL ORCHESTRA

In addition to AMPs, triggering of the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in response to infection has been described in several arthropods, such as *Drosophila* (107) and mosquitoes (108). ROS have an essential role in infection-related physiological as well as pathophysiological processes, such as signaling, regulation of tissue injury and inflammation, cell survival, proliferation, differentiation, and apoptosis (109, 110).

In ticks, there is still little available information on ROS metabolism and their impact on pathogen control. Nonetheless, it is recognized that hemocytes produce ROS under stimulation. Gram-positive bacteria, zymosan, and phorbol 12-myristate 13-acetate elicit the production of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) by hemocytes of *R. microplus* (111). In contrast, stimulation with lipopolysaccharide (LPS), the major component of the Gram-negative bacterial outer membrane, failed to induce ROS generation, indicating that different mechanisms or roles for ROS upon infection with either Gram-positive or Gram-negative bacteria may exist (111). Further studies with *R. microplus* showed that cytochrome c oxidase subunit III (COXIII), an enzyme of mitochondrial electron transport complex IV involved in mitochondrial ATP and ROS generation, is important for the transmission of *A. marginale* to calves (112). It is possible that COXIII knockdown imbalances tick redox metabolism, affecting its ability to transmit this pathogen (112). The peroxiredoxin Salp25D from *I. scapularis* had no effect on the transmission of *B. burgdorferi* but instead played a role in spirochete acquisition by the tick (113). RNAi-mediated silencing of Salp25D affects bacterial acquisition by ticks fed on *B. burgdorferi*-infected mice. The same effect was obtained when ticks were fed on Salp25-immunized mice (113). It is possible that Salp25 may detoxify ROS at the tick feeding site and gut, thus affording a survival advantage to *B. burgdorferi*.

In the mosquito *An. gambiae*, an extracellular matrix crosslinked by dityrosine covalent bonds catalyzed by dual oxidase (DUOX) and heme peroxidase is located in the gut ectoperitrophic space (between the epithelial cell layer and the peritrophic matrix). This extracellular matrix acts as an additional physical barrier to decrease gut permeability to bacterial PAMPs, impairing immune response activation by the resident microbiota (114). Importantly, the dityrosine network also provides a favorable environment for *Plasmodium* development, as it prevents the activation of nitric oxidase synthase (iNOS), a nitric oxide-generator enzyme (114). iNOS is responsible for parasite nitration, a key step in the action of the antiplasmodium complement-like molecule TEP1. Later, it was shown that the heme peroxidase 2/NADPH oxidase 5 system plays a central role in epithelium nitration, therefore potentiating the antiparasitic effect of nitric oxide (115). Similar to *An. gambiae* (114), an extracellular matrix was described in the tick *I. scapularis*, which acts as a shield that favors *B. burgdorferi* survival and indirectly prevents the induction of borreliacidal agents in the tick gut (116).

Intriguingly, *A. marginale* upregulated the genes encoding antioxidant enzymes, including superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase, thioredoxin, thioredoxin reductase, and peroxiredoxin, whereas genes encoding ROS-generating enzymes, such as DUOX and endoplasmic reticulum oxidase, were downregulated in *R. microplus*-derived BME26 cells (117). Conversely, *R. rickettsii* and heat-killed *S. cerevisiae, E. cloacae* or *M. luteus* triggered the opposite gene expression pattern (117). Furthermore, simultaneous RNAi knockdown of catalase, thioredoxin, and glutathione peroxidase, three representative members of the tick antioxidant enzymatic system, as well as the oxidation resistance 1 (OXR1), which regulates the expression of ROS detoxification enzymes, decreased *A. marginale* infection (117). Therefore, while BME26 cells respond to infection, producing an oxidant environment, *A. marginale* seems to subvert this response to create an antioxidant environment, which is required for its survival (117). It is possible that *A. marginale* manipulates *R. microplus* redox metabolism (and production of immune signaling pathway effectors, as aforementioned) to favor its proliferation. Additional studies are required to elucidate the mechanisms that this bacterium uses to subvert tick immune responses.

CELL-MEDIATED IMMUNITY IN TICKS

Hemocytes, which are sessile or circulating cells from arthropod hemolymph, are responsible for several immune responses. The nomenclature of hemocytes varies considerably depending on the arthropod species and/or the approaches of the study (118). Earlier morphological, ultrastructural, and physiological studies of the hemocyte repertoire in different tick species consistently reported the presence of three basic types of hemocytes, namely, phagocytic plasmatocytes and granulocytes and nonphagocytic granulocytes (119–121). These cells apparently differentiate from rarely occurring prohemocytes (120, 122). More recent studies have described additional types of tick hemocytes, namely, adipohemocytes in *Rhipicephalus sanguineus* (123) and spherulocytes and oenocytoids in *R. microplus* (122). The most important immune responses of arthropod hemocytes are phagocytosis, encapsulation, nodulation (which involves melanization by the PPO cascade), coagulation, and production of immune-related molecules.

The role of tick hemocytes in the phagocytosis of a variety of microbes, including bacteria, yeast, spirochetes, and foreign particles, has been investigated by several studies [for example, see (111, 124–127)]. By contrast, very little is known about the encapsulation and nodulation mechanisms. Indeed, there is only one report on encapsulation (128) and one on nodulation (129), both in *D. variabilis*. After the inoculation of ticks with *Escherichia coli*, hemocytes did not form circular layers but aggregated around the bacteria, which is a characteristic feature of nodule formation (129). As the encapsulation study was performed using an implant of Epon–Araldite under the tick

cuticle, it is still unknown whether it also occurs against microorganisms (128).

In invertebrates such as insects and crustaceans, hemocytes produce components of the melanization response, which involves an enzymatic cascade referred to as the PPO activating system, ultimately resulting in the production of melanin (130, 131). This process can be locally activated by cuticle injury or systemically triggered by microbial invasion of the hemocoel. Interestingly, in the cotton bollworm *Helicoverpa armigera*, infection with the baculovirus HearNPV decreased the levels of the majority of PPO cascade components, while serpin-9 and serpin-5 (which were also shown to regulate the proteases cSP4 and cSP6, respectively) were increased (132). In addition, *in vitro* assays showed that hemolymph melanization can kill baculovirus, an effect abolished by the specific PO inhibitor phenylthiourea. Together, the results suggest that baculovirus inhibits the melanization response to ensure its survival in *H. armigera* (132). There is no evidence of the existence of the PPO cascade in ticks based on available genomic and transcriptomic data. In line with this, no PPO activity has been reported to be present in the hemolymph of the hard ticks *Amblyomma americanum, D. variabilis*, and *I. scapularis* (133). In contrast, two studies reported PPO-like activity using L-DOPA as a substrate in the hard tick *R. sanguineus* (134) and in the soft tick *Ornithodoros moubata* (135). However, the enzymes responsible for such activity have not yet been identified, and enzymatic assays did not employ phenylthiourea as a control.

Coagulation is another important immune response of arthropods. The final product of coagulation is a protein clot, which is essential to avoid the loss of hemolymph in cases of an injury and the spread of an invader microorganism throughout the hemocoel (136). In horseshoe crabs, the clotting process involves a serine-protease cascade that leads to the activation of the clotting enzyme that converts the coagulogen into the insoluble clot (137), while in crayfish, the process depends on direct transglutaminase (TG)-mediated cross-linking of a specific plasma protein homologous to vitellogenins (19, 136). TG is also involved in the final step of coagulation in horseshoe crabs, cross-linking coagulin with hemocyte surface proteins named proxins (138). Interestingly, factors of the coagulation cascade interact with hemocyanin, causing it to present PO activity in the horseshoe crab *Tachypleus tridentatus*, demonstrating crosstalk between melanization and coagulation cascades (139). In *Drosophila*, coagulation and PO activity were also described to be tightly associated (140). Wound sealing in flies involves two steps: in the first step, TG-mediated crosslinking of hemolymph proteins occurs, and in the second step, PO-dependent crosslinking takes place, hardening the clot and producing melanin. In ticks, putative coagulation was uniquely reported for *D. variabilis*, where a fibrous matrix was observed around an inert implant (128). TGs and proclotting enzyme precursors have been detected in tick genomes (33). Moreover, an injury-responsive multidomain serine protease homologous to *Limulus* Factor C has been characterized in *I. ricinus* (141). Therefore, additional studies based on appropriate *in vitro* assays are needed to ultimately resolve the question of the existence of hemolymph clotting in ticks.

Tick hemocytes (83), as well as the hemocytes of other arthropods, such as mosquitoes (142), also produce a series of immune-related molecules. Intriguingly, the hemocytome of *I. ricinus* showed that only 1.48% of the 15,716 coding sequences (CDSs) identified were related to immune factors (143). Of the identified CDSs, 327 were five times more highly expressed in hemocytes than in salivary glands and the gut, among which 11 encode immune factors, including AMPs and proteins involved in pathogen recognition. As presented in this section, hemocytes are versatile components of the arthropod immune system that play diverse and key roles. The principal insect tissue that produces the majority of soluble immune molecules in hemolymph is the fat body (44). The role of tick fat body in the tick immune system requires further investigation.

THE PRIMORDIAL COMPLEMENT SYSTEM OF TICKS

One important branch of both cellular and humoral innate immunity in vertebrate and invertebrate metazoan organisms is carried out by the complement system. In higher vertebrates, the complement system is composed of approximately thirty components arranged in classical, lectin, and alternative pathways, which recognize foreign cells (microbes), specifically tag them *via* opsonization, and ultimately, eliminate them *via* phagocytosis or cell lysis (144). The common denominator of all three pathways is the proteolytic activation of the central C3 complement component. The occurrence of this molecule can be traced back in most ancient invertebrates, such as horseshoe crabs (subphylum Chelicerata, class Merostomata), implying that an ancestor of the complement system existed on Earth for more than 500 mil. years (145, 146). For ticks, which are also chelicerates, advanced knowledge of the primitive complement system of horseshoe crabs gathered during the past two decades presents the best matching comparative model (137, 145, 147).

Microbial pattern recognition by the vertebrate lectin pathway is mediated by multimeric mannose-binding lectins (MBLs) or ficolins. The horseshoe crab counterparts of mammalian ficolins are lectins named tachylectin-5 or carcinolectin-5 (148–150). These lectins share a fibrinogen-related protein (FRED) with ficolins but lack the N-terminal collagen-like domain responsible for forming complexes with MBL-associated serine proteases (MASPs) (151), which are absent in arthropods (146). The lectin Dorin M, purified from the plasma of the soft tick *O. moubata* (152), was shown to be a clear ortholog of the horseshoe crab tachylectins-5 (153), and similarly to ficolins and tachylectins, it forms high molecular weight multimers in the native state (152). The search for homologous lectins in *I. ricinus* (154) and in the genome of *I. scapularis* (155) revealed the existence of two phylogenetically distinct families, further referred to as ixoderin A and ixoderin B (**Figure 4**). Ixoderin A is mainly present in plasma and is responsible for the hemagglutination of mouse

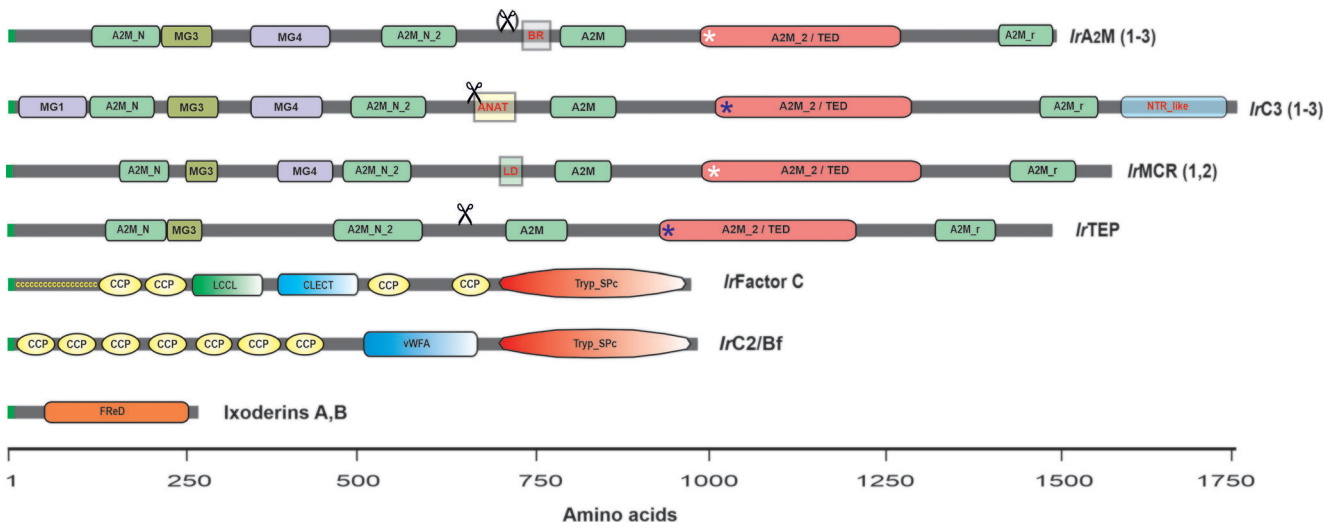

FIGURE 4 | Schematic representation of tick complement-related molecules (TEPs, convertases, lectins). Invertebrate TEPs are divided into four groups: panprotease inhibitors of the α_2-macroglobulin type (α_2M); C3-like complement components (C3); macroglobulin complement-related (MCR); and insect-type TEPs (iTEPs). IrA2M(1–3) represents *I. ricinus* α_2M: IrA2M-1, 2 and 3; IrC3(1-3) represents *I. ricinus* C3: IrC3-1, IrC3-2, and IrC3-3; *Ir*MCR (1,2) represents *I. ricinus* MCR: IrMcr-1 and IrMCR-*2;* and IrTEP represents *I. ricinus* iTEP. Other components of the *I. ricinus* primitive complement system are two putative convertases: IrFactor C, which shows the domain organization of the *I. ricinus* injury-responsive convertase related to *Limulus* Factor C (141), and IrC2/Bf, which shows the domain organization of the *I. ricinus* convertase related to the complement components C2 and/or Bf (156). Ixoderins A and B show the monomer structure of *Ixodes* sp. lectins related to ficolins (155). Domain abbreviations and nomenclature according to the NCBI conserved domain database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and symbols used in the figure: MG1, 3, 4 – macroglobulin domains 1, 3, 4; A2M_N – MG2 domain of α_2-macroglobulins; A2M_N_2 – α_2-macroglobulin family N-terminal region; Scissors – indicate the posttranslational cleavage site (not present in IrA2M-3); BR – bait region of α_2-macroglobulins (variable by alternative splicing); ANAT – anaphylatoxin homologous domain (signature domain of C3-complement components); LD – low density lipoprotein class A domain (signature domain of MCRs); A2M_2/TED – thioester containing domain; Blue asterisks – thioester bond present; White asterisks – thioester bond absent in IrA2M-2 and IrMCR-1; A2M_r – α_2-macroglobulin receptor domain; NTR_like – the signature C-terminal domain of C3,C4, and C5 complement components; cccccccc – the cysteine-rich N-terminal region of *Limulus* Factor C; CCP – complement control protein module (aka short consensus repeats SCRs or SUSHI repeats); LCCL – LCCL domain; CLECT – C-lectin domain; Tryp_Spc – Trypsin-like serine protease; vWFA – Von Willebrand factor A domain; FReD – Fibrinogen-related domain.

erythrocytes (155). On the other hand, the salivary gland transcriptomes of *I. ricinus* (157, 158) indicate that ixoderin B represents a highly variable multigene family that is preferentially expressed in the salivary glands and secreted into saliva. The function of these FREPs in tick saliva is still obscure, but we can hypothesize that they may play a role in the recognition of a specific tick host.

The central effector molecules of vertebrate and invertebrate complement systems are proteins belonging to the thioester-containing protein (TEP) family, formerly referred to as proteins of the α_2-macroglobulin superfamily (144, 159, 160). The TEP designation is given due to the presence of a highly reactive β-cysteinyl-γ-glutamyl thioester (TE) bond within a thioester domain. Invertebrate TEPs are divided into four major phylogenetically distinct groups: (i) panprotease inhibitors of the α_2-macroglobulin type (α_2M), (ii) C3-like complement components (C3), (iii) insect-type TEPs (iTEPs), and (iv) macroglobulin complement-related proteins (MCRs) (124, 146, 161). Genome-wide screening of the *I. scapularis* genome (124) and the recently available horseshoe crab genomes (162, 163), together with transcriptome data from a variety of arthropods, reveal that all these major groups of TEPs are present in chelicerates, but C3-like molecules are absent in crustaceans and hexapods, while α_2Ms were lost in the evolution of some insect lineages, such as *Drosophila* and mosquitoes (146).

Orthologs of nine TEPs present in the *I. scapularis* genome (124) were identified in closely related *I. ricinus* (125), and their full CDSs were recently deposited in GenBank: IrA2M-1 (MT779788); IrA2M-2 (MT779789); IrA2M-3 (MT779790); IrTep (MT779791); IrC3-1 (MT779792); IrC3-2 (MT779793); IrC3-3 (MT779793); IrMcr-1 (MT779795); and IrMcr-2 (MT779796). The domain structure of tick TEP representatives is shown in **Figure 4**. The hallmark domain of α_2Ms is the presence of the bait region (BR), which is cleaved by the target protease. Several bait region alternative splicing variants were reported in the α_2M region of the soft tick *O. moubata* (164) as well as in IrA2M-1 of *I. ricinus* (165). IrTEP has a domain architecture quite similar to that of IrA2Ms; however, this molecule is phylogenetically more closely related to insect TEPs (124). Tick C3-like molecules (IrC3-1, IrC3-2, IrC3-3) possess two signature domains, namely, the anaphylatoxin domain and C-terminal NTR complement_C345C domain. The MCRs can be clearly identified based on the presence of the short low-density lipoprotein receptor domain (LD), which occurs in the central part of the molecule (**Figure 4**). Tick TEPs are specifically expressed in tick fat body (IrA2M-1, IrA2M-3,

IrC3-1, IrC3-2, IrC3-3), tick hemocytes (IrA2M-2, IrA2M-3), salivary glands (IrC3-2, IrMcr-1), and ovaries (IrTEP) (125).

Other characterized components of the *I. ricinus* primitive complement system are two putative convertases: (i) IrC2/Bf (156), which is related to the vertebrate complement components C2 and/or FactorB (Bf) (144) and homologous to convertases from horseshoe crabs (145, 166), and (ii) IrFC (141), homologous to *Limulus* Factor C, which plays a dual function as the factor that triggers the clotting cascade upon sensing Gram-negative bacterial endotoxins and as an LPS-sensitive convertase of the horseshoe crab C3 complement component (147, 167). Both IrC2/Bf and IrFC are multidomain convertases that share the N-terminal trypsin-like domain and numerous CCP modules (complement control protein, aka sushi domains) (**Figure 4**). While IrC2/Bf is mainly expressed in the tick fat body and its expression is responsive to injection of the yeast *Candida albicans* and a variety of *Borrelia* species (156), IrFC is produced by tick hemocytes, and its expression is responsive to any injury, including injection of sterile phosphate-buffered saline, implicating its role in hemolymph clotting and wound healing (141).

RNAi-based functional studies of *I. ricinus* complement components successively deciphered their nonredundant roles in the phagocytosis of different microbes by tick hemocytes (124, 125, 141, 155, 156, 165). Phagocytosis of Gram-negative bacteria represented by the tick pathogen *Chryseobacterium indologenes* (168) depends mainly on the convertase IrFC, which seems to be linked to the IrC3-3 component. Interestingly, phagocytosis of this bacterium is also clearly mediated by α_2Ms IrAM2-1 and IrAM2-2 by a yet unknown mechanism that likely involves the interaction of these macromolecular protease inhibitors with the potent metalloprotease secreted by the bacterium (168).

A distinct phagocytic pathway dependent on the convertase IrC2/Bf is responsible for the phagocytosis of the yeast *C. albicans* and spirochete *Borrelia*. Phagocytosis of *C. albicans* is further facilitated by IrC3-1 and IrMcr-2, consistent with the reported role of its related molecule MCR (DmTep6) in the phagocytosis of this yeast by *Drosophila* S2 cells (161). Similar to other Gram-negative bacteria, phagocytosis of *Borrelia* is also mediated by IrC3-3. Ixoderins A and B were found to be involved in the phagocytosis of all the tested microbes, except *Borrelia*. Although *Borrelia afzelii* (the principal Lyme disease-causative agent in Europe) is actively phagocytosed by tick hemocytes; neither RNAi-mediated silencing of any tick complement-related molecules nor the total elimination of phagocytosis by preinjection of latex beads have shown any effect on the transmission of these spirochetes to the host (126). These results indirectly support the recent finding that the transmission of *B. afzelii* from infected *I. ricinus* nymphs to naive mice avoids the tick hemocoel and salivary glands and occurs by a direct gut-to-mouthpart route (169). However, it is possible that the tick complement plays a role in the transmission of other tick-borne pathogens, such as intracellular bacteria, including *Anaplasma* spp. and *Rickettsia* spp., or protozoan parasites, including *Babesia* spp. These objectives await an intensive research focus in the future.

REGULATED CELL DEATH AS AN IMMUNE DEFENSE

Regulated cell death (RCD) is widely distributed in nature, occurring in both unicellular and multicellular organisms (170). As extensively stated above, the arthropod innate immune system must coordinate pathogen recognition with effector mechanisms to successfully control infection. Nonetheless, over the past decade, several studies have established RCD processes as important mechanisms for the regulation of the immune response as well as the control of infections (171–173). Autophagy, apoptosis, and necrosis are the main types of RCD that have been described to be related to insect immunity in the last few years (173). In this section, we focus on autophagy and apoptosis and their interconnections with immune signaling pathways.

Autophagy is a highly conserved process in which endogenous material (misfolded proteins and aggregates, damaged organelles, and other macromolecules) or exogenous material (such as invading pathogens) are selectively recognized and sequestered within autophagosomes (double-membrane vesicles) that subsequently fuse with lysosomes, leading to cargo degradation (174). Autophagy is executed by a series of evolutionarily conserved autophagy-related (ATG) proteins that have orthologs in eukaryotic organisms ranging from yeasts to humans (174). Studies on *Drosophila* have provided excellent insights into the importance of autophagy during microbial infection (175). For instance, infection with the intracellular bacterium *Listeria monocytogenes* induces autophagy in both hemocytes and a hemocyte-derived *Drosophila* cell line (176). Interestingly, the IMD pathway receptor PGRP-LE is involved in bacterial recognition for autophagy activation. In addition, RNAi-mediated silencing of the autophagy genes *atg5* and *atg1* increases the bacterial load within cells, showing that this pathway is important to control infection. Autophagy is also important to control infection by vesicular stomatitis virus (VSV) in *Drosophila* (177, 178). In this process, the viral glycoprotein VSV-G is recognized by Toll-7, activating autophagy *via* a still unknown pathway that is independent of the canonical Toll, IMD, and JAK/STAT pathways (178). The role played by autophagy in the protection of mosquitoes against viruses is somewhat controversial, with reports suggesting both pro- and antiviral effects (179). In ticks, the expression of *atg* genes was upregulated under starvation in *Haemaphysalis longicornis* (180, 181), *I. scapularis* (182), *R. microplus*, and *A. sculptum* (183), correlating with the classical role of autophagy in stress. However, studies correlating tick autophagy with immune responses still need to be performed.

Apoptosis is another highly conserved RCD that is essential for removing damaged and infected cells to maintain homeostasis. There are two major apoptotic signaling pathways: extrinsic, also called death receptor pathway, and intrinsic, in which mitochondria play a central role (**Figure 5**). Both of these

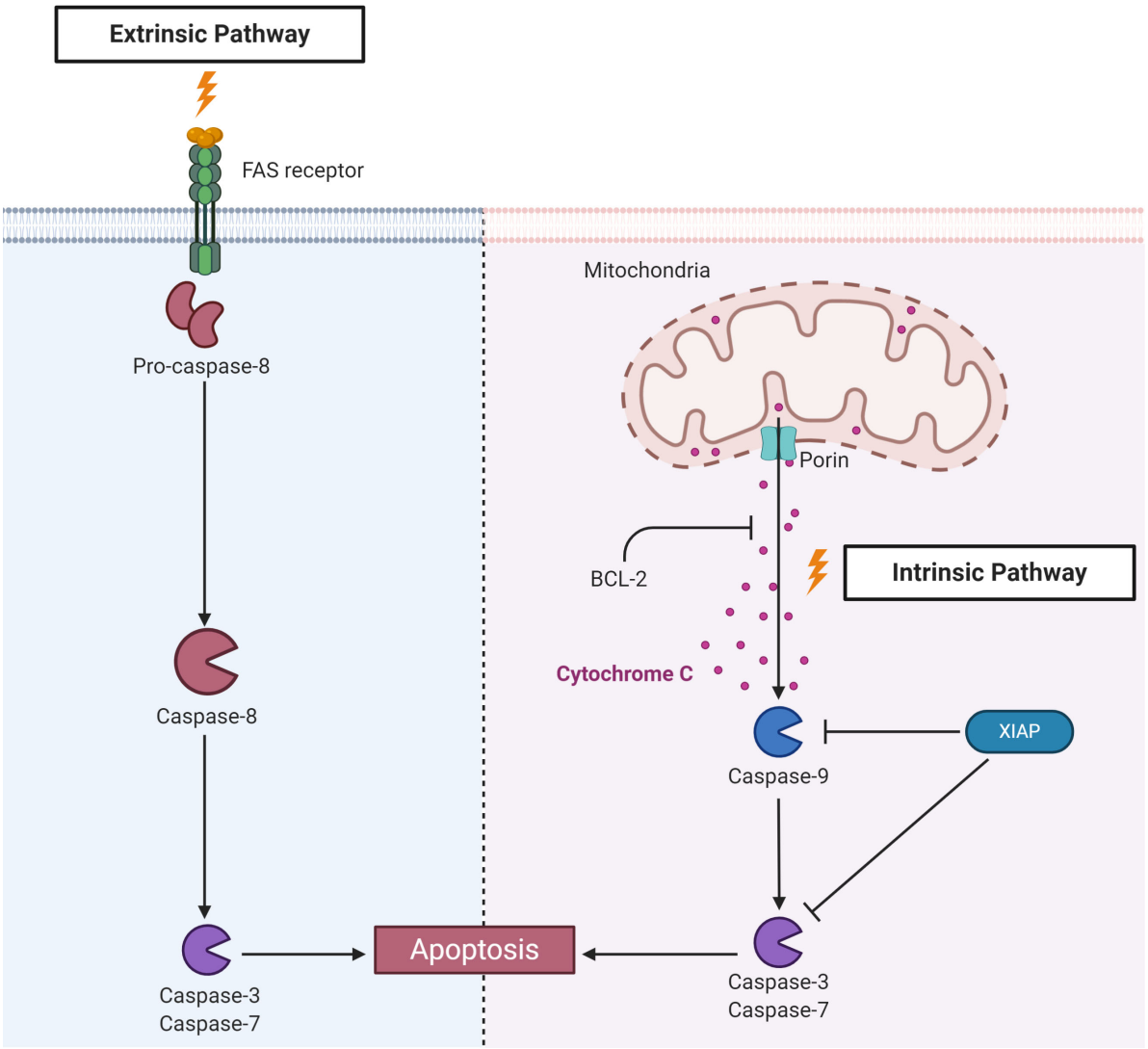

FIGURE 5 | Components of apoptosis activation pathways identified in ticks. Apoptosis is triggered by two main pathways. The extrinsic pathway is activated by recognition of external stimuli by transmembrane death receptors, such as fatty acid synthase (FAS), leading to the activation of caspase-8. The intrinsic pathway, also known as the mitochondrial pathway, is activated by internal stimuli. Subsequently, mitochondrial channels composed, for example, of porins, allow the release of mitochondrial components, such as cytochrome c, to the cytosol, activating the initiator caspase-9. B-cell lymphoma protein 2 (Bcl-2) can inhibit cytochrome c release from mitochondria. Both pathways culminate in the activation of effector or executioner caspases, such as caspases -3 and -7, resulting in chromatin condensation, DNA fragmentation, degradation of nuclear and cytoskeletal proteins and protein cross-linking, which ultimately cause cell death.

pathways culminate in the activation of executer caspases that are key for eliminating apoptotic cells (184). Apoptosis is activated in *Drosophila* by infection with *Drosophila* C virus (DCV), and infected cells are phagocytized by hemocytes in a phosphatidylserine-mediated process (185). Apoptosis can also control viral infection in mosquitoes (186, 187). Interestingly, the expression of proapoptotic genes was significantly higher in the refractory strain Cali-MIB of *Ae. aegypti* than in the susceptible strain Cali-S upon experimental infection with DENV-2, suggesting that apoptosis is involved in the distinct susceptibility of mosquitoes to infection (186). Apoptosis is also involved in the control of the proliferation of WNV in the midgut of a refractory strain of the mosquito *Culex pipiens pipiens* (187). Studies on the apoptotic response upon pathogen infection in ticks are also scarcer than those in insects. Infection of the *I. ricinus* cell line IRE/CTVM20 with the bacterium *A. phagocytophilum* and the flaviviruses TBEV and LIV upregulated the expression of apoptosis-associated components, such as cytochrome c and fatty-acid synthase (FAS) (54, 188).

To guarantee their replication and survival within the host cell, many pathogens, including viruses, bacteria and protozoa, subvert apoptosis induced by infection (189). For instance,

infection with Zika virus (ZIKV) inhibits apoptosis in *Ae. aegypti* through the action of sfRNAs (190). Until very recently, the unique example of a pathogen that inhibits apoptosis in tick cells was *A. phagocytophilum* (188, 191, 192). This bacterium inhibits the intrinsic apoptosis pathway in *I. scapularis* salivary glands and ISE6 cells by porin (voltage-dependent anion-selective channel) downregulation, resulting in the inhibition of cytochrome c release. Nonetheless, while the intrinsic pathway is inhibited, the extrinsic pathway seems to be activated through the inhibition of FAS by an unknown mechanism as a possible attempt to limit bacterial infection (192, 193). Conversely, in the *I. scapularis* gut and *I. ricinus* IRE/CTVM20 cells, *A. phagocytophilum* supposedly inhibits apoptosis through upregulation of the JAK/STAT pathway (191). However, these conclusions were mostly based on transcriptomics and proteomics data, and only a few genes were functionally characterized by RNAi. In addition, the effectors that *A. phagocytophilum* uses to inhibit tick apoptosis have not been elucidated to date, as they have been for the manipulation of apoptosis in human neutrophils (194). Recently, it was reported that *R. rickettsii* downregulates negative regulators of apoptosis in the initial phase of BME26 cell infection, which are upregulated later. Infection also prevents the fragmentation of DNA and decreases the activity of caspase-3 as well as the exposure of phosphatidylserine. Remarkably, bacterial growth is higher in apoptosis-inhibited tick cells, suggesting that such an inhibitory effect is important to guarantee cell colonization (195).

Apoptosis is closely regulated by apoptosis inhibitor proteins (IAPs) (**Figure 5**) (196, 197). IAPs present at least two conserved motifs: baculoviral IAP repeat (BIR) motifs, which are represented from one to three tandem repeats in the N-terminus, and the C-terminal really interesting new gene (RING) motif; this last motif presents E3-ubiquitin ligase activity. In *Drosophila*, the E3-ubiquitin ligase activity of DIAP-2 has been described as being important for the activation of Relish after recognition of Gram-negative bacteria (198–201). Knockdown of the XIAP of *I. scapularis* from the IMD pathway, which also possesses E3-ubiquitin ligase activity, increased colonization by *A. phagocytophilum*, showing that E3 is important for the control of infection (202) (see the above section "The unconventional IMD pathway"). However, it is still unknown whether XIAP plays a role in tick apoptosis.

Additional studies are warranted to better understand the role played by tick apoptosis pathway components in infection control and their interconnections with immune signaling pathways as well as the mechanisms that pathogens use to subvert the death of tick cells, thereby guaranteeing their survival and proliferation.

THE ROLE OF TICK MICROBIOTA IN VECTOR COMPETENCE

Ticks, as well as most multicellular eukaryotes, possess associated bacteria, viruses, fungi and archaea, mainly in mucosal organs, composing their microbiota (203). In the last decade, several studies have focused on the bacterial composition of different genera of ticks and have explored the interaction of TBPs with nonpathogenic tick endosymbionts to elucidate the impact of microbiota on their vector competence (62, 64, 204–206). The tick immune responses to microbiota, despite its importance for a more comprehensive understanding of tick biology, is a field that requires attention since little is known in comparison with other arthropods. Therefore, in this section, we summarize the *Ae. aegypti* immune responses to the gut microbiota and relate this knowledge to ticks.

In adult mosquitoes, the IMD pathway is activated in response to microbiota proliferation induced by the blood meal, limiting Sindbis infection (207). ROS production is mediated by DUOX, whose expression is regulated by a gut membrane-associated protein named Mesh (208). However, a reduction in ROS due to heme release upon blood digestion protects the gut microbiota (209). To counteract the action of Relish-dependent AMPs, the gut microbiota stimulates the expression of C-type lectins (CLTs) in *Ae. aegypti*, which bind to bacterial cell walls, thereby protecting the bacteria (210).

In addition to the immune response to microbiota, there is interest in the impact of microbiota on the vector capacity of mosquitoes and ticks. In *Ae. aegypti*, several studies have shown that larval microbiota can influence vector competence in adult mosquitoes, playing a critical role in their response to viral infections (211). For instance, *E. coli* infection during the larval stage stimulates the production of AMPs and nitric oxide, protecting the mosquito from other infections (212). In addition, when Enterobacteriaceae bacteria are the only members of the larval microbiota, DENV infection in adults is reduced in comparison to *Salmonella* sp. as the only member (213). Conversely, exposure to pathogenic *Bacillus thuringiensis* subsp. *israelensis* in resistant larvae increases adult susceptibility to DENV [but not to Chikungunya (CHIKV)] (214), possibly due to changes in the microbial community (215).

Of all the bacteria present in insect microbiota, *Wolbachia pipientis* may be the most ubiquitous symbiont, as it is naturally present in 40% of all terrestrial arthropod species (216). Intracellular and maternally transmitted *Wolbachia* can cause pathogen interference (PI; the ability to reduce the chance of pathogen infection and decrease pathogen load) and cytoplasmic incompatibility (CI; when infected males mate with uninfected females, the hatch of eggs is heavily reduced), manipulating host reproduction and working as a genetic driver (the ability to spread through a population in a non-Mendelian way) (217). In *Ae. aegypti*, *Wolbachia* strongly reduces CHIKV, DENV and ZIKV infection and vector competence *via* the PI phenotype (217–220). For this reason, there is an ongoing program, the World Mosquito Program, to infect mosquito eggs with *Wolbachia* (from *Drosophila* – *w*Mel) in the laboratory and release them in dengue-endemic areas, such as the city of Rio de Janeiro in Brazil (221, 222).

Despite being the most common bacteria in the microbiota of insects, *Wolbachia* has been reported only in a few species of ticks (205, 223, 224). In fact, the adult tick microbiota is mostly composed of *Coxiella*, *Rickettsia*, *Francisella*, *Spiroplasma*,

Midichloria and *Rickettsiella* (203, 205). Most of these bacteria are intracellular and noncultivable in the laboratory, which hampers the manipulation of tick microbiota. Some studies on the larval microbiota of *I. scapularis* (62, 64, 204) showed a wide variety of bacterial genera, including cultivable extracellular bacteria. A deep bioinformatics analysis of the raw data of these studies suggested a taxonomic core composed of 61 bacterial taxa for *I. scapularis* larvae (225). However, this high number of bacterial genera in the larval microbiota of *I. scapularis* has been questioned, and the possibility of contamination potentially due to the low biomass of tick samples has been raised (226, 227). Interestingly, some tick species, such as *R. microplus* and *I. ricinus*, present a poor and unstable microbiota in the gut (228). On the other hand, these two species harbor a more abundant and stable microbiota in their ovaries that is composed mostly of *Midichloria* spp. in *I. ricinus* (228) and *Coxiella* spp. in *R. microplus* (229). The authors hypothesized that the reduced microbiota in the tick gut might be due to the action of immune factors, such as AMPs and ROS (228).

As described for mosquitoes, the tick microbiota can exert an effect on vector capacity; bacterial infection can modify the microbiota of its host. In *I. scapularis*, perturbation of the normal gut microbiota decreased the expression of STAT, which, in turn, reduced the expression of peritrophin-1. Since the integrity of the peritrophic matrix is essential to *B. burgdorferi* infection, as previously discussed, alteration of the microbiota reduces borrelial colonization (62). In addition, infection with *B. burgdorferi* promotes the expression of the *I. scapularis* gene *pixr*, which encodes a gut secreted protein with functions in tick biology, such as larval molting and inhibition of biofilm formation (preferentially by Gram-positive bacteria), facilitating the colonization of *B. burgdorferi* in ticks (204). These studies suggest a mutual influence or interconnection between the gut microbiota and *B. burgdorferi* in *I. scapularis*. Conversely, *A. phagocytophilum* infection in this same tick species promotes the expression of an antifreeze protein, which perturbs the gut microbiota and reduces the integrity of the peritrophic matrix (64). In contrast to *B. burgdorferi* (62), an extracellular bacterium that benefits from a preserved peritrophic matrix, *A. phagocytophilum*, which is an obligate intracellular bacterium, reduces the thickness of the peritrophic matrix to colonize the tick gut (64). In the tick *Dermacentor andersoni*, a microbiota alteration was induced by feeding on calves treated with oxytetracycline. Although this treatment did not change the microbiota composition, the proportion of its components was altered, negatively impacting the acquisition of *A. marginale* and *Francisella novicida* (230). Importantly, perturbation of the *D. andersoni* microbiota exerted a negative impact on the reproductive fitness of the tick, thereby identifying the microbiota as an important target for the development of control strategies (231).

A recent study compared the microbiota of two *R. rickettsii* tick vectors in Brazil, *Amblyomma sculptum* and *A. aureolatum*, which present significant differences regarding their susceptibility to infection (232). Interestingly, *A. aureolatum* is highly susceptible to *R. rickettsii* infection and harbors a robust intestinal microbiota, mainly composed of the *Francisella* genus. *A. sculptum*, on the other hand, is less susceptible to *R. rickettsii* infection and harbors a reduced intestinal microbiota (206). Additionally, *R. rickettsii* causes a slight reduction in the microbiota load without changing its composition. It has been reported that the transcriptional gut response of these two ticks to *R. rickettsii* infection is also distinct: while the majority of genes of *A. sculptum*, including immune factors, were upregulated by infection, *A. aureolatum* genes were mostly downregulated (233). Together, these data suggest that the *A. aureolatum* gut microbiota somehow desensitizes the immune system and promotes *R. rickettsii* infection. Interestingly, the presence of *Francisella* endosymbionts positively impacted the establishment of *F. novicida* in *D. andersoni*, and the authors hypothesized that these endosymbionts may suppress the tick immune system, favoring *F. novicida* acquisition (230). Additional studies are needed to confirm this hypothesis and to identify the mechanisms by which microbiota delineate tick susceptibility to infection.

Studies focused on the immune response to tick microbiota are necessary to elucidate this important feature, which is involved in many aspects of tick biology, including its vector capacity, representing a question of public health interest. These responses may involve only one immune signaling pathway or crosstalk of the different pathways, as is suggested to occur in *R. microplus* in response to *A. marginale* infection (49). In addition, we can raise a possible role of DUOX in the control of tick microbiota since this enzyme is present and functional in *I. scapularis* (116).

CONCLUSIONS

To date, studies on the interactions between ticks and TBPs have shown that both the IMD and JAK/STAT pathways are key for the control of bacterial infections (*B. burgdorferi*, *A. marginale* and *A. phagocytophilum*), while the Toll and RNAi pathways might be involved in tick defense against viral infections. Studies on the identification of tick immune system-specific effectors are warranted to describe the mechanisms involved in these signaling pathways. In addition, this review has provided several lines of evidence of interconnections between immune signaling pathways, as well as links among several elements from the innate immunity of arthropods, such as the RNAi system, redox metabolism and microbiota. This review also highlights the importance of bearing in mind a widely integrated, versatile, and complex immune system as a response to infection in ticks, far beyond a canonical and linear pathway.

As mentioned above, most of our knowledge on arthropod immunity comes from *Drosophila* as well as other arthropod studies. Nevertheless, the search for a direct correlation between immune signaling and effector specificity in ticks may result in the absence of new and important mechanisms of the tick immune system. For instance, recent works have suggested that ticks express different types of effectors from immune

signaling pathway activation, such as peritrophins, which are key structural components of the gut peritrophic matrix. Moreover, it is extremely important to keep in mind that *Drosophila* is not a vector model and, as such, some aspects of the pathogen-vector interaction cannot be fully modeled. Of relevance, the studies on the innate responses of *Drosophila* follow infections by an intrathoracic injection with large loads of artificial pathogens, greatly contrasting those from ticks, which experience natural pathogens, doses, and routes of infection.

Phagocytosis, AMPs, complement-like molecules and ROS production are also considered important factors for protecting ticks from infection. The microbiota can interfere with tick colonization by pathogens as well. Therefore, it is important to identify the microorganisms that compose the microbiota of different organs of ticks and to determine their influence on the tick immune system as well as on tick vector competence. Interestingly, in some insects, the immune system can be primed by nonpathogenic microorganisms, protecting the animal from subsequent infection with a pathogenic microbe (234). Nonetheless, there is only one report on tick immune system priming to date (48). The authors showed that immune priming with POPG and PODAG protects *I. scapularis* against infection by *A. phagocytophilum* and *D. andersoni* against infection by *A. marginale*. In addition, it was shown that the lipid immune-priming effect is abolished only by the silencing of IMD pathway components but not of the Toll or JAK/STAT pathways, excluding an off-target effect (48). Therefore, the tick immune system and its relationship with microorganisms is a wide and unexplored field to be pursued.

In summary, every aspect concerning the tick immune system and its relation with microorganisms - endosymbionts or pathogens - remains far from completely understood. Despite the vast advances made in recent decades, which have helped us to build parts of this puzzle, working with ticks is still a bright and open field full of possibilities. We expect more groups to work with ticks due to their importance to public health and await the discovery of new knowledge in the next few years. In this way, we will all be able to assemble this extraordinary and complex puzzle.

AUTHOR CONTRIBUTIONS

All authors searched the literature and wrote the manuscript. SD and AF conceived the work and the final edition. GS, DP, EE, LM, and PK elaborated the figures. All authors contributed to the article and approved the submitted version.

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Multiple Country and Breed Genomic Prediction of Tick Resistance in Beef Cattle

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Ticks cause substantial production losses for beef and dairy cattle. Cattle resistance to ticks is one of the most important factors affecting tick control, but largely neglected due to the challenge of phenotyping. In this study, we evaluate the pooling of tick resistance phenotyped reference populations from multi-country beef cattle breeds to assess the possibility of improving host resistance through multi-trait genomic selection. Data consisted of tick counts or scores assessing the number of female ticks at least 4.5 mm length and derived from seven populations, with breed, country, number of records and genotyped/phenotyped animals being respectively: Angus (AN), Brazil, 2,263, 921/1,156, Hereford (HH), Brazil, 6,615, 1,910/2,802, Brangus (BN), Brazil, 2,441, 851/851, Braford (BO), Brazil, 9,523, 3,062/4,095, Tropical Composite (TC), Australia, 229, 229/229, Brahman (BR), Australia, 675, 675/675, and Nguni (NG), South Africa, 490, 490/490. All populations were genotyped using medium density Illumina SNP BeadChips and imputed to a common high-density panel of 332,468 markers. The mean linkage disequilibrium (LD) between adjacent SNPs varied from 0.24 to 0.37 across populations and so was sufficient to allow genomic breeding values (GEBV) prediction. Correlations of LD phase between breeds were higher between composites and their founder breeds (0.81 to 0.95) and lower between NG and the other breeds (0.27 and 0.35). There was wide range of estimated heritability (0.05 and 0.42) and genetic correlation (-0.01 and 0.87) for tick resistance across the studied populations, with the largest genetic correlation observed between BN and BO. Predictive ability was improved under the old-young validation for three of the seven populations using a multi-trait approach compared to a single trait within-population prediction, while whole and partial data GEBV correlations increased in all cases, with relative improvements ranging from 3% for BO to 64% for TC. Moreover, the multi-trait analysis

was useful to correct typical over-dispersion of the GEBV. Results from this study indicate that a joint genomic evaluation of AN, HH, BN, BO and BR can be readily implemented to improve tick resistance of these populations using selection on GEBV. For NG and TC additional phenotyping will be required to obtain accurate GEBV.

Keywords: beef cattle, genomic selection, ticks, tropical adaptation, host resistance

INTRODUCTION

Ticks and tick-borne diseases are among the most important causes of production losses for beef and dairy cattle. Recent estimates of those losses range from US$22 to 30 billion per year (1). Cattle host resistance to ticks is one of the most important factors affecting the economics of tick control, with host resistance being moderately to highly heritable and representing a permanent solution requiring no extra labor or resources (2). However, breeding for host resistance is largely neglected in tick control programs due to the challenge of phenotyping for this trait and costs associated with identifying individual animal variation in resistance.

Genomic selection is typically suggested as a solution for improvement of traits that are hard or costly to measure. However, in the case of tick resistance, the trait is so labor intensive and expensive to measure that only small reference populations have been recorded in countries where ticks prevail (3–5). Therefore, for most cases pooling reference populations across breeds and countries may be the only effective way to achieve genomic estimated breeding values (GEBV) with sufficient accuracy to be useful. Pooling reference populations across countries has previously been demonstrated to improve accuracy for traits such as dry matter intake (6). In that study, differences in trait measurement were accounted for by treating dry matter intake as different, but potentially correlated traits between countries. Most studies pooling trait and genotype data across countries have attempted to do so only where the same breed of cattle is considered. For tropical beef cattle, this is difficult and would restrict the size of the reference population greatly, as so many different breeds, crossbreds and composites are used across the different countries.

In this study, we pool tick resistance phenotyped reference populations from beef cattle breeds in Australia, Brazil, and South Africa. Firstly an assessment is made of the extent of phase of linkage disequilibrium shared between the breeds, as a predictor of how much information might be transferred from breed to breed in genomic predictions when a high density SNP array is used [e.g. (7, 8)]. We then jointly analyze existing tick infestation datasets to assess the possibility of improving host resistance in cattle through multi-population, multi-trait genomic selection.

MATERIALS AND METHODS

Phenotype, Genotype and Pedigree Data

Cattle Populations and Tick Data

Tick datasets were obtained from seven different cattle populations generated in Brazil, Australia and South Africa (**Table 1**). Tick species infesting cattle in Brazil and Australia are from the same genus (*Rhipicephalus microplus* and *R. australis*), whereas cattle in South Africa are additionally infested with the multi-host tick species *Amblyomma hebraeum* and *Hyalomma rufipes* and *H. truncatum*. Tick counts in South Africa were obtained from the *Rhipicephalus* (53%), *Amblyomma* (42%) and *Hyalomma* (5%) species.

Brazilian data consisted of log-transformed tick counts. Measurements were performed on occasions when large phenotypic variation existed in tick numbers, by manually counting adult female ticks that were at least 4.5 mm length on one whole side of the animal's body (9). One to three subsequent tick counts on one side of each animal were obtained from Angus (AN) cattle between 2012 and 2017 from five different herds associated with the Promebo Breeding Program; from 9 Hereford (HH) and 10 Braford (BO) cattle herds between 2010 and 2018 in the Delta G Breeding Program; and from the Embrapa South Livestock Brangus (BN) experimental herd between 2013 and 2018.

TABLE 1 | Tick resistance data according to population.

Population	Country of origin	Phenotype available	Number of observations	Mean ± S.D.	Min	Max	Number of genotyped/ phenotyped animals[1]	Number of animals in validation set
Angus (AN)	Brazil	Log_{10} tick counts	2,263	1.54 ± 0.46	0.00	2.49	921/1,156	344
Hereford (HH)	Brazil	Log_{10} tick counts	6,615	1.47 ± 0.50	0.00	2.78	1,910/2,802	684
Brangus (BN)	Brazil	Log_e tick counts	2,441	4.32 ± 1.20	1.00	7.69	851/851	300
Braford (BO)	Brazil	Log_{10} tick counts	9,523	1.32 ± 0.43	0.00	2.72	3,062/4,095	1,267
Trop.Comp. (TC)	Australia	Tick scores	229	2.52 ± 0.93	0.00	5.00	229/229	74
Brahman (BR)	Australia	Tick scores	675	0.67 ± 0.74	0.00	4.00	675/675	216
Nguni (NG)	South Africa	Averaged log_e tick counts[2]	490	0.50 ± 0.17	0.02	0.95	490/490	157

[1]*All genotyped animals had phenotype.* [2]*Animal average solution from log transformed Tick counts.*

For South African Nguni (NG) cattle, adult ticks were counted from the perineum body part under natural grazing for a continuous period of two years (2012 to 2014) from 490 Nguni animals. At least 23 tick counts were conducted for each animal throughout a two-year period, meaning at times there was little phenotypic variation for tick counts across animals. Tick counts (x) were log transformed using log10 (x + 1) to approximate normality. Data available for NG cattle was summarized as the average animal tick effect obtained in ASREML (10) after accounting for the following fixed effects: farm, month, year, sex, interaction between farm and month, and age, fitted as a covariate.

The Australian Brahman (BR) and Tropical Composite (TC) animals had estimates of tick counts derived from tick scores. Tick scores of adult female ticks that were > 4.5 mm in diameter on the left side of each animal, were on a 0 - 5 scale where 0 was no ticks, 1 was ≤ 10 ticks, 2 was 11 - 30 ticks, 3 was 31 - 80 ticks, 4 was 81 - 150 ticks, and 5 > 150 ticks. Tick scores are less accurate and less informative than tick counts, however there is a high genetic correlation between the two (11). Statistics of the different datasets (numbers, means and distributions) are also presented in **Table 1**.

Genotypes and Pedigree

Pedigree information were available and used in the analyses of the Brazilian populations only. All populations were genotyped using the Illumina SNP BeadChip technology (Illumina Inc., San Diego, CA, USA) with marker densities varying from 27k to 150k given by commercially available chips. Genotype quality control (QC) was implemented for all populations. In the case of Brazilian data QC was performed by R/SNPStats package (12). Samples with genotyping rate (call rate - CR) < 0.90, heterozygosity rate – calculated as the proportion of heterozygote genotypes within all autosomal markers of an animal – with 3 SD above or below the observed population mean, mismatching sex, and duplicate records were removed. These per animal QC criteria were applied to assure sample DNA high quality, lack of contamination or misidentification. Only SNPs mapped to autosomes with CR > 0.98, minor allele frequencies (MAF) > 0.03, and not in highly significant deviation Hardy–Weinberg equilibrium ($P > 10^{-7}$) were considered in the analyses. In addition, only the SNP with highest MAF was retained when SNPs were observed in the exact same position or the genotypes were highly correlated (r > 0.98). Similar quality control steps were applied to the Australian populations and NG, with the addition that genotype calls with a GC score below 0.6 were set to missing and were filled in with imputation using FImpute (13). After quality control, genotypes from all populations were imputed to a common high-density panel of 332,468 markers distributed throughout the 29 bovine autosomal chromosomes. Brangus, Braford and Hereford populations were imputed using the FImpute software (13) and an HD sample of 340 animals available at Embrapa datasets for these breeds. Angus, Brahman, Nguni and Tropical Composites were imputed using the 1,000 bull genome project reference, which includes 305, 122, 0 and 30 sequences respectively from those imputed breeds and 2,603 in total from 107 breeds (13), and findhap software (14).

Population Genomic Parameters

Linkage Disequilibrium

Linkage disequilibrium (LD) was estimated for each chromosome between adjacent pairs of SNPs as the squared correlation statistic (r^2) (15), which can be calculated as follows:

$$r^2 = \frac{(\rho_{AB}\rho_{ab} - \rho_{Ab}\rho_{aB})^2}{(\rho_A\rho_a\rho_B\rho_b)} \quad (1)$$

where ρ_A, ρ_a, ρ_B and ρ_b are the frequencies of alleles A, a, B and b, respectively; ρ_{AB}, ρ_{ab}, ρ_{Ab} and ρ_{aB} are the haplotype frequencies among alleles in the population.

Persistence of Phase Across Breeds

To investigate the LD phase between two specific breeds, the Pearson correlation $r_{ij(A)}$ and $r_{ij(B)}$ for a common set of adjacent SNPs between populations A and B was calculated using the following equation (16):

$$R_{A,B} = \frac{\Sigma_{(i,j)\in l}\left(r_{ij(A)} - \bar{r}_A\right)\left(r_{ij(B)} - \bar{r}_B\right)}{S_A S_B} \quad (2)$$

where $R_{A,B}$ is the correlation of phase between $r_{ij(A)}$ in population A and $r_{ij(B)}$ in population B, S_A and S_B are the standard deviation of $r_{ij(A)}$ and $r_{ij(B)}$ respectively, and $\bar{r}_A$ and $\bar{r}_B$ are the average r_{ij} across adjacent SNP i and j within the interval l for populations A and B for a common set of markers. The r^2 and r values were estimated using adjacent SNPs with the ld_estimate R scripts (16).

Allele Frequencies and Principal Components

Additionally, Pearson correlations were calculated between allele frequencies of all populations across the 332,468 SNP markers used in the present study and a principal components analysis (PCA) plot of all animals by breed was obtained from genotype data using PreGSf90 software (17).

Statistical Models and Analysis

Multivariate Genomic BLUP

Data quality checks for the Brazilian populations were performed using R program (18). Contemporary groups (CG) were formed by animals from the same farm, sex, year and season of birth, sex and management group and date of tick count evaluations. Contemporary groups with less than five animals and data exceeding 3.5 SD above or below the mean of the CG were excluded.

The statistical models for all populations except NG included the fixed effect of contemporary groups; the linear covariate effects of individual zebu breed composition and heterozygosity, according to their expected values based on pedigree information, and the linear and quadratic covariate effects of animal age. For pre-adjusted NG data only an overall mean was fitted as fixed effect. Additionally, direct additive genetic, permanent environmental and residual random effects were

included for the Brazilian populations that had repeated tick count measures and only the direct additive genetic and residual effects were considered for Australian and South African populations with single measurements. The models can be represented in matrix notation by the following equations:

$$\begin{bmatrix} y_{AN} \\ y_{HH} \\ y_{BN} \\ y_{BO} \\ y_{TC} \\ y_{BR} \\ y_{NG} \end{bmatrix} = \begin{bmatrix} X_{AN} & 0 & \cdots & 0 \\ 0 & X_{HH} & \cdots & 0 \\ \cdots & \cdots & \ddots & \cdots \\ 0 & 0 & \cdots & X_{NG} \end{bmatrix} \begin{bmatrix} \beta_{AN} \\ \beta_{HH} \\ \beta_{BN} \\ \beta_{BO} \\ \beta_{TC} \\ \beta_{BR} \\ \beta_{NG} \end{bmatrix} + \begin{bmatrix} Z_{AN} & 0 & \cdots & 0 \\ 0 & Z_{HH} & \cdots & 0 \\ \cdots & \cdots & \ddots & \cdots \\ 0 & 0 & \cdots & Z_{NG} \end{bmatrix} \begin{bmatrix} u_{AN} \\ u_{HH} \\ u_{BN} \\ u_{BO} \\ u_{TC} \\ u_{BR} \\ u_{NG} \end{bmatrix} + \begin{bmatrix} W_{AN} & 0 & \cdots & 0 \\ 0 & W_{HH} & \cdots & 0 \\ \cdots & \cdots & \ddots & \cdots \\ 0 & 0 & \cdots & 0 \end{bmatrix} \begin{bmatrix} p_{AN} \\ p_{HH} \\ p_{BN} \\ p_{BO} \\ 0 \\ 0 \\ 0 \end{bmatrix} + \begin{bmatrix} e_{AN} \\ e_{HH} \\ e_{BN} \\ e_{BO} \\ e_{TC} \\ e_{BR} \\ e_{NG} \end{bmatrix}, \quad (3)$$

where: the $\mathbf{y}_b$'s are vectors of the tick infestation trait for each bth breed, b=AN, HH, BN, BO, TC, BR, and NG, respectively for Angus, Hereford, Brangus, Braford, Tropical Composite, Brahman and Nguni breeds. Similarly, for each bth breed, $\boldsymbol{\beta}_b$'s are the vectors of systematic effects, $\mathbf{u}_b$'s are the vectors of random direct additive genetic effects, $\mathbf{p}_b$'s are the vector of random permanent environmental effects (only pertaining to AN, HH, BN and BO that have repeated measures), and the $\mathbf{e}_b$'s are the corresponding vectors of random residual effects. Additionally, each bth breed also has its own incidence matrices of systematic, direct additive genetic, and animal permanent environmental effects, respectively represented by $\mathbf{X}_b$'s, $\mathbf{Z}_b$'s, and $\mathbf{W}_b$'s.

As Brazilian populations had ungenotyped individuals with phenotype, we used a multi-trait single step genomic BLUP (ssGBLUP) approach (19, 20), with the following assumptions about the prior distributions of the model random parameters:

$$\begin{bmatrix} u_{AN} \\ u_{HH} \\ u_{BN} \\ u_{BO} \\ u_{TC} \\ u_{BR} \\ u_{NG} \end{bmatrix} \sim N\left(\begin{bmatrix} 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \end{bmatrix}, \begin{bmatrix} \sigma^2_{u_{AN}} & \sigma_{u_{AN,HH}} & \sigma_{u_{AN,BN}} & \sigma_{u_{AN,BO}} & \sigma_{u_{AN,TC}} & \sigma_{u_{AN,BR}} & \sigma_{u_{AN,NG}} \\ & \sigma^2_{u_{HH}} & \sigma_{u_{HH,BN}} & \sigma_{u_{HH,BO}} & \sigma_{u_{HH,TC}} & \sigma_{u_{HH,BR}} & \sigma_{u_{HH,NG}} \\ & & \sigma^2_{u_{BN}} & \sigma_{u_{BN,BO}} & \sigma_{u_{BN,TC}} & \sigma_{u_{BN,BR}} & \sigma_{u_{BN,NG}} \\ & & & \sigma^2_{u_{BO}} & \sigma_{u_{BO,TC}} & \sigma_{u_{BO,BR}} & \sigma_{u_{BO,NG}} \\ & & & & \sigma^2_{u_{TC}} & \sigma_{u_{TC,BR}} & \sigma_{u_{TC,NG}} \\ & & Symm. & & & \sigma^2_{u_{BR}} & \sigma_{u_{BR,NG}} \\ & & & & & & \sigma^2_{u_{NG}} \end{bmatrix} \otimes H \right), \quad (4)$$

where $\sigma^2_{u_b}$ is the additive genetic variance of the bth breed, $\sigma_{u_{b,c}}$ the additive genetic covariance between the bth and cth breeds, $\otimes$ denotes the direct product between the matrices, and $\mathbf{H}$ is a relationship matrix constructed by combining the pedigree and genomic relationship matrices (20–22). Although $\mathbf{H}$ is complex (22), its inverse, which is needed in the computations, has the simpler form (19):

$$H^{-1} = A^{-1} + \begin{bmatrix} 0 & 0 \\ 0 & (0.95G + 0.05A_{22})^{-1} - A_{22}^{-1} \end{bmatrix} \quad (5)$$

Here $\mathbf{G}$ is the genomic relationship matrix constructed as shown in the first method proposed by VanRaden (23) using current allele frequencies averaged across breeds. While theoretically correct for multiple breed populations, adjusting for breed specific allele frequencies was not performed because it has been shown to have negligible impact on prediction accuracy (24). Moreover, $\mathbf{A}^{-1}$ is the inverse of the numerator relationship matrix and A_{22} is the numerator relationship matrix for genotyped animals only. Since, there were no genetic ties between populations through pedigree, all the relationship between populations was genomic and given by $\mathbf{G}$.

Furthermore,

$$\begin{bmatrix} p_{AN} \\ p_{HH} \\ p_{BN} \\ p_{BO} \end{bmatrix} \sim N\left(\begin{bmatrix} 0 \\ 0 \\ 0 \\ 0 \end{bmatrix}, \begin{bmatrix} \sigma^2_{p_{AN}} & 0 & 0 & 0 \\ & \sigma^2_{p_{HH}} & 0 & 0 \\ & & \sigma^2_{p_{BN}} & 0 \\ Symm. & & & \sigma^2_{p_{BO}} \end{bmatrix} \otimes I \right) \quad (6)$$

and

$$\begin{bmatrix} e_{AN} \\ e_{HH} \\ e_{BN} \\ e_{BO} \\ e_{TC} \\ e_{BR} \\ e_{NG} \end{bmatrix} \sim N\left(\begin{bmatrix} 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \end{bmatrix}, \begin{bmatrix} \sigma^2_{e_{AN}} & 0 & 0 & 0 & 0 & 0 & 0 \\ & \sigma^2_{e_{HH}} & 0 & 0 & 0 & 0 & 0 \\ & & \sigma^2_{e_{BN}} & 0 & 0 & 0 & 0 \\ & & & \sigma^2_{e_{BO}} & 0 & 0 & 0 \\ & & & & \sigma^2_{e_{TC}} & 0 & 0 \\ & Symm. & & & & \sigma^2_{e_{BR}} & 0 \\ & & & & & & \sigma^2_{e_{NG}} \end{bmatrix} \otimes I \right), \quad (7)$$

where $\sigma^2_{p_b}$ and $\sigma^2_{e_b}$ are respectively the permanent environmental and residual variances of the bth breed, and $\mathbf{I}$ represents an identity matrix. These permanent environmental and residual effects were necessarily uncorrelated between traits due to the mutually exclusive assignment of individuals to breeds.

The (co)variance components and genetic parameters were estimated using Bayesian inference by Gibbs sampling, with the Gibbs2f90 program (25) in multi-trait analysis and using a linear animal model, considering the phenotypic measurement of tick infestation in each population as a different trait that is potentially genetically correlated among populations. Analyses consisted of a single chain of 1,000,000 cycles, with a burn-in period of 100,000 cycles and a thinning interval of 50 cycles. The posterior estimates were obtained using the Postgibbsf90 program (25) and the R/coda package (26). These estimated (co)variance components were used to obtain best linear unbiased predictions (BLUP) of tick resistance breeding values under multi- and single-trait scenarios using the Blupf90 software (25).

Univariate Genomic BLUP

Univariate breed-specific analyses were performed considering the records and the marginal model for each *b*th breed derived from multi-trait model described above (Equation [3]), as follows:

$$y_b = X_b\beta_b + Z_b u_b + W_b p_b + e_b . \quad (8)$$

Similarly, the marginal distributional assumptions were derived from equations [4], [6], and [7] as:

$$u_b \sim N\left(0, H\sigma^2_{ub}\right), p_b \sim N\left(0, I\sigma^2_{p_b}\right), \text{ and } e_b \sim N\left(0, I\sigma^2_{eb}\right). \quad (9)$$

These single trait/breed analyses were used as controls to check the advantages of jointly analyzing all breeds, and they used the same variance component estimates as the multi-trait analyses to maintain equivalent dispersion of breeding values for each breed under both strategies (single and multi population predictions).

Validation of Genomic Predictions

The utility of our reference populations to predict tick resistance and future phenotypes in single and multiple trait/breed genomic analyses was evaluated using the linear regression (LR) approach proposed by Legarra and Reverter (27). This method measures the correlation of estimated breeding values ($\hat{u}$) between whole (w) and partial (p) datasets between subsequent genetic evaluations when phenotypes are added for validation animals,

$$\rho_{w,p} = \frac{cov\left(\hat{u}_w, \hat{u}_p\right)}{\sqrt{var\left(\hat{u}_w\right)var\left(\hat{u}_p\right)}},$$

which is a function of the prediction accuracy with expected value of $E(\rho_{w,p}) \approx acc_p/acc_w$. Here *acc* is the "population accuracy", i.e. the correlation between true and estimated breeding values in the candidates for selection, which is a property of a population, not of an individual (27). Here, the whole dataset w included the combined set of all genotyped and phenotyped animals for all breeds (ranging from 229 to 3,062 individuals) in the multivariate analyses and the full set of genotyped and phenotyped animals for each breeds for univariate analyses. The partial datasets were derived for each population by two strategies, the first was the old-young where only 2/3 of the phenotypes pertaining to the older animals were retained in the partial data and the remaining 1/3 younger animals had their phenotypes set to missing and served as the validation group in both, uni and multivariate analyses. Additionally, for multivariate analyses only, a second strategy referred as other-pops consisted of removing from the analysis all phenotypes of the target population for validation and deriving predictions exclusively from the genetic correlations of the target with the other populations/breeds with full datasets included. When the $P_{w,p}$ is large (closer to one), the partial data reliably predicts the whole data. As additional validation statistics, we calculated the predictive ability defined as the correlation between phenotypes adjusted for fixed and permanent environmental effects ($\mathbf{y}^{\star}=\mathbf{y}_b-\mathbf{X}_b\beta_b-\mathbf{W}_b\mathbf{p}_b$) and $\hat{\boldsymbol{u}}_p$ ($r(\mathrm{y}^{\star},\hat{\mathrm{u}}_p)$) (28), where $\hat{\mathbf{u}}_p$ is the GEBV with partial data; and the slope of the regression of $\hat{\mathbf{u}}_w$ on $\hat{\mathbf{u}}_p$ ($\beta_{w,p}$), which was used to evaluate the degree of inflation/deflation of the genomic predictions.

RESULTS AND DISCUSSION

Population Genomic Structure and Diversity

Genomic Diversity

Based on the dispersion of individuals according to the first and second principal components (PC) of the **G** matrix (**Figure 1**), it is possible to identify the distinct genotypic constitution of the breeds included in the present study and the magnitude of genetic distance among them. If we analyze this PCA plot (**Figure 1**) from a perspective of a triangular form, we would place BR, HH and AN animals at the vertexes, respectively located at the lower left, the lower right and upper right regions of the plot. The composites BO and BN animals are respectively scattered on the lower and upper sides of the triangle that connect their founder breeds vertexes. Therefore the first PC mostly discriminates the percentage of indicine origin while the second PC genetically distinguished the AN and HH origin. The TC animals that are an admixture of Brahman, Sanga (represented mainly by Afrikaner but also some Tuli) and British/European (primarily Shorthorn and Hereford with some Charolais) breeds were scattered at the center of the triangle. Finally, the NG that is also part of the Sanga breed grouping fell in the PCA plot relatively close to TC samples towards the center upper left of our perspective triangle.

The clusters for the NG and AN samples have low dispersion reflecting their genetic homogeneity as opposed to more scattered and therefore heterogeneous samples of composite breeds (BN, BO, BR and TC) and HH (**Figure 1**). However, this could also reflect some ascertainment bias in the SNP on the Bovine HD array. The BO was the most genetically diverse breed group and had partial overlap with the HH samples. This was not surprising because the Delta G population from which records

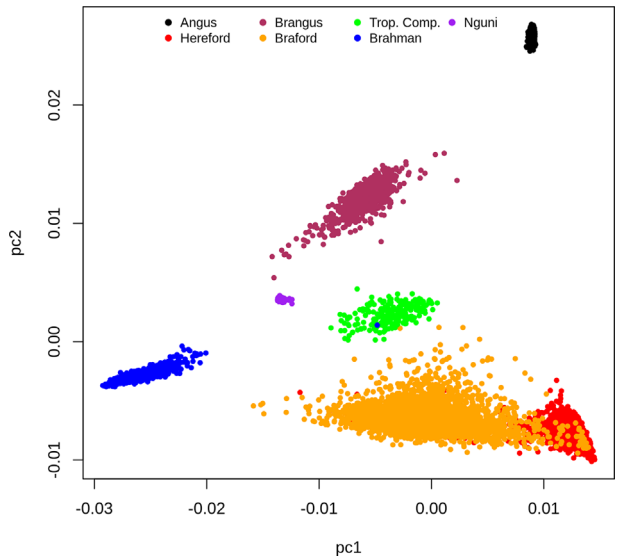

FIGURE 1 | Dispersion of individuals according to the first and second principal components of the **G** matrix, colored by breed.

were derived for the present study is a joint Breeding Program for purebred (as opposed to full blood) Herefords and Brafords that range from 1/16 to 7/8 of zebu proportion (29).

Linkage Disequilibrium

The mean ± standard deviation r^2 between adjacent SNPs ranged from 0.24 ± 0.34 to 0.37 ± 0.35 across all chromosomes for cattle populations from Brazil, Australia, and South Africa. The r^2 among chromosomes was similar within all breeds as observed in **Figure 2**.

The Brazilian populations of British origin, AN (0.33 ± 0.35) and HH (0.37 ± 0.35), had higher LD values than the other populations. The composite breeds from Brazil, BN (0.30 ± 0.28) and BO (0.31 ± 0.27) and TC (0.30 ± 0.29) from Australia had intermediate r^2 values. Conversely, the NG (0.24 ± 0.34) and BR (0.24 ± 0.27) breeds had lower r^2 among the studied breeds. Lower LD estimates at short distances are an indication of large ancestral population sizes and have been reported for indicine cattle compared to taurine cattle (30–32). This is consistent with LD estimates in the present study and in the case of NG, an African taurine population of the Sanga group, a previous report has also found lower short distance LD compared to European taurine cattle (33).

Furthermore, the r^2 was > 0.3 for more than 40% of neighboring SNPs only in HH and BO breeds (data not shown). In relation to the other breeds, the mean r^2 > 0.3 were about 30% for NG and BR and, around 38% for BN, AN, and TC.

Genomic selection relies on LD between QTLs and flanking SNPs and simulation results demonstrated that, to obtain sufficiently accurate GEBVs to be useful for breeding decisions, an average r^2 between adjacent markers of 0.20 would suffice [e.g. (34)]. This was achieved for all chromosomes within all studied breeds with our 332k SNP panel (**Figure 2**).

Persistence of Phase Across Breeds

The correlations ($R_{A,B}$) of linkage phase were used to estimate the haplotype-sharing between pairs of adjacent SNPs across breeds (**Table 2** and **Figure 3**). The $R_{A,B}$ statistic is useful because the accuracy of genomic selection across breeds relies on persistence of the LD phase, though not actually between pairs of SNPs but between SNP and QTL (7, 8). If the correlation between pairs of adjacent SNPs is high, then the correlation between the QTL and SNP should be high as well. In general, if two populations have a high positive $R_{A,B}$ value, it suggests high LD and the same haplotype phase in both populations; however, a high negative value indicates high LD but with reverse linkage phase (7).

The $R_{A,B}$ correlation between adjacent SNP pairs across chromosomes among Brazilian populations ranged from 0.77 (AN *vs*. BO) to 0.95 (HH *vs*. BO) (**Table 2**). The correspondence of linkage phase among the Brazilian composites (BN and BO) and Australian populations (BR and TC) was on average 0.80 and the highest $R_{A,B}$ value was 0.89 between TC *vs*. BO (**Table 2**). Among AN, HH and Australian populations, the $R_{A,B}$ values varied from 0.63 (AN *vs*. BR) to 0.87 (HH *vs*. TC). The smallest values were found for NG vs. all other breeds (**Figure 3**). As observed in the LD (**Figure 2**), the average $R_{A,B}$ values also vary across chromosomes within population pairs (**Figure 3**). This information is useful to choose marker density that should be determined according to the lower bound of the chromosome $R_{A,B}$ averages, particularly if those chromosomes harbor mutations potentially associated with traits of interest.

De Roos et al. (7) pointed out that finding markers in LD with QTL across divergent breeds, such as Australian Angus and New Zealand Jersey, would require a panel of approximately 300,000 markers. This is aligned with our choice of marker density (332k), but even so, $R_{A,B}$ was relatively low for several breed pairs, especially those including the NG breed, and between taurine AN and HH

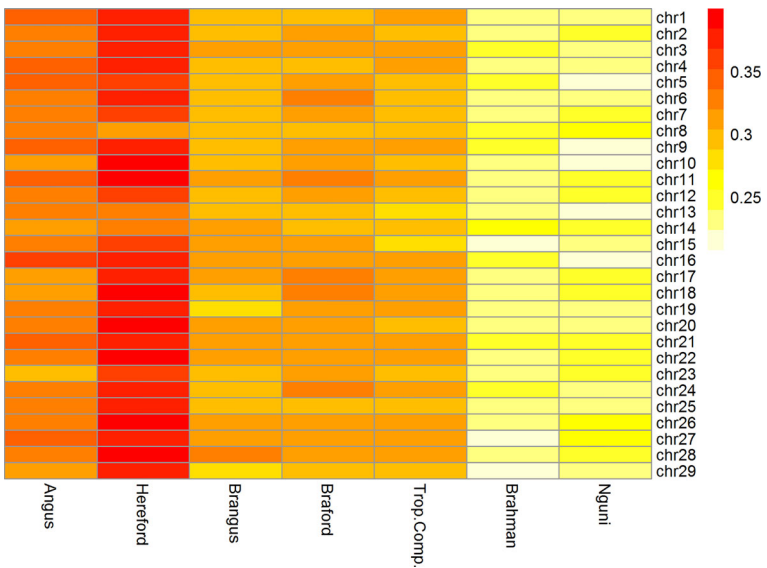

FIGURE 2 | Heatmap of linkage disequilibrium (r^2) between adjacent markers of the 332k SNP panel by breed and chromosome.

TABLE 2 | Average persistence of phase for adjacent markers (above the diagonal) and correlation of allele frequencies (below the diagonal) between different populations.

Population	Angus	Hereford	Brangus	Braford	Tropical Composite	Brahman	Nguni
Angus		0.81	0.81	0.77	0.81	0.63	0.27
Hereford	0.72		0.87	0.95	0.87	0.69	0.28
Brangus	0.77	0.60		0.92	0.88	0.82	0.31
Braford	0.69	0.88	0.77		0.89	0.81	0.32
Tropical Composite	0.67	0.69	0.76	0.81		0.83	0.32
Brahman	0.21	0.15	0.60	0.54	0.55		0.35
Nguni	0.48	0.43	0.66	0.64	0.67	0.67	

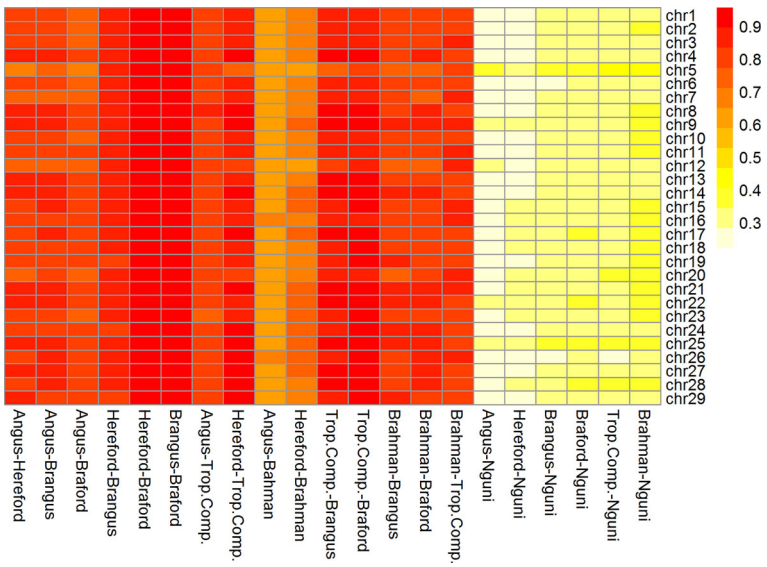

FIGURE 3 | Heatmap of correlation of phase between adjacent markers among breeds and chromosomes (332k panel by chromosome).

and indicine BR (**Table 2**), which are the most divergent breed groups. The highest $R_{A,B}$ value found between HH and BO indicates the highest proportion of SNP sharing the same linkage phase for these breeds and was in agreement with previous findings within the same populations and a 50k panel (32). It is important to point out however that genomic prediction across-population or across-breed accuracies rely not only on the persistence of LD phase across populations, but also on the trait genetic architecture and size of the reference populations (35).

Allele Frequency Correlations

Differences across populations were seen in terms of allele frequencies (**Table 2**) with correlations between populations ranging from 0.15 (HH and BR) to 0.88 (HH and BO). Composite breeds had high correlations among themselves and with their taurine founder breeds (AN with BN and HH with BO). As expected low correlations were found between the indicine BR and the taurine AN and HH, moderately high correlations were observed between BR and composites (BN, BO and TC) and medium to moderately high correlations of NG with all the other breeds.

Genomic Selection Parameters

Genetic Correlations and Heritabilities

All 39 estimated variance components passed a convergence test based on the Geweke's criterion (36). The mean ± standard deviation effective number of independent samples (37) for the genetic parameters was 402 ± 857, ranging from 46 to 4,594. This wide range of values reflects distinct data information content to estimate the posterior means of genetic correlations and heritabilities across the different populations (**Table 3**). Hereford showed the lowest h^2 for tick counts among the studied breeds, while the other Brazilian commercial populations had low to moderate values, in line with heritabilities typically found for this trait (5). The Australian BR and TC scores and South African NG averaged counts had larger estimated values around 0.40 in our multi-trait analysis, and were are considerably higher than previous results obtained within-population under single trait analyses of 0.15 for BR (11). Nonetheless, a similarly high h^2 value of 0.42 was reported for another Tropical Composite Australian population, the Belmont Red (38) and depending on the time of the year h^2 for perineum tick counts ranged between 0.00 and 0.58 (39). The wide range of

TABLE 3 | Posterior mean and time series standard errors for genetic correlations (above diagonal) and heritabilities (diagonal) of tick resistance measures across different populations.

Population	Angus	Hereford	Brangus	Braford	Tropical Composite	Brahman	Nguni
Angus	0.27 ± 0.001	0.32 ± 0.03	0.65 ± 0.001	0.42 ± 0.03	0.15 ± 0.04	0.17 ± 0.03	0.17 ± 0.03
Hereford		0.05 ± 0.001	0.39 ± 0.01	0.35 ± 0.01	0.22 ± 0.04	0.01 ± 0.03	0.05 ± 0.06
Brangus			0.21 ± 0.003	0.87 ± 0.01	0.32 ± 0.02	0.47 ± 0.01	0.29 ± 0.05
Braford				0.17 ± 0.001	0.48 ± 0.02	0.59 ± 0.01	0.14 ± 0.05
Tropical Composite					0.42 ± 0.01	0.28 ± 0.02	-0.01 ± 0.05
Brahman						0.39 ± 0.01	0.18 ± 0.03
Nguni							0.37 ± 0.02

h^2 estimates for tick resistance in cattle found in the present and other studies is related to differences in phenotyping, environmental control and intrinsic population characteristics. It is also important to highlight that the NG trait is the average of log transformed tick count over multiple observations. This may have lowered the environmental variance and, consequently, inflated the estimated h^2 for the NG breed. The estimated repeatability for the AN, HH, BN, BO populations that had repeated measures were, respectively 0.30, 0.16, 0.37, 0.28, with corresponding standard errors (SE) of 0.001 or less.

The largest genetic correlation among all studied populations was observed between BN and BO. These two breeds are composites with about 3/8 of zebu composition, mostly Nelore in our samples, and the other 5/8 being taurine of British origin, Angus or Hereford. This result indicates a very similar additive genetic mechanism for tick resistance in both populations. The second largest relationship was observed between AN and its composite with zebu, the BN breed, and this is not surprising because the average expected contribution of AN to BN is approximately 62.5%. Even though AN and BN are more closely related than BN and BO, the higher genetic correlation between the latter pair could be related to greater indicine impact on tick resistance (5, 40, 41). Braford and BR had the third largest genetic association for tick resistance and the only other with a value above 0.5. Brangus and BR, BO and TC, and BO and HH had values around 0.4 showing some level of additive genetic association, but not strong enough to decisively contribute to the sharing of information among reference populations designed for tick resistance prediction across breeds. All other breed pairs showed weak genetic correlations between tick phenotypes, particularly for the NG breed where there was no useful association pertaining to the improvement of resistance.

Genomic Predictive Ability

Predictive ability, as a measure of the GEBV to predict the observed phenotype, was improved under the old-young validation for three of the seven populations using the multi-trait approach compared to a single trait within-population prediction. Moreover, we observed improvement for the partial and whole data GEBV correlation in all cases by using multi-trait analysis under old-young (**Table 4**), with relative improvements ranging from 3% for BO to 64% for TC. Moreover, the multi-trait analysis was useful to correct typical over-dispersion of GEBV in all populations except for the BO breed that had no such issue in both analyses – uni or multivariate for the old-young validation strategy (**Table 4**).

The multivariate validations based on data of other populations only (other-pops), which were included to evaluate the possibility of predicting tick resistance for populations that do not have a reference population for this trait, had in general a poorer predictive performance compared to uni and multivariate old-young validations for all parameters evaluated (**Table 4**). These results emphasize the importance of having consistent phenotyping strategies and genotypes for populations in which improving tick resistance is a goal. Nonetheless, prediction ability retained estimated values that can be considered useful

TABLE 4 | Predictive ability[1] [$r(y^*,\hat{u}_p)$], regression coefficient ($\beta_{w,p}$) and correlation between genomic breeding values ($\hat{\mathbf{u}}$) predicted from whole (w) and partial[2] (p) data using uni and multivariate ssGBLUP population analyses.

Population	$r(y^*, \hat{u}_p)$			$\beta_{w,p}$			$\Delta_{w,p}$		
	Uni old-young	Multi old-young	Multi other-pops	Uni old-young	Multi old-young	Multi other-pops	Uni old-young	Multi old-young	Multi other-pops
Angus	0.15	0.16	0.07	0.92	1.06	1.06	0.50	0.58	0.22
Hereford	0.05	0.05	0.04	0.99	1.01	0.65	0.56	0.58	0.40
Brangus	0.22	0.25	0.22	0.88	0.93	1.46	0.67	0.72	0.57
Braford	0.24	0.24	0.17	1.01	1.00	1.45	0.76	0.78	0.56
Tropical Composite	-0.06	0.00	0.21	0.32	0.53	1.74	0.14	0.23	0.35
Brahman	0.13	0.13	0.20	0.77	0.83	1.44	0.57	0.64	0.43
Nguni	0.04	0.04	-0.04	0.79	1.00	-2.11	0.18	0.20	-0.04

[1]*Correlation between phenotypes adjusted for fixed and permanent environmental effects and* $\hat{u}_p$.

[2]*Partial datasets derived by two strategies: old-young = excluding phenotypes of 1/3 younger animals as validation group; and other-pops = removing all phenotypes of the target population for validation.*

for applied purposes for BO and BN, and was improved for TC and BR (**Table 4**). These results are an indication that a breed without reference population for tick resistance but with high genomic relationship and persistence of LD phase with one or more of our measured populations could be targeted for selection through such predictions. The greatest challenge for such application however is to estimate meaningful trait genetic correlation parameters with the reference populations.

Braford was the breed with highest predictive ability in all criteria in all analyses: uni or multivariate (**Table 4**). This was not surprising since BO has the largest reference population in our study and moderate trait heritability. Furthermore, the viability of implementing genomic selection for this breed has been previously demonstrated with a subset of our BO/HH data (4). Despite being highly correlated with BN and BR, there was minor improvement for BO in the old-young multi-trait analysis, as there was already considerable information for this breed. In fact BN and BR were the breeds that benefit most in terms of accuracy in the multi-breed multi-trait analyses, likely through their genetic linkage to BO tick resistance.

The HH breed with the second largest reference population in our sample had a low predictive ability (**Table 4**) in agreement with the very low h^2 for tick counts of this breed (**Table 3**). Nonetheless, the estimated correlations of GEBV for whole and partial data can be considered of medium value and useful enough to allow practical use of genomic selection to improve tick resistance of this breed. There was a minor improvement from old-young uni to multivariate analysis basically attributable to a medium genetic correlation with the BO breed (**Table 3**) and other breeds in the study. In both old-young analyses, HH predictions can be considered unbiased given the $\beta_{w,p}$ values close to 1 in **Table 4**.

Angus and BN breeds had similar population sizes close to 1,000 animals and over 2,200 records, their tick count h^2 were in the medium range, and in both populations we observed prediction results that ensure the possibility of improving tick resistance through genomic selection (**Table 4**). Brangus had, however, slightly better predictive abilities and GEBV correlations for uni- and multi-variate analyses than AN. The estimated genetic correlations were positive and of strong magnitude between BN and BO, AN and BN, and AN and BO, resulting in improvements of practical importance for all prediction measures in the old-young multivariate validations for these populations. These results support a joint evaluation to implement genomic selection for tick resistance. A similar strategy has been suggested for an international genetic evaluation of feed intake in dairy cattle for high-input production systems (6).

The largest improvements were observed for the TC population (**Table 4**), which had the smallest reference population with only 229 individuals. Nonetheless, the genetic correlation of their tick score phenotypes with other larger populations (**Table 3**), particularly the medium value with Brafords, was not strong enough to yield prediction with useful correlations to be immediately implemented in practical genomic selection. The results, however, indicate that perhaps even a modest additional effort of phenotyping in this population could suffice for future adoption of genomic prediction for tick resistance of Tropical Composites.

Even though the BR breed had a modest reference population of 675 animals, it had the third highest GEBV correlations in old-young uni and multivariate analyses (**Table 4**) due to the high heritability of their tick scores. With a high genetic correlation with the BO and a medium genetic correlation with BN, BR prediction accuracies and dispersions were improved when using a joint multi-population evaluation of tick phenotypes.

Finally, the NG breed had low predictive ability and GEBV correlation (**Table 4**) likely reflecting modest sample size. These results were not substantially improved when using the multivariate analysis due to overall low genetic (**Table 3**) and phase (**Table 2**) correlations of Ngunis with the other populations in our study. The poorer results and relationships for Nguni may reflect factors other than the genetic mechanisms of host tick resistance. For example there are multi-host tick species in South Africa [e.g. (3)] that are not present in either Brazil or Australia, so the counts may simply be a reflection of those different tick species possibly having different mechanisms of resistance (42).

Another factor that could also explain this lower accuracy of prediction for NG could be the time and body location of counting. In the Australian and Brazilian data, tick counts only occurred at times of the year when there was large phenotypic variation and assessing one whole side of the animal, while for the NG population perineum counts occurred throughout the year and the averaged data used in the present study included counting times that would not meet the phenotyping requirement of at least 20 ticks per side of each animal, averaged over at least 15 animals (42). Therefore, additional phenotyping and genotyping must be pursued within this breed before practical genomic selection can be implemented to increase its tick resistance.

A recent review of the scientific literature identified possibly simpler, more cost-effective phenotype(s) for tick resistance, which if developed and validated, could be used to greatly enlarge the reference populations for genomic prediction and to improve the accuracy of GEBV for this trait, as well as potentially improving tick control through cattle management (42).

Even though more extensive phenotyping should be a continuous effort to improve the accuracy of GEBV for tick resistance, old-young validation results from this study (**Tables 3**, **4**) indicate that a joint genomic evaluation of Angus, Hereford, Brangus, Braford and Brahman using multivariate genomic BLUP can be readily implemented to improve tick resistance of these populations using genomic predictions. The extent of improvement of accuracy of GEBV for a breed from the multi-population approach largely reflect the extent of LD phase between the breeds, except for cases such as BO where the reference population is already relatively large. Even for these breeds, the accuracy from using multi-breed information may be further improved if sequence data is used, provided the same mutations are segregating across the breeds (which is quite likely in composite breeds), such that correlations would be essentially 1 (43).

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: The raw datasets cannot be made available because they are the property of the Breeders and Institutions involved in generating them and this information is commercially sensitive. For scientific research purposes, the data requests should be forwarded along with the research proposal to the corresponding author email: fernando.cardoso@embrapa.br.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because it was developed using already existing datasets. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

FC, OM, AD, NM, HB, MY, RP-W and BH conceived and designed the study. FC, OM, MY, RP-W, GC, and BH performed data analyses. FC wrote the manuscript draft. C-GG, NM and AM participated in the design and acquisition of data. All authors contributed to the article and approved the submitted version.

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Allelic Variation in Protein Tyrosine Phosphatase Receptor Type-C in Cattle Influences Erythrocyte, Leukocyte and Humoral Responses to Infestation With the Cattle Tick *Rhipicephalus australis*

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The protein tyrosine phosphatase receptor type-C (*PTPRC*) gene encodes the common leukocyte antigen (CD45) receptor. CD45 affects cell adhesion, migration, cytokine signalling, cell development, and activation state. Four families of the gene have been identified in cattle: a taurine group (Family 1), two indicine groups (Families 2 and 4) and an African "taurindicine" group (Family 3). Host resistance in cattle to infestation with ticks is moderately heritable and primarily manifests as prevention of attachment and feeding by larvae. This study was conducted to describe the effects of *PTPRC* genotype on immune-response phenotypes in cattle that display a variable immune responsiveness to ticks. Thirty tick-naïve Santa-Gertrudis cattle (a stabilized composite of 5/8 taurine and 3/8 indicine) were artificially infested with ticks weekly for 13 weeks and ranked according to their tick counts. Blood samples were taken from control and tick-challenged cattle immediately before, then at 21 d after infestation and each subsequent week for 9 weeks. Assays included erythrocyte profiles, white blood cell counts, the percentage of cellular subsets comprising the peripheral blood mononuclear cell (PBMC) population, and the ability of PBMC to recognize and proliferate in response to stimulation with tick antigens *in vitro*. The cattle were *PTPRC* genotyped using a RFLP assay that differentiated Family 1 and 3 together (220 bp), from Family 2 (462 bp), and from Family 4 (486 bp). The *PTPRC* allele frequencies were Family 1/3 = 0.34; Family 2 = 0.47; Family 4 = 0.19. There was no significant association between *PTPRC* genotype and tick count. Each copy of the Family 1/3 allele significantly decreased total leucocyte count (WCC) and $CD8^+$ cells. Increasing dosage of Family 2 alleles significantly increased red blood cell count (RCC), haematocrit

(PCV), and haemoglobin (Hb) concentration in blood. Increasing dosage of the Family 4 allele was associated with increased WCC, reduced RCC, reduced PCV and reduced Hb. Homozygote Family 1/3 animals had consistently lower IgG1 in response to tick Ag than homozygote Family 2 animals. The *PTPRC* genotype influences the bovine immune response to ticks but was not associated with the observed variation in resistance to tick infestation in this study.

Keywords: ticks & TBDs, host resistance, immunity, parasite, immunoglobulin, erythron, leukocytes

INTRODUCTION

PTPRC or protein tyrosine phosphatase receptor-type C, also known as CD45, or leukocyte common antigen (LCA) is a key component of the signal transduction cascade in immune cells (1). Throughout this report, we refer to *PTPRC* as the gene encoding CD45, although the gene as annotated for human and mouse has several aliases: *B220*, *CD45*, *CD45R, Cd45, GP180, LCA*, *L-CA, Ly-, LY5*, *Ly-5, Lyt-, Lyt-4,T200*. CD45 was initially investigated in cattle for its potential involvement in pathogen tolerance in African cattle (2). They found that allelic polymorphisms in CD45 constituted the basis for differential antibody staining in peripheral blood leukocytes from cattle of African, European, and Indian origin, and suggested that polymorphism might be associated with tolerance to regionally endemic pathogens.

CD45 is an abundant cell surface glycoprotein found in the plasma of all nucleated hematopoietic cells and controls the immune response by dephosphorylating molecules that initiate antigen receptor signalling in T- and B-cell cells, such as the Src family kinases (SFKs) (3, 4). There are many isoforms of differing molecular weight due to the alternative splicing of exons 4, 5 and 6 (referred to as A, B and C) in the extracellular domain. The smallest isoform is CD45RO of approximately 180 kDa, lacking all of the alternatively spliced exons, whereas the largest isoform that includes all three exons – CD45RABC is about 240 kDa and heavily glycosylated (1, 3, 5). In addition to these variably spliced domains, the protein comprises three fibronectin type III (FN3) repeats, a short transmembrane domain, and a cytoplasmic region of two tandemly duplicated PTPase homology domains (D1 and D2), in which only D1 is catalytically active (3). The expression of *PTPRC* is tightly regulated depending on the cell type, maturation, and activation state. Although nucleotide sequence in the extracellular domains is highly variable, the isoform structures are largely conserved across species (3, 6). In *Bos taurus* cattle, *PTPRC* is on chromosome 16, has at least 30 exons and nine characterized isoforms (Gene ID: 407152, NCBI, 2021). Human and *B. taurus PTPRC* sequences show approximately 70% sequence identity. In humans five CD45 isoforms are well characterized (6). Ballingall et al. (2) initially considered *PTPRC* as one of several genes that might influence the diverse responses of African and Asian cattle to endemic pathogens in Africa. They noted that peripheral blood leukocytes from African and European taurine cattle had similar CD45RO antibody staining patterns whereas in indicine cattle, the pattern was variable. The pattern of staining corresponded with four distinct allelic families of *PTPRC*: *B. taurus*, *Bos indicus* (×2), and cattle of African origin (2, 7).

Ballingal et al. (2) showed that there appeared to be strong natural selection on extracellular domains of CD45 protein and proposed that it was likely to be a determinant of the immunity of cattle to endemic pathogens. Loss-of-function mutations of *PTPRC* have consequences related to immunodeficiency and malignancy in humans and mice (4) and CD45 has been associated with disease in cattle. A microarray-based study showed that *PTPRC* expression in the mesenteric lymph nodes of cattle with high resistance to gastrointestinal nematodes was increased, which was subsequently confirmed by qRT PCR (8). In a study on the reactivity of subsets of leukocytes present in the skin of *B. taurus* and *B. indicus* cattle infested with *R. australis*, antibodies specific for CD45 and CD45RO epitopes bound differentially in taurine and indicine cattle (9). In a follow-up study using tick resistant and susceptible Santa Gertrudis cattle, the reactivity of cells to CD45 and CD45RO mAbs also differed between resistant and susceptible cattle of the same breed (10). It was proposed that CD45 variants of *B. indicus* lack the epitopes recognized by mAb raised against CD45 and CD45RO in taurine cattle, and that CD45 might therefore have potential as a biomarker for resistance to infestation with cattle ticks.

We hypothesised that sequence variation in *PTPRC* in cattle affects resistance to ticks and immune phenotype. Our aim here was to take observations on erythrocytes, leukocytes and immunoglobulins obtained from cattle that were experimentally infested with *R. australis* in a previous experiment (10, 11), genotype the animals for the major *PTPRC* variants, and determine whether variation in these observations was associated with the presence of *PTPRC* variants.

MATERIALS AND METHODS

Background Experimental Design, Animals, Tick-Counts, and Immunological Assays

The experimental methods are described in detail in the earlier articles (10, 11) and summarized briefly here. Thirty-five tick-naïve Santa-Gertrudis cattle (a stabilized composite of 5/8 taurine – Shorthorn – and 3/8 indicine – Brahman) were used in this study, conducted near Brisbane, in Queensland, Australia. The cattle were from a single property of origin and were selected such that their parentage was as far as possible an even admixture

of sires. Five cattle were held as control animals on a separate, tick-secure property within 5 km of the experimental farm, and the remaining 30 were artificially infested by application to the neck and withers of 10 000 (0.5 g) *Rhipicephalus australis* tick larvae weekly for 13 weeks. Tick larvae were of the Non-Resistant Field Strain (NRFS) that is maintained free of *Babesia* and *Anaplasma* pathogens at the Queensland Department of Agriculture and Fisheries' Biosecurity Science Laboratories (12). Tick counts were conducted weekly using the standard tick count method of Utech et al. (13, 14). Each infestation consisted of larvae applied to the neck and withers. Blood samples were taken from control and tick-challenged cattle immediately before the first infestation, then at 21 d post primary infestation (PPI) and each subsequent week for 9 weeks. The study was conducted with approval from the University of Queensland Animal Ethics - Production and Companion Animals Committee (Approval numbers: SVS/864/06/CRC and SVS/872/07/CRC).

Tick count data recorded over 13 weeks were originally analysed using a mixed effects model applied to data summarized over time (median, area under the curve, final count) fit by restricted maximum likelihood (REML), to rank each animal on its ability to resist tick infestation.

Erythrocyte profiles and white blood cell counts were conducted using a VetABC animal blood cell counter (ABX Hematologie). The percentages of cellular subsets comprising the peripheral blood mononuclear cell (PBMC) population were determined using the Ab listed in **Table 1** with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems), as described in detail by Piper et al. (15). The ability of PBMC to recognize tick antigen (Ag) and proliferate in response to stimulation with antigens *in vitro* was quantified for concanavalin-A (ConA), and Ag mixtures derived from soluble fractions of salivary gland (SS), mid-gut (GS) or larvae (LS), or membrane-bound fractions of salivary gland (SM) or mid-gut (GM) in triplicate using the method described by Piper et al. (11). Results of PBMC proliferation are expressed in terms of optical density (OD) of microplate photometric readings at 450 nm. IgG1 and IgG2 responses to tick infestation were conducted in triplicate using an indirect ELISA, in wells coated with fractionated tick Ag (salivary soluble – SS; gut membrane – GM; gut soluble – GS; larval soluble – LS) as described in detail in Piper et al. (15). Microtiter plates were coated with diluted tick antigens. Sera were diluted and added to the microtiter plates in triplicate. Monoclonal antibodies (mouse anti-bovine IgG1 and mouse anti-bovine IgG2) were added to all wells. The conjugated antibody (goat anti-mouse IgG heavy and light chain specific, conjugated to horseradish peroxidase) was then added to each well. A tetramethylbenzidine-peroxidase substrate was used to develop the signal, and the reaction was stopped with orthophosphoric acid. The absorbance was read at 450 nm and the mean OD of each biological sample from triplicate wells was used for statistical analysis.

TABLE 1 | Monoclonal antibodies used and the cell subsets labelled in flow cytometric analysis of cellular subsets.

Specificity	Cell Subset	Identity	Source	Isotype
Isotype control		IgG1	Dako	IgG1
CD3	T cells	IgG1	VMRD[b]	
CD4	T helper	IL-A11	Cell culture[a]	IgG2a
CD8	T cytotoxic	IL-A51	Cell culture[a]	IgG1
CD14	Monocytes	MM61A	VMRD[b]	IgG1
CD25	Activated (IL-2Rα)	IL-A111	Cell culture[a]	IgG1
MHCII	Macrophages, dendritic cells, B cells, activated T cells	IL-A21	Cell culture[a]	IgG2a
WC3	B cells	CC37	Cell culture[a]	IgG1
WC1	γδ T cells	IL-A29	Cell culture[a]	IgG1
Goat anti-mouse		IgG-FITC	Calbiochem	IgG

[a]*Monoclonal antibodies obtained from cell culture were derived from hybridomas sourced from the International Livestock Research Institute in Kenya.*
[b]*VMRD, Veterinary Medical Research and Development Inc.*

Genotyping Assays

Thirty-four cattle were genotyped for *PTPRC* families using a restriction-enzyme fragment length-polymorphism (RFLP) assay that differentiated Family 1 and 3 together (220 bp amplicon - taurine and African taurindicine families), from Family 2 (462 bp - indicine), from Family 4 (486 bp - indicine). Accession numbers of publicly available sequences are shown in **Table 2**. Genotyping and sequencing assays assessed the region of *PTPRC* previously identified as exon-9 by Ballingal et al. (2), but which we now consider to most likely correspond with exon-5 or exon-6 (data not shown). The distinguishing features of the 4 families are shown in **Table 3**. We used a modification of their genotyping assay using the primers *CD45ex9_F*: TCCTGGGGCTATTTTTGTTGGTGTT and *CD45ex9_R*: AGGCTGCTCCGAGGTCACCA, with annealing temperature of 59°C, and an expected fragment size of 486 bp. The restriction site enzyme DdeI was used to cut only the *B. taurus* (Family 1 & Family 3) reference sequence at

TABLE 2 | Accession numbers and references for nucleotide sequences used in this study.

Accession No.	Species	Exon/Region	Genome Scaffold	Reference
NC_037343.1 (77540526-77670102)	*Bos taurus*		ARS-UCD1.2 Chromosome 16	NCBI Nucleotide
NC_032665.1 (75903959-76032820)	Bos *indicus*		Bos_indicus_1.0 Chromosome 16	
NC_040091.1 (76794293-76923526)	Bos *taurus* x *indicus*		UOA_Brahman_1 Chromosome 16	
AJ278876	*Bos indicus*	Partial Exon 9		Ballingal et al. (2)
AJ278877	*Bos indicus*	Partial Exon 9		
AJ278878	*Bos indicus*	Partial Exon 9		
AJ278879	*Bos indicus*	Partial Exon 9		
AJ400864	*Bos taurus*	Partial mRNA PTPRC gene		

TABLE 3 | Major discriminating features of nucleotide sequence used for defining the four distinct *PTPRC* families.

Family	Constant Variant Nucleotides	Insertions or deletions	Genotype in RFLP assay (fragment length)
Family 1 Taurine	Reference sequence	Reference sequence	220 bp
Family 2 Indicine	G<A 68,992 T<A 68,995 A<G 69,001 (shared Family 2 & 4) plus 2 unique SNP G<A 68,876 T<A 68,964	24 bp deletion 68,932	462 bp
Family 3 Taurindicine	9 unique SNP AG<TT 68,798-9 G<A 68,850 G<A 68,852 A<G 68,865 G<A 68,894 T<A 68,897 C<G 68,899	ACA insertion at 68,895 An insertion at 68,761 4 bp deletion at 68,792	220 bp
Family 4 Indicine	G<A 68,992 T<A 68,995 A<G 69,001 (shared Family 2 & 4) Plus 3 unique SNP G<C 68,890 A<G 68,928 A<G 68,930	Nil	486 bp

location 68,989. Genotyping was conducted by capillary electrophoresis using a 3130 XL Genetic Analyzer (Thermofisher, Australia). The amplicons generated for Family 1 and Family 3 were the shortest, at 220 bp, whereas Family 2, with the 24-bp deletion, is 462 bp, and Family 4 is the complete amplicon from forward to reverse primer of 486 bp (**Figure 1**).

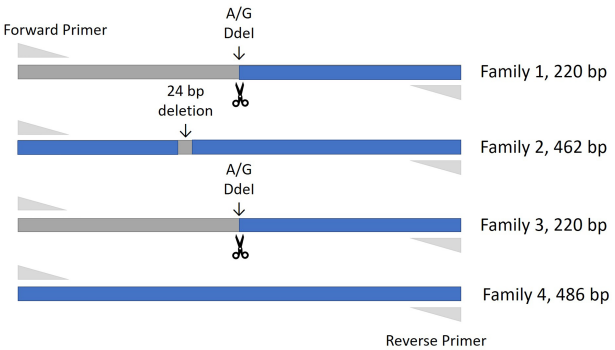

FIGURE 1 | Amplification fragment sizes obtained by the genotyping assay for each of the PTPRC families. The restriction site enzyme DdeI was used to cut the *Bos taurus* (Family 1 & Family 3) reference sequence at location 68,989. Neither the Family 2 nor Family 4 alleles are cut at this location, and these alleles are differentiated by the 24 bp deletion that is the main characteristic of the Family 2 allele.

Statistical Analysis

All statistical analysis was conducted using R version 4.0.3 [(16) R Core Team, 2018]. Data consisted of 14 or 15 successive time-series observations for each variable for each individual. Only a subset of samples from resistant and susceptible animals had originally been subjected to IgG quantification, so the representation of each of the genotypes was uneven, with some genotypes missing completely. Therefore, only those animals with 220/220 (n = 3) and 462/462 (n=7) genotypes were included in the analysis for IgG1 and IgG2. All dependent variables were checked for normality by plotting as histograms and application of the Shapiro-Wilk test of normality. Variables with non-normal distributions were tested for compliance after natural log and square root transformations, and if these did not yield normally distributed data, they were then transformed using the Johnson family of distributions using the "ls" procedure from the R package "jtrans" (version 0.2.1). Given the highly skewed time-responses of IgG1 and IgG2, only the distributions of the residuals of the GAMs were checked and all were found to approximate normal distributions. Time was expected to be an important explanatory variable, but there was no *a priori* reason to expect any particular response function for any of the dependent variables over time. Therefore, a generalized additive model was used, with time as a smoothed effect, the allele dosage as a fixed effect (for each of the three alleles, any animal can have the value 0,1,2), and individual animal as a random effect. The R function "gam" from the package "mgcv" (version 1.8-33) was used (17), and models were tested using the "gam.check" function (18). Residuals were plotted for each model and checked for deviations from normality. Estimates of p-values are presented in tables as obtained from the models, but a statistical significance level (α) was set at 0.00083, consistent with Bonferroni correction for testing of 60 variables. For the re-analysis of resistance to ticks, a similar approach was taken to make more efficient use of the non-summarized time-series data.

RESULTS

The most frequent allele was the 462, indicine Family 2, with a relative frequency of 0.47 (32/68 possible alleles), followed by the taurine Family 1/3 allele 220 at 0.34 (23/68 possible alleles), with the 486 allele of the indicine Family 4 being least frequent at 0.19 (13/68 possible alleles). The distribution of genotypes and alleles was uneven, the most common genotype being 462/462, the indicine Family 2 (**Table 4**, 10/34 animal genotypes). However, the observed frequencies of genotypes did not differ from expectations under Hardy-Weinberg equilibrium (**Table 4**, χ^2 = 3.314, $p > 0.1$).

Neither tick burden nor resistance category was significantly influenced by the *PTPRC* genotype. Linear regressions of total or median tick count against genotype were not significant (p = 0.46, 0.64, 0.74 for the 222, 462 and 486 genotypes respectively). The GAMs for tick count considered each of 12 weekly

TABLE 4 | *PTPRC* allele and genotype frequencies.

Allele	Allele Count	Allele Frequency	Genotype	Genotype Count	Expected Genotype Frequency	Expected Genotype Count	χ^2	*p*-value
220	23	0.34	D220/D220	5	0.11	4	3.314, df = 3	> 0.1
462	32	0.47	D220/D462	7	0.32	11		
486	13	0.19	D220/D486	6	0.13	4		
			D462/D462	10	0.22	8		
			D462/D486	5	0.18	6		
			D486/D486	1	0.037	1		

Genotype	Controls	Medium Resistance	Resistant	Susceptible	Total
220/220	2	0	1	2	5
220/462	1	6	0	0	7
220/486	0	4	1	1	6
462/462	1	5	2	2	10
462/486	1	3	1	0	5
486/486	0	1	0	0	1
Total	5	19	5	5	34

Part A: overall allele and genotype frequencies and assessment of Hardy-Weinberg equilibrium of alleles and genotypes. Part B: genotypes according to their resistance or experimental status. (Control animals were not infested; the 5 animals with the lowest and highest tick counts were designated Resistant and Susceptible respectively, and the remainder (n=13) were designated as medium).

timepoints for each of 30 animals, commencing at three weeks after initial infestation. Neither the effect of (smoothed) time nor of dosage of any of the alleles was significant ($p > 0.00083$, **Figure 2** and **Table 5**).

Almost all the immunological and haematological assay results were significantly affected by time (**Table 5**, **Table S1** and **Figures S1**–**S3**). Only Hb, platelet count and the response to larval soluble Ag did not vary significantly ($p > 0.00083$) over time. White cell count (WCC) was significantly affected by the doses of alleles 220 and 486. Each dose of allele 220 decreased WCC ($p = 7.08 \times 10^{-10}$), whereas each dose of allele 486 increased WCC ($p = 4.63 \times 10^{-5}$, **Figure 3A**). Red cell count (RCC) increased significantly ($p = 1.39 \times 10^{-8}$, **Figure 3B**) with each dose of the 462 allele and decreased significantly with each dose of the 486 allele ($p = 0.000369$). PCV and Hb followed this same pattern of significant increase with each dose of the 462 allele and significant reduction with each dose of the 486 allele (**Table 5**). For the red blood cell variables, there were distinct response patterns for 462 heterozygotes and 462/462 homozygotes (**Figure 4**). Among the immunolabelled cells, only $CD8^+$ cells were significantly associated with allele, being reduced in cattle with each additional copy of the 220 allele ($p = 0.000197$, **Figure 5**). Immunoglobulin responses were affected by genotype; 220/220 animals had consistently lower IgG1 in response to tick Ag than the 462/462 animals. Most of the models failed to explain a large proportion of the deviance – with the best model explaining 48% and the worst model explaining 4% of the deviance.

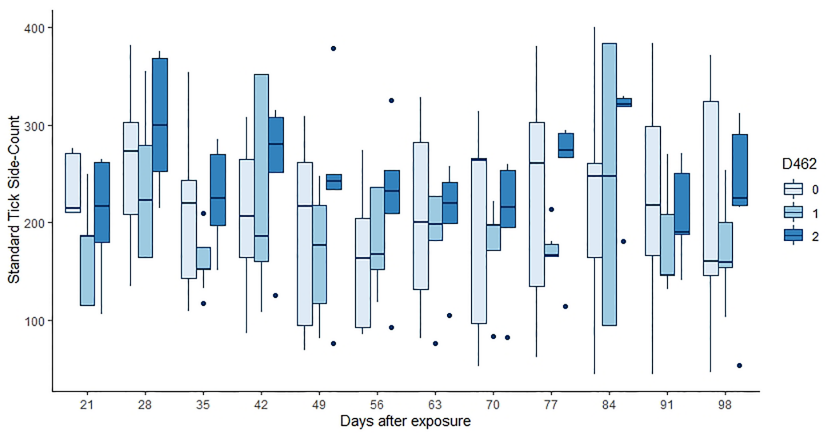

FIGURE 2 | Tick counts by days after exposure, commencing at 21 d post infestation and continuing for 11 weeks. Data for the number of copies of the 462 allele are shown, those animals without the allele in pale blue, and animals that were 462/462 in the darkest blue. Neither the count day nor the allele dose were significant in the GAM ($p > 0.00083$, **Table 5**).

TABLE 5 | Summary of GAM outputs for each of the models for tick count and each of the variables for which the main effect of allele frequency was considered to be statistically significant ($p < 0.00083$).

Outcome variable	Transformation	Explanatory variable	Intercept	Effect estimate	*t*	*p*-value	s(time) *F*-value	S(time) *p*-value	Deviance explained
Tick Count (ticks)	None needed	Allele 220	220.978	-16.038	-2.618	0.00928	1.83	0.0536	7.8%
		Allele 462	199.867	11.344	2.002	0.0461	1.807	0.0572	6.9%
		Allele 486	205.721	5.612	0.621	0.535	1.57	0.114	5.5%
White cell count (cells $\times 10^3/mm^3$)	Johnson	Allele 220	0.23839	-0.32141	-5.777	1.39e-08	11.1	<2e-16	20.6%
		Allele 462	-0.04420	0.08412	1.570	0.117	10.52	<2e-16	15.3%
		Allele 486	-0.11413	0.24091	4.112	4.63e-05	10.78	<2e-16	17.9%
Red cell count (cells $\times 10^6/mm^3$)	sqrt	Allele 220	2.80477	-0.04098	-3.092	0.00211	12.02	3.53e-07	9.3%
		Allele 462	2.71928	0.07549	6.295	7.08e-10	12.74	<2e-16	14.7%
		Allele 486	2.80425	-0.04905	-3.587	0.000369	12.21	<2e-16	9.9%
PCV (%)	Johnson	Allele 220	0.13281	0.13281	2.215	0.0272	7.408	1.08e-06	8.9%
		Allele 462	-0.20056	0.31662	5.753	1.59e-08	7.696	8.7e-07	14.1%
		Allele 486	0.17799	-0.24550	-3.948	9.08e-05	7.548	1.24e-06	10.9%
Hb (g/dl)	$\log_e$	Allele 220	2.461282	-0.018942	-2.408	0.0164	1.128	0.361	3.1%
		Allele 462	2.418265	0.039476	5.52	5.65e-08	1.178	0.319	7.9%
		Allele 486	2.464186	-0.028318	-3.502	0.000507	1.148	0.343	4.4%
CD8 (% gated cells)	Johnson	Allele 220	3.66767	-0.12974	-3.753	0.000197	29.96	<2e-16	34.6%
		Allele 462	3.50044	0.10380	3.21	0.00142	29.76	<2e-16	34.1%
		Allele 486	3.57392	0.01088	0.3	0.764	29.16	<2e-16	32.6%
IgG1 – gut membrane – OD	None	Allele 220	1.25063	-0.16825	-3.711	0.000281	27.3	<2e-16	40.6%
IgG1 – gut soluble – OD	None	Allele 220	0.80045	-0.04853	-4.221	3.96e-05	79.52	<2e-16	40.6%
IgG1 – salivary soluble – OD	None	Allele 220	0.73197	-0.11101	-4.081	6.61e-05	30.54	<2e-16	48.7%

In all cases, the model includes measurement time as a smoothed variable, allele dosage as a fixed effect with three levels (that represent the number of copies of that allele that the individual has: 0,1,2) and animal ID as a random effect. Data have not been back-transformed – model intercepts and effect estimates represent the intercept and effect sizes on the transformed data. Results for the full set of outcome variables are shown in ***Table S1****.*

DISCUSSION

The study on which this project is based (10, 11) was intended to contrast local and systemic immune responses and haematology between cattle of high resistance and those of low resistance to tick infestations. An incidental finding of the original studies was that highly resistant animals were less likely to have detectable $CD45^+$ or $CD45RO^+$ cells in skin (10). However, that observation was based on an extreme-group comparison of the 6 most resistant and 6 least resistant animals. In the present study, we genotyped *PTPRC* (CD45) for all the original animals in the trial and found that although there was no significant relationship between tick count and the dosage of any one of the three differentiable alleles, large differences in erythrocyte, leukocyte and humoral responses were observed among *PTPRC* genotypes: the indicine Family 2 (462) allele was associated with a more robust erythron; the "taurindicine" Family 1 allele (220) was associated with lower leukocyte count, lower % gated $CD8^+$ cells, and lower IgG1 recognition of tick-specific Ag. Given that these alleles are believed to have tick-resistant and tick-susceptible origins respectively, there is some potential confounding of the apparent allelic effects by alleles at other loci that are in linkage disequilibrium (LD) with them. The Santa Gertrudis breed was selected for this study intentionally to reduce confounding by genetic background. The breed was established in Texas about 100 years ago as a hybrid between *B. taurus* and *B. indicus* cattle, so it is expected that over 30-40 generations of breeding LD should have been reduced among the linked genes and eliminated among the unlinked genes. It follows that caution is required in extrapolating from contrasts among the genotypes in this study to contrasts between indicine and taurine animals from previous studies. It cannot be inferred that differences between *B. indicus* and *B. taurus* cattle can be attributed to variation in *PTPRC* genotype, nor that *PTPRC* genotype is necessarily consistent in populations of *B. taurus* and *B. indicus* cattle. Our unpublished sequence and genotyping data suggest that Brahman cattle in Australia are diverse and include members of all four families, whereas Holstein-Friesian cattle seem to be almost exclusively taurine Family 1.

The most pronounced differences among genotypes were in the variables relating to red blood cells. Cattle with the indicine Family 2 allele for *PTPRC* (462) had higher RCC, PCV and Hb. The Family 2 heterozygotes had significantly higher RCC than the Family 2 homozygotes during the pre-infestation and early infestation periods, but by 11 weeks the homozygote was also high. Similar patterns were noted for PCV and Hb. At the end of the study period, MCH was lowest in Family 2 homozygotes, which, taken with the increase in RCC in these animals, is consistent with a stronger regenerative response to blood loss. Red cell counts have previously been reported to be higher in tick-infested indicine than taurine cattle, in the absence of *Babesia* and *Anaplasma* haemoparasites (15), and greater resistance to reduction in erythrocyte counts of *B. indicus* cattle that have been exposed to *Babesia* has also been demonstrated (19). All nucleated haematopoietic cells express CD45, the dominant isoforms being RO and RB (3). Although

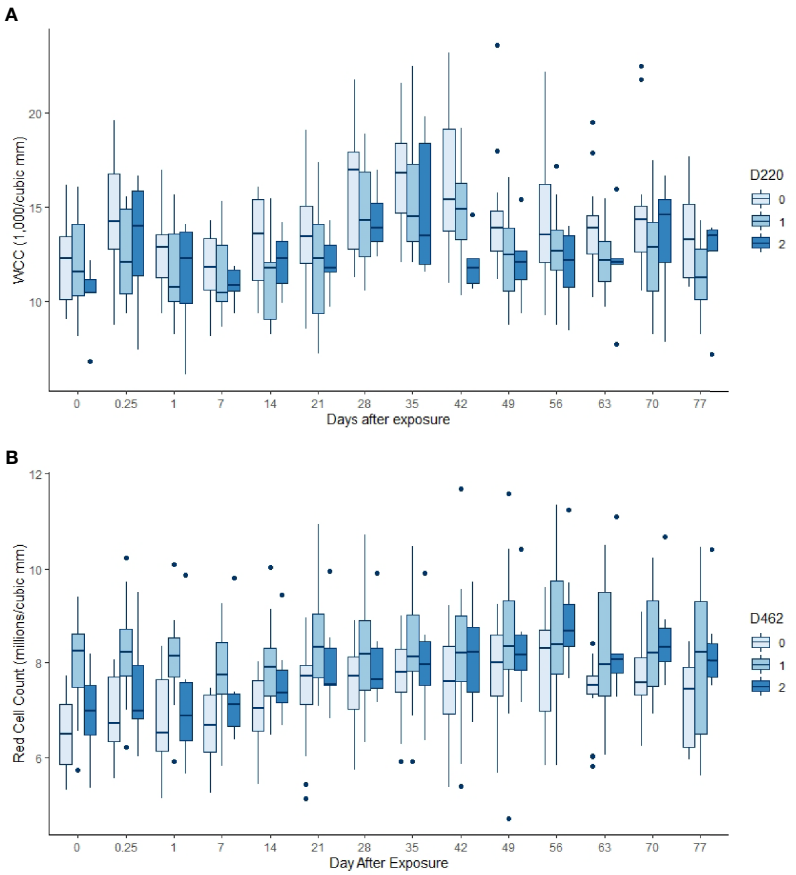

FIGURE 3 | **(A)** White cell counts by day, commencing pre-infestation and continuing for 11 weeks. Data for the 220 allele are shown, those animals without the allele in palest blue, and animals that were 220/220 in the darkest blue. Both day and the allele dose were highly significant in the GAM ($p < 0.00083$, **Table 5**). **(B)** Red cell counts by day, commencing pre-infestation and continuing for 11 weeks. Data for the 462 allele are shown, those animals without the allele in palest blue, and animals that were 462/462 in the darkest blue. Both day and the allele dose were highly significant terms in the GAM ($p < 0.00083$, **Table 5**).

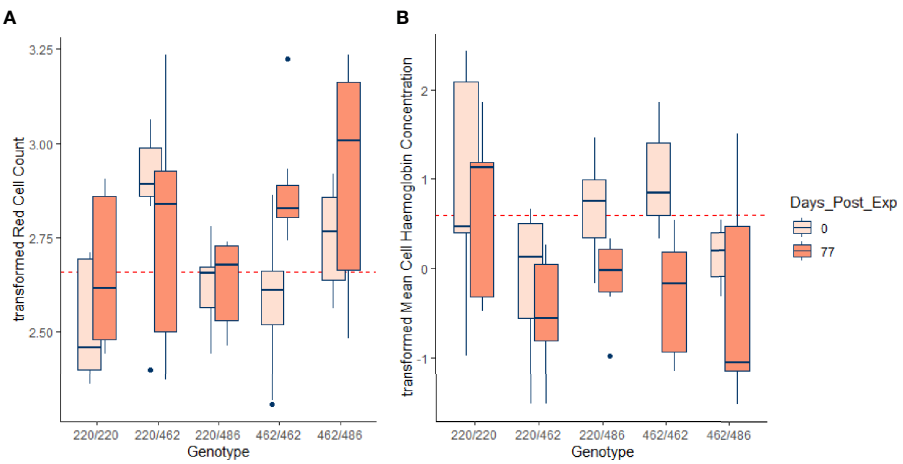

FIGURE 4 | Transformed RCC (tRCC - **A**) and transformed MCH (tMCH - **B**) for the initial (pre-infestation) and end (77 d post-infestation) time points, for each of the genotypes. Horizontal dotted lines in red are the mean pre-infestation values for each of the transformed variables. For RCC, the effect of 462 allele dosage was highly significant in the GAM ($p < 0.00083$, **Table 5**) but for MCH the effect approached significance ($p = 0.00726$, **Table S1**).

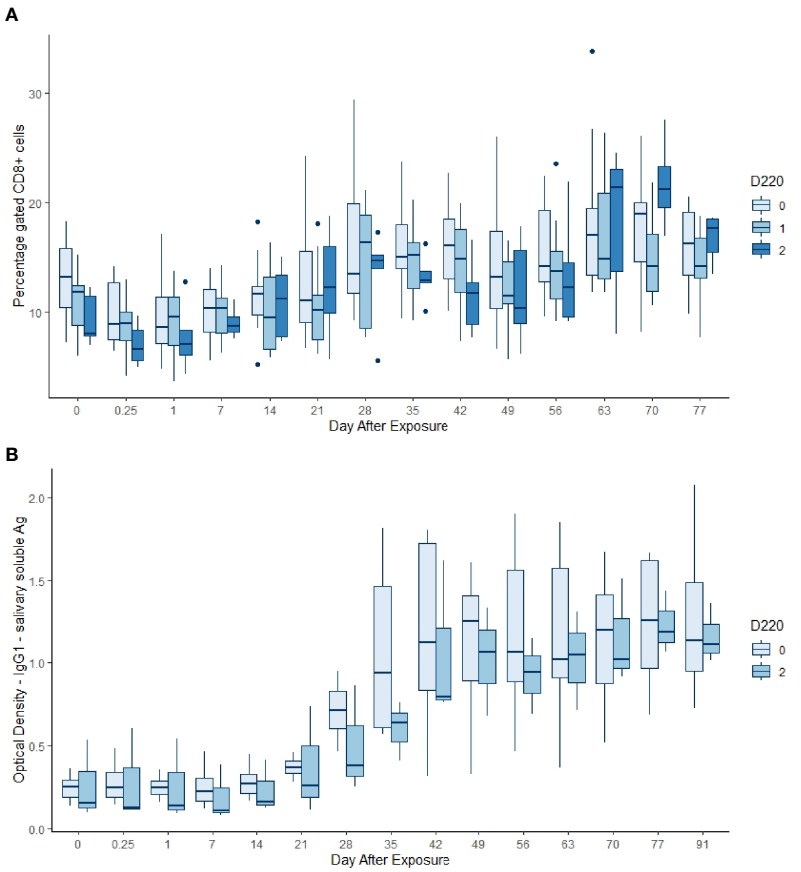

FIGURE 5 | **(A)** Gated percentage of CD8+ cells by day, commencing pre-infestation and continuing for 11 weeks. Data for the 220 allele are shown, those animals without the allele in palest blue, and animals that were 220/220 in the darkest blue. Both day and the allele dose were highly significant in the GAM ($p < 0.00083$, **Table 5**). **(B)** IgG1 optical density (OD) in response to soluble salivary tick Ag by day, commencing pre-infestation and continuing for 15 weeks. Data for the 220 allele are shown and those animals without the allele (pale blue) are all 462/462 (dark blue). Both day and the genotype were highly significant terms in the GAM ($p < 0.00083$, **Table 5**).

most investigations on CD45 function have focused on immune signalling, it has been shown that CD45 is an important regulator of splenic erythropoiesis (20). Although the bulk of erythropoiesis occurs in the bone marrow, splenic erythropoiesis, supported by red pulp macrophages (RPM) makes an important contribution to the expansion of the erythron in response to diverse stressors including hypoxia, endotoxins, bacterial and viral infections. Mice that are deficient in CD45 show abnormal erythropoiesis and accumulate progenitor forms of erythrocytes (20). It has also been shown that CD45 is a negative regulator of erythropoietin-dependent haematopoiesis through its inhibition of Janus kinase (JAK) signalling pathways (21). Therefore, there are several mechanisms by which variation in CD45 genotype could influence haematopoiesis, and the observations from our study are consistent with the pathogen-driven selection hypothesis advanced by Ballingal et al. (2).

Cattle with the taurine Family 1 (220) allele for *PTPRC* had lower WCC and lower gated percentages of $CD8^+$ cells (T cytotoxic cells) in circulation. Immunoglobulins specific to three of five tick Ag mixtures differed highly significantly between homozygotes of the Family 1/3 (220) and the Family 2 (462) genotypes. Among the cell proliferation assays conducted in our study, genotype did not have a significant effect, using α corrected for multiple comparisons to 0.00083. However, several of the GAMs estimated *p*-values approaching this level (Family 1/3 allele 220: $p = 0.00174$ for ConA stimulation, and $p = 0.00181$ for larval soluble Ag). Diverse leukocytic responses to tick infestation have been reported in tick-infested cattle of indicine and taurine origins. Rechav (22) reported that Simmental (*B. taurus*) cattle had higher leukocyte counts than Brahmans (*B. indicus*) when infested with diverse species of African ticks. We previously found a similar result in a contrast between tick-infested Holstein-Friesian (*B. taurus*) and Brahman (*B. indicus*) cattle (15). Immunoglobulin production in response to tick Ag has been shown to differ between taurine and indicine cattle exposed to ticks although the directions of the associations have not been consistent among studies and experimental

conditions (15, 23). Rocha Garcia et al. (24) confirmed that there were clear differences between taurine and indicine cattle in their ability to recognize and respond to tick Ag. The lymphoproliferative, phagocytosis and oxidative burst activity of neutrophils and monocytes differs between indicine and taurine cattle, each responding differently to co-culturing with *R. microplus* salivary gland extract (25). Ramachandra and Wikel (26) found substantial differences in taurine and indicine leukocyte biology – T cells from *B. indicus* cattle had a stronger proliferative response to ConA and peripheral blood mononuclear cells from *B. indicus* cattle produced more IL-1 in response to lipopolysaccharide (LPS). Given the many mechanisms by which CD45 is known to modulate leukocyte proliferation and cytokine responses to various stimuli (4, 21), the divergent leukocyte biology evident in animals of the different genotypes in our study is not surprising.

The immunological observations used in our study were selected with a view to better understanding the mechanisms underlying the differences in host resistance to tick infestation rather than for the characterization of the complete immunological phenotypes of animals of each of the *PTPRC* genotypes. As such, we have an incomplete set of observations on a relatively small dataset of animals that is not balanced by genotype. However, our population does have the advantage of being drawn from a breed in which we expect some of the confounding effects of linkage disequilibrium to have been reduced or eliminated. The effects of CD45 are mediated largely by variation in isoform expression and glycosylation rather than by variable ligand binding or variable enzyme expression, and most of the clinically relevant polymorphisms in humans influence isoform expression (4). At present there is not enough information on the full genomic sequence variants of *PTPRC* in cattle or isoform expression variants to confidently relate the cattle genotypic families to any studies on human or murine variants of *PTPRC*. Nonetheless, it seems safe to conclude that variation in *PTPRC* is likely to contribute to variation in the profiles and functions of leukocytes and erythrocytes of cattle. In human medicine, CD45 isoform expression is used as an important component of clinical immunological profiles (27). In cattle, there are relatively few reports on its application, although it has been used as one of several markers of immune response to mastitis (28) and rumen fluke (29), among others. In our study, *PTPRC* polymorphism was strongly associated with divergent erythrocytic, leukocytic and humoral responses to tick infestation. The extent to which this might be useful to aid in the selection of adapted cattle will depend on better knowledge of the variants in populations of cattle, the link between polymorphism of *PTPRC*, form and function of CD45, and possible interactions with other genes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Queensland Animal Ethics - Production and Companion Animals Committee.

AUTHOR CONTRIBUTIONS

NJ – conceived study, coordinated original field work, undertook original field work, undertook data analysis, and drafted paper. DC – undertook genetic analysis. EP – undertook original field work, and conducted laboratory work including immunological assays. EM – undertook genetic analyses. CC – carried out immunological assays. LJ – conceived study, undertook original field work, and undertook immunological assays. MS – contributed to genetic analyses. AT – conceived study, and coordinated original project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.675979/full#supplementary-material

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Immunomic Investigation of Holocyclotoxins to Produce the First Protective Anti-Venom Vaccine Against the Australian Paralysis Tick, *Ixodes holocyclus*

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Venom producing animals are ubiquitously disseminated among vertebrates and invertebrates such as fish, snakes, scorpions, spiders, and ticks. Of the ~890 tick species worldwide, 27 have been confirmed to cause paralysis in mammalian hosts. The Australian paralysis tick (*Ixodes holocyclus*) is the most potent paralyzing tick species known. It is an indigenous three host tick species that secretes potent neurotoxins known as holocyclotoxins (HTs). Holocyclotoxins cause a severe and harmful toxicosis leading to a rapid flaccid paralysis which can result in death of susceptible hosts such as dogs. Antivenins are generally polyclonal antibody treatments developed in sheep, horses or camels to administer following bites from venomous creatures. Currently, the methods to prevent or treat tick paralysis relies upon chemical acaricide preventative treatments or prompt removal of all ticks attached to the host followed by the administration of a commercial tick-antiserum (TAS) respectively. However, these methods have several drawbacks such as poor efficacies, non-standardized dosages, adverse effects and are expensive to administer. Recently the *I. holocyclus* tick transcriptome from salivary glands and viscera reported a large family of 19 holocyclotoxins at 38-99% peptide sequence identities. A pilot trial demonstrated that correct folding of holocyclotoxins is needed to induce protection from paralysis. The immunogenicity of the holocyclotoxins were measured using commercial tick antiserum selecting HT2, HT4, HT8 and HT11 for inclusion into the novel cocktail vaccine. A further 4 HTs (HT1, HT12, HT14 and HT17) were added to the cocktail vaccine to ensure that the sequence variation among the HT protein family was encompassed in the formulation. A second trial comparing the cocktail of 8 HTs to a placebo group demonstrated complete protection from tick challenge. Here we report the first successful anti-venom vaccine protecting dogs from tick paralysis.

Keywords: cocktail vaccine, anti-paralysis vaccine, paralysis tick, *Ixodes holocyclus*, vaccine

1. INTRODUCTION

Venom producing animals are ubiquitously distributed among vertebrates and invertebrates like fish, snakes, scorpions, spiders, and ticks (1, 2). Snake venoms are amongst the most highly characterized of animal venoms and are conformed by a complex mixture of pharmacologically active proteins and peptides (3) conferring their toxicological property. Due to its incidence and human impact, the World Health Organization recently recognized snakebite as a neglected tropical disease that affects ~ 2.7 million per annum (3).

However, hematophagous invertebrates such as ticks are not as well recognized as venomous animals (1). There are approximately 890 tick species worldwide with 73 species confirmed to be associated with host paralysis (4). These include 27 species with evidence of paralysis, such as soft tick species (n=8) from the genera *Argas* and *Ornithodoros* which paralyze mostly livestock, and also hard tick species (n=19) from the genera *Ixodes*, *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma* and *Rhipicephalus* which paralyze a broader list of hosts including humans, livestock, reptiles, companion animals and rabbits (1, 4, 5). *Ixodes holocyclus* and *Ixodes cornuatus* cause paralysis in humans, domestic animals, and wildlife with an eastern coast and southern Australian distribution respectively (6). The Australian paralysis tick (*I. holocyclus*) is the most important tick species associated with paralysis in Australia distributed along the eastern coast from North Queensland to the Lakes Entrance in the southern state of Victoria (6). It has been reported from an unpublished survey to cause tick paralysis in approximately 10,000 to 100,000 animals annually with a death rate of around 5% (7). The toxicity of *I. holocyclus* is caused by a family of neurotoxins named 'holocyclotoxins' present in the tick saliva. The only toxin molecules that have been characterized for any tick species is for the Australian paralysis tick (8, 9), including demonstrated specific paralysis activity in the mouse neonate model (8, 10). Additionally, there are several bacterial pathogens transmitted by Australian ticks such as *I. holocyclus* including *Rickettsia australis* (Queensland tick typhus) and *Coxiella burnetii* (Q fever). Recently, *I. holocyclus* tick bites have been confirmed as the trigger for a mammalian meat allergy in humans (11). Despite evidence from scientific studies (transcriptomic, sentinel studies and antibody screens) failing to show that the causative agent of Lyme disease *Borrelia burgdorferi* sensu lato is present in Australia (10, 12), a similar multisystem disorder has been identified with similar symptoms in humans (13). Currently, in Australia, there is no formal reporting system of paralysis tick cases with incidence data only available from focused geographical or specific annual survey reports (14, 15).

Antivenoms based on polyclonal antibodies for treatments against venoms of ticks, snakes, spiders, scorpions and other species are produced in animals such as dogs, horses, camels and sheep with research focusing on identifying safer inoculation schedules (16–20). These antivenoms are the most effective method to abrogate and treat paralysis by reducing the effect of postsynaptic neurotoxins (21). Ticks are much smaller producing minute volumes of saliva and thus collecting venom, or salivary gland secretions requires a large number of ticks. For ticks, where their size precludes physical extraction of venom, antivenoms are produced commercially by feeding ticks on canine hosts to obtain hyper-immune tick anti-serum (22, 23). The antivenoms applied for presynaptic neurotoxins (such as those present in the South American rattlesnake and the Australian paralysis tick) is less successful if the paralysis has progressed prior to treatment (24, 25). The paralysis caused by these species are considered incidental and as such vaccines do not appear to be a priority as the delivery of treatment for bitten individuals deemed the best approach to control neurotoxic consequences (26, 27).

Chemical acaricides administrated topically or *via* collars have been the most common methods used to prevent and control paralysis ticks on companion animals (28). Oral acaricides based on isoxazoline chemicals sold as Bravecto™ and NexGard® have been introduced (29, 30) which aim to control both paralysis ticks and fleas. New long acting collars based on slow release Imidacloprid/flumethrin (Seresto®) have shown promise more recently (31). However, the risk of ticks developing resistance remains and the adverse reactions of these drugs warrant the treatments unsafe for some dogs and as such new drugs continue to be developed by companion animal product companies (32, 33). These preventative methods have drawbacks with efficacies of less than 100%, and a single surviving tick(s) can kill a large dog (34), however the risk is higher for smaller dogs as neurotoxin dose correlates with host weight (14). Cats are more sensitive to these drugs thus other options have been developed using dog orals as topicals with a recent study examining 2077 cat cases to conclude that the mortality risk for cats is low from paralysis caused by *I. holocyclus* (35). The current treatment for tick paralysis relies on the prompt removal of all attached ticks and the administration of a commercial tick antiserum (TAS). Morbidity and mortality rates decrease with TAS introduction. It also has many drawbacks including: inhumane production (*via* dog hyper-immunization), limited window of utility (needing to be administered in the early stages of disease), non-standardized dosage, side effects and varying potency between batches and manufacturers (reviewed by 26). In the last few years, commercial production of TAS has reduced to only one Australian company (36), suggesting that the use of new oral drugs in dogs has decreased the number for dog paralysis cases and also the TAS commercial production need.

The notion of an anti-paralysis tick vaccine and the protective attributes of hyperimmune dog serum to treat cases of paralysis were first developed more than 80 years ago in a controlled study with paralysis tick infected dogs (22, 23). Later studies also demonstrated tick infestation of dogs to produce hyperimmune dog serum (37), or to demonstrate protection from tick challenge, which correlated to serum anti-toxin antibody titers (38). Other studies using whole tick homogenates (39) or dissected salivary gland extracts 'toxoid' also demonstrated protection from challenge with *I. holocyclus* in controlled dog studies. These studies required large numbers of ticks and the preparation of

native protein for the production of a potential crude vaccine which was not commercially feasible. It was not until the 1990s that neurotoxins or holocyclotoxins bound to rat brain synaptosomes (pinched-off nerve terminals) were isolated as three polypeptides HT1, HT2 and HT39. Following N-terminal sequencing, a partial sequence of holocyclotoxin 1 (HT1) was obtained, and PCR technologies were utilized to obtain the gene sequence (40). It was not until 2014 and 2018 that the structure of a chemically synthesized HT1 demonstrated four disulphide bonds with three contributing to inhibitory cysteine knot motif, and transcriptome sequencing identified a large holocyclotoxin family of up to 19 neurotoxins respectively (10, 41). The holocyclotoxin sequence identities varied from 38-99%, suggesting the development of a vaccine may be challenging.

Currently, there are 19 characterized HTs (10), and identifying the holocyclotoxins critical to paralysis may assist future vaccine development. The present study aims to identify immunogenic HTs following screening with dog anti-paralysis tick serum. Here we describe the first successful anti-venom vaccine demonstrating the protection of dogs from *Ixodes holocyclus* challenge.

2. MATERIALS AND METHODS

2.1 Holocyclotoxins and Immunogenicity Screening With Polyclonal Tick Anti-Serum

A transcriptome database was produced following cDNA sequencing of organs dissected from *I. holocyclus* females collected from paralyzed dogs and cats as previously described (10). Subsequently to the publication in 2019, the database was deposited into Genbank as the transcriptome shotgun assembly (TSA) under accession GIBQ00000000 (see 'Data Availability Statement' for more details). Transcriptome sequence data was mined for homologues of HT1 (Accession AAV34602) and an additional 18 full length transcripts were described with amino acid sequences under accessions: HT2 (KP096302), HT3 KP096303), HT4 (KP963966), HT5 (KP096304), HT6 (KP096305), HT7 (KP096306), HT8 (KP096307), HT9 KP096308), HT10 (KP096309), HT11 (KP096310), HT12 (KP963967), HT13 (KP963968), HT14 (KP963969), HT15 (KP963970), HT16 (KT439073), HT17 (KT439074), HT18 (KT439075), and HT19 (KT439076), respectively. All HTs used in ELISAs and immunizations were synthesized as previously described using Fmoc chemistry (10).

Three different Summerland purified anti-tick serum batches (2012-2014) (42) containing purified dog anti-tick immunoglobulins at 500 anti-toxin units (ATU) per bottle were screened against 19 synthetic holocyclotoxins produced as previously described (10) using ELISA. Nunc-Immuno™ MicroWell™ 96 well plates (Sigma-Aldrich, Australia) were coated with 100 ng/well of each synthetic holocyclotoxin diluted in carbonate buffer (0.1M sodium carbonate-bicarbonate solution, pH 9.6) in duplicate and incubated overnight at 4°C. HT13 was not screened due to poor synthetic peptide preparation (data not shown). Control wells were coated with 100 ng of *I. holocyclus* salivary gland extract (positive control) or bovine serum albumin (BSA) (negative control). Plates were washed three times with 200 µL wash buffer (WB: 0.05% Tween 20 in 10mM phosphate buffer saline, pH 7.4) before blocking the wells with 200 µL blocking buffer (BB: Pierce™ Protein Free PBS Blocking Buffer, Thermo Scientific, Australia) overnight at 4°C. After blocking, the plates were washed three times with 200 µL WB. Serial dilutions of Summerland TAS starting at 1:500 in BB were added across each row of the plates as the primary antibody and incubated for 1hr at room temperature (RT) on a platform shaker (Ratek Instruments, Australia). After three washes, 100 µL of horseradish peroxidase (HRP) conjugated- Sheep Anti-Dog IgG (abcam®, Australia) diluted 1: 10,000 in BB were added to the plates and incubated for 30 minutes at room temperature. Subsequently, plates were washed five times with WB before 100 µL of 3,3',5,5'- Tetramethylbenzidine (TMB) was added to each well (KPL, USA) and allowed to develop for 10 minutes. The reaction was stopped with 100 µL 1M phosphoric acid. Absorbance was measured using the BioTek Epoch Spectrophotometer with λ= 450nm filter (Millennium Science, Australia) and the end point titer was calculated as the reciprocal of the dilution that reached the background absorbance of the negative control (OD λ= 450nm).

2.2 Dog Challenge Trials

These studies were conducted under the Australian Pesticide and Veterinary Medicine Authority (APVMA) Small-scale Trials Permit PER7250 at an R & D Centre, New South Wales, Australia, approved by the Elanco Animal Ethics Committee. Peptides for the dog trials were synthesized as previously described using fmoc chemistry and confirmation of peptide folding (10, 41).

2.2.1 Trial 1

An exploratory immunization experiment was carried out to determine if HT peptide structure was important for immunogenicity using three animal groups consisting of one adult dog per group. The 'unfolded' preparation contained 1mM Dithiothreitol (DTT) to reduce the disulphide bonds forming between cysteine residues of the HT peptides. Three adult female kelpie cross Labrador dogs from the same litter of 5.25 years of age at 18.9-20.2kg in weight were randomized into three treatment groups. Group II (Dog #62469) and III (Dog #63458) were immunized with unfolded and folded peptides respectively, and Group I was the placebo control (Dog #70084) treated with PBS and Incomplete Freund's adjuvant (IFA). The immunization was subcutaneous in the neck with 1 mL of IFA containing 60 µg (~10.83 nmol) each of 5 HTs (HT1, HT2, HT3, HT4 and HT12) – thus a total of 300 µg (54.15 nmol) of protein). The first immunization was Day 0 with two booster injections at 14 and 28 days after the first dose. The dogs were observed for signs of tick paralysis as summarized in **Supplementary Table 1**.

2.2.2 Trial 2

A double blinded, placebo-controlled pilot study designed to compare the efficacy and safety of the peptide cocktail vaccine and determine the antibody responses. The dog cohort consisted

of three Beagles, one Beagle cross, two Huntaways, one Huntaway cross and one Kelpie cross as two de-sexed males, four de-sexed females and two fertile females. The dogs were two years and six months old with one dog eight years old. The eight dogs were randomized into two groups and treated with either the vaccine (HT cocktail, Group 2, Dog IDs: 34295, 55891, 64799, 68888; #55891 included the single 8-year-old dog) or a placebo control (adjuvant only, Group 1, Dog IDs: 62458, 63061, 66324, 99768). Eight HT peptides (HT1, HT2, HT4, HT8, HT11, HT12, HT14 and HT17) were formulated in IFA at 30μg (5.42 nmol) per peptide with a total of 240μg (43.32 nmol) per dose per dog. These were administered in three subcutaneous doses on days 0, 28 and 49. The dogs were observed for signs of tick paralysis as determined by clinical observation and evaluated against a matrix of clinical and subclinical signs, as summarized in **Supplementary Table 1**.

2.3 Sera Collection

Sera were collected on days 0 (baseline), and 14, 28, 42 and 50 (Trial 1), and 28, 42, 63 and 66 (Trial 2) with antibody levels to the HTs determined by ELISA. Each dose was delivered to a new site (left and right shoulder) to allow for the monitoring of injection site reactions. After each vaccination the dogs were observed for signs of tick toxicosis every 3 hours (± 30 mins) for a period of 12 hours post vaccination. Personnel evaluating the dogs were blinded.

2.4 Tick Challenge

The ticks were sourced from the Tweed Heads region of Northern New South Wales, Australia (Latitude 28.1787° S, Longitude 153.5370° E). Two weeks after the third vaccination (trial 1 day 42, trial 2 day 63), in both trials the dogs were challenged with one unfed adult female *I. holocyclus* ticks to induce symptoms of toxicosis. At three days after tick attachment (trial 1 day 45, trial 2 day 66, 72h post tick attachment) the dogs were observed every 3 hours (± 30 mins) for signs of toxicity until the study end (trial 1 day 50, trial 2 day 70). Signs of tick toxicosis were determined by clinical observation performed by an appropriately trained person and evaluated against a matrix of clinical and subclinical signs (**Supplementary Table 1**). A total score of 20 was considered a diagnosis of toxicosis, however, the attending veterinarian had the freedom to use their clinical discretion to diagnose toxicosis using fewer variables. Diagnosis of an individual dog with paralysis occurs when the dog reaches a total score of 20 across all 10 variables, or when the attending or consulting veterinarian uses their discretion to make a diagnosis based on one or more serious clinical signs. Once a dog was diagnosed with paralysis, the dog was removed from the study for clinical intervention.

2.5 ELISA Analysis of Sera From Trials 1 and 2

Sera from dogs at days 0, 14, 28, 42 and 50 from Trial 1 and at days 0, were screened in the HT ELISA as described above with the following changes. Following the blocking step, serial dilutions starting at 1:50 in BB of sera collected from each treatment group was dispensed across the plates and incubated for 1hr at room temperature (RT) on a platform shaker (Ratek Instruments, Australia). The negative control wells were coated with HT and probed with day 0 sera pooled from all dogs in experiment #1. Similarly, the positive control wells coating with HTs and tested with commercial Summerland purified anti-tick serum (TAS).

To determine the whole IgG and IgG1:IgG2 ratios from trial 2, the sera were screened using the HT ELISA with the following amendments. Sera were tested in triplicate, altering only the secondary antibody utilized each time. The secondary antibodies were: HRP conjugated- Sheep Anti-Dog IgG (abcam®, Australia); HRP conjugated- Sheep Anti-Dog IgG2 (Bethyl Laboratories, USA); and HRP conjugated- Goat Anti-Dog IgG1 (Bethyl Laboratories, USA), each diluted at 1: 10, 000 in BB.

The final titers for the ELISAs were determined as the reciprocal of the dilution that reached the background absorbance of the negative control.

2.6 Statistical Analyses

Data was transformed using the natural log (Ln2) to normalize before analysis using statistical software. All analyses were performed using GraphPad Prism software v6.0 (http://www.graphpad.com/scientific-software/prism/). Two-way ANOVA with a Tukey's multiple comparisons post-test determined the variation between batches of TAS.

3. RESULTS

3.1 Identification of HTs From the *Ixodes holocyclus* Transcriptome

Using the HT1 sequence (AAV34602) identified in the 1990s, our study identified a further 18 HT homologues (HT2-HT19) from the *I. holocyclus* internal organ transcriptome data11. Cocktail vaccine discovery described here screened all HT peptides except HT13 due to poor synthesis (data not shown).

3.2 Holocyclotoxin Immunogenicity Using Commercial Sera

Production of different batches of commercial tick antiserum (TAS) obtained in 2014, 2015 and 2016 were used to determine the antibody titer against each synthetic holocyclotoxin. In ELISAs, these sera displayed a similar anti-HT IgG recognition pattern, as presented in **Figure 1**. The most significant anti-HT IgG titers were obtained for HT2, HT3, HT4, HT6, HT8, HT11 and HT19 when compared to all other toxins ($p < 0.05$) with IgG titers ranging from 1: 80,000 to 1: 256,000. The rest of the HTs had significantly higher titers ranging from 1: 16,000 to 1: 32,000 when compared to HT16 and HT17 ($p < 0.0001$). The lowest IgG titers were observed for HT16 and HT17 with 1: 2,000 and 1: 1,500, respectively.

3.3 Dog Immunization Trials

3.3.1 Trial 1 – Folded *vs* Unfolded Holocyclotoxin Cocktail Vaccine (5 HTs) Tick Challenge in Dogs

Trial 1 aimed to investigate the safety of dog vaccination using folded *versus* unfolded HTs mixed in a cocktail of: HT1, HT2,

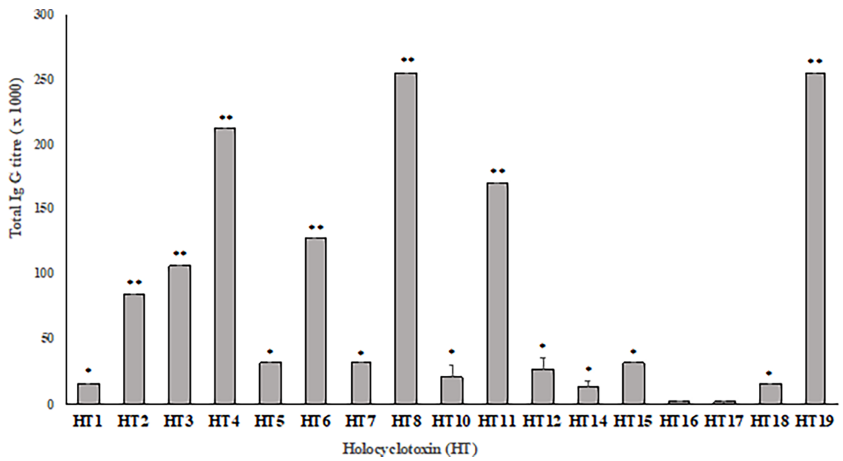

FIGURE 1 | Average IgG titer against synthetic holocyclotoxins present in three different production batches of commercial tick anti-serum (Summerland). Results displayed as Mean± SD of end point titer (n = 3). The end point titer was determined as the reciprocal of the dilution that reached the background absorbance (OD λ = 450nm). Significance was determined by two-way ANOVA with a Tukey's multiple comparisons post-test. ** ($p < 0.05$), * ($p < 0.0001$).

HT3, HT4 and HT12 using a single dog for each group. The dog immunization was conducted on Days 0, 14 and 28 with only one dog per treatment including folded HTs (62469), unfolded HTs (63458) and a placebo (70084). The analysis of the paralysis tick symptoms in the folded HT dog (62469) showed only mild signs of tick toxicosis with tick removal or treatment not required (**Table 1**). This dog (62469) had an adult *I. holocyclus* tick feeding throughout the whole challenge. Similar results were observed for the placebo however, on final observations (144 hours post tick attachment), no ticks were found on this dog. However, the dog immunized with the unfolded HTs (63458) showed symptoms of tick paralysis, and the tick was removed at 84 hours post tick attachment (**Table 2**).

Specific HT immunoglobulin induction was observed in the dogs receiving the folded and unfolded formulations of the pilot vaccine (**Table 1**). In dogs immunized with folded HTs and unfolded HTs formulations, the IgG titers against HT1 and HT2 increased after the first immunization, but no IgG titer was detected for HT3, HT4, HT12. A boost of the IgG titer occurred after the second and third immunization for all HTs in both formulations, at 28 and 42 days, see **Table 1**. Following tick infestation (at 50 days) the IgG titers were boosted for all HTs with a maximum increase for HT2, HT3, HT4 and HT12 in the folded HT (62469) and the unfolded HT (63458) immunized dogs. At this time, the anti-HT1 IgGs appeared to not change in both dogs immunized with folded and unfolded formulations. Two-way ANOVA with Tukey's post-test identified a significant increase in the end point titers for all HTs compared to day 14 titer ($p<0.001$) excluding HT1. This trial had only a single dog per group, the placebo group tick challenge was not successful. However, it can be concluded that a folded HT formulation protected the dog from tick paralysis after an active challenging with an adult *I. holocyclus* tick. The unfolded peptide formulation induced non-protective antibodies as paralysis symptoms were observed after tick challenge. The tick paralysis symptoms in this dog (63458) decreased following removal of the *I. holocyclus* tick.

TABLE 1 | IgG titers from dog Trial 1 with individual dogs immunized with Folded and Unfolded HT formulations consisting of HT1, HT2, HT3, HT4 and HT12.

HTs	1st Dose	2nd Dose	3rd Dose	Tick Infestation	Post Tick Infestation
	Day 0	Day 14	Day 28	Day 42	Day 50
Folded					
HT1	0	3200	6400	800	400
HT2	0	200	6400	3200	51200
HT3	0	0	200	1600	12800
HT4	0	0	800	12800	51200
HT12	0	0	400	3200	51200
Unfolded					
HT1	0	200	1600	800	800
HT2	0	50	800	3200	25600
HT3	0	0	1600	1600	25600
HT4	0	0	400	800	12800
HT12	0	0	800	3200	25600

Immunization schedule and tick infestation days are described.

TABLE 2 | Trial 1 clinical data for cocktail vaccine HT1, HT2, HT3, HT4, HT12 as folded *vs* unfolded immunizations.

Treatment group	Clinical observations post tick attachment (PTA)		Score[a]	Treatment
	Hours PTA	Observations		
Group 1 #70084 Placebo	102	slightly unwell	8/40	–
	105	Mild signs of tick toxicosis	5/40	–
	120	Mild signs of tick toxicosis	3/40	–
	123	Mild signs of tick toxicosis	3/40	–
	144	(no attached tick found)	3/40	–
Group 2 #62469 Folded HTs	102	slightly unwell	8/40	–
	105	Mild signs of tick toxicosis	5/40	–
	120	Mild signs of tick toxicosis	4/40	–
	123	Mild signs of tick toxicosis	1/40	–
Group 3 #63458 Unfolded HTs	81	Unwell, sore feet, did not jump, reduced gag reflex, bright and alert	6/40	–
	84	Quiet, did not jump, stumbled, appetite maintained	11/40	Tick removed
	87	Similar to 84hrs	8/40	–
	90	Similar to 84hrs	9/40	–
	93	Improvement noted	5/40	–
	96	Almost normal	0/40	–
	102	Sore on back, reluctant to walk	NA	–

[a]*Maximum score is 40 which describes severe paralysis and poor prognosis. Total score for developing toxicity which required withdrawal from the study was >12 (or <12 at the veterinarian's discretion); NA, not applicable.*

3.3.2 Trial 2 – Cocktail Vaccine (8 HTs) Tick Challenge in Dogs

Trial 2 included a combination of eight HTs with four HTs selected due to high immunogenic recognition by polyclonal dog antisera, HT2, HT4, HT8 and HT11, see **Figure 1**. The subsequent four HTs were selected to guarantee that sequence variation of the entire HT protein family was included within the cocktail, HT1, HT12, HT14 and HT17. Note that HT19 was also highly immunogenic, yet its synthetic production was unsuccessful, and thus HT19 was not added to the cocktail. Immunizations and placebos (4 dogs per group) were delivered on days 0, 28 and 49 followed by challenge with one unfed adult female *I. holocyclus* tick at day 62. **Table 3** summarizes the clinical observations for Trial 2.

TABLE 3 | Trial 2 clinical data for cocktail vaccine HT1, HT2, HT4, HT8, HT11, HT12, HT14 and HT17 as the cocktail immunization compared to the placebo group.

Treatment group	Dog ID	Clinical observations post tick attachment (PTA)		Score[b]	Treatment
		Hours PTA[a]	Observations		
Placebo Group I	62458	117	Overall clinical toxicity 1st quartile Overall disease judgment 1st quartile	2/40	
		153	Walks in circles with difficulty; climbs stairs with difficulty Overall toxicity in the 1st quartile; Paralysis recorded In the first quartile, NMJ[c] test showed mild weakness ataxia. Overall toxicity judgment in the first quartile. Hind leg weakness (wobbly when walking/standing)	7/40	
		156	Signs worsened drastically; Scored 3 out of 4 on 7 of the 12 tests	25/40	Tick removed, treated with TAS, fully recovered
	63061	168	Mild decrease in appetite, no other signs of toxicosis, ticks still attached	0	
	66324	144	Lost engorged tick during assessment	0	
		150	Climbed stairs with difficulty, mild weakness/ataxia in NMJ test Slight signs of hind leg weakness	3/40	
		156	Climbed all stairs but did not jump down Slight signs of hind leg weakness	1/40	
		168	Climbed stairs with difficulty, NMJ[c] test showed mild weakness/ataxia toxicity score in first quartile, paralysis score in first quartile. Overall judgment of toxicity score in first quartile. Ataxic and weak hind legs; Episode of labored breathing.	6/40	Tick removed, fully recovered without treatment
	99768	96	Tick could not be located, did not develop signs of toxicosis	0	
Treatment Group II	34295 64799 68888	168	Engorged tick detached before final health check, no signs of toxicosis	0	
	55891		Still had engorged tick at health check; dog quiet and low body temperature otherwise no signs of toxicosis	0	

[a]*Time points where all observations were zero are not shown, except the last day of observations.*

[b]*Maximum total score is 41 which describes severe paralysis and poor prognosis. Total score for developing toxicity which required withdrawal from the study was >12 (or <12 at the veterinarian's discretion).*

[c]*NMJ, neuromuscular junction.*

Administration of the vaccines was well tolerated. After the first vaccination on day 0, approximately 3 hours after the injection, a mild swelling at the injection site was observed in one of four placebo dogs (63061) but did not appear to cause pain and quickly resolved. Two of the four dogs (62458 and 66324) in the placebo group developed clinical signs of tick toxicosis on day 69 at approximately 150 hrs post tick attachment (PTA). At 156 hrs PTA, one control dog (62458) required intervention and was treated with tick anti-serum (TAS) resulting in a full recovery. For the other placebo dog (66324) that displayed signs of toxicosis, removal of the tick was sufficient to reverse the clinical signs and no further treatment was necessary. At 96 hrs PTA, the tick attached to dog 99768 could not be located and this dog did not develop signs of toxicosis. Dog 63061 in the placebo group did not develop signs of toxicosis despite the tick remaining attached until the final health check on day 70, although a mild decrease in appetite was observed on days 69 and 70. **Table 2** describes the clinical observations that were made for the placebo treated animals (Group 1) from day 67 where there was a clinical observation greater than 0. **Table 2** also summarizes the clinical observations that were made on the last day of trial 2 for the immunized animals (Group 2), no signs of toxicosis were identified at all previous health checks for the immunized group. Ticks stayed attached to all of the dogs in the immunized group (Group 2) until the final day (Day 70-final day of assessment, 168 hrs post tick attachment). None of the immunized dogs developed any signs of toxicosis.

Trial 2 observed an induction of HT specific IgGs in the dogs receiving the HT cocktail vaccine (**Figure 2**). The IgG titer induced against the HTs included in the cocktail increased during the course of the trial, except for HT2, whose IgG titer decreased between days 63 and 66. Spikes in IgG titers were observed after each inoculation. The most antigenic toxins were HT8 and HT11 with average titers between 232,000 and 480,000 at day 63. Two-Way ANOVA with Tukey's post-test determined HT8 and HT11 to be significantly increased at days 63 and 66 compared to all other HTs ($p<0.05$). At day 66, the least antigenic holocyclotoxins were HT14 and HT17 with average titers of 32,000 and 42,000, respectively. Analysis of anti-HT IgG titers for individual dogs are shown in **Supplementary Figure 1**. IgG ELISAs of the dog serum samples analyzed at day 14 showed that IgG2 was the principal IgG subclass developed against all HTs under experimentation (**Table 4**). The IgG1:IgG2 ratios ranged from 1:1 to 1:64, however HT vaccinated dogs 34295 and 68888 had identical IgG1:IgG2 ratios for HT2 (1:1) and HT4 (1:1).

4. DISCUSSION

The primary host of *I. holocyclus* are Australian native marsupials such as bandicoots, whereas livestock, companion animals and humans are considered as secondary or incidental hosts. The adult female *I. holocyclus* produces the most harmful form of tick paralysis compared to its counterparts *Dermacentor andersoni* and *Dermacentor variabilis* in North America and *Rhipicephalus evertsi* in Africa (reviewed by 4, 5, 43). The severity of this toxicosis is observed principally in pets and domestic animals but there have been reports of fatal pediatric/pediatric cases (14, 26, 44–49). The clinical symptoms of paralysis ticks are loss of appetite, voice alteration and loss of limb coordination. Also, there is an ascending flaccid paralysis, excessive salivation, asymmetric pupillary dilation and respiratory distress, reviewed by (26).

Purification of neurotoxic components present in the paralysis tick's saliva were elusive since the first report by Thurn et al. in 1992 (8) and a proteomics study undertaken in 2008 failed to identify holocyclotoxins (50). Up to twelve years ago, the resources of tick protein databases were limited and thus

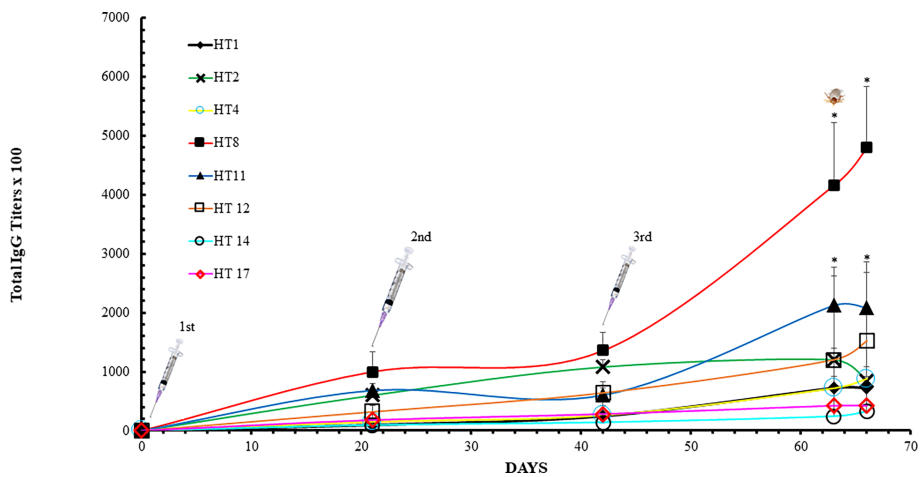

FIGURE 2 | ELISA analysis with dog sera collected in Trial 2. Dog anti-holocyclotoxins IgG titer average displayed as mean ± SD after immunization with HT cocktail: HT1, HT2, HT4, HT8, HT11, HT12, HT14 and HT17. The titers were determined as the reciprocal of the last dilution that gave a positive signal in comparison to the background absorbance from the placebo dog IgG values. Arrows represent dog immunization dates (Days 0, 28 and 49). The tick identifies the date dogs were challenged with *I. holocyclus* unfed adult female ticks. Data were analyzed by Two-Way ANOVA with Tukey's post-test (*$p < 0.05$).

TABLE 4 | The IgG1:IgG2 ratios against each holocyclotoxin within the cocktail vaccination at 14 days post immunization in trial 2.

Dog ID	IgG1:IgG2 titer ratio							
	HT1	HT2	HT4	HT8	HT11	HT12	HT14	HT17
34295	1:4	1:4	1:1	1:2	1:4	1:4	1:8	1:2
55891	1:16	1:16	1:16	1:16	1:64	1:32	1:8	1:4
64799	1:8	1:4	1:4	1:8	1:8	1:4	1:16	1:4
68888	1:2	1:1	1:1	1:4	1:4	1:2	1:16	1:4

it was a bioinformatics hurdle to identify tick proteins using *de novo* sequencing and mass spectrometry. Currently, the situation is quite different with approximately 375 *I. holocyclus* proteins reported in NCBI (txid65647[Organism:noexp]), 149,746 proteins from *Ixodes scapularis*, and the *I. holocyclus* database deposited into Genbank (transcriptome shotgun assembly GIBQ00000000) associated with the manuscript originally describing the HT family published in 2018 (10). Holocyclotoxins are described as a ~ 5kDa peptide that in SDS-PAGE migrate at 40 – 80kDa (8, 40). It is not known if these large HTs from extracted gel fragments are produced only by HTs or whether HT carrier proteins are involved. This phenomenon has previously been described for *I. holocyclus* with 'anti-toxin' monoclonal antibodies recognizing 100-200 kDa proteins through western blot analysis (51). Similar inconsistencies have been identified for other paralysis causing tick species with different size toxic fractions found in *R. evertsi evertsi* (80-100kDa) and *R. evertsi evertis* monoclonal antibodies showed cross reactivity with *A. walkerae* toxins of different sizes (5, 52, 53). Transcriptomic studies on these other tick species to identify the coding regions of the corresponding toxins are yet to be reported. With the advent of improved tick genome sequencing combining improved long and short read technologies (54, 55), it may be feasible to sequence genomes which will assist to identify homologues of toxins in more tick species. The genome sequence of a paralyzing tick species is yet to be reported. Toxin or venom research reviews do not always mention ticks most likely due to the fact that only toxins for *I. holocyclus* have been recently characterized (10). Nonetheless, this study reports the development of an anti-paralysis tick vaccine based on a cocktail of neurotoxins produced in the venom of the adult female *I. holocyclus* tick.

Recently, toxin-related sequence descriptions were identified within the *I. holocyclus* dataset. Data showed that holocyclotoxins belong to a multigene family of a highly conserved inhibitor cysteine knot (ICK) motif (10, 56). These cystine-rich peptides are present in the venoms of scorpions and spiders and ICK motif conferred remarkable stability to these peptides (57). Following the discovery in *I. holocyclus* of a large holocyclotoxin (HT) family of ~19 peptides (10), this report confirms the identification of HTs included in a vaccine cocktail able to protect dogs from tick challenge. Our study demonstrates for the first time that folded HTs (cystine-rich peptide) induced a strong and protective immune response compared to unfolded holocyclotoxins. These toxins are cystine-rich peptides with disulphide bridges that provide conformational rigidity to the molecule, extreme stability to degradation by heat or enzymes (40, 41, 57). There is a previous report of anti-toxin peptide vaccination obtained using a linear epitope of the *Loxosceles intermedia* (recluse spider) dermonecrotic protein isoform (LiD1) showing a modest level of protection (58). However, a continuous B-cell epitope of 27 amino acid related to a fragment of the Smase D protein induced 75% protection *in vivo* for lethal doses of *L. intermedia* venom (59, 60).

The administration of an anti-paralysis tick serum (TAS) has demonstrated neutralizing potency after envenomation with the paralysis tick neurotoxins. It also has many drawbacks, including inhumane production (through dog hyper-immunization), limited window of efficacy (needing to be administered in the early stages of paralysis), non-standardized dosages, side effects associated with serum sickness, and varying potencies between batches and manufacturers (reviewed by 26). The paralysis caused by holocyclotoxins appears to be pre-synaptic (25), hence, the anti-tick serum (TAS) used in Australia is ineffective once the paralysis has progressed. Acaricides have been the most dominant form of preventative treatment. However, the development of tick resistance to chemicals and evidence of adverse reactions to certain chemicals for some pets demonstrate the need for a safe preventative such as a vaccine. In an attempt to remediate these circumstances for other anti-venoms, studies (20, 61–63) have used new biotechnological tools to improve the efficacy, safety, and cost-effective management of antivenin production, such as immunization with synthetic peptide epitopes, recombinant toxins (or toxoids), or DNA. These methodologies reveal the potential for producing antivenins with high therapeutic antibody titer and broad neutralizing capacity (20, 61). Also, these approaches avoid the use of venom in the production process, thus preventing the difficulties related with animal captivity and venom collection (20, 61–64). This study describes for the first time the development of a neurotoxin cocktail vaccine demonstrating dog protection from paralysis induced by female adult *I. holocyclus* ticks, which could also enable the production of safe anti-paralysis antivenins. Further larger trials would be necessary to confirm the protective efficacy of the vaccine described by this study using, for example, different breeds of dogs and a challenge with a higher number of ticks.

The high titers of anti-holocyclotoxin IgGs induced after immunizing dogs with the HT cocktail vaccine neutralized the symptoms of the paralysis tick. In these experiments, the IgG subclass predominantly stimulated was canine IgG2 (or IgG- B and C, as reported by Bergeron et. al., 2014 (65). Dog IgGs have been divided into four subclasses, A-D (66), and subsequently Bergeron et al. (65) showed that commercial anti-canine IgG1

recognized the dog IgG subclasses A and D while anti-canine IgG2 reacts with subclasses B and C. Based on the effector function, canine IgG subclasses are analogous to their human IgG counterparts. Canine immunoglobulins B and C are similar to human IgG1 and IgG3, but A and D IgGs are most similar to human IgG2 and IgG4 (65). These classes are known to be stimulated by soluble proteins, corresponding with our vaccine composition, and are potent triggers of effector mechanisms due to strong FcγR engagement in anti-toxin activity (67, 68). Canine IgG A and D are associated with more subtle responses caused by a weaker FcγR engagement (68, 69). In addition, canine IgG2 has previously correlated to the successful protection against diseases and parasites in dogs. For example, high IgG2 titers in dogs immunized with a cocktail peptide-based vaccine has been associated with protection against leishmaniasis (70). The evidence suggested that a preferential induction of the IgG2 subclass in dogs is correlated with a highly protective immune response. In this study, the immunization with HTs in an oil-based adjuvant developed hyper immunity in dogs without inducing symptoms of paralysis. Stone et al. reported different results, as they detected paralysis symptoms in partially immunized dogs (71, 72). These previous studies in optimizing the production of anti-tick IgGs in dogs and rabbits reported a very slow development and affinity maturity of protective immunity caused by using toxin preparations of questionable purity, and an inoculation regime of up to two-years to develop sufficient protection (72). Thus, in the pursuit of a tick-paralysis vaccine, synthetic or recombinant HTs offer a safe and cost-effective vaccine delivery method.

In conclusion, we report the successful immunization of dogs using a synthetic peptide toxin cocktail derived from a family of holocyclotoxins identified in the Australian paralysis tick *I. holocyclus*. Future research should focus on recombinant production of the HT peptides, determining optimal dosages, and vaccine longevity studies to ensure adoption of the vaccine to protect companion pets from paralysis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, GIBQ01000000.1.

ETHICS STATEMENT

The animal study was reviewed and approved by Elanco Animal Health.

AUTHOR CONTRIBUTIONS

MR-V and AT (equal contributions and coordination of the research) conceived the project with support from KA, AK, and MB (project initiation and research management). Dog trial planning undertaken by AK, followed by CE and KV with the above acknowledged trial team to execute the trials. ELISA screening and the analysis of dog immune responses undertaken by SM and MR-V. The Honours thesis of SM contributed methods and results to this manuscript. Manuscript was equally prepared by MR-V and AT. Bioinformatics analyses (research component coordinated by MIB) and assistance towards the submission of the TSA to the Genbank database undertaken by MB and PM and coordinated by AT. Vaccine doses were prepared by MR-V. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.744795/full#supplementary-material

Supplementary Table 1 | Clinical Diagnosis Matrix for Tick Paralysis in Dogs. VAS, Visual Analog Scale.

Supplementary Figure 1 | Dog anti-holocyclotoxin IgG titers of the animals immunized in Trial 2. Titers against each holocyclotoxins per dog are shown. The average SD titer of three replicates per time point of each dog serum are represented.

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