LYME DISEASE: RECENT ADVANCES AND PERSPECTIVES

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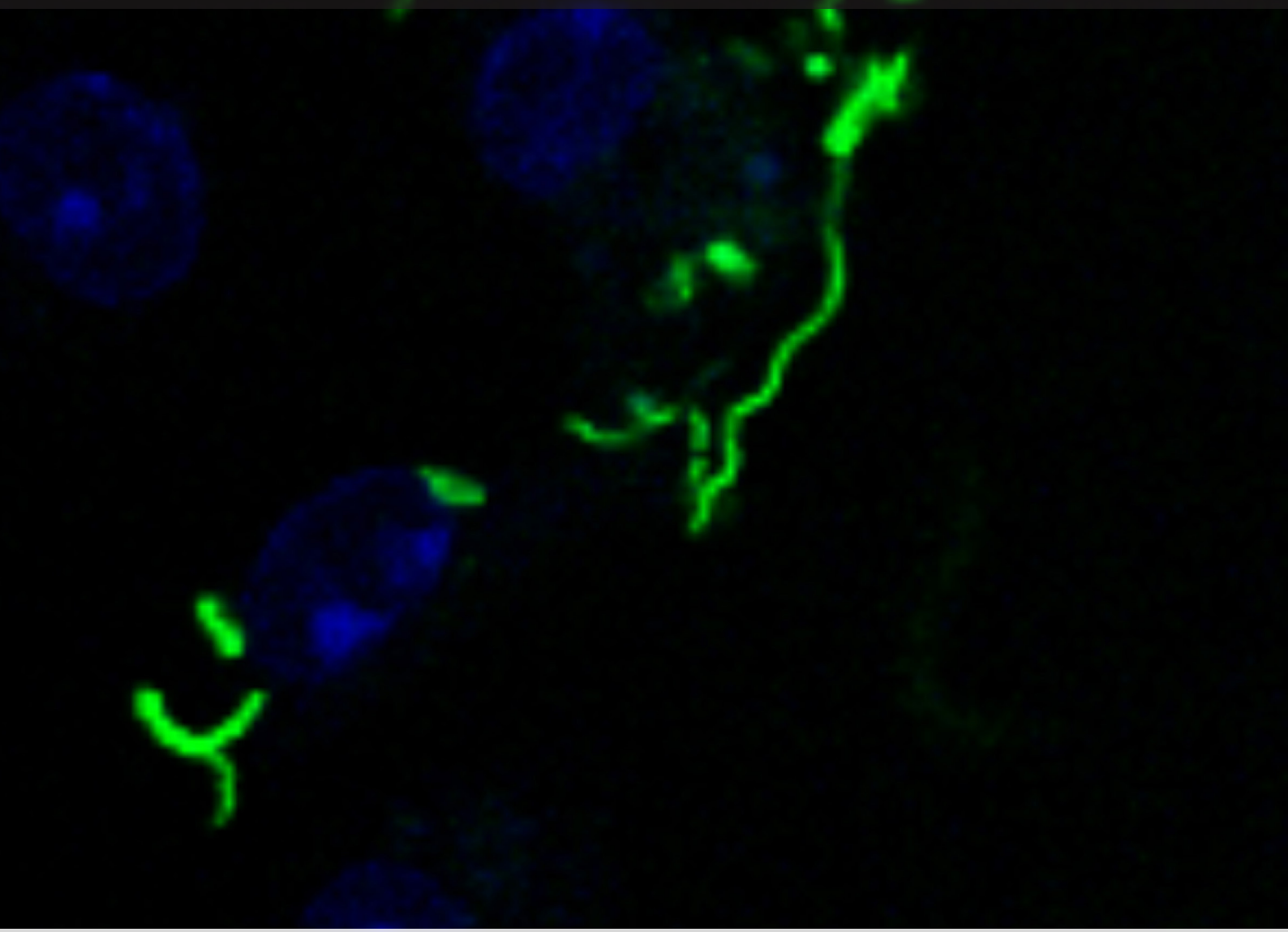

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LYME DISEASE: RECENT ADVANCES AND PERSPECTIVES

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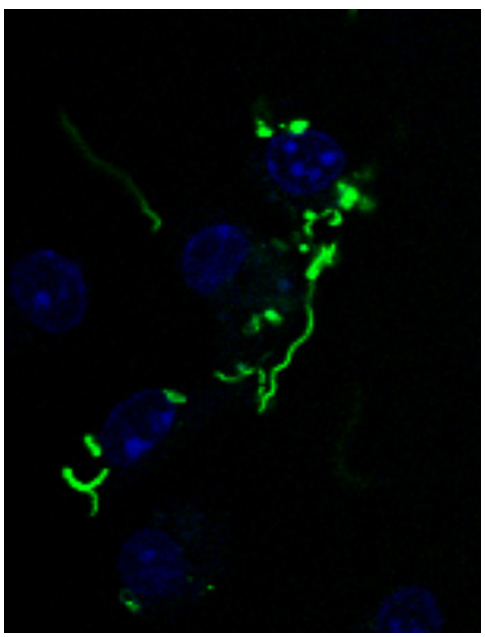

Borrelia burgdorferi interacting with mammalian cells. Image by: Tanja Petnicki-Ocwieja

The interplay between host and pathogen is a complex co-evolutionary battle of surveillance and evasion. The pathogen continuously develops mechanisms to subvert the immune response in order to establish infection while the immune system responds with novel mechanisms of detection. Because the majority of Lyme disease pathology is due to an over-exuberant immune response, much research in Borrelia burgdorferi pathogenesis has been devoted to understanding the mammalian host response to the bacterium. Immunological studies continue to be an active area of research employing emerging techniques, such as intra-vital imaging. These studies have furthered our understanding of inflammatory processes during long-term infection and provided some surprising insights, such as the continued presence of bacterial products after clearance. The field of Lyme disease has long debated the etiology of long-term inflammation and recent studies in the murine host have shed light on relevant cell types and inflammatory mediators that participate in the pathology of Lyme arthritis. Live imaging and bioluminescent studies have allowed for a novel view of the bacterial life cycle, including the tick mid-gut, tick-to-mammal transmission and dissemination throughout a mouse. A number of tick and bacterial proteins have been shown to participate in the completion of the enzootic cycle. Novel mechanisms of gene regulation are continuously being identified. However, B. burgdorferi lacks many traditional virulence factors, such as toxins or specialized secretion systems. Many genes in the B. burgdorferi genome have no known homolog in other bacteria. Therefore, studies focusing on host-pathogen interactions have therefore been limited by an incomplete understanding of the repertoire of bacterial virulence factors. Questions such as how the pathogen causes disease, colonizes the tick

and evades host immune-surveillance have been difficult to address. Genetic studies involving single gene deletions have identified a number of important bacterial proteins, but a large-scale genomics approach to identify virulence factors has not been attempted until recently. The generation of a site-directed mutagenesis library is an important step towards a detailed analysis of the B. burgdorferi genome and pathogenome. Using this library, high-throughput genomic studies, utilizing techniques such as massively parallel sequencing have been promising and could be used to identify novel virulence determinants of disease in the mammalian host or persistence in the tick vector. Continued research on this unique pathogen and its specific interaction with host and vector may have far reaching consequences and provide insights for diverse disciplines including ecology, infectious disease, and immunology. Here, several reviews will discuss the most recent advances and future studies to be undertaken in the field of B. burgdorferi biology.

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EDITORIAL
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Lyme disease: recent advances and perspectives

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Keywords: Lyme Disease, *Borrelia burgdorferi*, *Ixodes scapularis*, innate immunity, Lyme arthritis, adhesins, c-di-GMP, Tnseq

Lyme Disease, caused by the spirochete *Borrelia burgdorferi* and transmitted by *Ixodes scapularis* (deer tick or blacklegged tick), has been gaining in incidence over the past decade. Without treatment, it is a long-term infection characterized by inflammation of the joints, heart and nervous system. The Centers for Disease Control and Prevention (CDC) classifies it as an Emerging Infectious Disease with an expanding geographical area of occurrence and have recently revised their incidence cases in the United States by 10 fold.

In this Lyme Disease Research Topic we have gathered reviews and original research in the fields of microbiology and immunology of *B. burgdorferi* infection. Included in this Topic are the abstracts from the 13th International Congress on Lyme Borreliosis and we thank the organizers Dr. Linda Bockenstedt and Dr. Linden Hu for publishing them in this issue at the following link http://www.frontiersin.org/books/13th_International_Conference_on_Lyme_Borreliosis_and_other_tick_Borne_Diseases_/357.

Increased Geographical Distribution

As an emerging infectious disease the incidence and geographical distribution of Lyme disease cases are being monitored by a number of US states. Based on the CDC, Ohio is considered a non-endemic area for Lyme Disease, largely due to the low incidence of the arthropod vector *I. scapularis*. A tick surveillance program established by the Ohio Department of Public Health indicated a sharp increase in the prevalence of this tick in the state. Here, Wang et al. provide data that suggest an establishment of the enzootic cycle in Ohio (Wang et al., 2014).

Vaccine Development—a Different Approach

The development of a Lyme disease vaccine has been a hot topic for researchers and the public. Currently, the Food and Drug Administration has not given its approval for a human Lyme Disease vaccine. Therefore, researchers have sought alternate approaches to address control of *B. burgdorferi* infection in humans through pest management intervention. These methods have mainly consisted of vector-targeted or reservoir-targeted vaccines aiming to reduce tick density or control different aspects of the enzootic cycle. Gomes-Solecki writes a detailed review of the current status of those studies (Gomes-Solecki, 2014).

Host Immunity—Novel Pathways and Genetic Approaches

Because the majority of Lyme disease pathology is due to an over-exuberant immune response, much research in *B. burgdorferi* pathogenesis has been devoted to understanding the mammalian host response to the bacterium. A significant focus of immune

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studies has been the innate immune response as an initiator of inflammation. Recent studies have elucidated novel components of the innate immune response and intracellular pathways that participate in *B. burgdorferi* induced inflammation. Here, Cervantes et al. and Petnicki-Ocwieja and Kern review the most recent studies dissecting the numerous innate immune response pathways involved in *B. burgdorferi* recognition (Cervantes et al., 2014; Petnicki-Ocwieja and Kern, 2014).

Although much progress has been made in identifying immune pathways that participate in the *B. burgdorferi* induced response, it is unclear how these pathways lead to arthritis resistance or susceptibility. The field of Lyme disease has long debated the etiology of long-term inflammation and recent studies in the murine host have shed light on relevant cell types and inflammatory mediators that participate in the pathology of Lyme arthritis. Pratt and Brown review the role of eicosanoids as important mediators of arthritis (Pratt and Brown, 2014). Also, Bramwell et al. review the challenges of genome wide association studies for studying complex genetic traits in humans and the power of forward genetic approaches in model animals leading to the identification of genetic loci responsible for arthritis severity phenotypes (Bramwell et al., 2014). Finally, in a research paper, Belperron et al. present data that implicate the FcRY receptor as an important contributor to acute phase Lyme arthritis (Belperron et al., 2014).

Lessons from the Tick

Much of the research in Lyme Disease immunology has focused on understanding the immune response in the mammalian host. However, the importance of the enzootic cycle and the mode of survival of the bacterium in its arthropod host cannot be understated. The *I. scapularis* tick maintains the bacterium in its mid-gut, but precisely how tick immunity functions and influences persistence of pathogens remains unknown. Here, Smith and Pal discuss exciting insights that could be gained from mining the sequenced Ixodes genome and highlight future areas of investigation (Smith and Pal, 2014).

What about the Bug?

In nature, *B. burgdorferi* cycles between the vastly different environments of the *Ixodes* tick vector and mammalian host. *B. burgdorferi* must be able to detect changes in its environment, and rapidly respond to these changes. c-di-GMP, a second messenger unique to bacteria, is a global regulator that facilitates adaption to changing environmental circumstances. Novak et al. provide up-to-date information on how c-di-GMP signaling is instrumental in orchestrating the adaptation of *B. burgdorferi* to the tick environment (Novak et al., 2014).

Adhesion is the initial event in the establishment of any infection. *B. burgdorferi* modulates adhesion to host tissues in order to colonize, disseminate, and persist in its mammalian host. Brissette and Gaultney update current knowledge on the structure, function, and role of borrelial adhesins in Lyme disease pathogenesis (Brissette and Gaultney, 2014).

The Next Generation: Massively Parallel Sequencing Tnseq

B. burgdorferi lacks many traditional virulence factors, such as toxins or specialized secretion systems. Therefore, studies focusing on host-pathogen interactions have been limited by an incomplete understanding of the repertoire of bacterial virulence factors. Questions such as how the pathogen causes disease, colonizes the tick and evades host immune-surveillance have been difficult to address.

Genetic studies involving single gene deletions have identified a number of important bacterial proteins, but a large-scale genomics approach to identify virulence factors has not been attempted until recently. Lin et al. review the generation of a site-directed mutagenesis library as an important step toward a detailed analysis of the *B. burgdorferi* genome and pathogenome (Lin et al., 2014). Using this library, high-throughput genomic studies, utilizing techniques such as massively parallel sequencing or Tnseq have shown to be a powerful tool in understanding the pathogen.

Lyme disease is endemic through much of the Northern hemisphere, including North America, Europe, and Asia, and will continue to be a public health concern for the foreseeable future. This Topic highlights the important work that is currently being done to understand the pathogen, the vector, and the disease. We hope that you find this Topic as enlightening and thought-provoking as we did.

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Mechanisms of *Borrelia burgdorferi* internalization and intracellular innate immune signaling

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Lyme disease is a long-term infection whose most severe pathology is characterized by inflammatory arthritis of the lower bearing joints, carditis, and neuropathy. The inflammatory cascades are initiated through the early recognition of invading *Borrelia burgdorferi* spirochetes by cells of the innate immune response, such as neutrophils and macrophage. *B. burgdorferi* does not have an intracellular niche and thus much research has focused on immune pathways activated by pathogen recognition molecules at the cell surface, such as the Toll-like receptors (TLRs). However, in recent years, studies have shown that internalization of the bacterium by host cells is an important component of the defense machinery in response to *B. burgdorferi*. Upon internalization, *B. burgdorferi* is trafficked through an endo/lysosomal pathway resulting in the activation of a number of intracellular pathogen recognition receptors including TLRs and Nod-like receptors (NLRs). Here we will review the innate immune molecules that participate in both cell surface and intracellular immune activation by *B. burgdorferi*.

Keywords: *Borrelia burgdorferi*, Lyme disease, Toll-like receptor signaling, Nod-like receptor signaling, phagocytosis, endosomal signaling

INTRODUCTION

Lyme disease is caused by the spirochete *Borrelia burgdorferi* transmitted through a tick bite. The disease manifests as early localized skin inflammation (erythema migrans) occurring at the site of the tick bite. Late stage disease is characterized by inflammation of the heart, the joints, the nervous system or the skin. The incidence of human infections has risen steadily over the last 15 years and Lyme disease is the most common tick-borne disease in the United States and Europe. More than 30,000 cases are reported annually in the US and the number of cases is estimated around 85,000 yearly in Europe (Lindgren et al., 2006; Centers for Disease Control and Prevention, 2013).

Innate immune responses are the first responders to infection and the catalyst of inflammation causing much of Lyme disease pathology. Recently, studies have shown that phagocytosis plays a role in initiating inflammatory responses (Moore et al., 2007; Shin et al., 2008; Salazar et al., 2009). Because of the requirement for pathogen internalization, much interest has been generated in studying intracellular signaling pathways. As a result, in addition to pathogen recognition by Toll-like receptors (TLRs), intracellular receptors such as the NOD-like receptors (NLRs) have been shown to participate in *B. burgdorferi* signaling. These families of receptors do not act in isolation and there is considerable cross talk among the innate immune signaling pathways activated (Takeuchi and Akira, 2010). TLR2 has been shown to initiate a significant portion of the inflammatory output in response to *B. burgdorferi*. The TLR2 ligands are *Borrelia* cell surface lipoproteins, the best-characterized being Outer Surface Protein A (OspA) (Hirschfeld et al., 1999; Lien, 1999; Takeuchi and Akira, 2010).

Cellular compartmentalization is increasingly recognized as having a significant role in the regulation of innate immune signaling. Although this concept has been broadly understood as distinguishing between cell surface receptors, such as TLRs 2, 4, and 5 and intracellular sensors, such as TLRs 3, 7, and 9, as well as NLRs, it has recently become apparent that intracellular trafficking to different sub-cellular compartments and organelles, such as mitochondria and peroxisomes, plays a more intricate role in innate immune regulation than previously thought (Eisenbarth and Flavell, 2009; Blasius and Beutler, 2010; Dixit et al., 2010; Kagan, 2012). Here we will review various intracellular innate immune pathways activated by *B. burgdorferi* and how they may collectively contribute to inflammatory signaling.

TOLL-LIKE RECEPTORS

CELL SURFACE SIGNALING

Recognition of B. burgdorferi at the cell surface

Due to the importance of bacterial phagocytosis into host cells, significant research in recent years has been devoted to understand the involvement of cell surface molecules in the internalization of *B. burgdorferi*. Spirochete internalization involves attachment or tethering of the bacteria to the host cell followed by engulfment into the host cell. These two processes, although linked, often involve different sets of cells surface molecules. *B. burgdorferi* contains a number of molecules known to function as adhesins and participate in the attachment of the bacterium to the host cell. One group of receptors shown to participate in tethering of *B. burgdorferi* to the cell surface is the integrin family, specifically integrins $\alpha_v\beta_3$, $\alpha_5\beta_1$,and $\alpha_M\beta_2$(CD18/CD11b, Mac-1, CR3) (Cinco et al., 1997; Coburn et al., 1998). Integrin $\alpha_v\beta_3$has

been shown to bind to the p66 protein of *B. burgdorferi,* but has not been shown to play a direct role in the initiation of immune responses (Coburn et al., 1998; Coburn and Cugini, 2003). It does, however, participate in adherence and potentially internalization of the bacterium into the host cell. Interestingly, integrin $\alpha_v\beta_3$ has been suggested to tether TLR2 ligands to the host cell via interaction with the serum protein vitronectin (Gerold et al., 2008). This has not been explored in the context of *B. burgdorferi* adhesion. Integrin $\alpha_M\beta_2$ has been shown to participate in the attachment of *B. burgdorferi* to the cell surface (Cinco et al., 1997) and together with the TLR2 associated GPI anchored receptor, CD14, $\alpha_M\beta_2$ mediates the internalization of the bacterium into the host cell (Hawley et al., 2012).

Signal transduction from the cell surface

Signaling from the plasma membrane is a necessary component of the *B. burgdorferi* response, the mechanisms of which remain to be completely described. Understanding how cell surface accessory molecules contribute to the recognition of the ligand by the TLR is an active area of research. In the model of TLR4 signaling, integrin $\alpha_M\beta_2$ participates in the recruitment of the Toll-interleukin 1 receptor (TIR) domain-containing adapter protein (TIRAP) to Phosphatidylinositol 4,5-bisphosphate (PIP2) rich membranes, where TIRAP interacts with PIP2 and initiates cells surface signaling by recruiting the MyD88 signaling adaptor (Kagan and Medzhitov, 2006). TLR2 is the only other TLR that has been shown to utilize TIRAP for signaling and thus is likely that interaction with PIP2 is also important for TLR2 signaling (Yamamoto et al., 2002). However, there is significant interest in uncovering the signaling mechanism behind the ability of $\alpha_M\beta_2$ to traffic ligands and the TLR receptor to PIP2 rich locations and recruit signaling molecules.

B. burgdorferi does not contain lipopolysaccharide (LPS) and does not activate TLR4 (Takayama et al., 1987; Berende et al., 2010). However, it contains other ligands that activate different TLRs. Specifically, *B. burgdorferi* activates TLR2/1 heterodimers through recognition of the triacylated lipid moiety on its cell surface localized lipopeptides (Hirschfeld et al., 1999; Alexopoulou et al., 2002). *B. burgdorferi* has been shown to activate TLR5, potentially through the spirochetal flagellin (Shin et al., 2008). Recruitment of MyD88 to the plasma membrane by TIRAP for TLR2 signaling or directly to TLR5 results in a signaling cascade which proceeds through the recruitment of the IRAK kinases, the E3 ubiquitin ligase, TRAF6, and TAK1 leading to the activation of MAP kinases and of NF-kB through the IKK complex (Takeda and Akira, 2004). This signaling pathway leads to the activation of pro-inflammatory cytokines such as IL-6, IL-12, TNF-α, and pro-IL1β. Type I IFNs have not been shown to be activated from this signaling cascade initiated at the plasma membrane.

INTRACELLULAR SIGNALING

Internalization of B. burgdorferi into host cells

Although there are a number of cell surface molecules that mediate phagocytosis of *B. burgdorferi*, the signaling events during the process of internalization are under investigation. Early studies into the mechanisms behind *B. burgdorferi* internalization indicate that it occurs through coiling, rather than conventional, phagocytosis, in which the bacteria attach to the host cell surface and are rolled into a single fold of the plasma membrane (Rittig et al., 1992). In studies with primary human macrophages it was shown that interaction with the bacteria leads to the formation of f-actin rich structures. The actin polymerization is mediated by the Wiskott-Aldrich syndrome family protein (WASP) and Arp2/3 complex, particularly during integrin $\alpha_M\beta_2$and Fcγ receptor mediated phagocytosis (Linder et al., 2001; Shin et al., 2009). The regulatory pathways influencing actin polymerization and internalization of *B. burgdorferi* are mediated by the small GTPases Cdc42 and Rac1 (Linder et al., 2001). In addition, PI3K signaling was shown to be required for phagocytosis of *B. burgdorferi* by murine macrophages (Shin et al., 2009).

Although some cell surface molecules may serve as tethers, other molecules seem to have a more direct role in internalization. As described, CD14 is a molecule that mediates endocytosis of *B. burgdorferi* (Hawley et al., 2012). However, this is in itself a confusing finding in that CD14 is not known to have any cytoplasmic signaling domains that could initiate a signaling cascade leading to phagocytosis. CD14 has been shown to bind integrin $\alpha_M\beta_2$ and localize it to lipid rafts and it is possible that it then interacts with other molecules that can mediate endocytosis (Hawley et al., 2013). The primary role of this integrin seems to be in the attachment of the *B. burgdorferi*, rather than its internalization, and the signaling pathways activated by the integrin to mediate phagocytosis have not been described in the context of *B. burgdorferi*. Interestingly, integrin $\alpha_3\beta_1$ has been shown to mediate *B. burgdorferi* and TLR2/1 ligand signaling (Marre et al., 2010). However, unlike $\alpha_M\beta_2$, $\alpha_3\beta_1$ was not shown to mediate the attachment of *B. burgdorferi* or other TLR2 ligands to the cell surface but rather to participate in internalization (Behera et al., 2006; Marre et al., 2010). Overall, the $\beta_1$integrin is required for internalization of *B. burgdorferi* into murine fibroblasts and to utilize the Src kinase signaling pathway for internalization (Wu et al., 2011). It is unclear if $\alpha_3\beta_1$directly activates the Src signaling cascade, nor if CD14 and integrin $\alpha_3\beta_1$ cooperate to mediate phagocytosis of *B. burgdorferi*.

The complexity of molecule involvement in the phagocytic process is increased by the added role of scavenger receptors, which have also been shown to participate in TLR2 and *B. burgdorferi* signaling. CD36, a type B scavenger receptor, has been shown to be important for the internalization of TLR2 ligands and numerous studies have been devoted to understanding the mechanisms behind CD36 cooperation with TLR2. However, its role in *B. burgdorferi* internalization has not been explored (Shamsul et al., 2010). The scavenger receptor Macrophage Receptor with Collagenous Structure (MARCO), which plays a role in the internalization of a variety of microbial ligands, does mediate *B. burgdorferi* phagocytosis. In our studies we showed that MARCO was significantly up-regulated upon *B. burgdorferi* stimulation. The up-regulation of MARCO was dependent on MyD88 and MARCO deficient macrophage showed a decrease in the phagocytosis of *B. burgdorferi* (Petnicki-Ocwieja et al., 2013). These findings offered one possible explanation for the partial phagocytic defect previously observed in MyD88 deficient macrophage (Shin et al., 2008; Petnicki-Ocwieja et al., 2013). It is

yet unclear if MARCO participates in *B. burgdorferi* attachment to the cell surface. Significant work still needs to be done to determine how these cell surface accessory molecules: integrins, scavenger receptors and the GPI anchored receptor, CD14, cooperate to mediate signaling and internalization of *B. burgdorferi* into the host cell.

Signal transduction from the endosomal compartment

TLR signaling can be initiated from both the plasma membrane and intracellular compartments. TLR5 has not been shown to signal from intracellular compartments and thus will not be discussed further in this context. Interestingly, we and others have recently shown that in addition to being localized at the plasma membrane, TLR2 mediates signaling from endosomal vesicles in response to *B. burgdorferi* and TLR2 synthetic ligands. Inhibition of endosomal acidification upon *B. burgdorferi* stimulation results in a decrease in type I IFN and pro-inflammatory cytokine activation, such as IL-6 (Marre et al., 2010; Cervantes et al., 2011). TLR2 cooperates with other endosomal TLRs to generate a *B. burgdorferi* specific inflammatory response. *B. burgdorferi* activates TLR7/8 and TLR9, which are endosomally localized TLR receptors (Shin et al., 2008; Petzke et al., 2009; Cervantes et al., 2011, 2013). Adaptor molecules, such as MyD88, are recruited to the endosomal compartment to transduce signals for the activation of inflammatory cytokines and type I IFN from these endosomal TLRs (for a detailed review on TLR 7/8 and 9 in *B. burgdorferi* signaling, please see Cervantes, Hawley and Salazar in this issue).

The localization of TLR2 at two different cellular locations requires that signaling molecules are able to distinguish the cellular localization of TLRs and assemble cell location specific signaling complexes. The only other TLR that has been shown to signal from two different cellular locations is TLR4. In the model of TLR4 signaling, signaling pathways from the cell surface vs. the endosome are clearly distinguished. TLR4 mediates signaling from the plasma membrane via TIRAP/MyD88 for MAP kinase and NF-kB activation resulting in pro-inflammatory cytokine activation. From the endosome TLR4 utilizes an entirely different set of adaptors, TRAM and TRIF, which are the signaling platform used to signal for type I IFNs, although TRAM/TRIF also mediate a delayed wave of NF-kB and pro-inflammatory cytokine activation (Kawai and Akira, 2011).

Investigating the TLR2 signaling complex at the plasma membrane in comparison to the endosome, we found that TLR2 was also able to utilize the adaptor TRIF. TRIF deficient macrophage showed a reduction in type I IFN activation and secretion of IL-6 (Petnicki-Ocwieja et al., 2013). This was an unexpected result as TRIF was previously thought not to participate in TLR2 signaling. Interestingly, as opposed to the clear separation of signaling pathways in the TLR4 model, TLR2/TRIF signaling was dependent on MyD88, suggesting that the MyD88 and TRIF signaling pathways were interconnected. In addition to participating in TLR7/8 and 9 signaling, MyD88 may also participate in TLR2 signaling at the endosome. *In vivo*, TRIF deficient mice did not show any deficiencies in the ability to control bacterial loads in the joints of *B. burgdorferi* infected mice in comparison to wild type mice (Petnicki-Ocwieja et al., 2013). However, TRIF deficient mice did have increased levels of inflammatory cytokines in the joints, suggesting that TRIF has an important role in controlling immune responses leading to inflammation but not responses leading to control of pathogen burden. This might in part be due to the fact that, unlike MyD88 deficient cells, TRIF deficient cells do not have any observable phagocytic defects.

Intracellular activation of immune pathways has been extensively studied in the case of viral infections. The intracellular activation of type I interferons (IFNs) was for a long time considered to be strictly a viral response. Recently, type I IFN activation has been shown to play an important role in a large number of bacterial infections (Katze et al., 2002; Perry et al., 2005). In *B. burgdorferi* infection, the type I IFN response has also been shown to be important for the development of murine Lyme arthritis (Miller et al., 2008; Petzke et al., 2009; Salazar et al., 2009; Cervantes et al., 2011). Type I IFN activation initiated by TLRs is mediated by interferon regulatory factors (IRFs). In studies with TLR2 signaling, IRF1, and IRF7, both of which have been shown to bind MyD88, participate in TLR2 signaling (Dietrich et al., 2010). Downstream of the adaptor TRIF, TRAF3 is responsible for localizing an IRF3 signaling complex to the endosome leading to type I IFN activation. *B. burgdorferi* stimulation has also been shown to proceed through IRF7 via TLR2 and TLR7 and 9 (Petzke et al., 2009; Petnicki-Ocwieja et al., 2013). Interestingly, studies have also shown that IRF3 is required for the type I IFN response to *B. burgdorferi* (Miller et al., 2010).

NUCLEOTIDE BINDING OLIGOMERIZATION DOMAIN RECEPTORS (NOD-LIKE RECEPTORS)

TLRs sense the extracellular and the endosomal compartments whereas RIG-like receptors (RLRs) and Nod-like receptors (NLRs) are intracellular sensors. In addition to TLRs, NLRs also participate in *B. burgdorferi* mediated intracellular signaling. From the receptors in the NLR family, Nod1, and 2 and the inflammasome complex are the best studied.

NOD1 AND NOD2

Nod1 and Nod2 multi-domain proteins in the NLR family and are involved in the recognition of intracellular pathogens. NLRs contain an N-terminal effector domain which is thought to participate in protein-protein interactions with downstream signaling molecules, a central nucleotide-binding oligomerization domain (NBD or NACHT), and a C-terminal leucine-rich repeat domain for ligand recognition (LRR). Members of the NLR family can be sub-divided based on their N-terminal domain, which can be a Caspase Recruitment Domain (CARD), a pyrin domain (PYR), or a baculovirus IAP repeat domain (BIR) (Kanneganti et al., 2007) (Wilmanski et al., 2007). Many NLRs are complexed into the inflammasome, a signaling platform which activates caspase-1. Others, such as Nod1 and Nod2 are not considered to be inflammasome components and instead recruit the RIP2 kinase (or RICK) through a CARD-CARD interaction leading to the expression of pro-inflammatory cytokines (Moreira and Zamboni, 2012). Nod1 and Nod2 cytoplasmic receptors sense intracellular bacteria through muropeptides derived from bacterial peptidoglycans although some data suggest they are also able to sense viruses and protozoan parasites (Strober et al.,

2005). *In vitro* stimulation of Nod2 deficient macrophage with *B. burgdorferi* results in a decrease in pro-inflammatory and type I IFN induction, suggesting Nod2 plays a role in the activation of inflammatory signals (Sterka and Marriott, 2006; Chauhan et al., 2009; Oosting et al., 2010a; Petnicki-Ocwieja et al., 2011). Nod1 does not seem to play a significant role in *B. burgdorferi* signaling.

In contrast to the pro-inflammatory effect of Nod2 *in vitro*, the opposite pattern is observed *in vivo*. Nod2 deficiency results in increased inflammation in the heart and joints after *B. burgdorferi* infection *in vivo.* This *in vivo* finding suggests that Nod2 is also involved in the suppression of the inflammatory signal during *B. burgdorferi* infection (Petnicki-Ocwieja et al., 2011). Nod2 has been extensively studied in other *in vivo* inflammation models and in some systems, Nod2 deficiency results in increased rather than decreased pathology, as we have observed for *B. burgdorferi*. Such cases include chronic *M. tuberculosis* infection (Gandotra et al., 2007) and the Inflammatory Bowel Disorder–Crohn's disease, to which Nod2 mutations have been genetically linked (Hugot et al., 2001; Netea et al., 2004). A possible explanation for our *in vivo* observations may lie in the long-term nature of the infection. Prolonged stimulation of Nod2, similar to endotoxin/LPS mediated tolerance, results in the downregulation of pro-inflammatory cytokines in the intestinal environment, thus maintaining homeostasis (Hedl et al., 2007). In the context of *B. burgdorferi* infection, we hypothesized that Nod2 might play a role in controlling over exuberant inflammation *in vivo*, possibly via a mechanism of tolerance through stimulation of Nod2 or other PRRs (Petnicki-Ocwieja et al., 2013). Thus, Nod2 may have roles in both resistance to microorganisms and also in tolerance to microbial stimulation. The signals, which lead Nod2 to switch from its inflammatory role to its tolerance function, remain unknown.

INFLAMMASOME

Inflammasomes, described in 2002 (Martinon et al., 2002), are multi-protein complexes that recognize diverse stimuli such as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). These complexes are formed in response to inflammation and control the production of pro-inflammatory cytokines especially interleukin-1β (IL1β) and interleukin-18 (IL18). Their formation is initiated in the cell cytosol and comprises a NLR receptor, an adaptor protein ASC (Apoptosis-associated Speck-like protein containing a C-terminal CARD) and the cysteine protease caspase-1. The recruitment of caspase-1 to the inflammasome leads to its proteolytic activation. The activated caspase cleaves pro-IL1β and pro-IL18 into their active secreted form (Couillin et al., 2011; Strowig et al., 2012). Caspase-1 is also known to be involved in the activation of the NF-kB pathway resulting in expression of other pro-inflammatory cytokines and in cell death. Among the inflammasomes, the NLRP3 complex is the best characterized and has been looked at in the context of *B. burgdorferi*, although no significant inflammatory phenotype *in vivo* was observed (Eisenbarth and Flavell, 2009; Oosting et al., 2012). NLRP3 has been shown to recognize a large number of microbial and non-microbial stimuli (MDP, nucleic acids, toxins, silica, urate crystals etc.) but other proteins of the NLR family that recognize flagellin (NLRC4/IPAF) might also play a role (Franchi et al., 2010).

Clinical manifestations of Lyme disease can involve inflammation of the skin, joints and heart (Steere, 2001). Cytokine levels measured in erythema migrans lesions from Lyme patients have revealed the presence of IFNγ but also IL1β and IL6 (Müllegger et al., 2000). In addition, IL1β and IL18 levels are elevated in serum of patients presenting Lyme arthritis (Pietruczuk et al., 2006). *In vitro* it has been shown that IL1β is secreted by human PBMC after phagocytosis of live *B. burgdorferi* (Cruz et al., 2008). Despite the presence of cytokines that are the result of inflammasome activation, the role of the inflammasome in the host immune response to *B. burgdorferi* is not clear.

Mice deficient in caspase-1 and ASC, had no effect on host defense against *B. burgdorferi in vivo* (Liu et al., 2009). However, Oosting et al. demonstrated that *B. burgdorferi* activates the inflammasome and the production of IL-17 through a caspase-1-dependent mechanism in an *in vivo* model of Lyme arthritis (Oosting et al., 2010b). Both studies were done in knockout mice that were later described as double knockout for caspase-1 and caspase-11, suggesting that some of the phenotypes observed may be attributed to the deficiency in caspase-11 (Kayagaki et al., 2012). Recently a paper using mice lacking only caspase-1 showed that acute Lyme arthritis seems to be dependent on the adaptor ASC and caspase-1 (Oosting et al., 2012). These different results regarding the role of caspase-1 and ASC may be due to the fact that the infection models used in the studies differed in the route of inoculation and the time points examined. As a result, the role of the inflammasome in *B. burgdorferi* infection remains unclear. One hypothesis that may resolve some of the discrepancies is that the inflammasome may have a different role during acute and chronic phases of infection.

OTHER INTRACELLULAR PATHWAYS

While studies have found that the activation of type I IFNs is driven by endosomal TLRs and Nod2, other studies have found that type I IFN signaling is TLR and Nod2 independent (Miller et al., 2008). Although differences in the types of knockout cells or activation state of the macrophage are possible explanations for such discrepancies, it is likely that at least part of the type I IFN response generated occurs via other intracellular sensors. A recent study suggests that TLR independent type I IFN signals may be responsible for naïve B cell accumulation in Lyme disease (Hastey et al., 2014). The involvement of members of the RLR family, such as the dsRNA receptors RIG-1 or MDA5, or the DNA-sensing AIM2 inflammasome complex, remains to be explored in the context of *B. burgdorferi* infection. Alternatively, it is possible none of the above mentioned sensors may be responsible for a type I IFN response. Miller et al. showed in their studies that supernatants from log phase cultures of *B. burgdorferi* were still able to induce IFN gene-related transcripts even when the supernatants were filtered and treated with DNase and RNase, excluding recognition by RLRs or AIM2 (Miller et al., 2010). Thus, it is possible that there is a soluble and possibly secreted *B. burgdorferi* non-nucleic acid ligand that activates type I IFNs via IRF3. In this case, both the sensor and the ligand remain to be identified.

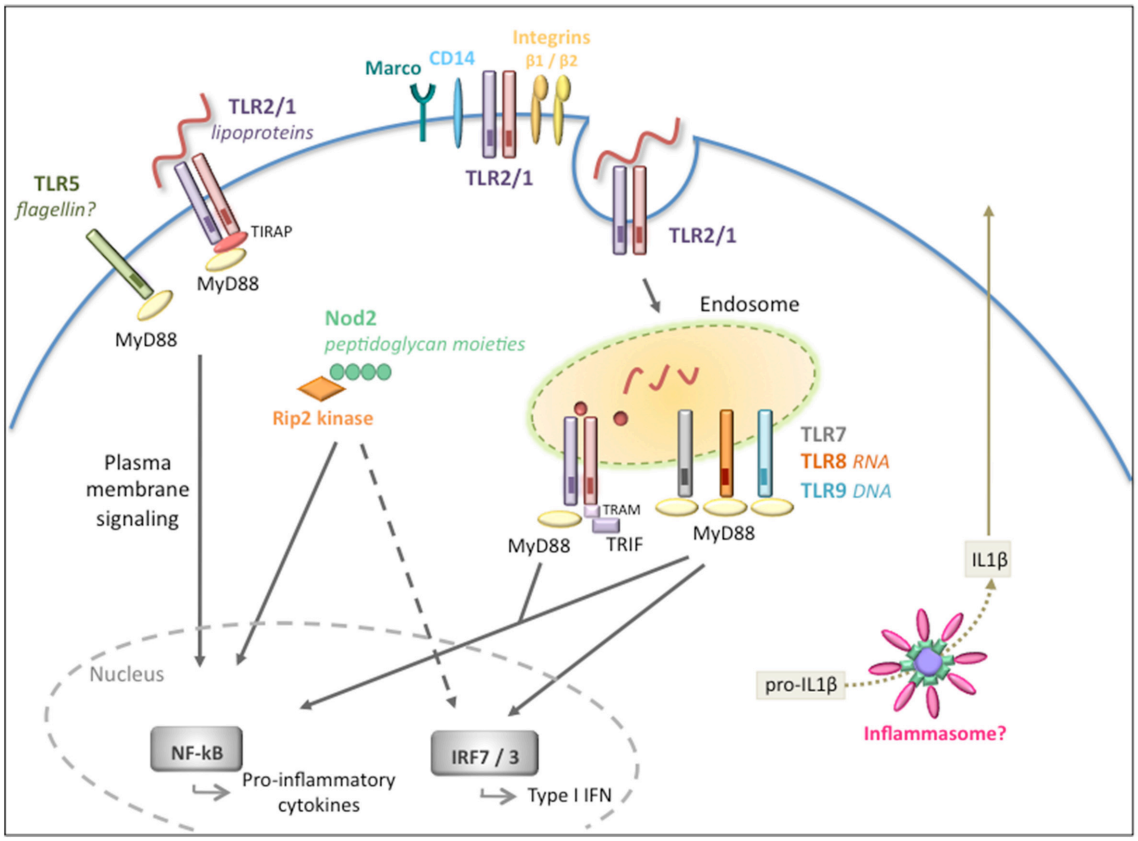

FIGURE 1 | Extracellular and intracellular signaling pathways mediated by *B. burgdorferi*. Overview of the pathogen recognition receptors (PRRs) involved in the recognition and signaling in response to *B. burgdorferi*. Cell surface signaling is primarily mediated by TLR2/1 leading to pro-inflammatory cytokine production. Integrins and CD14 are known to recognize and internalize the spirochete but their role in intracellular signaling is not fully understood. Intracellular receptors located at the endosome, in particular TLR2/1, TLR7/8, and TLR9, are activated by different *B. burgdorferi* ligands and recruit adaptors such as MyD88 and/or TRIF to transduce signals for the activation of inflammatory cytokines and type I IFNs. The Nod2 receptor also plays a role in recognition of *B. burgdorferi* and in the induction of inflammatory responses, but it might have a dual regulatory role depending on the stage of infection. The inflammasome is likely to be involved, however, *in vivo*, it is unclear whether the inflammasome is required for the development of host responses to the pathogen.

CONCLUSIONS

The innate immune system rapidly responds to the presence of pathogens. Although there are a number of receptors localized at the cell surface that detect invasive pathogens before they enter the cells, there is also an entire network of "second-wave" intracellular receptors that alert the host to a microbial presence. Thus, immune responses to microbial invasion are comprised of a signaling network involving multiple receptors (**Figure 1**).

The contribution of a number of these intracellular receptors to pathogen infection may not be in the aspect of bacterial clearance but rather in exacerbating the inflammatory response. We have seen evidence of this in mice deficient in Nod2 and TRIF, which do not affect pathogen burden but do affect severity of inflammation (Petnicki-Ocwieja et al., 2011, 2013). Treatment of Lyme arthritis requires the use of antimicrobials to clear the pathogen (Wormser et al., 2006). However, due to the fact that much of the pathology is a result of excessive inflammation, there could be a clinical application for immunomodulatory drugs. Thus, understanding intracellular signaling pathways engaged by *B. burgdorferi* could lead to the development of anti-inflammatory treatments that, when necessary, could be used in combination therapy for Lyme arthritis.

One receptor we have focused on is TLR2, which has been shown to be responsible for a significant portion of *B. burgdorferi* initiated pro-inflammatory signaling. However, studies have shown that other TLRs, such as TLR7/8 and TLR9 also signal for the activation of inflammatory responses, including type I IFNs (Petzke et al., 2009; Salazar et al., 2009; Cervantes et al., 2011, 2013). Futhermore, TLR2 and TLR8 were shown to cooperate in response to *B. burgdorferi* (Cervantes et al., 2011). In addition, Nod2 has also been shown to play a role in mediating some of the *B. burgdorferi* inflammatory response. Thus, the *B. burgdorferi*-induced inflammatory signature is a result of contribution of multiple receptors.

It is unclear how the cell directs this signaling network. In recent years it has been shown that localization to different cellular compartments plays a significant role in coordinating the

activation of innate immune pathways. The broad classification of cell surface being distinct from intracellular receptors is no longer sufficient when describing the complexity of signaling pathways. Although "intracellular" is used to broadly imply endosomal localization it is now known that immune signaling can be orchestrated from intracellular vesicles and organelles (Kagan, 2012). In addition, some immune signaling complexes previously thought to be strictly cytoplasmic may in fact be recruited to specific compartments containing ligands. In support of this hypothesis, recent data has shown that caspase-1 can accumulate at the phagosome, suggesting that molecules of the inflammasome are also recruited to phagosomal compartments. In the *B. burgdorferi* model, we have made strides in understanding the cellular distribution of different receptors at the cell surface as well as inside the host cell. However, the role of vesicular trafficking to different compartments in the *B. burgdorferi* host response and *B. burgdorferi* pathogenesis remains to be explored.

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Blocking pathogen transmission at the source: reservoir targeted OspA-based vaccines against *Borrelia burgdorferi*

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Control strategies are especially challenging for microbial diseases caused by pathogens that persist in wildlife reservoirs and use arthropod vectors to cycle amongst those species. One of the most relevant illnesses that pose a direct human health risk is Lyme disease; in the US, the Centers for Disease Control and Prevention recently revised the probable number of cases by 10-fold, to 300,000 cases per year. Caused by *Borrelia burgdorferi*, Lyme disease can affect the nervous system, joints and heart. No human vaccine is approved by the Food and Drug Administration. In addition to novel human vaccines, new strategies for prevention of Lyme disease consist of pest management interventions, vector-targeted vaccines and reservoir-targeted vaccines. However, even human vaccines can not prevent Lyme disease expansion into other geographical areas. The other strategies aim at reducing tick density and at disrupting the transmission of *B. burgdorferi* by targeting one or more key elements that maintain the enzootic cycle: the reservoir host and/or the tick vector. Here, I provide a brief overview of the application of an OspA-based wildlife reservoir targeted vaccine aimed at reducing transmission of *B. burgdorferi* and present it as a strategy for reducing Lyme disease risk to humans.

Keywords: *Borrelia burgdorferi*, Lyme disease, enzootic cycle, oral vaccination, transmission cycles, wildlife reservoir

Given the unabated increase in Lyme disease in the US, public health officials are searching for effective and creative solutions to quell the epidemic. The immunization of animals as a means of preventing disease in humans and in domesticated animals of agricultural economic interest was comprehensively reviewed by Monath (2013). The use of reservoir targeted vaccines to control disease is an attractive but under-explored concept. This approach benefits primarily zoonotic diseases in which wild animals play a major role in transmitting the pathogen to humans such as plague, rabies and Lyme disease but it can be easily applied to other vector-borne and directly transmitted diseases. Here my goal is to provide an update on progress toward a wildlife targeted vaccine as a means to reduce risk of exposure to Lyme disease, while discussing its strengths, limitations and a framework for its application.

BORRELIA BURGDORFERI AND LYME DISEASE

Lyme disease was clinically characterized after an epidemic of asymmetrical arthritis affecting multiple joints was observed in children in Lyme, Connecticut, US in 1977 (Steere et al., 1977). The disease has steadily increased in incidence (Kugeler et al., 2008) and has expanded its geographic range (Kugeler et al., 2008; Diuk-Wasser et al., 2012). Caused by a spirochete, *B. burgdorferi* sensu stricto (s.s.) (Benach et al., 1983; Steere et al., 1983), Lyme disease accounts for >90% of all vector-borne diseases with nearly 30,000 confirmed cases reported each year to the Center for Disease Control and Prevention (CDC). Recently the CDC revised the number of probable infections upward 10-fold (to 300,000) to account for wide underreporting (http://www.cdc.gov/lyme/) (Young, 1998).

Lyme disease spirochetes are transmitted to reservoir hosts by hard-bodied ticks (Burgdorfer, 1989). Because the *B. burgdorferi* genome does not encode any known toxins or the machinery that would be required to secrete them (Fraser et al., 1997), tissue damage and disease are mediated by the inflammatory response that follows infection in the mammalian host (Weis and Bockenstedt, 2010). Erythema migrans is the most common clinical manifestation of *B. burgdorferi* infection (60–80%) (Stanek et al., 2012) and develops after an incubation period of 2–32 days (Wormser et al., 2006). Most cases of erythema migrans occur between June and August (Kugeler et al., 2008). Low level spirochetemia occurs in ∼50% of untreated patients presenting with signs of early Lyme disease (Wormser et al., 2005). Occasionally, Lyme disease affects the peripheral or central nervous system, joints or heart months after infection (Steere, 1989).

THE ENZOOTIC CYCLE AND TRANSMISSION

Transmission of *B. burgdorferi* occurs in rural areas used for forestry and recreational activities and in peridomestic suburban areas (Piesman and Gern, 2004; Stanek et al., 2012). Development and survival of the ticks depends on the level of humidity provided by a layer of decaying vegetation in the understory of deciduous or mixed woodlands which provide support and shelter to a range of vertebrate reservoir hosts that sustain transmission of the bacterium (Stanek et al., 2012) in states endemic for Lyme disease.

Borrelia burgdorferi is transmitted by at least four species of tick within the *Ixodes ricinus-persulcatus* complex: *I. scapularis* and *I. pacificus* in eastern and western North America, respectively; *I. ricinus* in Europe, and *I. persulcatus* in Asia (Burgdorfer, 1989). These ticks go through a four-stage life cycle (egg, larvae, nymph, and adult) feeding only once per active stage. Unfed ticks attach to the skin of a host as the animal passes through vegetation. After feeding for 3–7 days the ticks drop off their host and take shelter under leaf litter where they need a minimum relative humidity of 80% to survive (Piesman and Gern, 2004). They take several months to molt into the next stage of development (Stanek et al., 2012). Larvae are uninfected as they hatch—there is no transovarial transmission (Patrican, 1997)—and *B. burgdorferi* is acquired upon feeding on an infected reservoir host. After molting to the nymphal stage, the tick transmits the pathogen to a susceptible animal or human providing its next blood meal. Transmission of Lyme borreliae occurs through injection of tick saliva during feeding (Ribeiro et al., 1987). A feeding period of at least 36 h is needed for transmission of *B. burgdorferi* by *I. scapularis* or *I. pacificus* ticks (Peavey and Lane, 1995; des Vignes et al., 2001) and infection becomes increasingly likely after the tick has been attached for 48 h (Piesman et al., 1987).

The life cycle of all four species of ticks have distinct seasonality. *I. scapularis* nymphs are active in early summer and adults become active in the fall, remain so until winter and become active again in the spring; furthermore, after the fall and winter, nymphal ticks from the following tick cohort undergo questing in the spring (Piesman and Gern, 2004). In the case of *I. ricinus* and *I. persulcatus*, nymphs and adults become active in early spring and continue to seek hosts until mid-summer; both stages can be active until later in the year in sheltered humid environments. With *I. ricinus* a second peak of activity can occur in the fall. Patterns of activity of *I. pacificus*, prevalent in the western US, seem more like those of *I. ricinus* than *I. scapularis* (Piesman and Gern, 2004). In all species, peak activity usually occurs slightly later in larvae than in nymphs, especially for *I. scapularis* in the eastern US. The 3 month difference between one cohort of *I. scapularis* nymphal activity and the following cohort of larval activity allows plenty of time for transmission from infected reservoir hosts to larvae and could explain the high transmission rate in the eastern US (Eisen et al., 2002). Most transmission to humans occurs from May to September, coinciding with the activity of nymphs and with the increasing recreational use of tick habitats by the public (Kugeler et al., 2008; Stanek et al., 2012).

Field studies in North America and Eurasia have identified a variety of small-mammal and avian reservoirs in enzootic transmission cycles (**Table 1**). The white-footed mouse, *Peromyscus leucopus,* is considered to be the main reservoir in the northeastern US (Anderson, 1989). In most tick habitats, deer are essential for the maintenance of the tick population (Anderson, 1989) because they are one of the few wild hosts that can feed sufficient numbers of adult ticks and keep the cycle ongoing, but they are not competent reservoirs for spirochetes (Matuschka et al., 1993). Cattle are non-competent hosts and sheep also appear to be non-competent reservoirs (Matuschka et al., 1993; Ogden et al., 1997). Different pathogenic genospecies of *B burgdorferi* sensu lato favor some vertebrates as reservoir hosts, though this host specificity is not absolute. One factor thought to be relevant to reservoir competence is the resistance of the particular genospecies of Lyme borreliae to complement-mediated killing by the animal host (Bykowski et al., 2008). Populations of deer in a tick habitat can be regarded as a good indication of Lyme disease

Table 1 | The reservoir competence of vertebrate species.

	Species	Geographical area	References
Competent	Mouse	US/Europe	De Boer et al., 1993; LoGiudice et al., 2003
	Voles	Europe	De Boer et al., 1993
	Chipmunk	US	LoGiudice et al., 2003
	Shrew	US	LoGiudice et al., 2003; Dykhuizen et al., 2008
	Squirrel	US/Europe	LoGiudice et al., 2003; Mannelli et al., 2012
	Ground birds	US/Europe	LoGiudice et al., 2003; Mannelli et al., 2012
	Striped skunk	US	LoGiudice et al., 2003
Weakly/Non-competent	Deer	US/Europe	Gray et al., 1992; LoGiudice et al., 2003
	Cattle	Europe	Mannelli et al., 2012
	Opossum	US	LoGiudice et al., 2003
	Raccoon	US	LoGiudice et al., 2003
	Lizards	US/Europe	Casher et al., 2002; Eisen et al., 2004; Salkeld and Lane, 2010; De Sousa et al., 2012
	Catbirds	US	Mather et al., 1989; Ginsberg et al., 2005
	Sheep	Europe	Gray et al., 1995; Ogden et al., 1997
Not confirmed	Hare	Europe	Jaenson and Talleklint, 1996
	Hedgehog	Europe	Gern et al., 1997
	Badgers	Europe	Mannelli et al., 2012
	Red foxes	Europe	Mannelli et al., 2012

Reservoir competence is the ability of a host to become infected, remain systemically infected and then transmit the pathogen to a feeding vector (Gray et al., 2002); from a population level, it is the probability that an infected host transmits B. burgdorferi to feeding ticks (Brunner et al., 2008). Reservoir competence is the product of (1) the probability the host is infected (infection prevalence), and (2) the probability an infected host will transmit the infection to a feeding vector (infectivity). Prevalence varies in space and time, whereas infectivity is a property of the host species and it depends on the strain of B. burgdorferi.

risk because an array of other hosts, including reservoir competent animals, are likely to be present. If most animals in a habitat are those which do not act as reservoirs for Lyme borreliae, such as deer or cattle, Lyme disease risk may decrease because ticks will feed mostly on these animals and therefore will reinforce a positive feedback loop by not becoming infected (LoGiudice et al., 2003). However, this is a highly debated issue. The presence of diversionary hosts may reduce the proportion of ticks infected, but may actually increase the overall abundance of infected ticks because they augment the entire tick population, allowing more larvae to feed on reservoir competent hosts vs. a smaller population (Ogden and Tsao, 2009; Mannelli et al., 2012; Randolph and Dobson, 2012; Wood and Lafferty, 2013). The specific effect probably varies with the hosts involved and with the degree non-competent hosts "out-compete" the reservoir hosts for juvenile ticks (Ogden and Tsao, 2009).

THE HUMAN OspA VACCINE AND OTHER METHODS OF PREVENTION

There are multiple strategies for prevention of Lyme disease. Direct vaccination of humans could have been the gold standard for prevention of Lyme disease, as vaccination in general is the gold standard for prevention of a multitude of infectious diseases. A vaccine based on OspA protected mice against *B. burgdorferi* infection after tick challenge (Fikrig et al., 1992a,b; de Silva et al., 1996); this work led to successful clinical trials that culminated in the approval of the human vaccine by the FDA in 1998. Individuals vaccinated subcutaneously showed approximately ∼76% protection against *B. burgdorferi* infection after receiving three vaccine doses (Steere et al., 1998). Despite adequate efficacy results, the human vaccine was removed from the market. Other vaccines based on assorted combinations of four to eight OspC types appear to be promising candidates as preventive measures against Lyme disease (Seinost et al., 1999; Earnhart and Marconi, 2007; Earnhart et al., 2007).

However, disease expansion into new geographical areas (Burgdorfer, 1989; Ogden et al., 2005; Kugeler et al., 2008; Mannelli et al., 2012) can not be controlled by promoting only direct human vaccination programs. Given that humans are only incidental hosts of *B. burgdorferi*, even mandatory human vaccination will not reduce *B. burgdorferi* from its enzootic cycle; *B. burgdorferi* will simply cycle through its reservoir hosts and vectors until optimal environmental conditions and breaches in vaccine effectiveness allow for its recurrence. Integrated approaches that build upon effective methods to contain *B. burgdorferi* infection within its enzootic cycle as well as direct vaccination of humans would lead to a synergistic effect in public health more likely to yield sustainable positive outcomes (Monath, 2013).

CONTROL OF TICK DENSITY

Human risk of Lyme disease from an environmental perspective is measured in terms of density of infected nymphs (Diuk-Wasser et al., 2012). Other methods of tick control have been explored to reduce the risk of Lyme disease ranging from avoiding tick infested environs, covering exposed skin, using tick repellents, bathing within 2 h of tick exposure, to daily inspections of the entire skin surface to remove attached ticks (Connally et al., 2009), to chemoprophylaxis after removal of attached ticks (Wormser et al., 2007; Warshafsky et al., 2010). Management of wood chips where lawns are adjacent to forests, application of acaricides, and the construction of fences to keep out deer are also effective, as these measures disturb the habitat where the density of host seeking ticks is high (Piesman, 2006). Acaricide self-treatment of white-tailed deer resulted in reduction of tick density (Hoen et al., 2009; Stafford et al., 2009) and a rodent-targeted acaricide (fipronil) delivered to white-footed mice (*P. leucopus*) in modified commercial bait boxes was also effective in reducing nymphal and larval tick infestations as it reduced tick density (Dolan et al., 2004). A decrease in tick density is expected to reduce human exposure to Lyme disease as it would reduce density of infected ticks.

RESERVOIR TARGETED VACCINES

Vaccines targeted to the host reservoir have been developed as strategies to reduce transmission of pathogens (Piesman, 2006). Baits and baiting systems have proven effective for delivery of rabies and plague vaccines (Pastoret et al., 1988; Creekmore et al., 2002; Knobel et al., 2002). An example of a successful application is the oral baited vaccine Raboral™ currently used by local governments in the United States to create barriers between infected wildlife and highly populated areas to prevent transmission of rabies (Blanton et al., 2012).

Ixodes scapularis and I. pacificus ticks and its competent reservoirs are distributed across the US in many areas where Lyme disease is not endemic as appears to be the case in California (Eisen et al., 2009) and in the Southern states of the US (Oliver et al., 2003). In the presence of both competent reservoir and tick vector, one reason for the lack of Lyme disease in these areas could be that environmental weather conditions dramatically affects the ecology of the habitat that sustains the enzootic cycle of *B. burgdorferi* and enable pathogen transmission (Eisen et al., 2002). Another way to achieve the same goal would be to remove *B. burgdorferi* from the enzootic cycle through the application of reservoir targeted vaccines.

Although eradicating the main reservoir host(s), deer and the vector are measures generally accepted as effective in disrupting transmission of *B. burgdorferi*, the fact that the competent reservoir and vector co-exist in areas where *B. burdgorferi* does not appear to be transmitted to humans argues against the necessity of implementing drastic measures to eliminate any. It further argues in favor of a solution that is less disruptive of an ecosystem that sustain life and lifestyles. Thus, preventing the vector from acquiring or transmitting an organism may be an effective strategy for preventing Lyme disease in humans (Clark and Hu, 2008). Although elimination of Lyme borreliae from nature is unrealistic, diminishing their threat to humans is an achievable goal (Radolf et al., 2012).

The reservoir host that is most competent for transmission of *B. burgdorferi* in the US is the white-footed mouse (*Peromyscus leucopus*) (Anderson et al., 1987; Anderson, 1989). However, chipmunks, squirrels, shrews, and other small vertebrates are increasingly recognized as important hosts (Brisson et al., 2008). Further, birds may also play a role in spreading *B. burgdorferi* (Burgdorfer, 1989; Comstedt et al., 2006; Ogden et al.,

2010). The principle behind development of reservoir targeted vaccines (RTV) is to reduce *B. burgdorferi* from the reservoir host(s) and from the ticks that feed on them. In other words, the ultimate objective of RTV vaccination is to turn competent reservoir hosts into incompetent hosts while at the same time depleting *B. burgdorferi* from the vector without eradicating either (the vertebrate host or the tick) from the ecosystem (**Figure 1**).

OspA was previously used to vaccinate mice orally (Fikrig et al., 1991; Luke et al., 1997). Two groups have been developing baited OspA-based oral delivery systems to vaccinate reservoir hosts against Lyme disease. One delivery system is based on Vaccinia virus (VV) (Scheckelhoff et al., 2006) and the other is based on *E. coli* expressing OspA (Gomes-Solecki et al., 2006). Both systems are equally effective in eliciting production of protective levels of anti-OspA antibodies in inbred *M. musculus* and in outbred *P. leucopus* mice that receive the vaccine orally either via oral gavage or via *ad libitum* feeding. Furthermore, both vaccines are equally effective in decreasing *B. burgdorferi* from infected ticks that fed on vaccinated mice (Gomes-Solecki et al., 2006; Scheckelhoff et al., 2006; Bhattacharya et al., 2011; Meirelles Richer et al., 2011). While VV-based vaccines require a desirable low number of doses to induce protective immune responses in the host, they are also potentially infectious to people who are immunocompromised or suffer from eczema (Reed et al., 2012). The bacteria-based delivery system, on the other hand, requires that a higher number of vaccine doses be deployed to induce protective immune responses in the host, which burdens any distribution plan, but it is also considered a safer approach. In an alternative approach, a doxycycline rodent bait formulation prevented tick transmission of *B. burgdorferi* to vertebrate hosts as well as cured established infections in mice (Dolan et al., 2008; Zeidner et al., 2008). A decrease in tick infection with *B. burgdorferi*, as well as the decrease of vertebrate host infection is expected to result in an overall decrease in human risk of Lyme disease.

A 1-year field study testing the effect of a reservoir targeted vaccine in Lyme disease risk was done in Connecticut in 1998 and it was repeated in 2002 (Tsao et al., 2004). Mice were trapped, injected subcutaneously with an OspA-based suspension, and in the following year ticks were collected in immediate surrounding areas to test for infection with *B. burgdorferi*. The year after deployment of the RTV, nymphal infection prevalence was reduced by ∼24%. A comprehensive 5-year field study of an oral RTV against *B. burgdorferi* was reported recently (Richer et al., 2014). The second study differed from the first in vaccine formulation (oral bait delivery of vaccine comprised of bacteria previously induced to produce OspA) and duration of vaccine treatment (treatment up to 5 years). The field study results demonstrated that a bacteria-based oral bait vaccine delivered

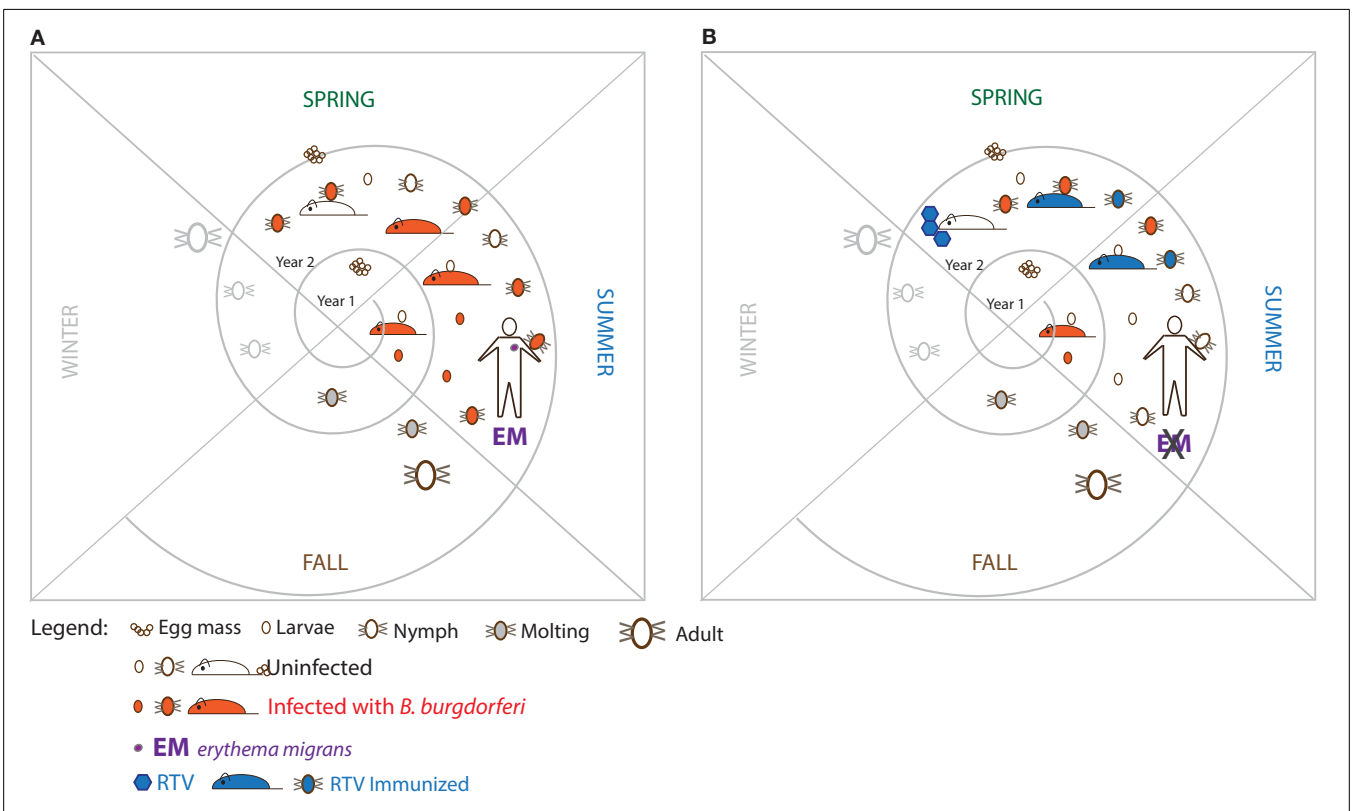

FIGURE 1 | Proposed strategy to break the enzootic cycle of the Lyme disease spirochete. (A) The triad which comprises the enzootic cycle: the tick vector (*Ixodes* sp.), the major reservoir host (white-footed mouse) and *Borrelia burgdorferi*. **(B)** Hypothesis: immunizing wild white-footed mice with oral reservoir targeted vaccine (RTV) could break the enzootic cycle of *B. burgdorferi*.

yearly to trapped wild white-footed mice resulted in reduced infection rates of nymphal ticks the year after RTV deployment (23%) and that 5 years of consecutive treatment caused a substantial disruption of the enzootic cycle of *B. burgdorferi* with reductions in nymphal infection prevalence as high as 76%. The VV based RTV was not tested in field studies. These data offer empirical support to an analysis using a dynamic model of *B. burgdorferi* transmission which predicted that mouse-reservoir vaccination was expected to reduce infection prevalence amongst ticks by 56% (Tsao et al., 2012).

Such decreases in the prevalence of infected *I. scapularis* vectors could significantly reduce the risk to humans and other accidental mammalian hosts (such as dogs) of acquiring Lyme disease. Implementation of a reservoir targeted vaccine (RTV) as part of integrated pest management interventions to block transmission of *B. burgdorferi* should consider deployment both in sylvatic as well as in suburban areas and broad distribution of such vaccines should take into account issues of toxicity to humans.

STRENGTHS, LIMITATIONS, AND FRAMEWORK FOR APPLICATION

A major strength of a reservoir targeted vaccine approach is to bypass immunizing humans instantly avoiding potential vaccine failures and side effects (Monath, 2013); additionally, for Lyme disease, the RTV objective is to remove only the pathogen (*B. burgdorferi*) from the ecosystem while leaving all other living components of the enzootic cycle undisturbed (Richer et al., 2014). RTVs control infections acquired from wild animals, which leads to collaborations between animal and human health industries that, in turn, should lead to implementation of more robust public health measures. Furthermore, accelerated regulatory pathways should lead to faster licensing of new vaccines (Monath, 2013).

One of the limitations of the anti-*Borrelia* RTVs described is that both are based in a single immunogen, OspA, which neutralizes the spirochete in the tick midgut (Fikrig et al., 1992a,b; de Silva et al., 1996). A vaccine based on OspA supplemented with assorted OspC types (Earnhart and Marconi, 2007; Earnhart et al., 2007) may be more efficacious given that targeting both the vector (OspA) and the host (OspC) would contribute to protection. Only one of the reservoir species that contribute *B. burgdorferi* to the enzootic cycle—mice—was targeted for treatment (Tsao et al., 2004; Gomes-Solecki et al., 2006; Scheckelhoff et al., 2006; Dolan et al., 2008; Zeidner et al., 2008; Bhattacharya et al., 2011; Meirelles Richer et al., 2011; Richer et al., 2014). Other small vertebrates such as chipmunks, squirrels, and shrews also transmit *B. burgdorferi* effectively to the tick (LoGiudice et al., 2003; Dykhuizen et al., 2008; Mannelli et al., 2012) and should be included in integrated pest management interventions. RTVs are not designed to reduce tick density, which is considered to be an important eco-epidemiological measure of Lyme disease risk (Ogden and Tsao, 2009; Mannelli et al., 2012). A multipronged integrated pest management intervention comprised of RTV treatment to reduce pathogen burden in the ticks supplemented by acaricide treatment to reduce tick density and by vaccination of humans at risk of infection maybe the most effective route to drastically reduce incidence of Lyme disease in human populations.

SUMMARY

Reservoir targeted vaccines are under development for prevention of transmission of *B. burgdorferi* as a means to reduce Lyme disease risk. Oral vaccines administered to mice using virus-based or bacteria-based delivery vehicles expressing OspA reduced *B. burdorferi* from the tick vector in the laboratory. Dynamic models of *B. burgdorferi* transmission predicted that mouse-reservoir vaccination was expected to reduce infection prevalence amongst ticks by 56%. OspA based RTVs tested in short-term (1-year) and long-term (5-year) field trials lead to reductions of tick infection prevalence ranging from 24 to 76%, respectively. Other reservoir targeted approaches reduced infection prevalence in the tick vector as well as cured infection in the reservoir host. A multipronged integrated pest management intervention combined with human vaccination maybe the most effective route to drastically reduce Lyme disease incidence. Lastly, eco-epidemiological factors should be studied to understand the relationship between the pathogen, the tick, the hosts, and its environs to be able to assess the long-term effects of any strategies that may affect the ecosystem.

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Conflict of Interest Statement: Maria Gomes-Solecki has a relevant patent and is a co-founder of US BIOLOGIC.

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Immunity-related genes in *Ixodes scapularis*—perspectives from genome information

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Ixodes scapularis, commonly known as the deer tick, transmits a wide array of human and animal pathogens including *Borrelia burgdorferi*. Despite substantial advances in our understanding of immunity in model arthropods, including other disease vectors, precisely how *I. scapularis* immunity functions and influences persistence of invading pathogens remains largely unknown. This review provides a comprehensive analysis of the recently sequenced *I. scapularis* genome for the occurrence of immune-related genes and related pathways. We will also discuss the potential influence of immunity-related genes on the persistence of tick-borne pathogens with an emphasis on the Lyme disease pathogen *B. burgdorferi.* Further enhancement of our knowledge of tick immune responses is critical to understanding the molecular basis of the persistence of tick-borne pathogens and development of novel interventions against the relevant infections.

Keywords: ticks, *Ixodes scapularis, Borrelia burgdorferi*, immunity-related genes, innate response, genomics

INTRODUCTION

Although several hundred tick species are known to exist (Jongejan and Uilenberg, 2004), only a handful transmit human diseases. *Ixodes scapularis* is one of the predominant tick species that spread a wide array of serious human and animal pathogens, including *Borrelia burgdorferi*, which causes Lyme borreliosis (Burgdorfer et al., 1982; Anderson, 1991). Our understanding of arthropod innate immune responses, primarily involving the fruit fly and mosquito, has advanced over the past decades (Vilmos and Kurucz, 1998). However, our knowledge of tick immune responses, especially the occurrence of immune-related genes, pathways, and specifically how these components respond to invading pathogens remains under-explored. Notably, many pathogens that persist in and transmit through ticks are evolutionarily distinct and possess unique structures (Hajdusek et al., 2013). For example, key pattern recognition molecules (PAMPs), such as peptidoglycan (PG) and lipopolysaccharides (LPS), are structurally different or completely absent, respectively, in major tick-borne pathogens, such as in *B. burgdorferi* (Schleifer and Kandler, 1972; Takayama et al., 1987; Fraser et al., 1997). Thus, the wealth of knowledge generated in other model arthropods, especially regarding the genesis of host immune responses against classical Gram-positive or Gram-negative bacterial pathogens, might not be readily applicable for tick-borne pathogens, like *B. burgdorferi.* The primary goal of this review is to present a general overview of tick immune components, as gathered from the sequenced genome and published data, and discuss their potential for modulating infection, with a focus on a major tick-borne pathogen, *B. burgdorferi*. A better understanding of the *I. scapularis* immune response to invading pathogens could contribute to the development of new strategies that interfere with relevant pathogen persistence and transmission.

While a number of studies detailed characterization of *I. scapularis* proteins, predominantly salivary gland proteins, that influence immunity and pathogen persistence in the vertebrate hosts (Wikel, 1996; Das et al., 2001; Gillespie et al., 2001; Narasimhan et al., 2002, 2004, 2007; Hovius et al., 2008; Dai et al., 2010; Pal and Fikrig, 2010; Kung et al., 2013), relatively limited information is available on how tick proteins shape vector immunity and influence pathogen persistence. In order to generate a list of tick immune genes and related pathways, we sought to perform a comprehensive analysis of the recently sequenced *I. scapularis* genome data that are available through several publicly accessible databases (Hill and Wikel, 2005; Pagel Van Zee et al., 2007). To accomplish this, we initially searched the National Institute of Allergy and Infectious Diseases Bioinformatics Resource Center (www.vectorbase.org) for annotated *I. scapularis* immune-related genes. In addition, we also reviewed the relevant literature to identify additional innate immune genes, including those discovered in related tick species (Rudenko et al., 2005) or in fruit fly, mosquito, and mammalian genomes (Sonenshine, 1993; Hoffmann et al., 1999; Dimopoulos et al., 2000; Christophides et al., 2002; Hoffmann and Reichhart, 2002; Janeway and Medzhitov, 2002; Govind and Nehm, 2004; Osta et al., 2004; Saul, 2004; Tanji and Ip, 2005; Dong et al., 2006; Ferrandon et al., 2007; Tanji et al., 2007; Jaworski et al., 2010; Kopacek et al., 2010; Yassine and Osta, 2010; Valanne et al., 2011). The latter information was then used to search for possible *Ixodes* orthologs via BLASTP against the VectorBase database. In total, 234 genes were identified and categorized into one of the following nine major immune pathways or components (number of unique genes): gut-microbe homeostasis (17), agglutination (37), leucine-rich repeat (LRR) proteins (21), proteases (33), coagulation (11), non-self recognition and signal transduction via Toll, IMD, and

JAK-STAT pathways (55), free radical defense (13), phagocytosis (33), and anti-microbial peptides (14). These genes are listed in **Tables 1–9**; unless stated otherwise, all annotations are based on the VectorBase database. We recognize that although our list might not be comprehensive as there might be additional published data inadvertently overlooked in our literature/database searches or yet-to-be identified genes involved in tick immune defense, we believe that it still represents the majority of genes that are potentially involved in the tick immune response. In the following sections, occurrence of these components and pathways are systematically discussed for their occurrence in the tick genome; we also highlighted their potential influence on the persistence and transmission of tick-borne pathogens like *B. burgdorferi*.

I. SCAPULARIS GENOME

The *I. scapularis* genome is relatively large, approximately 2.1 Gb in size and contains nearly 70% repetitive DNA (Ullmann et al., 2005). Recently it was completely sequenced by the *I. scapularis* genome project - a partnership between a number of tick research communities and institutions (Hill and Wikel, 2005; Pagel Van Zee et al., 2007). Toward the end of 2008, sequencing centers announced the annotation and release of the whole genome sequence data (IscaW1, 2008; GenBank accession ABJB010000000). The sequence data were derived from purified genomic DNA preparations isolated from an in-bred tick colony and sequenced to approximately 6-fold coverage using a combined whole genome shotgun and clone-based approach. The genome information are organized and displayed by a bioinformatics resource center focused on invertebrate vectors of human disease called VectorBase (www.vectorbase.org), which is funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health. The *I. scapularis* gene counts included 20,486 high confidence protein-coding genes, 316 non-coding genes and 20,771 transcripts. While the most recent release (IscaW1.3; 2014) reported no modifications of protein-coding loci, it incorporated a new prediction for 285 non-coding RNAs.

Table 1 | Gut-microbe homeostasis.

Annotation	Accession number
Dual oxidase	ISCW007865
Phospholipid-hydroperoxide glutathione peroxidase, putative	ISCW019584
Phospholipid-hydroperoxide glutathione peroxidase, putative	ISCW022517
Glutathione peroxidase, putative	ISCW020571
Peroxidase	ISCW017070
Peroxidase	ISCW022537
Glutathione peroxidase, putative	ISCW008495
Oxidase/peroxidase, putative	ISCW002528
Phospholipid-hydroperoxide glutathione peroxidase, putative	ISCW015098
Oxidase/peroxidase, putative	ISCW017368
Oxidase/peroxidase, putative	ISCW005828
Peroxidase	ISCW024650
Glutathione peroxidase, putative	ISCW020569
Oxidase/peroxidase, putative	ISCW018825
Peroxidase	ISCW013159
Thioredoxin peroxidase, putative	ISCW013767
Glutathione peroxidase, putative	ISCW001759

IMMUNITY-RELATED GENE/PATHWAYS IN *I. SCAPULARIS*

GUT MICROBE HOMEOSTASIS

Gut microbiota serve a critically important function in shaping host immunity in a number of organisms, including model arthropods (Dillon and Dillon, 2004; Round and Mazmanian, 2009; Hooper et al., 2012; Buchon et al., 2013; Kamada et al., 2013; Schuijt et al., 2013). Characterization of gut microbiota

Table 2 | Agglutination.

Annotation	Accession number
Ferritin	ISCW015079
Beta-galactosidase	ISCW000651
Ubiquitin associated domain containing protein	ISCW023764
Chitin bindin peritrophin A	ISCW006076
Beta-galactosidase precursor	ISCWOI9676
galectin, putative	ISCW008553
Manose binding ER-golgi comparment lectin	ISCW016179
Ixoderin precursor	ISCW002664
Ixoderin precursor	ISCW022063
Ixoderin B	ISCW013797
Ixoderin B	ISCW003711
Hemelipoglycoprotein precursor	ISCW012423
Ferritin	ISCW023334
galectin, putative	ISCW020268
Beta-galactosidase	ISCW019681
Beta-galactosidase precursor	ISCW019677
Beta-galactosidase precursor	ISCW019679
Beta-galactosidase precursor	ISCW0I6637
Hemelipoglycoprotein precursor	ISCW024299
fatty acyl-CoA elongase, putative	ISCW010899
Galectin	ISCW020268
Ixoderin Precursor	ISCW024686
Hemelipoglycoprotein precursor	ISCW0I2424
Sodium/proton exchanger	ISCW008652
C-Type Lectin, Putative	ISCW010467
Ixoderin Precursor	ISCW013746
Double sized immunoglobulin g binding protein A	ISCW021766
Galectin	ISCW008553
Hemelipoglycoprotein precursor	ISCW021704
Beta-galactosidase precursor	ISCW019678
Lectin, Putative	ISCW012623
Galectin	ISCW020586
Hemelipoglycoprotein precursor	ISCW014675
Hemelipoglycoprotein precursor	ISCW021709
Ixoderin precursor	ISCW012248
Ubiquitin associated and SH3 domain containing protein B	ISCW02I035
Galectin, putative	ISCW020586

Table 3 | Leucine-rich repeat (LRR) proteins.

Annotation	Accession number
Lumicans	ISCW001027
LRR (in flii) interacting protein	ISCW016609
Lumicans	ISCW005645
F-Box/LRR protein, putative	ISCW000110
F-Box/LRR protein, putative	ISCW010598
F-Box/LRR protein, putative	ISCW010597
F-Box/LRR protein, putative	ISCW010599
LRR protein, putative	ISCW008095
LRR protein	ISCW014626
F-Box/LRR protein, putative	ISCW010347
F-Box/LRR protein, putative	ISCW005273
LRR and NACHT domain containing protein	ISCW004678
LRR and NACHT domain containing protein	ISCW001292
LRR protein	ISCW012038
F-Box/LRR protein, putative	ISCW016452
F-Box/LRR protein, putative	ISCW018961
F-Box/LRR protein, putative	ISCW013925
LRR containing G-protein coupled receptor	ISCW015788
F-Box/LRR protein, putative	ISCW018134
F-Box/LRR protein, putative	ISCW008236
LRR protein, putative	ISCW003174

Table 4 | Proteases/Protease inhibitors.

Annotation	Accession number
Serpin 4 precursor	ISCW023622
Serpin 2 precursor	ISCW010422
Serpin 2 precursor	ISCW018607
Serpin 1 precursor	ISCW023618
Serpin 7 precursor	ISCW024109
PAP associated domain containing protein	ISCW014870
Serpin 7 precursor	ISCW009616
Serine proteinase inhibitor serpin-3	ISCW015204
Heparan sulfate 2-O sulfotransferase, putative	ISCW000208
Secreted salivary gland peptide	ISCW023621
Serpin	ISCW016489
Hypothetical protein	ISCW017929
Secreted salivary gland peptide	ISCW023620
Serpin 4 precursor	ISCW023623
Protein disulfide isomerase 1	ISCW002080
Alkaline phosphatase	ISCW023785
Alkaline phosphatase	ISCW003801
Alkaline phosphatase	ISCW004677
Hypothetical protein	ISCW021544
Zinc metalloprotease	ISCW008637
Zinc metalloprotease	ISCW005798
Zinc metalloprotease	ISCW005687
Zinc metalloprotease	ISCW005854
Serpin 8 precursor	ISCW014652
Serpin	ISCW014100
Zinc metalloprotease	ISCW012815
Conserved hypothetical protein	ISCW006169
Serpin 8 precursor	ISCW015349
Zinc metalloprotease	ISCW021286
Serpin 2 precursor	ISCW021417
Serpin 2 precursor	ISCW014779
Secreted serine protease	ISCW014551
Alkaline phosphatase	ISCW000162

Table 5 | Coagulation.

Annotation	Accession number
Proclotting enzyme precursor	ISCW013112
Thrombin inhibitor	ISCW000427
Proclotting enzyme precursor	ISCW000320
Proclotting enzyme precursor	ISCW011206
Proclotting enzyme precursor	ISCW001322
Keratinocyte transglutaminase	ISCW019475
Proclotting enzyme precursor	ISCW011961
Proclotting enzyme precursor	ISCW003779
Proclotting enzyme precursor	ISCW010999
Prostate-specific transglutaminase	ISCW009303
Prostate-specific transglutaminase	ISCW011739

in ticks, including *I. scapularis*, as well as their influence on the persistence of tick-borne pathogens like *B. burgdorferi* has been a focus of a number of recent studies (Clay et al., 2008; Carpi et al., 2011; Narasimhan et al., 2014). As many of these gut microbes play a beneficial role in the physiology of the host, the immune system therefore must be able to differentiate between commensal microbes and pathogenic microorganisms (Macpherson and Harris, 2004). While mechanisms that contribute to the microbial surveillance and pathogen elimination while tolerating the indigenous microbiota remain obscure in ticks, these are well-researched in many arthropods, particularly in *D. melanogaster* (Buchon et al., 2013). Studies have established that immune reactivity within the fly gut ensures preservation of beneficial and dietary microorganisms, while mounting robust immune responses to eradicate pathogens (Buchon et al., 2013). There are at least two models of fly immunity for sensing and preserving beneficial bacterial associations while eliminating potentially damaging ones (Lazzaro and Rolff, 2011). The first occurs by recognition of non-self molecules (invading microbes), while the second involves the recognition of "danger" signals that are released by damaged host cells. However, it is also likely that they work together to maintain effective gut microbe homeostasis. Recent studies suggest that dual oxidase (DUOX) and peroxidases enzymes play a key role in this process (Kim and Lee, 2014). While a number of other regulatory molecules may participate in gut homeostasis, we classified 17 different genes within the *I. scapularis* genome to this pathway, including a single dual oxidase (DUOX) and several peroxidase proteins (**Table 1**).

Additional studies have recently detailed how DUOX plays an essential role in gut mucosal immunity and homeostasis (Bae et al., 2010; Deken et al., 2013). DUOX, a member of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase NOX family (Geiszt and Leto, 2004), has previously been shown to be a key source of local microbicidal reactive oxygen species (ROS) production within the fly gut (Kim and Lee, 2014).

Table 6 | Non-self recognition (Toll, IMD, and JAK-STAT pathways).

Annotation	Accession number
Regulator of ubiqutin pathway, putative	ISCW015648
NF-kappaB inhibitor IkappaB, putative	ISCW007030
Peptidoglycan recognition receptor protein	ISCW022212
Embryonic polarity dorsal, putative	ISCW000140
Ankyrin repeat-containing protein	ISCW018861
Peptidoglycan recognition receptor protein	ISCW024175
Netrin receptor DSCAM	ISCW016844
Netrin receptor DSCAM	ISCW016100
Caspase, apoptotic cysteine protease, putative	ISCW003039
Netrin receptor DSCAM	ISCW020406
Peptidoglycan recognition receptor protein	ISCW004389
Netrin receptor DSCAM	ISCW022828
Scavenger receptor class B	ISCW003371
Scavenger receptor class B	ISCW010934
Scavenger receptor class B	ISCW002412
Netrin receptor DSCAM	ISCW003295
UBX domain-containing protein, putative	ISCW011870
NF-kappaB inhibitor IkappaB, putative	ISCW019520
Nuclear factor nf-kappa-B P105 subunit, putative	ISCW018935
Peptidoglycan recognition receptor protein	ISCW024689
N-CAM Ig domain containing protein	ISCW022144
Toll	ISCW018193
Secreted protein, putative	ISCW021005
Secreted protein, putative	ISCW024521
Toll	ISCW018363
Serine-threonine protein kinase, plant-type, putative	ISCW001463
Toll	ISCW004495
Toll	ISCW020989
Spatzle	ISCW022569
Fibrinogen	ISCW009412
Tartan protein, putative	ISCW016292
Secreted protein, putative	ISCW024389
Toll	ISCW007724
Toll	ISCW007726
Myd88, putative	ISCW008802
Fibrinogen	ISCW009412
Toll	ISCW009512
Toll	ISCW022740
Serine/threonine protein kinase	ISCW020049
Toll	ISCW017724
Tartan protein, putative	ISCW021508
Kekkon 1, putative	ISCW018006
Toll	ISCW020221
Tolkin	ISCW022120
Membrane glycoprotein LIG-1, putative	ISCW005558
Slit Protein	ISCW018651
Toll	ISCW008289
Adenylate cyclase	ISCW012040
Toll	ISCW006897
Fibrinogen	ISCW024309
Toll	ISCW007727
Spatzle	ISCW022732
Fibrinogen	ISCW001478
JAK	ISCW016158
STAT 3	ISCW005692

Table 7 | Free radical defense.

Annotation	Accession number
Manganese superoxide dismutase	ISCW016585
Superoxide dismutase	ISCW015027
Manganese superoxide dismutase	ISCW016737
Superoxide dismutase Cu-Zn	ISCW012382
Nitric oxide synthase interacting protein, putative	ISCW017590
Superoxide dismutase	ISCW018077
Superoxide dismutase	ISCW024422
Cu^{2+}/Zn^{2+} superoxide dismutase SODI	ISCW011852
Manganese superoxide dismutase	ISCW012767
Superoxide dismutase Cu-Zn	ISCW008219
Ras responsive element binding protein	ISCW009132
Decarboxylase	ISCW021675
Nitric oxide synthase	ISCW018074

Table 8 | Phagocytosis.

Annotation	Accession number
Integrin beta-3	ISCW024103
Cadherin- repeats domain containing protein	ISCW013741
Rho GTPase activating protein	ISCW015851
Protocadherin fat	ISCW017319
Thioester containing protein	ISCW020822
Cadherin	ISCW005817
Rho GTPase activating protein	ISCW015201
Protocadherin beta-6	ISCW016805
Integrin alpha-ps	ISCW005672
GTPase Rho	ISCW004349
Rho guanine nucleotide exchange factor	ISCW014238
GTPase Rho	ISCW004348
Protocadherin fat	ISCW016765
Rho GTPase activating protein RICH2	ISCW003282
Integrin alpha	ISCW003186
Integrin beta-3	ISCW010037
GTPase Rho	ISCW006741
Rho GTPase activating protein	ISCW003559
Integrin beta subunit	ISCW008948
p116 Rho-interacting protein	ISCW007121
Integrin alpha repeat domain containing protein	ISCW019648
Rho GTPase activating protein	ISCW018998
Rho guanine exchange factor	ISCW008875
Rho	ISCW002009
Integrin alpha-ps	ISCW022321
Rho GTPase activating protein	ISCW019271
GTPase Rho	ISCW018929
Rho GTPase activating protein	ISCW001560
Rho GDP dissociation inhibitor	ISCW020878
Rho associated kinase	ISCW011682
GTPase Rho	ISCW015794
Integrin beta subunit	ISCW002553
Integrin alpha	ISCW003185

Table 9 | Antimicrobial peptides.

Annotation	Accession number
Putative secreted salivary gland peptide	ISCW005928
Secreted protein, putative	ISCW018425
Secreted salivary gland peptide	ISCW002695
TAK 1 putative	ISCW009364
Secreted salivary gland peptide	ISCW001310
Beta transducin Trp-Asp domain containing protein	ISCW014204
Map kinase activating death domain protein	ISCW017494
Secreted salivary gland peptide	ISCW018541
Defensin	ISCW022102
Preprodefensin putative	ISCW016747
Secreted salivary gland peptide	ISCW002331
Secreted salivary gland peptide	ISCW016466
Secreted salivary gland peptide	AAV63544*
Arsenite-resistance protein	ISCW011320

*Based on annotation in NCBI database (http://www.ncbi.nlm.nih.gov).

Targeted depletion of DUOX in flies has resulted in the overproduction of commensal gut bacteria and renders the flies susceptible to infection (Buchon et al., 2013; Kim and Lee, 2014). As originally discovered in *Caenorhabditis elegans* (Edens et al., 2001), in addition to ROS generation, DUOX is also implicated for catalysis of protein cross-linking that contributes to maintenance of gut microbiota in *Anopheles gambiae* (Kumar et al., 2010). In mosquitoes, DUOX, along with a specific heme-peroxidase, catalyzes the formation of an acellular molecular barrier, termed dityrosine network (DTN), which forms in the luminal space along the gut epithelial layer during feeding (Kumar et al., 2010). The DTN decreases the gut permeability to various immune elicitors protecting the gut microbiota, both commensal and pathogenic species. Another recent study revealed that an ovarian dual oxidase is essential for insect eggshell hardening through the production of H_2O_2, which ultimately promotes protein cross-linking (Dias et al., 2013). Further studies on how DUOX and peroxidase systems maintain gut microbiota in *I. scapularis* could give novel insight into how pathogens that are transmitted through ticks are able to evade the immune system and persist within the vector.

AGGLUTINATION

Agglutination, the biological phenomenon by which cells or particles clump together, has been described within various tick species (Uhlir et al., 1996; Kibuka-Sebitosi, 2006). A group of carbohydrate-binding proteins called lectins (Grubhoffer et al., 1997, 2004), which are often produced in a tissue specific manner within arthropods, especially in the gut, hemocytes, or fat bodies, could be key mediators of the process (Grubhoffer et al., 2004, 2005). Agglutination of pathogens by lectins, which also function as host recognition receptors for pathogen-associated molecular patterns (Dam and Brewer, 2010), has been reported in many arthropod vectors, including mosquitoes and tsetse flies, where they play an important role in the pathogen-host relationship (Abubakar et al., 1995, 2006; Barreau et al., 1995; James, 2003). While lectins can function as signaling factors for the maturation of the African trypanosome or as lytic factors (Abubakar et al., 1995, 2006), in mosquitoes they act as agonists of the development of malarial parasites within the vector (Barreau et al., 1995; James, 2003) While tick lectins, particularly those in hard ticks (*Ixodidae*), have not been studied as extensively as other arthropod lectins, previous reviews summarized available information on lectins of *I. ricinus* (Grubhoffer and Jindrak, 1998; Grubhoffer et al., 2004). Since most lectins isolated from arthropods are the ones from the hemocoel, studies have focused on their localization or hemagglutinating activity in the hemolymph (Sonenshine, 1993; Kuhn et al., 1996). In *I. ricinus*, this activity was characterized as Ca^{2+} dependent binding activity (Grubhoffer et al., 2004). A 85 kDa lectin produced by the granular hemocytes and basal laminae surrounding the hemocoel was identified to have a strong binding affinity for sialic acid (Grubhoffer et al., 2004). This immunoreactivity supports the idea that lectins may function as a recognition molecule of the immune system in ticks, implying that they could influence the persistence of tick-borne pathogens like *B. burgdorferi*. In fact, the hemocytes in *I. ricinus* can also phagocytize *B. burgdorferi* through the coiling method, which has previously been though to be a lectin-mediated process (Grubhoffer and Jindrak, 1998). Specifically, two agglutinins/lectins were isolated from the gut, one 65 kDa and the other 37 kDa in size; the former was shown to be the main agglutinin with a binding affinity for mucin, while the latter protein was found to have a strong affinity for a specific glucan (Grubhoffer and Jindrak, 1998; Grubhoffer et al., 2004). It is also suggested that a gut agglutinin has the potential to bind LPS that in cooperation with other digestive enzymes thought to affect the persistence of Gram-negative bacteria and spirochetes that pass through the gut lumen (Uhlir et al., 1996; Grubhoffer et al., 2004). In addition to hemolymph and gut, lectin activities are also documented in the salivary gland; a 70 kDa protein has been identified as being responsible for the hemagglutinating activity in this organ (Grubhoffer et al., 2004). It is thus possible that lectin or a related protein in the salivary glands could influence pathogen transmission. In fact, a tick mannose-binding lectin inhibitor that is produced in the salivary glands has been shown to interfere with the human lectin complement cascade, significantly impacting the transmission and survival of *B. burgdorferi* (Schuijt et al., 2011). Taken together, it is likely that lectins could play a role in the immunity of *I. scapularis*, which encodes for at least 37 lectins or related proteins (**Table 2**).

LEUCINE-RICH REPEAT PROTEINS

LRR have previously been shown to occur in more than 2000 proteins throughout the plant and animal kingdom, including Toll-like receptors, and are thought to play an essential role in host defense (Boman and Hultmark, 1981; Kobe and Kajava, 2001; Bell et al., 2003; Enkhbayar et al., 2004). LRR proteins typically contain 20–29 amino acid residues (with repeats ranging from 2 to 42) that are involved in protein-protein interactions with diverse cellular locations and functions. While the biological significance of LRR containing proteins in ticks remains unknown, notably, the *I. scapularis* genome encodes at least 22 potential LRR proteins (**Table 3**). Unlike in ticks, the roles of LRR proteins in the immunity of other arthropods, including blood-meal

seeking arthropods, however, are relatively well-characterized (Povelones et al., 2009, 2011). For example, in *Anopheles gambiae*, LRR-containing proteins, such as LRIM1 and APL1C, have been identified as a potent antagonist of malarial parasites, limiting *Plasmodium* infection by activating a complement-like system (Fraiture et al., 2009; Povelones et al., 2009, 2011; Baxter et al., 2010). In *Manduca sexta,* an LRR-containing protein, termed leureptin, is shown to bind lipopolysaccharide and is involved in hemocyte responses to bacterial infection (Zhu et al., 2010). Further studies into how tick LRR-containing proteins contribute to vector immunity and influence pathogen persistence are warranted.

PROTEASES/PROTEASE INHIBITORS

A number of immune cascades that serve to recognize and control invading pathogens are dependent on the activity of specific proteases or protease inhibitors (Janeway and Medzhitov, 2002; Sojka et al., 2011). Proteases, specifically serine proteases, have previously been shown to be a key regulating molecule for several of these immune response pathways, including coagulation, antimicrobial peptide synthesis, and melanization of pathogens (Gorman and Paskewitz, 2001; Janeway and Medzhitov, 2002; Jiravanichpaisal et al., 2006). Such serine protease-dependent cellular response, for example, as demonstrated for coagulation in the horseshoe crab, manifests through the rapid activation of immune pathways in response to pathogen detection (Hoffmann et al., 1999; Fujita, 2002). Activation of this pathway has been shown to be controlled by three serine proteases: factor C, factor B, and a pro-clotting enzyme (Tokunaga et al., 1987). When LPS is present, clotting factors that are stored within hemocytes are readily released into the hemolymph, which ultimately results in the immobilization of the invading pathogen.

Protease inhibitors also control a variety of proteolytic pathways and are known to play an important role in arthropod immunity (Kanost, 1999). A group of serine protease inhibitors, termed serpins, have been the focus of many recent studies that demonstrate the critical contribution of these proteins to the regulation of inflammation, blood coagulation, and complement activation in mammals (Kanost, 1999). Serpins are also shown to contribute to immunity and physiology in arthropods, as shown in mosquitoes (Gulley et al., 2013) and flies (Reichhart et al., 2011). A detailed characterization of serpins in ticks, including *I. scapularis*, has been reported by Mulenga et al. (Mulenga et al., 2009). These authors reported the presence of at least 45 serpin genes within the *I. scapularis* genome, interestingly, most of which are differentially expressed in the gut and salivary glands of unfed and partially fed ticks (Mulenga et al., 2009). It is speculated that ticks could utilize some of these serpins to manipulate host defense to facilitate tick feeding and subsequent disease transmission, although the precise role of serpins in the physiology and immunity within the tick vector awaits further investigation. More recently, a novel serpin, termed IRS-2, was described in *I. ricinus* (Chmelar et al., 2011). IRS-2 was shown to inhibit cathepsin G and chymase, thereby inhibiting host inflammation and platelet aggregation. This particular protein was also thought to act as a modulator of vascular permeability. Although whether serpins play a role in host microbe interactions remains unknown, studies also explored their potential as target antigens for development of a tick vaccine (Muleng et al., 2001).

COAGULATION

Injury as well as the presence of microbes in arthropods could result in the induction of two major proteolytic pathways - coagulation and melanization (Theopold et al., 2004). Key enzymes for these processes that cross-link the clot or induce a proteolytic pathway similar to the vertebrate clotting cascade include transglutaminase and phenoloxidase, respectively. Studies in the horseshoe crab have provided a breakthrough in our understanding of the coagulation pathway in arthropods (Theopold et al., 2004). This pathway is characterized by a rapid sequence of highly localized serine proteases and culminates in the generation of thrombin; the process is tightly regulated to ensure excessive clot formation does not occur (Crawley et al., 2011). The *I. scapularis* genome encodes for at least 11 genes that may be part of the coagulation pathway (**Table 5**), although precisely how this pathway controls wound healing or affects microbial survival remains unknown. Notably, while the *I. scapularis* genome lacks genes related to the melanization (phenoloxidase) pathway, phenol oxidase activity was detected in the hemolymph of the soft ticks, *Ornithodoros moubata* (Kadota et al., 2002).

NON-SELF RECOGNITION AND SIGNAL TRANSDUCTION PATHWAYS (TOLL, IMD, AND JAK-STAT)

Three major pathways, namely Toll, immune deficiency (IMD), and Janus kinase (JAK)- signaling transducer and activator of transcription (STAT) pathways, contribute to the activation of the immune response within arthropods, as previously detailed (Belvin and Anderson, 1996; De Gregorio et al., 2002; Hoffmann and Reichhart, 2002; Govind and Nehm, 2004; Lemaitre, 2004; Rawlings et al., 2004; Kaneko and Silverman, 2005; Tanji and Ip, 2005; Zambon et al., 2005; Tanji et al., 2007; Xi et al., 2008; Souza-Neto et al., 2009; Valanne et al., 2011; Liu et al., 2012). Notably, the *I. scapularis* genome encodes many representative genes from all three pathways (**Figure 1**). While Toll pathways are activated in the presence of bacterial, viral, and fungal pathogens, the IMD pathway is induced by Gram-negative bacteria. The arthropod JAK-STAT pathway, analogous to a cytokine-signaling pathway in mammals (Shuai et al., 1993), has also previously been shown to be activated in the presence of bacterial or protozoan pathogens (Buchon et al., 2009; Gupta et al., 2009; Liu et al., 2012). The Toll pathway is most extensively studied in *Drosophila*, which encodes nine Toll receptors (Valanne et al., 2011). Cell wall components in Gram-positive bacteria stimulate this pathway, whereas the precise fungal component that induces specific Tolls is not well-defined. In both cases, stimulation of the Toll pathway causes cleavage of the protein Spätzle, which eventually leads to the activation of NF-κB transcription factor family members Dif and Dorsal, which are homologous to mammalian c-Rel and RelA, resulting in the production of different antimicrobial peptides (AMPs) (Irving et al., 2001; Christophides et al., 2002; Hetru et al., 2003). Specifically, research in *Drosophila* has shown that Gram-positive bacteria induce the Toll pathway, leading to the generation of Toll-specific AMPs, such as drosomycin (Zhang and Zhu, 2009). While roles of Toll pathways in *I. scapularis* remain

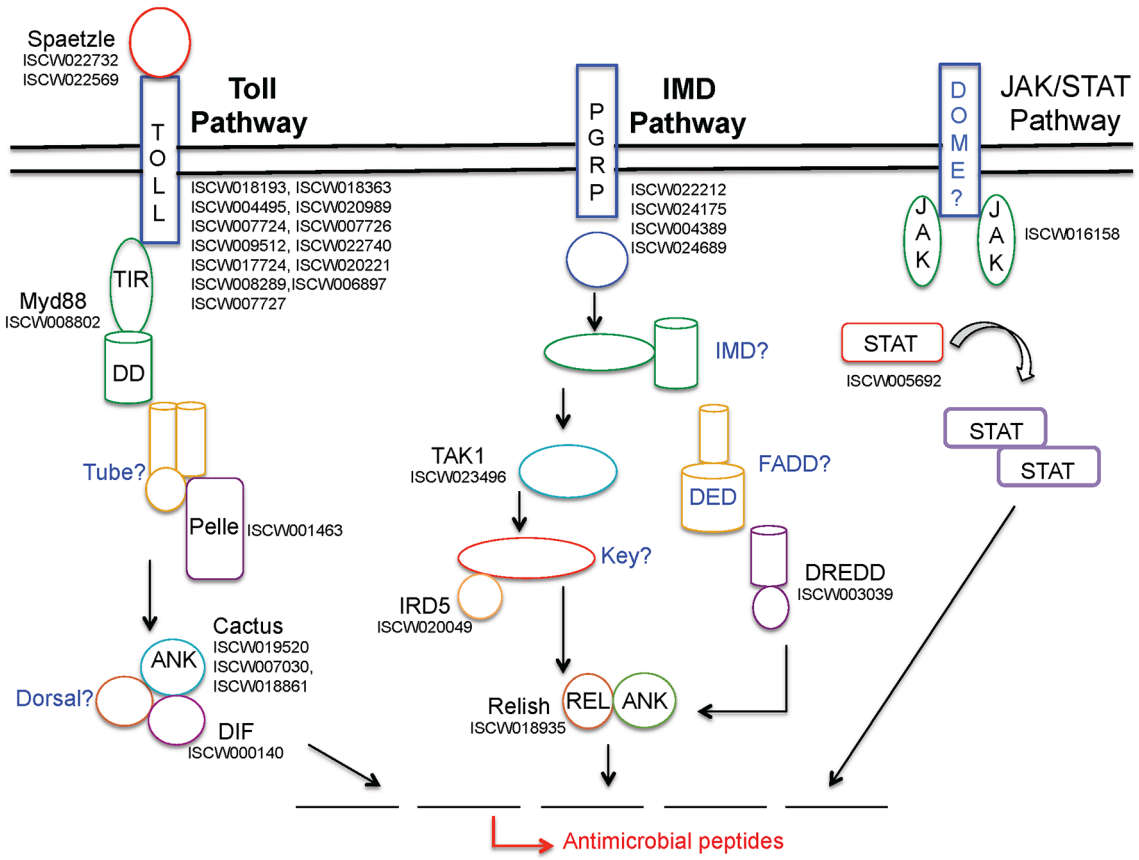

FIGURE 1 | Schematic representation of *I. scapularis* immune-related genes potentially representing three major signaling pathways that lead to anti-microbial responses. While annotated tick genes from each pathway are shown in black font, the apparently missing genes are indicated in blue. For further details on these pathways, including abbreviations, please refer to the text and earlier publications (Lemaitre, 2004; Liu et al., 2012).

obscure, we list at least 33 genes that potentially belong to this pathway (**Table 6**). The IMD pathway, on the other hand, is activated by the peptidoglycan molecules present on the surface of Gram-negative bacteria that are recognized by host cells via peptidoglycan recognition receptors (PGRP) (Ferrandon et al., 2007). This recognition leads to the activation of an adaptor protein and further downstream signaling molecules, such as transcription factor Relish, a compound Rel-Ank protein homologous to mammalian p100 and p105, ultimately resulting in the production of AMPs (Matova and Anderson, 2006; Ferrandon et al., 2007). Although the tick genome encodes at least 20 potential genes from this pathway (**Table 6**), similar to Toll, how the IMD pathway affects Gram-negative pathogens, including *B. burgdorferi* is unknown. A critical and common aspect in the response of both pathways is the ability to induce a specific AMP to combat microbial infections through the recognition of non-self. Interestingly, it is also thought that these two pathways can work synergistically to activate the expression of the same AMP (Tanji et al., 2007).

FREE RADICAL DEFENSE

Free radicals, such as ROS, which include superoxide radicals (O·2), hydroxyl radicals (·OH), and other compounds, are able to react with biomolecules and cause damage to DNA, proteins, and lipids, playing as critical role in cell signaling (Thannickal and Fanburg, 2000). While ROS are important in arthropod development (Owusu-Ansah and Banerjee, 2009), they are indispensible in arthropod immunity, including activation of specific immune pathways (Pereira et al., 2001; Bubici et al., 2006; Molina-Cruz et al., 2008; Morgan and Liu, 2011). For example, mosquitoes that were previously infected with *Wolbachia* bacteria were observed to produce much higher levels of ROS (Pan et al., 2012). Nitric oxide (NOS), a highly unstable free radical gas, is another component of free radical defense shown to be toxic to both parasites and pathogens (James, 1995; Wandurska-Nowak, 2004). In insects, NOS is known to be induced following parasite infection (Dimopoulos et al., 1998; Davies, 2000).

A family of superoxide dismutases (SOD) that catalyze the conversion of these free radicals to non-toxic O_2 and less toxic hydrogen peroxide (H_2O_2) are responsible for destroying any free radicals generated in the hosts. Glutathione-S-transferases (GST) also detoxify stress-causing agents, including toxic oxygen free radical species (Sharma et al., 2004). The genes encoding GSTs are shown to be induced in model arthropods upon oxidative stress and microbial challenge, including in ticks infected with *B. burgdorferi* (Rudenko et al., 2005). Despite these studies, how different free radicals or SOD detoxification systems play

roles in pathogen persistence or clearance within *I. scapularis*, which encodes at least 13 genes of this pathway (**Table 7**), remains uncharacterized.

PHAGOCYTOSIS

Cells recognize, bind, and ingest relatively large particles in phagocytosis (Walters and Papadimitriou, 1978). This process is considered a major evolutionarily conserved cellular immune response in arthropods, mostly studied in model insects (Sideri et al., 2008), and is mediated by hemocytes, also known as blood cells, which are primarily present in the hemolymph as well as infrequently exist within various organs. Phagocytosis of microbes plays a critical role in arthropod defense, as blocking of phagocytosis in *Drosophila* mutants significantly impairs the flies' ability to survive subsequent bacterial infection (Elrod-Erickson et al., 2000). Hemocytes within the hemolymph have previously been shown to phagocytize various pathogens (Inoue et al., 2001). Whereas, although solid experimental evidence of phagocytosis of *B. burgdorferi* within *I. scapularis* is lacking, certain cell lines derived from ticks have been shown to be phagocytic to spirochetes (Mattila et al., 2007). Further studies into the phagocytic pathway of *I. scapularis*, which encodes 33 potentially related genes (**Table 8**), would provide insight into whether or how pathogens, such as *B. burgdorferi,* are phagocytized**,** as well as how tick-borne pathogens are able to escape this cellular immune response. Notably, the *I. scapularis* genome encodes for five small GTPases belonging to the Rho family that in addition to other cellular functions, are shown to play central roles in phagocytosis (Etienne-Manneville and Hall, 2002; Bokoch, 2005).

ANTI-MICROBIAL PEPTIDES

The production of AMPs, a hallmark of systemic humoral immune responses, is an important aspect of host defense in arthropods (Bulet et al., 1999). At least eight different classes of AMPs have been observed in the fruit fly, *Drosophila*. These AMPs, are mainly produced by fat bodies and secreted into the hemolymph and can then be further grouped into three different families based on their intended target: Gram-negative bacteria, Gram-positive bacteria, and fungi (Imler and Bulet, 2005). In arthropods, specific AMPs are produced as a result of activation of the Toll, IMD, or JNK-STAT pathway by the presence of bacteria, fungi, or viruses.

Among effector molecules of innate immune defense, AMPs are relatively well-studied in ticks, which likely generate classical AMPs in the gut and hemocoel (Hynes et al., 2005; Saito et al., 2009). AMPs have been found to be produced in hard ticks, such as *I. scapularis* and *Dermacentor variabilis*, as well as in the soft tick *Ornithodoros moubata* (Nakajima et al., 2002; Sonenshine et al., 2002; Hynes et al., 2005; Rudenko et al., 2005; Saito et al., 2009). *I. ricinus* induces a defensin-like gene in response to *B. burgdorferi* in a tissue-specific manner that is not capable of clearing the infection (Rudenko et al., 2005). *I. scapularis* encodes for at least 14 AMPs (**Table 9**). The exact role of defensin or other AMPs in clearance of tick-borne pathogens remains unclear. In addition, ticks may also produce non-classical AMPs. Although gastric digestion in ticks is primarily intracellular, degradation of blood components, such as hemoglobin, could create peptides with antimicrobial activities (Sonenshine et al., 2005). Whether these fragments would protect against pathogenic bacteria has currently not been reported.

CONCLUDING REMARKS

I. scapularis ticks are known to transmit a diverse set of disease agents ranging from bacterial to protozoans to viruses. A number of studies explored the immunomodulatory activities of tick saliva or components of the salivary gland in mammalian hosts or how these activities benefit tick-transmitted pathogens (Hovius et al., 2008; Pal and Fikrig, 2010). However, limited investigation addressed how vector immune responses influence the survival or persistence of specific pathogens within the tick. It is rather surprising that although ticks are known to encode components of a number of immune effector mechanisms, including humoral (classical AMPs) or cellular (phagocytosis) immune responses as well as evolutionary conserved signaling molecules or potentially active pathways (Toll, IMD, or JAK/STAT), their contribution in shaping *I. scapularis* immunity remains largely obscure. Tick-borne pathogens are evolved to persist and be transmitted by a specific tick species. Thus, it is conceivable that these pathogens coevolved and developed a successful and intimate relationship with the host. Additionally, to be successful in nature, these pathogens must have also evolved specific mechanisms to persist in the vector and evade innate immune insults. For example, when artificially challenged with the Lyme disease pathogen, *I. scapularis* ticks mount slower phagocytic responses and therefore, remain practically immunotolerant against spirochete infection (Johns et al., 2001). In contrast, another hard tick species, *D. variabilis*, when challenged with the same pathogen, generates a rapid and effective increase in phagocytic cells and clears the infection and thus, is highly immunocompetent against spirochete infection. With the availability of *I. scapularis* genome information and development of robust functional genomics and bioinformatics as well as the advent of efficient high-throughput genome sequencing tools, we expect exciting future research enhancing our knowledge of *I. scapularis* immunity and hope to address specific questions on the biology of tick immune responses against a diverse group of human pathogens. Together, these studies will contribute to a better understanding of the special biology of vector-microbe interaction and specific aspects of tick immunity and at the same time, contribute to the development of new strategies to combat pathogen transmission.

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Dual role for Fcγ receptors in host defense and disease in *Borrelia burgdorferi*-infected mice

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Arthritis in mice infected with the Lyme disease spirochete, *Borrelia burgdorferi*, results from the influx of innate immune cells responding to the pathogen in the joint and is influenced in part by mouse genetics. Production of inflammatory cytokines by innate immune cells *in vitro* is largely mediated by Toll-like receptor (TLR) interaction with Borrelia lipoproteins, yet surprisingly mice deficient in TLR2 or the TLR signaling molecule MyD88 still develop arthritis comparable to that seen in wild type mice after *B. burgdorferi* infection. These findings suggest that other, MyD88-independent inflammatory pathways can contribute to arthritis expression. Clearance of *B. burgdorferi* is dependent on the production of specific antibody and phagocytosis of the organism. As Fc receptors (FcγR) are important for IgG-mediated clearance of immune complexes and opsonized particles by phagocytes, we examined the role that FcγR play in host defense and disease in *B. burgdorferi*-infected mice. *B. burgdorferi*-infected mice deficient in the Fc receptor common gamma chain ($Fc\varepsilon R\gamma^{-/-}$ mice) harbored ∼10 fold more spirochetes than similarly infected wild type mice, and this was associated with a transient increase in arthritis severity. While the elevated pathogen burdens seen in *B. burgdorferi*-infected $MyD88^{-/-}$ mice were not affected by concomitant deficiency in FcγR, arthritis was reduced in $Fc\varepsilon R\gamma^{-/-}MyD88^{-/-}$ mice in comparison to wild type or single knockout mice. Gene expression analysis from infected joints demonstrated that absence of both MyD88 and FcγR lowers mRNA levels of proteins involved in inflammation, including Cxcl1 (KC), Xcr1 (Gpr5), IL-1beta, and C reactive protein. Taken together, our results demonstrate a role for FcγR-mediated immunity in limiting pathogen burden and arthritis in mice during the acute phase of *B. burgdorferi* infection, and further suggest that this pathway contributes to the arthritis that develops in *B. burgdorferi*-infected $MyD88^{-/-}$ mice.

Keywords: Fc receptor, *Borrelia burgdorferi*, arthritis, mice, Lyme disease, toll-like receptors, MyD88

INTRODUCTION

Lyme disease, due to infection with *Borrelia burgdorferi sensu lato* spirochetes, is the most common arthropod-borne disease in the northern hemisphere (Kurtenbach et al., 2006). The disease usually manifests as the skin lesion erythema migrans at the tick bite site and can result in myocarditis, arthritis, and neurological abnormalities once infection has disseminated (Bockenstedt, 2013). In the United States, arthritis is the second most common clinical sign of confirmed cases of Lyme disease reported to the Centers for Disease Control and Prevention (CDC, 2013). A mouse model of Lyme disease has been developed that manifests primarily as myocarditis and arthritis, although spirochetes can be found in other, non-diseased tissues such as the skin (Barthold et al., 1992). Inflammation peaks in the heart and joints 2–4 weeks after infection introduced by needle inoculation of cultured spirochetes and resolves without treatment in wild type mice even though spirochetes remain in these tissues (Barthold et al., 1990). Histopathology of *B. burgdorferi*-infected mouse tissues reveals a dominance of innate immune cells at sites of inflammation, especially macrophages in the heart and neutrophils in the joints (Barthold et al., 1990, 1992; Ruderman et al., 1995). This pathology develops in the absence of B and T cells, but adaptive immunity is required for the spontaneous, immune-mediated regression of disease (Barthold et al., 1992; McKisic and Barthold, 2000; Bockenstedt et al., 2001).

B. burgdorferi express an abundant array of lipoproteins that are potent stimulators of inflammatory responses (Fraser et al., 1997). Lipoproteins activate macrophages and other innate immune cells through Toll-like receptor (TLR) pattern recognition molecules, especially TLRs 2, 5, 7, 8, and 9 (Aliprantis et al., 1999; Hirschfeld et al., 1999; Wooten et al., 2002; Shin et al., 2008; Petzke et al., 2009; Cervantes et al., 2011). These TLRs utilize the common intracellular adaptor molecule myeloid differentiation primary response gene 88 (MyD88) to initiate intracellular signaling events that culminate in NFκb activation and inflammatory cytokine production (Creagh and O'Neill, 2006). *In vitro* stimulation of macrophages by borrelia lipoproteins via TLR2 leads to the production of chemokines, cytokines, and the upregulation of costimulatory molecules (Hirschfeld et al., 1999; Shin et al., 2008; Salazar et al., 2009). While the TLR/MyD88 signaling pathway

is the main pathway contributing to *B. burgdorferi* lipoprotein mediated inflammation *in vitro*, arthritis and carditis still develop *in vivo* in the absence of TLR2 or MyD88 expression (Wooten et al., 2002; Liu et al., 2004; Behera et al., 2006b). This observation suggests that other MyD88-independent pathways contribute to the development of disease.

Receptors for the Fc region of IgG (FcγR) and immune complexes can also play a role in the development of inflammation (Nimmerjahn and Ravetch, 2006, 2008). FcγR couple innate and adaptive immune responses through their ability to activate effector cells (Nimmerjahn and Ravetch, 2008). Proinflammatory and anti-inflammatory mechanisms are linked to different FcγR, which share the common FcR γ-chain (Nimmerjahn and Ravetch, 2006). The high affinity FcγRI, intermediate affinity FcγRIV, and low affinity FcγRIII and FcεRI, which mediate the binding and internalization of mouse IgG1, IgG2a, IgG2b, IgG3, and IgE subclasses, are activating receptors that contain an immunoreceptor tyrosine-based activation motif (ITAM) (Nimmerjahn and Ravetch, 2007). FcγRII is an inhibitory receptor containing an immunoreceptor tyrosine-based suppression motif. FcγR are predominantly expressed on macrophages, neutrophils, dendritic cells, and other innate immune cells, and have limited expression on lymphocytes, including B cells, and NK cells and endothelial cells (Takai, 2005). The outcome from engagement of FcγR depends on the IgG subclass and the balance between activation and inhibitory FcγR stimulation.

Mice deficient in the common gamma chain (FcεRγ$^{-/-}$ mice) lack the ability to mediate IgG dependent phagocytosis and antibody-dependent cell-mediated cytotoxicity through FcγRI, FcγRIII, and FcγRIV (Takai et al., 1994). In some infection models, the ability to control infection is impaired in FcεRγ$^{-/-}$ mice, as has been reported for intracellular pathogens *Plasmodium* and *Pneumocystis* spp. (Yoneto et al., 2001; Wells et al., 2006). Yet, with other infections and with some autoimmune models, the absence of FcγR signaling ameliorates disease (Kima et al., 2000; Tarzi et al., 2002; Alexander and Scott, 2004; Kaneko et al., 2006a,b; Giorgini et al., 2008). Antigen-antibody complexes (immune complexes) can trigger inflammation by binding to and activating FcγR, and these receptors have been shown to play an integral role in immune complex-mediated tissue injury (Jancar and Crespo, 2005). When antigens encounter their cognate antibody in the presence of complement, binding of the complex to the complement C3 protein follows immune complex formation (Wessels et al., 1995; Baudino et al., 2008; Giorgini et al., 2008). This complex facilitates simultaneous interaction with both FcγR and complement receptors. Activation of complement can lead to clearance of pathogens as well as the immune complexes, thereby preventing them from causing further tissue damage. More recently it has been found that signaling through both FcγR and TLRs are modulated by ITAM-coupled receptors. ITAM receptors are activated by immune complexes that crosslink FcγR, and this high avidity activation synergizes with TLR signals to activate pro-inflammatory gene expression (Ivashkiv, 2008).

Immune complexes containing *B. burgdorferi* antigens have been found early after infection in human plasma samples (Benach et al., 1984; Schutzer et al., 1999; Lencáková et al., 2007). Here, we postulated that inflammation in *B. burgdorferi*-infected mice could be mediated in part by the formation of immune complexes, and that this FcγR-mediated inflammation may contribute to the pathology seen in mice deficient in TLR/MyD88-mediated responses. Our results using mice deficient in FcγR only or in both FcγR and MyD88 reveal that FcγR has a dual role in immune defense and arthritis development associated with *B. burgdorferi* infection.

METHODS

MICE

C57Bl/6 (B6);129P2-Fcer1^{gtm1Rav}/J (FcεRγ$^{-/-}$) mice, 6–8 weeks of age, were obtained from the Jackson Laboratory (Bar Harbor, ME, stock number 002847). The B6.129 MyD88$^{-/-}$ mice were the kind gift of Ruslan Medzhitov, Yale University School of Medicine (Schnare et al., 2001). FcεRγ$^{-/-}$ mice were bred with MyD88$^{-/-}$ mice and select progeny were intercrossed to produce FcεRγ$^{-/-}$ MyD88$^{-/-}$ double knockout mice; the F2 generation was used in these studies. Wild type and heterozygous littermates were used as controls. Mice were housed in filter frame cages and administered food and water *ad libitum* according to Yale University animal care and use guidelines. Mice were euthanized by carbon dioxide asphyxiation. The Yale University Institutional Animal Care and Use Committee approved all procedures.

INFECTION OF MICE

Frozen aliquots of low-passage *B. burgdorferi* N40 were grown to mid-logarithmic phase in BSK-H medium (Sigma-Aldrich, St. Louis, MO) and enumerated by dark-field microscopy using a Petroff-Hausser chamber. Each mouse was inoculated intradermally in the central lower back or in each hind limb between the knee and tibiotarsal joints with 10^4 spirochetes in 100 μl of BSK-H medium. These inoculation sites were chosen as we have found that arthritis may be more apparent in a disease-resistant mouse background such as C67BL/6 or 129 mice if spirochetes are inoculated closer to the joints examined for histopathology. Both inoculation sites still result in systemic disseminated infections. At the time of mouse sacrifice, infection was confirmed by culturing the blood or a portion of the urinary bladder in BSK-H medium for 14 days, after which the presence of spirochetes was determined by dark-field microscopy.

MACROPHAGE STIMULATION

Macrophages were isolated from WT and MyD88$^{-/-}$ mice by peritoneal lavage according to previously published methods (Liu et al., 2004). Cells were resuspended in α-minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum and aliquoted at 2×10^6 cells/ml into a 24-well plate. Cells were stimulated for 24 h with recombinant lipidated OspC protein (kindly provided by John Dunn, Brookhaven National Laboratories) or Osp C protein-immune complexes formed using rabbit polyclonal anti-OspC antisera. We confirmed that antibodies present in the rabbit antisera could bind to mouse FcγR using the J774 mouse macrophage cell line (data not shown). In order to create the immune complexes, concentrations of lipidated OspC protein ranging from 0.1 ng to 1 μg were incubated with 20 μl of rabbit polyclonal anti-OspC antisera (with an anti-OspC IgG titer of 1:192,000) and 80 μl of α-MEM media for

30 min prior to incubation with the cells. Negative control samples contained just the rabbit polyclonal anti-OspC antisera in media to insure that antisera alone did not stimulate production of TNFα. Cells were cultured for 24 h in a 37°C incubator with 5% CO_2, after which supernatants were harvested and analyzed for TNFα production by ELISA as described (Liu et al., 2004).

MEASUREMENT OF *B. BURGDORFERI*-SPECIFIC ANTIBODY TITERS

B. burgdorferi-specific enzyme-linked immunosorbent assays were performed using sera from 21-day infected animals as previously described (Liu et al., 2004). Briefly, 96-well microtiter plates were coated with *B. burgdorferi* lysate (3 μg in 50 μl 100% ethanol per well). After blocking, serial twofold dilutions of sera in PBS containing 0.5% bovine serum albumin and 0.5% Tween 20 were added to the wells and incubated for 1 h at room temperature. Secondary biotinylated anti-mouse IgM and IgG (Vector Labs, Burlingame, CA), IgG1, IgG2a, and IgG2b (Invitrogen, Carlsbad, CA), and IgG3 (BD Biosciences, Franklin Lakes, NJ) were used at a 1:1000 dilution, and bound Abs were detected with the ABC Elite peroxidase detection and ABTS [2,2′-azino-bis(ethylbenzthiazolinesulfonic acid)] substrate kits (Vector Labs). The mixed B6.129 mouse background permitted reactivity with IgG2a even though the B6 mouse strain lacks this subclass and expresses the IgG2c subclass instead (with 70% homology to IgG2a).

QUANTITATIVE PCR OF *B. BURGDORFERI* DNA

DNA was isolated from urinary bladder using the DNeasy kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. This tissue was chosen as representative of pathogen burden as the entire organ can be processed for DNA extraction and, as a non-diseased site, the immune response would not be expected to impact the spirochete quantification. The copy number of *B. burgdorferi* in each sample was determined by quantitative PCR of the *B. burgdorferi recA* gene using an iCycler (Bio-Rad, Hercules, CA), the Brilliant SYBR green kit (Stratagene, Cedar Creek, TX), and the following primers: 5′ primer 5′-GTGGATCTATTGTATTAGATGAGGCTCTCG-3′ and 3′ primer 5′-GCCAAAGTTCTGCAACATTAACACCTAAAG-3′. The *recA* copy number was normalized to the mouse *actin* gene amplified using the following primers and probe: 5′ primer 5′-ATCAGGTA GTCGGTCAGG-3′, 3′ primer 5′-GGTATCTATCTCGACTC-3′, and probe 6-carboxyfluorescein-TCCAGCAGATCTGGATCAGC AAGCA-carboxytetra-methylrhodamine (Applied Biosystems, Foster City, CA). A 60°C annealing temperature and 45 or 50 cycles were used for the *recA* and *actin* gene reactions, respectively. Standard curves were generated for both PCRs using known quantities of DNA. Reactions were performed in duplicate, and the quantities of PCR products generated were determined from the standard curves.

HISTOPATHOLOGY

Bilateral hind limb joints (knee and tibiotarsal joints) were fixed in formalin, embedded in paraffin and sections stained with hematoxylin and eosin by routine histologic techniques as previously described (Liu et al., 2004). The knee and tibiotarsal joints were scored for the presence and severity of periarticular inflammation and arthritis on a scale of 0 (negative) to 3 (severe) in a blinded fashion as described previously (Liu et al., 2004). Values for arthritis severity were reported as the mean scores of all the joints of individual mice in each group ± the standard error of the mean. Carditis was considered present when inflammatory cell infiltrates were present in the heart base in tissue sections.

QUANTITATIVE REAL-TIME ARRAY ANALYSIS FOR INFLAMMATORY CYTOKINES AND RECEPTORS

RNA was extracted from joints of uninfected and 21 day-infected WT, FcεRγ$^{-/-}$, MyD88$^{-/-}$and double knockout mice using TRIzol® and the PureLink™ micro-to-midi total RNA purification system (Invitrogen, Carlsbad, CA). The quality of RNA was verified in all samples with A_{260}:A_{230} ratios greater than 1.7, and A_{260}:A_{280} ratios between 1.8 and 2.0. RNA was combined from 4 mice in each group and was converted to complimentary DNA (cDNA) and real-time PCR was performed on the samples using the RT^2 First Strand Kit and the RT^2 qPCR Master Mix according the manufacturer's instructions (SABiosciences, Frederick, MD). The master mix containing the cDNA was loaded onto the RT^2 Profiler PCR Array for Inflammatory Cytokines and Receptors (SABiosciences, PAMM-011), and the amplification was performed according to the manufacturer's protocol using a Bio-Rad iCycler. The data were analyzed using the ΔΔCt method by the Keck facility (Yale University).

PCR CONFIRMATION OF SELECT MICROARRAY DATA

0.4 μl aliquots of cDNA (generated above) were used as templates for PCR reactions in order to confirm select results obtained with the microarray. A 60°C annealing temperature and 35 cycles were performed for each reaction using Taq polymerase (QIAGEN) and the following primers: TNFα, 5′ primer 5′atgagcacagaaagca tgatc 3′ and 3′primer 5′tacaggcttgtcaccgaatt 3′; IL-10, 5′ primer 5′atgcaggactttaagggttacttg3′ and 3′ primer 5′tagacaccttggtcttggag ctta 3′; IFNγ, 5′ primer 5′gacagaagttctgggcttctcc 3′ and 3′ primer 5′gcagcgactccttttccgctt 3′.

RESULTS

PRESENTATION OF *B. BURGDORFERI* ANTIGENS AS IMMUNE COMPLEXES ENHANCES MACROPHAGE PRODUCTION OF TNFα

To examine the response of mouse macrophages to a *B. burgdorferi* antigen alone or in the form of an immune complex, we stimulated resting peritoneal macrophages from WT or MyD88$^{-/-}$ mice with recombinant lipidated OspC alone or as an immune complex with anti-OspC antisera (**Figure 1**). As expected, recombinant lipidated OspC elicited TNFα in a dose-dependent fashion from WT macrophages, whereas there was little response of MyD88$^{-/-}$ macrophages to this stimulation. In the presence of OspC antibody, however, the dose of OspC required to stimulate TNFα from WT macrophages was reduced 100-fold, from 0.01 to 0.0001 μg/ml (**Figure 1**). MyD88$^{-/-}$ macrophages also produced TNFα when stimulated with OspC as an immune complex, at levels approaching those elicited from WT cells. OspC immune serum alone had no effect (data not shown). These findings indicate that in the absence of MyD88, macrophages can produce inflammatory cytokines when *B. burgdorferi* antigens are presented as immune complexes.

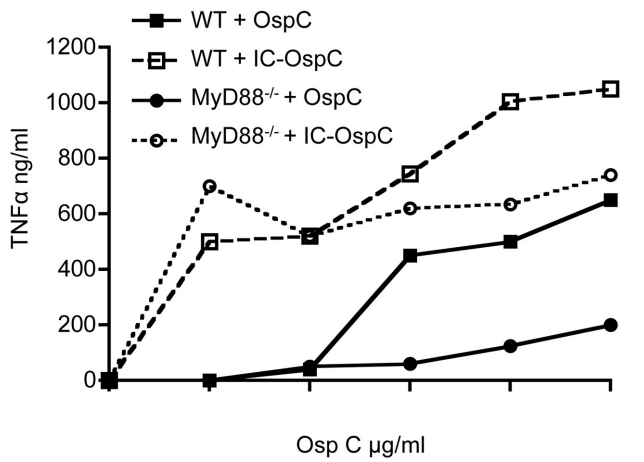

FIGURE 1 | TNFα secretion by macrophages stimulated with OspC and OspC-immune complexes. Peritoneal macrophages from naïve WT and MyD88$^{-/-}$ mice were stimulated *in vitro* with recombinant *B. burgdorferi* OspC protein or OspC-anti-OspC immune complexes for 24 h. The levels of secreted TNFα in the supernatants were measured by ELISA.

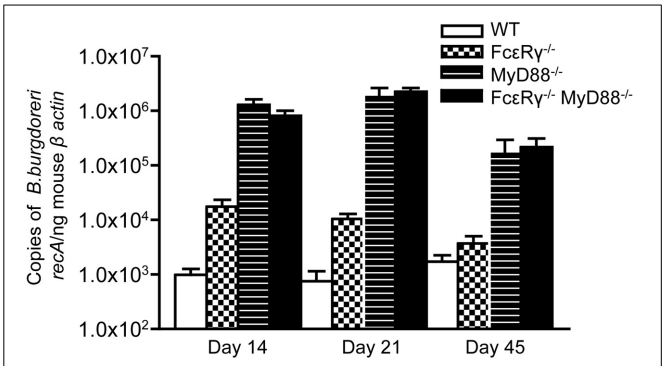

FIGURE 2 | Quantitative PCR of *B. burgdorferi* in urinary bladders of infected mice. The copy number of the *B. burgdorferi recA* gene was normalized to the mouse β-actin gene by real-time PCR. Values are averages of 5–11 individual mice. Results are combined from 2 separate experiments. At day 14, the burdens in the FcεRγ$^{-/-}$ mice are statistically different from those in the WT mice ($P = 0.0043$) and those in the MyD88$^{-/-}$ and FcεRγ$^{-/-}$ MyD88$^{-/-}$ mice ($P = 0.0029$ and $P = 0.0095$, respectively). At day 21 the FcεRγ$^{-/-}$ mice maintain statistically higher burdens than the WT mice ($P = 0.007$) but lower burdens than the MyD88$^{-/-}$ ($P = 0.001$) and the FcεRγ$^{-/-}$ MyD88$^{-/-}$ ($P \leq 0.0001$). At day 45 there is no difference between the WT and FcεRγ$^{-/-}$ mice but the MyD88$^{-/-}$ and FcεRγ$^{-/-}$ MyD88$^{-/-}$ burdens remain high. All *P*-values were calculated using the Mann-Whitney assay.

FCγR DEFICIENCY TRANSIENTLY INCREASES PATHOGEN BURDENS EARLY AFTER *B. BURGDORFERI* INFECTION

We next sought to determine the effects of FcγR deficiency on *B. burgdorferi* infection and disease *in vivo*. Although opsonization of *B. burgdorferi* is not required for its uptake by macrophages *in vitro* (Montgomery et al., 1994), FcγR could contribute to limiting pathogen burden by enhancing the uptake and lysosomal targeting of opsonized *B. burgdorferi*. We found that FcεRγ$^{-/-}$ mice have 10-fold higher pathogen burdens as compared to WT mice at day 14 after infection (**Figure 2**, checkered bars vs. open bars). MyD88$^{-/-}$ macrophages are known to have impaired uptake and degradation of *B. burgdorferi in vitro* (Liu et al., 2004; Behera et al., 2006a) and MyD88$^{-/-}$ mice exhibit elevated pathogen burdens after *B. burgdorferi* infection (Bolz et al., 2004; Liu et al., 2004). As expected, the pathogen burdens in mice deficient in MyD88 were about 100-fold higher than those in WT mice (**Figure 2**, hatched bars), and combined deficiency in both FcγR and MyD88 did not lead to a further increase (hatched bars vs. solid black bars). Pathogen burdens remained elevated in FcεRγ$^{-/-}$ mice through infection day 21, but by day 45, levels were comparable to those seen in WT mice. In contrast, MyD88$^{-/-}$ and FcεRγ$^{-/-}$ MyD88$^{-/-}$ still had significantly elevated pathogen burdens at this late time point (**Figure 2**).

DEFICIENCY IN FCγR REDUCES THE SEVERITY OF DISEASE IN MYD88$^{-/-}$ MICE

In *B. burgdorferi*-infected mice, absence of TLR signaling does not diminish the severity of arthritis and in fact, has even been reported to enhance inflammation (Bolz et al., 2004). In this situation, IgG opsonization of *B. burgdorferi* and/or its membrane blebs could enhance the uptake of spirochetes and their inflammatory components via activating FcγR, and promote release of inflammatory cytokines, similar to what we observed when MyD88$^{-/-}$ macrophages were stimulated with OspC immune complexes *in vitro*. We therefore determined the effects of FcγR deficiency alone or in combination with MyD88 deficiency on *B. burgdorferi*-induced arthritis. The severity of arthritis was highest in MyD88$^{-/-}$ mice at day 14, although FcεRγ$^{-/-}$ mice also exhibited a modest increase in joint inflammation relative to WT at this time point (**Figure 3A**). At day 21, WT mice had increased arthritis in comparison to the day 14 timepoint, whereas the arthritis detected in MyD88$^{-/-}$ was less pronounced. FcεRγ$^{-/-}$ mice had the highest arthritis severity scores relative to WT or MyD88$^{-/-}$ mice at 21 days of infection. Mice deficient in both FcγR and MyD88 had minimal arthritis at this time point (**Figure 3B**).

ARTHRITIS PREVALENCE IN THE FCεRγ$^{-/-}$ MYD88$^{-/-}$ DOUBLE KNOCKOUT MICE WAS LOWER THAN THAT OF WT OR SINGLE KNOCKOUT MICE EARLY AFTER INFECTION

To assess the effects of FcγR deficiency on prevalence of arthritis after *B. burgdorferi* infection, we determined the number of joints that had evidence of inflammation in WT and MyD88$^{-/-}$ mice for comparison with FcεRγ$^{-/-}$ and the double knockout FcεRγ$^{-/-}$ MyD88$^{-/-}$ mice (**Table 1**). At day 14 of infection, the prevalence of arthritis was higher in MyD88$^{-/-}$ and FcεRγ$^{-/-}$ mice than in WT or FcεRγ$^{-/-}$ MyD88$^{-/-}$ mice (**Table 1**). The prevalence remained higher in each of the single knockout strains as compared to the WT mice at day 21. At this time point the arthritis in the double knockout mice was similar in prevalence to what had been observed in each of the single knockout mouse groups (**Table 1**).

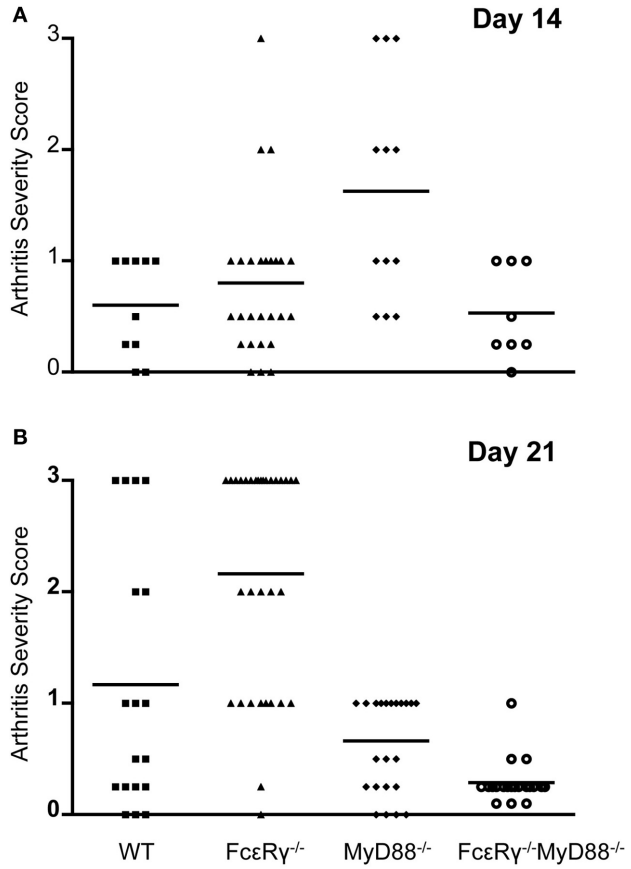

FIGURE 3 | Arthritis severity is diminished in the combined absence of both FcγR and MyD88. Hind limb joints were analyzed, and each data point represents one joint. Arthritis was scored on a scale of 0 (negative) to 3 (severe). Four to 14 mice were used in each group and results were combined from 2 to 4 separate experiments. **(A)** shows arthritis severity at day 14, and **(B)** shows arthritis severity at day 21. Significant differences were observed in **(A)**: WT vs. MyD88$^{-/-}$ $P = 0.007$, FcεRγ$^{-/-}$ vs. MyD88$^{-/-}$ $P = 0.004$, and MyD88$^{-/-}$ vs. FcεRγ$^{-/-}$ MyD88$^{-/-}$ $P = 0.009$. **(B)** FcεRγ$^{-/-}$ vs. WT $P = 0.0026$, FcεRγ$^{-/-}$ vs. MyD88$^{-/-}$ $P \leq 0.0001$, FcεRγ$^{-/-}$ vs. FcεRγ$^{-/-}$ MyD88$^{-/-}$ $P \leq 0.0001$, MyD88$^{-/-}$ vs. FcεRγ$^{-/-}$ MyD88$^{-/-}$ $P = 0.0003$, and WT vs. FcεRγ$^{-/-}$ MyD88$^{-/-}$ $P = 0.0013$. *P*-values were determined using the unpaired Student t test.

MYD88$^{-/-}$ MICE EXHIBIT A DELAY IN THE RESOLUTION OF KNEE ARTHRITIS THAT IS ABROGATED IN THE ABSENCE OF FCγR

We examined *B. burgdorferi*-infected mice at a later time point, day 45, when arthritis is normally subsiding. Mild inflammation was still present in tibiotarsal joints from each mouse group at infection day 45 (**Table 2**). MyD88$^{-/-}$ and WT mice exhibited comparable degrees of inflammation in these joints whereas arthritis scores were lower in FcεRγ$^{-/-}$ mice and FcεRγ$^{-/-}$ MyD88$^{-/-}$ (**Table 2**). We found that arthritis in the knees was completely resolved in the WT and FcεRγ$^{-/-}$ mice and a single joint in one FcεRγ$^{-/-}$ MyD88$^{-/-}$ mouse had a small amount of inflammation. This was not the case with MyD88$^{-/-}$ mice, which had the highest prevalence of arthritis, with 8 of 10 knees showing some degree of inflammation (**Table 2**). No differences in carditis were observed at day 21 and carditis had resolved by day 45 (data not shown).

Table 1 | Prevalence of arthritis in *B. burgdorferi* infected mic[a].

Day of infection	Mouse group			
	WT	FcεRγ −/−	MyD88$^{-/-}$	FcεRγ −/−MyD88$^{-/-}$
14	22/40	60/75[b]	36/44[c]	8/16
21	38/75	86/108[d]	46/58[e]	51/65[f]

[a] *Arthritis prevalence was reported as the number of joints with inflammation over the total number of joints examined in each group. The day 14 data are the prevalence combined from two separate experiments, and at day 21 the data for the prevalence are combined from four experiments. Statistically significant differences were found between the following groups.*

[b] *FcεRγ$^{-/-}$ vs. WT $P = 0.009$ and vs. FcεRγ$^{-/-}$MyD88$^{-/-}$ $P = 0.02$.*

[c] *MyD88$^{-/-}$ vs. WT $P = 0.01$ and vs. FcεRγ$^{-/-}$MyD88$^{-/-}$ $P = 0.02$.*

[d] *FcεRγ$^{-/-}$ vs. WT $P \leq 0.0001$.*

[e] *MyD88$^{-/-}$ vs. WT $P = 0.001$.*

[f] *FcεRγ$^{-/-}$MyD88$^{-/-}$ vs. WT $P = 0.0008$. P-values were determined using the Fisher's exact test.*

FCεRγ −/−MYD88$^{-/-}$ DOUBLE KNOCKOUT MICE HAVE HIGHER SERUM TITERS OF ANTI-*B. BURGDORFERI* IGG3 ANTIBODIES THAN MYD88$^{-/-}$ MICE

We have previously shown that *B. burgdorferi*-specific IgG in infected MyD88$^{-/-}$ mice exhibit a shift toward the IgG1 subclass, although IgG2a, IgG2b, and IgG3 subclass responses can still be detected, albeit at lower levels (Liu et al., 2004). At 21 days of infection, FcεRγ$^{-/-}$ mice had anti-*B. burgdorferi* IgG subclass responses similar to WT mice (**Figure 4**). MyD88$^{-/-}$ mice, as expected, had reduced IgG2a and 2b titers, reduced IgG3 titers, and increased IgG1 titers compared to WT mice. The FcεRγ$^{-/-}$MyD88$^{-/-}$ double knockout mice also had reduced levels of *B. burgdorferi*-specific IgG2a and 2b, and elevated IgG1, similar to the single knockout MyD88$^{-/-}$ mice (**Figure 4**). The *B. burgdorferi*-specific IgG3 levels in the double knockout mice however, were greater than titers in MyD88$^{-/-}$ single knockout mice and similar to those of WT and FcεRγ$^{-/-}$ mice (**Figure 4**).

GENE EXPRESSION PROFILES ARE ALTERED IN *B. BURGDORFERI* INFECTED FCεRγ −/−MYD88$^{-/-}$ MOUSE JOINTS

To globally assess the cytokines that may be involved with arthritis in our mouse groups, we measured the changes in expression levels of select cytokine and chemokines in RNA isolated from knee joints using the RT^2 Profiler PCR Array for Inflammatory Cytokines and Receptors (SA Biosciences). Each mouse group was analyzed compared to uninfected controls of the same phenotype (**Table 3**). Analysis of knee joint RNA demonstrated that FcεRγ$^{-/-}$ MyD88$^{-/-}$ mice exhibit a unique expression pattern that was not seen in either of the single knockouts or in WT mice. In WT mice, we found that the most abundantly up-regulated gene in infected joints was the B cell chemokine Cxcl13 (215 fold) (**Table 4**). It was also significantly upregulated in the FcεRγ$^{-/-}$ mice (129 fold) and FcεRγ$^{-/-}$ MyD88$^{-/-}$ (40 fold) infected joints, but not in the joints of MyD88$^{-/-}$ mice (0.39 fold).

Table 2 | MyD88-deficient mice exhibit knee arthritis at day 45 of infection that is reduced in prevalence and severity in the absence of FcγR.

Mouse strain	Joints	Arthritis prevalence[a]	Arthritis severity[b]
WT	Total	7/20	0.45 ± 0.8
	Tibiotarsal	7/10	0.9 ± 0.9
	Knees	0/10	0
FcεRγ$^{-/-}$	Total	4/20	0.24 ± 0.6
	Tibiotarsal	4/10	0.48 ± 0.8
	Knees	0/10	0
MyD88$^{-/-}$	Total	16/20[c]	0.8 ± 0.6[d]
	Tibiotarsal	8/10	1.1 ± 0.8[e]
	Knees	8/10[f]	0.58 ± 0.4[g]
FcεRγ$^{-/-}$MyD88$^{-/-}$	Total	7/20	0.18 ± 0.3
	Tibiotarsal	6/10	0.3 ± 0.3
	Knees	1/10	0.05 ± 0.16

[a] *Arthritis prevalence was reported as the number of joints with inflammation over the total number of joints examined in each group.*

[b] *Arthritis was scored on a scale of 0 (negative) to 3 (severe) and reported as the average score of all the joints examined in each mouse group. Five mice were used in each group. Statistically significant differences were found between the following groups.*

[c] *MyD88$^{-/-}$ vs. WT P = 0.01, vs. FcεRγ$^{-/-}$ P = 0.004, and vs. FcεRγ$^{-/-}$ MyD88$^{-/-}$ P = 0.01.*

[d] *MyD88$^{-/-}$ vs. WT P = 0.0.04, vs. FcεRγ$^{-/-}$ P = 0.001, and vs. FcεRγ$^{-/-}$ MyD88$^{-/-}$ P = 0.0008.*

[e] *MyD88$^{-/-}$ vs. FcεRγ$^{-/-}$ −MyD88$^{-/-}$ P = 0.02.*

[f] *MyD88$^{-/-}$ vs. WT P = 0.007, vs. FcεRγ$^{-/-}$ P = 0.007, and vs. FcεRγ$^{-/-}$ MyD88$^{-/-}$ P = 0.006.*

[g] *FKO vsWT P = 0.04, vs. FcεRγ$^{-/-}$ P = 0.001, and vs. FcεRγ$^{-/-}$ MyD88$^{-/-}$ P = 0.0008.*

[c,f] *Fisher's exact test.*

[d,e,g] *Mann-Whitney test.*

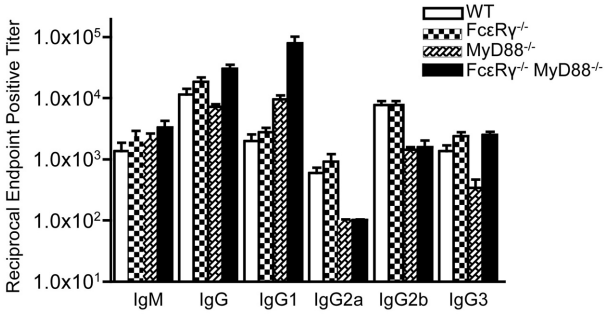

FIGURE 4 | Anti-*B. burgdorferi* IgG subclass titers are different in the single and double knockout mice. Anti-*B. burgdorferi* specific titers were determined by ELISA and are reported as the reciprocal of the endpoint positive titers. The FcεRγ$^{-/-}$ MyD88$^{-/-}$ anti-*B. burgdorferi* IgG1 titers are significantly higher than the MyD88$^{-/-}$ titers ($P = 0.0113$). The MyD88$^{-/-}$ IgG 3 titers are significantly lower than the FcεRγ$^{-/-}$ MyD88$^{-/-}$ titers ($P = 0.0025$) as well as the WT and FcεRγ$^{-/-}$ titers ($P = 0.01$ and.0025, respectively).

FcεRγ$^{-/-}$ MyD88$^{-/-}$ joints expressed less Cxcl1, Xcr 1, IL-1β, and C reactive protein than did the joint tissues from either single knockout or WT infected joints (**Table 3**). In order to confirm the results of the Superarray, RT-PCR reactions were performed on a select set of genes using the same RNA as was used on the array. Results using primers for TNFα, IL-10, and IFNγ show that the trends for increased or decreased expression or very low expression were consistent between the array and the PCR results (**Table 4**). The Superarray, however, was more sensitive than the PCR (**Table 4**).

DISCUSSION

B. burgdorferi lipoproteins have been shown to activate innate immune cells *in vitro* through interaction with TLR that signal the secretion of pro-inflammatory cytokines and chemokines (Weis and Bockenstedt, 2010). As deficiency in TLR2 or its intracellular signaling molecule MyD88 is sufficient to eliminate *B. burgdorferi* lipoprotein-induced innate immune cell activation, the observation that arthritis still occurs in TLR2- and MyD88-deficient mice suggests involvement of other inflammatory pathways. Here, we determined the contribution of FcγR to host defense and disease associated with *B. burgdorferi* infection. Our findings in *B. burgdorferi*-infected FcεRγ$^{-/-}$ mice reveal a modest role for FcγR in limiting pathogen burden early in infection, with spirochete numbers about 10-fold higher than those found in WT mice. This elevated pathogen burden was transient and associated with enhanced inflammation in joints at day 14 after infection, but not thereafter. In contrast, in later stages of infection when disease is resolving, our results suggest that FcγR may promote arthritis in MyD88$^{-/-}$ mice, as elimination of activating FcγR in MyD88$^{-/-}$ mice significantly attenuates this disease manifestation.

FcγR are thought to bridge innate and adaptive immunity through the relative engagement of activating and inhibitory FcγR. The subclasses of IgG produced have different binding affinities for FcγR, which influences the degree of inflammatory cytokine secretion (Nimmerjahn and Ravetch, 2006). IgG2a antibodies are the most potent at activating cells and preferentially bind activating FcγRI, whereas IgG1 antibodies are less activating and bind to low affinity FcγRIII. Our analysis of *B. burgdorferi*-specific IgG subclasses showed no significant difference between WT and FcεRγ$^{-/-}$ mouse groups, so arthritis susceptibility at 14 days of infection cannot be attributed to a shift toward a more inflammatory IgG subclass. In murine Lyme borreliosis, IgG is detectable within a week or so of infection and may serve to opsonize *B. burgdorferi* and augment phagocyte clearance of the pathogen. In FcεRγ$^{-/-}$ mice, phagocytes may rely on TLR/MyD88-dependent pathways for disposal of *B. burgdorferi*, with secondary consequences of enhanced inflammatory cytokine secretion and arthritis in the early stages of infection. A similar reliance on TLR/MyD88-dependent responses for pathogen clearance may explain the effects of complement component C3 deficiency on the course of murine Lyme borreliosis (Lawrenz et al., 2003). Activation

Table 3 | Microarray analysis of inflammatory genes expressed in mouse knee joints after *B. burgdorferi* infection.

Gene	WT Inf/Un	MyD88$^{-/-}$ Inf/Un	FcεRγ − /− Inf/Un	MyD88$^{-/-}$ FcεRγ − /− Inf/Un	MyD88$^{-/-}$ ratio/ WT ratio	FcεRγ − /− ratio/ WT ratio	MyD88$^{-/-}$ FcεRγ − /−ratio/ WT ratio
1-Ccl1	1.97	12.55	1.27	6.06	6.37	0.64	3.08
1-Ccl11	1.15	5.86	0.02	0.64	5.1	0.02	0.56
1-Ccl12	2.66	4.76	0.66	11.79	1.79	0.25	4.43
1-Ccl17	0.13	5.1	0.06	14.83	39.23	0.46	114.08
1-Ccl19	1.18	2.55	2.89	5.54	2.3	2.45	4.69
1-Ccl2	1.8	8.88	3.2	11.88	4.93	1.78	6.6
1-Ccl20	1.95	20.39	6.92	0.99	10.46	3.55	0.51
1-Ccl22	2.41	4.44	0.09	3.66	1.84	0.04	1.52
1-Ccl24	0.21	1.37	0.04	0.45	6.52	0.19	2.14
1-Ccl25	1.04	0.34	0.62	0.78	0.33	0.6	0.75
1-Ccl3	4.08	1.68	2.25	2.1	0.41	0.55	0.51
1-Ccl4	1.74	4.14	0.63	2.17	2.38	0.36	1.25
1-Ccl5	0.34	8.88	0.21	5.35	26.12	0.62	15.74
1-Ccl6	0.25	6.28	0.35	8.22	25.12	1.4	32.88
1-Ccl7	3.66	14.42	2.19	14.83	3.94	0.6	4.05
1-Ccl8	3.94	100.43	0.29	67.18	25.49	0.07	17.05
1-Ccl9	0.34	6.28	0.95	2.62	18.47	2.79	7.71
1-Cx3cl1	0.72	6.73	0.56	1.59	9.35	0.78	2.21
1-Cxcl1	5.46	2.55	2.1	0.54	0.47	0.38	0.1
1-Cxcl10	2.5	26.91	8.51	57.28	10.76	3.4	22.91
1-Cxcl11	N/A	8.88	0.29	5.1	N/A	N/A	N/A
1-Cxcl12	0.42	0.68	0.18	0.34	1.62	0.43	0.81
1-Cxcl13	215.27	0.39	128.89	40.5	0.002	0.6	0.19
1-Cxcl15	3.73	0.78	1.3	0.51	0.21	0.35	0.14
1-Cxcl9	0.75	26.91	0.98	22.47	35.88	1.31	29.96
2-Ccr1	0.2	3.36	0.11	0.59	16.8	0.55	2.95
2-Ccr2	N/A	2.93	0.07	0.69	N/A	N/A	N/A
2-Ccr3	0.63	5.1	0.1	0.68	8.1	0.16	1.08
2-Ccr4	0.75	1.46	0.47	1.67	1.95	0.63	2.23
2-Ccr5	1.12	38.05	0.07	1.16	33.97	0.06	1.04
2-Ccr6	1.03	0.84	0.05	0.66	0.82	0.05	0.64
2-Ccr7	0.32	0.9	0.15	0.33	2.81	0.47	1.03
2-Ccr8	0.54	15.45	0.31	0.95	28.61	0.57	1.76
2-Ccr9	0.8	1.04	0.04	0.16	1.3	0.05	0.2
2-Cxcr3	1.32	7.73	0.75	5.74	5.86	0.57	4.35
2-Il8rb	0.37	0.32	0.04	0.17	0.86	0.11	0.46
2-Xcr1	13.74	0.97	0.23	0.15	0.07	0.02	0.01
3-1-Il13	4.56	7.73	0.26	0.51	1.7	0.06	0.11
3-Cd40lg	1.09	1.46	0.04	0.7	1.34	0.04	0.64
3-Ifng	0.8	10.93	0.86	8.82	13.66	1.08	11.03
3-Il10	2.27	530.06	4.38	6.15	233.51	1.93	2.71
3-Il11	0.41	2.22	0.2	0.41	5.41	0.49	1
3-Il15	0.39	3.14	0.19	0.48	8.05	0.49	1.23
3-Il16	0.1	0.78	0.02	0.11	7.8	0.2	1.1
3-Il17b	0.34	1.04	0.23	0.25	3.06	0.68	0.74
3-Il18	0.56	0.84	0.86	1.01	1.5	1.54	1.8
3-Il1a	1.59	12.55	0.96	1.65	7.89	0.6	1.04
3-Il1b	5.74	3.36	1.21	0.86	0.59	0.21	0.15
3-Il1f6	2.45	1.04	2.99	1.34	0.42	1.22	0.55
3-Il1f8	1.41	0.84	1.26	0.46	0.6	0.89	0.33
3-Il20	2.13	0.84	6.41	1.21	0.39	3.01	0.57

(Continued)

Table 3 | Continued

Gene	WT Inf/Un	MyD88$^{-/-}$ Inf/Un	FcεRγ −/− Inf/Un	MyD88$^{-/-}$ FcεRγ −/− Inf/Un	MyD88$^{-/-}$ ratio/ WT ratio	FcεRγ −/− ratio/ WT ratio	MyD88$^{-/-}$ FcεRγ −/−ratio/ WT ratio
3-Il3	1.27	0.84	4.23	1.05	0.66	3.33	0.83
3-Il4	1.06	14.42	0.33	2.51	13.6	0.31	2.37
3-Itgam	1.44	9.51	0.67	0.58	6.6	0.47	0.4
3-Itgb2	N/A	10.2	3.36	2.81	N/A	N/A	N/A
3-Lta	0.74	0.26	0.97	0.55	0.35	1.31	0.74
3-Ltb	0.31	0.52	0.03	0.24	1.68	0.1	0.77
3-Mif	0.58	2.55	0.41	0.93	4.4	0.71	1.6
3-Scye1	N/A	0.97	0.64	0.81	N/A	N/A	N/A
3-Spp1	0.93	0.24	0.66	0.2	0.26	0.71	0.22
3-Tgfb1	0.16	0.9	0.1	0.27	5.63	0.63	1.69
3-Tnf	0.32	16.56	0.25	0.26	51.75	0.78	0.81
4-Il10ra	0.16	5.46	0.22	0.53	34.13	1.38	3.31
4-Il10rb	N/A	4.44	0.37	0.57	N/A	N/A	N/A
4-Il13ra1	0.82	2.38	0.58	1.36	2.9	0.71	1.66
4-Il1r1	N/A	2.38	0.32	0.41	N/A	N/A	N/A
4-Il1r2	1.85	0.78	1.36	0.99	0.42	0.74	0.54
4-Il2rb	0.18	2.22	0.02	0.45	12.33	0.11	2.5
4-Il2rg	0.29	10.93	0.15	1.03	37.69	0.52	3.55
4-Il5ra	0.99	0.45	0.09	0.44	0.45	0.09	0.44
4-Il6ra	0.56	2.73	0.21	0.74	4.88	0.38	1.32
4-Il6st	0.41	2.07	0.1	0.27	5.05	0.24	0.66
4-Tnfrsf1a	0.03	3.61	0.03	0.07	120.33	1	2.33
4-Tnfrsf1b	0.27	5.46	0.23	0.88	20.22	0.85	3.26
5-Abcf1	0.64	1.27	0.43	0.59	1.98	0.67	0.92
5-Bcl6	0.18	2.55	0.08	0.19	14.17	0.44	1.06
5-C3	0.68	19.03	0.1	2.55	27.99	0.15	3.75
5-Casp1	3.73	1.8	2.1	3.41	0.48	0.56	0.91
5-Crp	1.75	1.11	3.58	0.51	0.63	2.05	0.29
5-Cxcl5	1.12	0.28	0.57	0.3	0.25	0.51	0.27
5-Tollip	0.26	4.14	0.31	0.38	15.92	1.19	1.46
Actb	0.16	2.38	0.26	0.65	14.88	1.63	4.06
Ccr10	0.66	2.22	0.04	0.1	3.36	0.06	0.15
Cxcr5	1.01	0.3	0.14	0.66	0.3	0.14	0.65
Gapdh	0.2	1.68	0.09	0.22	8.4	0.45	1.1
Gusb	0.72	8.28	0.71	1.2	11.5	0.99	1.67
Hprt1	1.41	1.19	2.16	1.74	0.84	1.53	1.23
Hsp90ab1	0.71	0.84	0.65	0.48	1.18	0.92	0.68
Pf4	0.14	0.9	0.23	0.2	6.43	1.64	1.43

PCR-array PAMM—011 (SABiosciences). Values shown in the first four columns are ratios of ΔΔ ct values from uninfected and infected joints from each strain (pooled RNA from joints of four mice per group) calculated using the SABiosciences template for analysis. The last three columns show ratios comparing WT ratios to knockout ratios. The five genes at the end of the table, Gapdh, Gusb, Hprt1, Hsp90ab1, Pf4 are housekeeping genes. Ratios >2 or <0.5 are considered significant in this assay.

of complement by the classical, lectin, or alternative pathways results in deposition of C3b on the bacterial surface, enhancing its killing via C3b-mediated phagocytosis or by membrane attack complex formation. C3$^{-/-}$ mice infected with *B. burgdorferi* harbor ∼3-fold more spirochetes in joints in comparison to wild type mice and exhibit a trend toward more severe arthritis, which could be due to activation of TLRs (Lawrenz et al., 2003).

We and others have previously reported that *B. burgdorferi*-infected MyD88$^{-/-}$ mice have markedly elevated pathogen burdens, but paradoxically, the degree of arthritis is similar to that observed in WT mice (Bolz et al., 2004; Liu et al., 2004). MyD88$^{-/-}$ mice have a shift in *B. burgdorferi*-specific IgG subclass to IgG1. Opsonization of spirochetes or their remnants with this antibody subclass will preferentially activate FcγRIII, which in many model systems is negatively regulated by FcγRIIb

Table 4 | Detection of select cytokines by RT-PCR in 21-day infected mouse joints.

Mouse strain	TNFα	IL-10*	IFNγ
WT Inf/Uninf	0.4	0.0.35	N/A
MyD88$^{-/-}$ Inf/Uninf	136.4	3.5	4.9
FcεRγ$^{-/-}$ Inf/Uninf	3.5	1.3	0.13
FcεRγ$^{-/-}$ MyD88$^{-/-}$ Inf/Uninf	N/A	N/A	15.6

*RT-PCR was performed using the same RNA that was isolated for the PCR Super arrays. The amount of PCR product was estimated from the density of the bands in the agarose gels using ImageJ software. The data reported are the ratio of density values for Infected sample/Infected tubulin control to Uninfected sample/Uninfected tubulin controls. N/A, not available. * The levels of IL-10 PCR product were high for all of the samples thus the magnitude of the differences calculated by ImageJ are lower than what was observed in the PCR array.*

(Nimmerjahn and Ravetch, 2008). This pattern of FcγR engagement in MyD88$^{-/-}$ mice may provide one explanation for the lack of correlation between the high pathogen burden and disease. In addition, MyD88$^{-/-}$ mice express higher levels of the anti-inflammatory cytokine IL-10, which could also lead to reduced arthritis in the setting of elevated pathogen burdens (Wooten et al., 2002).

Our studies using FcεRγ$^{-/-}$ MyD88$^{-/-}$ mice have unveiled a contribution of FcγR and immune complexes to disease. *In vitro*, immune complexes can stimulate MyD88$^{-/-}$ macrophages to produce TNFα, supporting the possibility that FcγR contribute to arthritis in MyD88$^{-/-}$ mice. *In vivo* studies with FcεRγ$^{-/-}$ MyD88$^{-/-}$ double knockout mice reveal lower arthritis severity scores than either of the single knockout strains or in WT mice, suggesting that both FcγR and MyD88-dependent pathways may contribute to the development of arthritis, and each pathway may take on a more prominent role when the other is inactivated. The dominance of TLR/MyD88-dependent inflammation over lipidation-independent signaling via FcγR is supported by an earlier report that *B. burgdorferi*-induced TNFα production by bone-marrow derived macrophages from FcεRγ$^{-/-}$ mice was not significantly reduced compared to wild type macrophages (Talkington and Nickell, 2001).

To gain further insight into the immune molecules involved in arthritis development when FcγR or MyD88-dependent pathways were interrupted, genes expressed in joints from 21 days infected mice were determined using microarray and compared to uninfected control mice of the same genotype. We found differences in genes expressed among the uninfected control WT and the single and double knockout control mice, and each mouse group exhibited additional differences after infection. More striking differences were noted between WT and MyD88$^{-/-}$ mice than between WT and FcεRγ$^{-/-}$ mice. The FcεRγ$^{-/-}$ MyD88$^{-/-}$ mice showed decreased expression of several proteins known to be involved in inflammation as compared to WT and either of the single knockout mice. The most strongly upregulated gene in the joints of all the strains except for the MyD88$^{-/-}$ mice was Cxcl13, a B cell chemoattractant cytokine. Cxcl13 is significantly upregulated in the cerebrospinal fluid of patients with acute neuroborreliosis and may serve as a diagnostic marker for disease (Senel et al., 2010a,b). It has also been found that Cxcl13 mRNA levels are elevated in synovial tissues from rheumatoid arthritis patients, but not in osteoarthritic joints (Shi et al., 2001; Carlsen et al., 2004; Meeuwisse et al., 2011). Cxcl13 may mediate recruitment of immune cells to infected joints during infection, but since the MyD88$^{-/-}$ mice (which have reduced expression of Cxcl13) still develop arthritis, this suggests that other chemokines may also play significant roles. We did not isolate and quantify the cell populations in the joints, but histologically the predominant infiltrate in the inflamed joints was neutrophilic (data not shown). The MyD88$^{-/-}$ mice had large increases in Ccl8 expression (100 fold) and Cxcl 9 and 10 (both 27 fold). The reduced levels of arthritis incidence and severity in the FcεRγ$^{-/-}$ MyD88$^{-/-}$ mice correlated with reduced expression of several chemokines and cytokines compared to the three other strains. Cxcl1, reduced in FcεRγ$^{-/-}$ MyD88$^{-/-}$ mice, functions to recruit neutrophils (Coelho et al., 2008; Conte et al., 2008; Grespan et al., 2008; Ritzman et al., 2010), which are a main component of the cell infiltrates found in arthritic joints of the infected mice. A decrease in expression of Cxcl1 (also known as KC) may reduce neutrophil recruitment. WT and single knockout mice have modest increases in Cxcl1 (ranging from 2–5.5 fold), which may be sufficient in conjunction with other chemoattractants to recruit cells to the joint. IL-1β levels were also found to be lower in the FcεRγ$^{-/-}$ MyD88$^{-/-}$ mice. This may not play a major role in the reduced disease, however, as we have previously shown that caspase 1, which cleaves IL-1β into its active form, is not essential for the control of disease (Liu et al., 2009). The chemokine receptor Xcr1 was also selectively down regulated in the FcεRγ$^{-/-}$ MyD88$^{-/-}$ mice. Xcr1 is the receptor for Xcl1 and Xcl2, which were not part of our microarray, but it is known that Xcrl also plays a role in rheumatoid arthritis (Wang et al., 2004) and in the recruitment of cells to arthritic joints (Coelho et al., 2008; Grespan et al., 2008). Xcr1 was upregulated in the WT mice (13.7 fold) but not in the FcεRγ$^{-/-}$ or MyD88$^{-/-}$ mice. Taken together, these results suggest that multiple chemokines and their receptors contribute to the development of murine Lyme arthritis, and that the molecules involved are similar to those seen in other forms of antigen-induced and autoimmune-mediated arthritis.

In summary, our studies reveal a role for FcγR in limiting pathogen burden and disease in mice early after infection with the extracellular pathogen *Borrelia burgdorferi*. We also show that FcγR may contribute to prolonged arthritis in certain settings, such as in MyD88$^{-/-}$ mice when phagocytosis is impaired and pathogen burden is high. Polymorphisms in FcγR have been found in humans that affect IgG subclass binding and responses (Kapur et al., 2014), which could influence the outcome from Lyme disease. Additional studies are needed to determine whether FcγR and/or polymorphisms in these receptors affect the expression of human Lyme arthritis.

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Forward genetic approaches for elucidation of novel regulators of Lyme arthritis severity

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Patients experiencing natural infection with *Borrelia burgdorferi* display a spectrum of associated symptoms and severity, strongly implicating the impact of genetically determined host factors in the pathogenesis of Lyme disease. Herein, we provide a summary of the host genetic factors that have been demonstrated to influence the severity and chronicity of Lyme arthritis symptoms, and a review of the resources available, current progress, and added value of a forward genetic approach for identification of novel genetic regulators.

Keywords: Lyme disease, Lyme arthritis, forward genetics, pathogenesis, genome-wide association studies, innate immunity and responses, inflammation, beta-Glucuronidase

INTRODUCTION TO LYME DISEASE

Lyme Disease, caused by infection with the tick borne spirochete *Borrelia burgdorferi*, is a growing societal concern, especially in endemic regions of the United States and Europe. Approximately 30,000 case reports are filed by physicians each year in the United States (C.D.C, 2013a), while the CDC has upwardly revised their best estimate of the total incidence to 300,000/year, based on several complementary lines of evidence (Kuehn, 2013). Part of the societal concern is rooted in the uncertainty surrounding pathological outcomes associated with *B. burgdorferi* infection. A large percentage (70%) of infected individuals develop the characteristic bulls-eye rash *erythema migrans* at the site of the infected tick bite, with progression to further clinical complications following dissemination of the spirochete. Arthritis, the most common symptom occurs in 30–60% of infected individuals, while Bell's palsy and other neurological symptoms are seen in 10–12% of patients (Wormser et al., 2006; C.D.C, 2013b). Carditis has been considered a rare complication (<1%), however, 3 recent deaths with documented *B. burgdorferi* in autopsied heart tissue strongly argue for increased vigilance in detecting infection of this tissue (C.D.C, 2013c).

The wide variation in Lyme disease symptoms and severity observed within the patient population is thought to reflect unique features of individual *B. burgdorferi* isolates that influence invasive potential, as well as heritable factors in the patient population that contribute to clinical severity. Furthermore, although most patients resolve infection with appropriate antibiotic therapy, a small percentage of treated patients with severe clinical symptoms fail to resolve and develop a chronic disease termed Post Treatment Lyme Disease (Steere and Glickstein, 2004). Thus, there are compelling reasons to identify host genes that determine the severity of Lyme disease, both in understanding the pathogenic mechanisms of acute clinical disease and in characterizing predisposing features for chronic disease. This review will assess past and ongoing studies that have provided insight into genetic susceptibility to Lyme arthritis, with particular emphasis on studies using Forward Genetic approaches.

WHAT IS FORWARD GENETICS?

Forward genetics is an unbiased genetic approach that begins with a heritable trait of interest and attempts to determine the alleles responsible for the observed variability through a process of genetic mapping. In contrast, reverse genetics is a hypothesis-driven scientific approach that begins with a gene of interest and attempts to determine the phenotypic impacts caused by experimental manipulations of that gene. Classically, forward genetic studies were first performed through random mutagenesis screens. More recently, forward genetics has been used to map pre-existing genetic variations in human populations or experimental animal models. In practice, forward genetic studies involve three key steps: (1) Each individual in a population of mixed genetic composition is surveyed for the trait of interest; (2) The genetic makeup of each individual is assessed; (3) Statistical calculations predict the strength of association in the study population between the measured trait and each genetic locus in the genome. Forward genetic screens often produce a map across the entire genome with peaks and valleys denoting areas with strong or weak statistical association with the trait, respectively.

Scientific investigations into heritable genetic risk factors that contribute to complex disease have been conducted using a variety of approaches, including Genome Wide Association Studies (GWAS) and forward genetic screens in tractable animal models.

GENOME WIDE ASSOCIATION STUDIES AS A METHODOLOGY FOR STUDYING COMPLEX GENETIC TRAITS IN HUMANS - SUCCESSES AND LIMITATIONS

Genome Wide Association Studies were first proposed in the mid-1990s as a way to study association between human genetic polymorphisms and complex, multigenic traits. Rather than measure all genetic variation present in each individual, approximately one million Single Nucleotide Polymorphisms (SNPs) are assayed as genetic landmarks, and scored for association with the trait of interest. Based on linkage disequilibrium, genes in close proximity to associated SNP landmarks are potential candidates for further investigation.

This technique is well-suited to identify susceptibility genes that are of intermediate prevalence in the population, with a Mean Allele Frequency of greater than 0.05 (Risch and Merikangas, 1996). Although no such studies of Lyme arthritis severity have been conducted, GWAS has been extensively used to investigate genetic modulators of other inflammatory conditions including rheumatoid arthritis (RA). Thus far, almost fifty susceptibility loci have been identified for RA, accounting for approximately one-half of the total genetic variation expected in populations of European ancestry. Nineteen of these loci have been refined to a single candidate gene association, and the underlying causal polymorphism has been predicted for seven of these loci (Eyre et al., 2012).

Recent studies with RA and juvenile RA have involved cohorts of up to 10,000 patients and controls, pointing out the requirement for large populations of well-characterized patients for GWAS analysis (Hinks et al., 2013). If sufficiently large sample sizes could be achieved, these findings suggest that GWAS could be a successful strategy to investigate Lyme arthritis susceptibility loci, but also indicate that additional approaches are needed to capture the significant fraction of variation likely to be left unaccounted for.

It is also important to recognize that identification of regulatory loci is not the ultimate goal of a forward genetics study, but is only a first step. While association of a specific genetic landmark to disease susceptibility may have potential relevance to clinical diagnosis, there is added value in the formal investigation of candidate genes and predicted causal polymorphisms, and in further understanding the underlying mechanisms of pathogenesis. This type of mechanistic investigation frequently involves the use of animal models.

USE OF ANIMAL MODELS FOR IDENTIFICATION OF GENES REGULATING DISEASE SEVERITY

Animal models provide an alternative approach for identification of genes that regulate disease development. Inbred mouse lines are powerful genetic resources, which have been widely used to identify genes associated with disease severity. Visionary scientists began the breeding of inbred mice over a century ago. Each modern inbred line has fixed genetic composition, while the plentitude of inbred strains collectively capture a large amount of genetic variation. Several advances in the past decade have significantly added to their value, particularly the publication of the mouse genome, coupled with various efforts to define the genetic variation between inbred mouse strains (Gregory et al., 2002; Keane et al., 2011). More recently, the Collaborative Cross was developed, which represents an ambitious community effort by mouse geneticists to develop approximately 1000 additional recombinant inbred mouse strains with defined genetic composition. These recombinant mice were derived from 8 parental inbred and wild-derived strains through an intricate directed breeding process (Churchill et al., 2004). Due to the increased genetic diversity of mouse strains that can be interrogated simultaneously, the Collaborative Cross is expected to provide additional power to forward genetics screens for many diseases. Together, these more recent developments are expected to provide greater predictive power for identification of regulatory intervals underlying complex multigenic traits.

Prior to the availability of these more sophisticated modern resources, a seminal study by Dr. Stephen Barthold recognized that inbred mouse strains exhibit distinct genetic susceptibilities to Lyme arthritis, recapitulating the range of arthritis severity seen in patients (Barthold et al., 1990). However, mice do not recapitulate the full depth and breadth of symptoms experienced by human patients. This is evident in the inability of *B. burgdorferi* to elicit neurological symptoms or *erythema migrans* in mice (Garcia-Monco and Benach, 2013). Despite these limitations, the finding that C3H mice develop severe arthritis and carditis at reproducible times following intradermal infection with *B. burgdorferi* cultured in the laboratory was very important. The C3H mouse has been used extensively for studies of severe disease, and the involvement of a variety of cell types and signaling pathways have been evaluated. Equally important was the observation that C57BL/6 (B6) mice consistently develop less severe disease despite being equally susceptible to infection and having similar numbers of bacteria in joints. Since many mutant alleles have been crossed onto the B6 background, this has allowed identification of the contribution of numerous immunologically important genes to both host defense and modulation of arthritis and carditis severity.

Mice and other small mammals are essential reservoir species for *B. burgdorferi* in nature, and greater than 90% of trapped wild mice in some Lyme endemic areas have tested seropositive for infection (Bunikis et al., 2004; Radolf et al., 2012). Wild mice are generally resistant to Lyme arthritis, although recent work has shown that natural variants of known innate immune regulatory genes may be correlated with the prevalence of Lyme infection within the wild rodent population (Tschirren et al., 2013). These considerations make the mouse an excellent model for assessment of genetic factors contributing to Lyme arthritis development.

Barthold's findings were corroborated and expanded upon by others. Many groups then addressed specific facets of the immune response and pathogenesis of Lyme arthritis, using the mouse models developed by Barthold, and that important work continues today. Many such studies rely on reverse-genetic approaches, such as targeted genetic deletion, gene silencing, treatment with inhibitory or stimulatory molecules, or transgenic manipulation. For example, the importance of the innate immune response in Lyme pathogenesis was demonstrated by Schaible, Barthold, and Brown who collectively observed that mice with severe combined immunodeficiency (*scid/Rag*$^{-}$), lacking B and T cells,

retained the differential genetic severities in arthritis and carditis observed between inbred mouse strains (Schaible et al., 1989; Barthold et al., 1992; Brown and Reiner, 1999). This finding set a lasting framework for future studies into various facets of the innate immune response. The reverse genetic techniques used in these and other studies are powerful and conclusive, and have resulted in the identification of many genes with documented importance in the pathogenesis of Lyme arthritis and host defense to *B. burgdorferi*. However, these approaches are by nature biased to genes with known function and are not suitable for global analysis of the potential genetic contribution to disease. Selection of a candidate gene necessarily involves assessment of pathways that are suspected to influence the disease process, resulting in the rejection or delay of other non-candidate genes for study.

BRIEF REVIEW OF GENETIC ASSOCIATIONS WITH HUMAN LYME DISEASE AND RELATED MOUSE STUDIES

ROLE OF THE MHC IN IMMUNE RESPONSE TO *B. BURGDORFERI* AND IN ARTHRITIS SEVERITY: HUMAN AND MOUSE STUDIES

Several important studies have discovered natural genetic alleles that influence Lyme arthritis severity. Steere et al. first reported the influence of the human major histocompatibility complex (MHC) on Lyme arthritis severity, and provided important early evidence that Lyme arthritis has an immunogenetic basis (Steere et al., 1990). This pioneering work identified increased incidence of clinical Lyme arthritis, particularly that lasting longer that 12 months in a single joint, as associated with two serologically defined Class II alleles, HLA-DR4 and HLA-DR2. Importantly, the association of Class II alleles with Lyme arthritis was not supported by studies inclusive of all outcomes of Lyme arthritis patients. The advent of molecular characterization of Class II alleles allowed more precise analysis of associations with disease phenotype, and led to the conclusion that MHC alleles are not major determinants of early Lyme disease severity, a distinction from rheumatoid arthritis (Feng et al., 1995; Klempner et al., 2005). More recently, Steere and colleagues have confirmed the association of two Class II alleles (DRB1*0101 and 0401) for the subgroup of patients with treatment refractory Lyme disease but not in the larger group of patients that respond to antibiotic treatment, and have proposed an auto-immune mechanism in this treatment refractory group (Steere et al., 2006; Drouin et al., 2013).

A number of investigators found association of MHC haplotypes with antibody recognition of individual *B. burgdorferi* antigens using MHC congenic mouse lines. However, use of MHC congenics in our studies and in those of other investigators led to the conclusion that MHC alleles were not determinants for the differences in arthritis severity found 4 weeks following infection in C3H-$H2^k$, C57BL/6-$H2^b$, and DBA-$H2^d$ mice (Yang et al., 1992; Brown and Reiner, 2000). Thus, studies with mice are consistent with patient studies failing to show association with early Lyme arthritis. Interestingly, mice expressing the $H2^k$ allele do not develop collagen-induced arthritis, a contrast with their development of severe Lyme arthritis (Wooley et al., 1981).

IDENTIFICATION OF TLR1/TLR2 IN THE HOST RESPONSE TO *B. BURGDORFERI* IN HUMANS AND MICE

Early seminal studies into the host-pathogen interaction of *B. burgdorferi* revealed the potential of the spirochete and its lipoproteins to induce inflammatory cytokine production in a variety of human and mouse cell types (Radolf et al., 1991; Wooten et al., 1996; Sellati et al., 1998). The association of NF-κB with these inflammatory responses directed numerous laboratories to investigate the involvement of Toll-like receptors as these molecules were discovered as central components of inflammatory responses to microbial pathogens (Wooten et al., 1996; Sellati et al., 1998). These studies documented the interactions between *B. burgdorferi* lipoproteins with TLR2 and TLR1, both with mouse knock-out and cell culture transfection studies and in patients, and established a critical role for TLR signaling through MyD88 in host defense to this pathogen (Aliprantis et al., 1999; Brightbill et al., 1999; Hirschfeld et al., 1999, 2000; Alexopoulou et al., 2002). More recent studies by Schroder et al. identified a human variant in TLR2, Arg753Gln, with reduced pro-inflammatory signaling in patient samples (Schroder et al., 2005). Cells from mice heterozygous for this variant also displayed reduced inflammatory responses to *B. burgdorferi* lysate. Notably, this TLR2 allele was significantly underrepresented within a cohort of late stage Lyme disease patients, suggesting that it has a protective effect.

Oosting et al. found that N248S and S602I polymorphisms in TLR1 were associated with reduced *in vitro* responsiveness to *B. burgdorferi* and TLR1/TLR2 agonist stimulation (Oosting et al., 2011a). Using a similar experimental approach, the same group also reported that peripheral blood mononuclear cells (PBMCs) for individuals bearing an IL-23R Arg381Gln polymorphism exhibited a reduced Th17 response following *in vitro* stimulation with *B. burgdorferi* (Oosting et al., 2011b). However, there was no association between the IL-23R polymorphism and the persistence of symptoms among patients in the study population, arguing against a role for this SNP in disease pathogenesis.

Strle et al. recently described the frequency and impact of several polymorphisms in the TLR1 gene within a cohort of Lyme disease patients (Strle et al., 2012). This study found a skewed inheritance pattern of TLR1 1805GG polymorphisms within an antibiotic-refractory Lyme arthritis patient population. They also recognized a synergy between inheritance of this host polymorphism and infection with a particular invasive isolate (termed RST1) of *B. burgdorferi*. Importantly, patients carrying TLR1 1805GG exhibited higher serum levels of CXCL9 and CXCL10 chemokines, consistent with a functional role for this polymorphism. This effect was reproduced through *in vitro* activation of PBMCs with a *B. burgdorferi* RST1 isolate, arguing that heightened production of these IFNγ-inducible chemokines may set the stage for antibiotic refractory arthritis.

THE POWER OF FORWARD GENETICS

Forward Genetic approaches attempt to determine which genetic loci are responsible for a phenotype of interest. In general, individuals are generated with genotypes that have been altered in an unbiased way, followed by analysis to map inheritance of the phenotype of interest to specific genetic loci. This was made possible

by the development of genetic maps of microsatellite landmarks evenly distributed throughout the genome, the utility of which Paterson et al. first demonstrated for Quantitative Trait Locus (QTL) mapping in plants, later followed by Todd et al. in mice (Paterson et al., 1988; Todd et al., 1991). Model organisms are often studied through QTL analysis followed by the breeding of recombinant inbred congenic lines to isolate regulatory loci, and the Collaborative Cross is a more expansive modern variation of this theme that combines these two steps together. QTL mapping of disease susceptibility in mice has the potential to yield a veritable avalanche of information about complementary facets of disease initiation and pathogenesis. For example, efforts by Edward Wakeland and others to determine differential susceptibility to systemic lupus erythematosus (*sle*) between resistant and acutely lupus-prone inbred mouse strains led to the identification of *Ly108* and other SLAM family members as key modulators of B cell tolerance (Kumar et al., 2006), lack of proper *Fcgr2b* upregulation as a potentiator of IgG production (Rahman et al., 2007), *Cr2* or other closely linked genes as mediators of autoreactive B- and T-cell production (Chen et al., 2005; Tchepeleva et al., 2010), and hemostatic *kallikreins* as important regulators of kidney pathogenesis (Liu et al., 2009).

Other successes include the identification of genes important in the regulation of animal models of rheumatoid arthritis and autoimmunity by comparing disease susceptible and disease resistant mouse strains (Ma et al., 2002; Glant et al., 2004; Wicker et al., 2005; Ahlqvist et al., 2009). In some cases, QTL mapping efforts have bridged gaps between seemingly distinct experimental models of autoimmune and other inflammatory diseases through the identification of shared immunopathology loci (Teuscher, 1985; Meeker et al., 1995; Teuscher et al., 1996, 1997, 1998; Del Rio et al., 2008; Spach et al., 2009, 2010) and identification of the relevant functional polymorphisms (Sudweeks et al., 1993; Ma et al., 2002).

The first step of QTL analysis in mice is the direct interbreeding of two strains of interest to generate a large cohort of genetically distinct individuals (**Figure 1**). F_1 hybrids are genetically identical, carrying one copy of each chromosome from each parental line. These hybrids can be backcrossed to either parental strain (BC1), or interbred to generate F_2 hybrids. In each case, genetic variability among the offspring is generated by random recombination events between sister chromatids during meiosis. Each interbreeding strategy can identify regulatory alleles with a dominant, codominant, or additive effect. F_2 intercross populations allow the identification of alleles acting in recessive fashion that are capable of "standing alone," whereas the BC1 populations have the added advantage of allowing identification of genetic alleles whose effect is most apparent in the genetic context of a particular inbred background. However, hybrids backcrossed to a parental strain are not expected to detect any phenotype from recessive alleles bred back to a dominant parent.

CURRENT PROGRESS: MAPPING QUANTITATIVE TRAIT LOCI THAT REGULATE LYME ARTHRITIS SEVERITY IN MICE

Janis Weis, Cory Teuscher, and their collaborators performed the first murine Lyme arthritis QTL analysis (Weis et al., 1999). This initial study used an F_2 intercross between C57BL/6N (B6) and C3H/HeN (C3H) mice, with 150 total male and female mice included in the cohort. Each individual was assessed for seven quantitative traits and for genetic composition, which was determined using 195 microsatellite markers distributed throughout the genome. Permutation threshold analysis was then used for the entire cohort to determine the degree of association between these quantitative traits and the parental derivation of specific loci. Four distinct regions on chromosomes 4, 5, and 11 were found to regulate arthritis severity traits as measured by caliper measurement of ankle swelling and by blinded scoring of a number of microscopically assessed histopathology traits. Five additional loci on chromosomes 6, 9, 11, 12, and 17 were found to regulate *B. burgdorferi*-specific humoral IgM and IgG responses independently of arthritis severity.

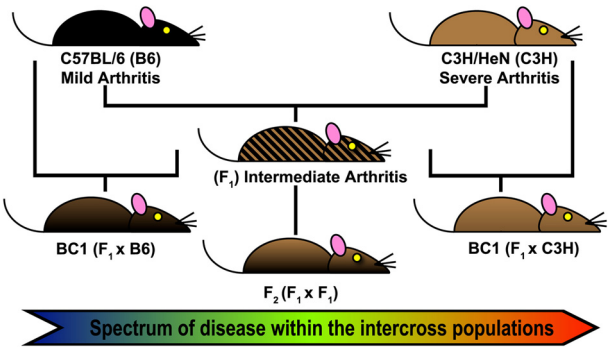

FIGURE 1 | Forward Genetics approach for Lyme arthritis severity. Intercross populations of B6 and C3H mice were used for identification of Quantitative Trait Loci (QTL) regulating *Borrelia burgdorferi* associated arthritis and other responses related to infection (*Bbaa*). Arthritis and other metrics of host response were assessed at 4 weeks of infection. A total genome scan was performed for each infected mouse ($n = 450$) and threshold permutation analysis identified loci associated with disease and response.

This foundational study was followed up by Roper et al. with additional QTL experiments using reciprocal $F_1 \times$ B6 and $F_1 \times$ C3H backcrosses and a (BALB/c $\times$ C3H) $F_1 \times$ C3H intercross that found 12 new QTL on Chromosomes 1, 2, 4, 6, 7, 9, 10, 12, 14, 15, 16, and 17 regulating a variety of traits (Roper et al., 2001). A total of twenty-three QTL were identified that regulate metrics of arthritis severity (**Figure 2**, red) or other traits related to the humoral response, inflammatory response, or host defense (**Figure 2**, blue). As predicted from previous MHC congenic studies, none of the arthritis-associated QTL identified in the three B6:C3H intercrosses identified the MHC locus on chromosome 17. Interestingly, ankle swelling did associate with this region in a single backcross (BALB/c $\times$ C3H) $F_1 \times$ BALB/c, with a lod score of 3.1, predicting an association with one or more of the numerous class I, class II, or class III genes in this region. Seven of the 23 QTL were reproduced in multiple crosses (*Bbaa2*, *Bbaa6*, *Bbaa8*, *Bbaa10*, *Bbaa12*, *Bbaa14*, and *Bbaa15*). The *Bbaa2* QTL on Chromosome 5 was reproduced in all four intercross experiments, and in every case the arthritis severity originated from the C3H parental strain. The lod scores identifying *Bbaa2* ranged from 3.5 to 10.2 for the four intercross populations, with the 10.2

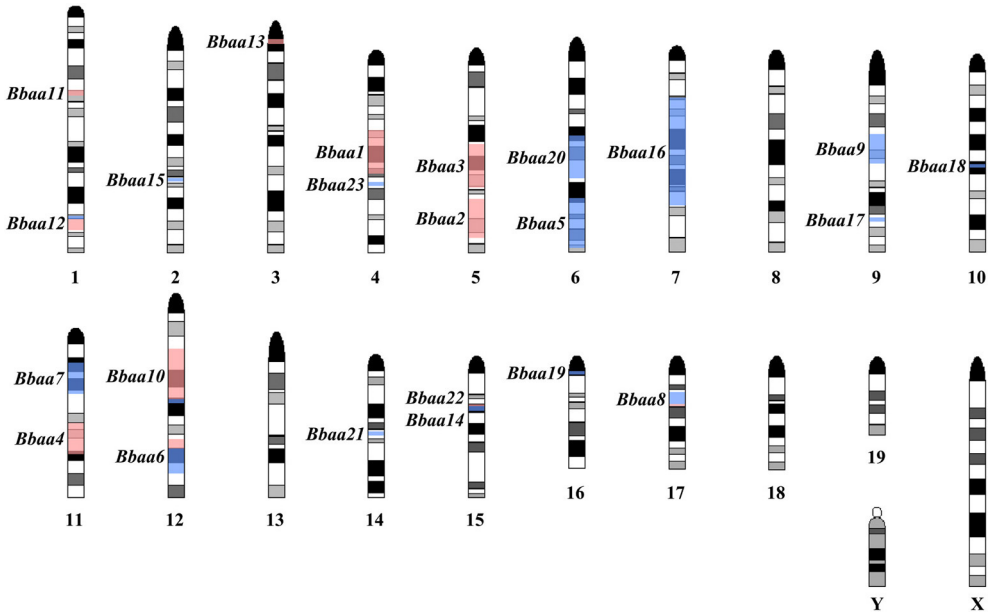

FIGURE 2 | Location of predicted Quantitative Trait Loci, summarized from Weis et al. (1999) and Roper et al. (2001). Black and gray bands on each chromosome denote an idealized representation of the Giemsa banding pattern found in a normal mouse karyotype. Red shaded regions denote QTL regulating arthritis severity (ankle swelling, histopathology score, tendon sheath). Blue shaded regions denote QTL regulating humoral immune response (Total/Specific IgM levels, Total/Specific IgG levels, serum IL-6) or host defense (*B. burgdorferi* bacterial burden).

lod value detected in the (BALB/c × C3H) F_1 × C3H intercross. This study also predicted that the combined *Bbaa2Bbaa3* locus contains at least four distinct regulatory genes. It is possible that some of these loci may be implicated in other QTL studies, but the use of different inbred strains of mice and the extensive polymorphism of this region of the genome among strains confounds the ability to directly extrapolate between studies (Lindvall et al., 2009).

Based on these and other data, several subsequent studies generated congenic mouse strains to isolate putative regulatory loci in the context of an otherwise uniform genetic background. This laborious and time-intensive process is essential to convert the statistically predicted loci derived from QTL analysis into physical genetic boundaries. Congenic lines can also be used to formally interrogate potential candidate genes, by determining if the phenotype of interest is retained after such a candidate gene is excluded from the congenic interval. The presence of a strongly penetrant phenotype within a congenic interval is a strong predictor of success in further steps of positional cloning.

Subsequent studies used congenic mice based on the initial QTL assignments. Crandall et al. described the phenotype of two B6 × C3H congenic lines, and the evaluation of a specific candidate gene (Crandall et al., 2005). B6 × C3H F_1 mice were backcrossed seven times onto each parental background, producing reciprocal congenic lines B6.C3H-*Bbaa2Bbaa3* and C3H.B6-*Bbaa2Bbaa3*. Of note, the congenic nomenclature in mice differs from other systems, with the background strain listed first, followed by the donor strain, followed by the introgressed locus (Jackson Laboratory, 2000). *Bbaa2Bbaa3* from the C3H donor strain was found to confer increased Lyme arthritis severity on a resistant B6 background, while B6 derived *Bbaa2Bbaa3* conferred reduced severity to susceptible C3H mice, in a reciprocal fashion. This publication also described a polymorphism carried by C3H mice in the *Ncf1* gene, but ruled out this candidate with a variety of studies, including the finding that B6 $Ncf1^{-/-}$ mice exhibited no increase in arthritis severity relative to wild type B6 controls.

Ma et al. reported the generation of additional B6xC3H reciprocal congenic lines for five intervals identified in the foundational QTL study (*Bbaa1*, *Bbaa2Bbaa3*, *Bbaa4*, and *Bbaa6*), plus another pair of reciprocal congenic lines for an interval on Chromosome 1 (*Bbaa12*) (Ma et al., 2009). Through marker-assisted selection over the course of seven iterative backcrosses, these intervals were isolated and found to be free of genetic contamination on other chromosomes, with congenic intervals ranging from 25 to 146 megabases in size. *Bbaa2Bbaa3* and *Bbaa4* were found to reciprocally transfer the ankle swelling and histopathology phenotypes, while the B6 allele of *Bbaa6* transferred protection from ankle swelling and histopathology to the C3H background. Other congenic intervals conferred no change in arthritis severity or gave inconsistent results. This study also demonstrated the added utility of congenic lines as an experimental resource through comparative microarray gene expression profiling.

In the process of further refining these congenic intervals, Bramwell et al. described the implementation of high throughput SNP genotyping and high resolution melting analysis (Bramwell et al., 2012). This improved genotyping methodology took advantage of the recently published Sanger high-resolution sequence of the C3H mouse, allowing enhanced comparison with the previously published genome of the B6 reference strain (Keane et al.,

2011). The Sanger database revealed the precise location of thousands of SNPs distinguishing B6 and C3H genomic sequences within the 20 Mbp *Bbaa2* interval, exponentially increasing the ability to discriminate donor sequences and define boundaries of congenic mice, and allowing the genetic composition of the congenic lines to be tested with greater precision (**Figure 3**). As an added benefit, the screening process was accelerated, helping to reduce expenses by allowing litters to be screened prior to weaning age.

This line of investigation has recently culminated in the identification of the first definitive natural regulator of Lyme arthritis severity in laboratory mice. With further backcrossing and refinement of the B6.C3H-*Bbaa2* congenics, Bramwell et al. describe the generation of 14 new advanced congenics that delimit the boundaries of several regulatory sub-intervals (**Figure 4**) (Bramwell et al., 2014). Notably, these intervals bear striking resemblance to several of the maximal linkage peaks predicted previously (Roper et al., 2001). One narrow 1.5 Mb C3H-derived interval, surrounding and including the highest peak of linkage predicted by QTL analysis at D5Mit30 on Chromosome 5, was able to independently confer an increased arthritis severity phenotype in the context of a resistant B6 genetic background. Close scrutiny of this interval revealed only a single coding-non-synonymous polymorphism between B6 and C3H mice. This point mutation in the lysosomal enzyme beta-Glucuronidase leads to a partially hypomorphic allele ($Gusb^{h}$) in the C3H, AKR, and CBA/J inbred strains. *Peromyscus* mice, which do not exhibit Lyme arthritis but serve as important reservoir hosts for *B. burgdorferi* in nature, appear to carry the wild-type B6 allele of *Gusb* (GenBank Accession XM_006971357). The exacerbated Lyme arthritis effect conferred by $Gusb^{h}$ was recapitulated in a spontaneous *Gusb* mutant mouse line ($Gusb^{Null}$), and transgenic overexpression of wild type $Gusb^{b}$ in C3H mice ($Gusb^{Tg}$) profoundly reduced ankle swelling and histopathology. The $Gusb^{h}$ congenic line was further tested in an experimental model of RA, the K/BxN serum transfer model (Monach et al., 2008). Disease severity in this model is induced by autoantibodies generated against glucose-6-phosphate isomerase, a ubiquitous glycolytic enzyme. Importantly, transfer of this serum induces a joint specific inflammatory arthritis that occurs independently of the MHC haplotype of the recipient and reflects the effector phase of arthritis development. Much greater arthritis severity was observed in $Gusb^{h}$ congenic mice than in wild type B6 control animals, revealing a common mechanism for the pathogenesis of Lyme arthritis and rheumatoid arthritis. Thus, the identification of genes important in Lyme arthritis also illuminated previously unrecognized pathways in RA. This linkage to a gene associated with Sly syndrome, an overt congenital lysosomal storage disease (LSD), strongly implicated a common pathogenic mechanism involving accumulation of undigested glycosaminoglycans (Tomatsu et al., 2009). This possibility was confirmed by detection of pronounced Alcian blue staining of sulfated GAGs in the inflamed joint tissues of *B. burgdorferi* infected and K/BxN treated mice with partial or severe *Gusb* deficiencies (**Figure 5**). The association of $Gusb^{h}$ with increased disease severity in both Lyme-associated and rheumatoid arthritis identifies *Gusb* as a shared immunopathology disease gene (Teuscher, 1985; Sudweeks et al., 1993; Ma et al., 2002).

The novelty of the beta-Glucuronidase polymorphism highlights the power and added value of forward genetic approaches. *Gusb* is most often cited in the recent scientific literature as a housekeeping gene, primarily used as a reference to study something more interesting, making it a most unlikely candidate for a hypothesis-driven reverse genetics study. Allelic variants of the *Gusb* gene were found not to be differentially expressed under

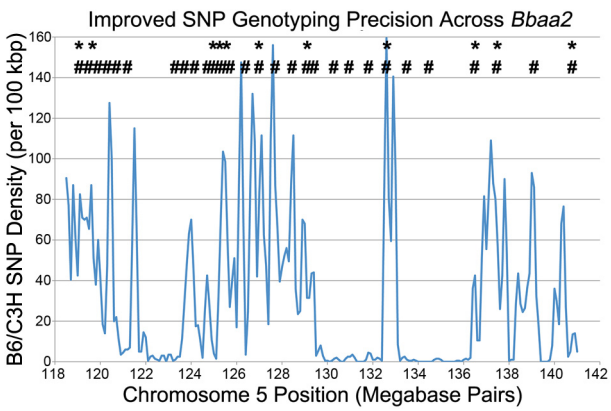

FIGURE 3 | A SNP genotyping methodology improves congenic mapping precision, adapted from Bramwell et al. (2012). * - Location of all 11 microsatellite markers that can differentiate between B6 and C3H DNA across *Bbaa2*. **#** - Of the thousands of SNPs available (blue line), the 28 positions that were developed into SNP genotyping assays for improved discrimination of sub-interval congenic lines (Bramwell et al., 2014).

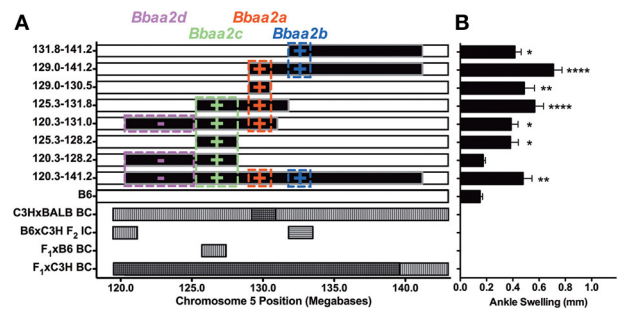

FIGURE 4 | Regulatory loci identified by analysis of advanced congenic lines, modified from Bramwell et al. (2014). (A) Each of the top 8 horizontal bars represents one B6.C3H-*Bbaa2* sub-interval congenic mouse line. The horizontal axis represents the position of *Bbaa2* on mouse Chromosome 5 (120.3–141.2 Mb). The black portions of each row are derived from the C3H genetic background and the white portions are derived from the B6 background. Colored boxes indicate the position boundaries and predicted effect (+/−) of multiple regulatory intervals identified by advanced congenic lines. The lower 4 rows indicate arthritis severity QTL intervals predicted by Roper et al. (2001) for the backcross/intercross populations listed on the left vertical axis. Horizontal hatching denotes QTL for ankle swelling, vertical hatching denotes QTL for histopathology or tendon sheath thickening, cross hatching denotes overlap of multiple predicted QTL. **(B)** Ankle swelling measurements for the eight congenic lines listed in A, with significance assessed relative to B6 negative control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

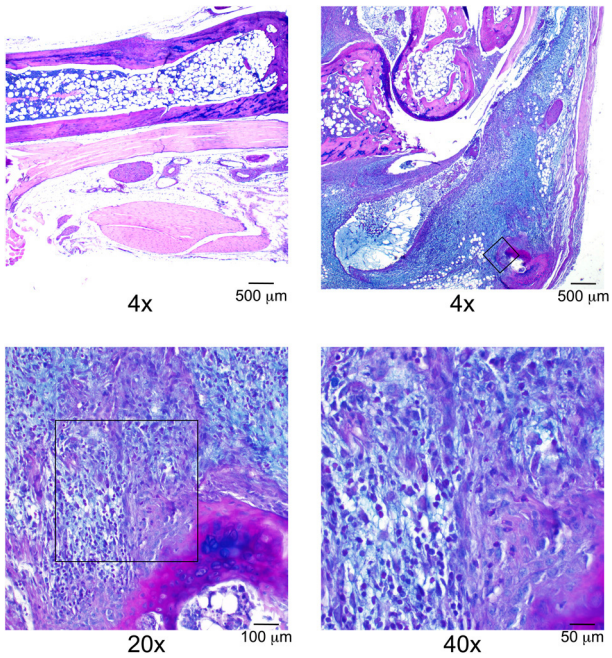

FIGURE 5 | Alcian blue staining reveals excess deposition of GAGs within severely arthritic ankle joints, modified from Bramwell et al. (2014). Top left panel: Ankle joint section from a day 7 K/BxN treated B6 mouse at ×4 magnification. Remaining panels: Ankle joint section from a day 7 K/BxN treated B6.C3H-*Gusb*^{h} mouse. Original magnification ×4, ×20, and ×40. Scale bars: 500, 100, 50 μm, respectively. Boxes on the top right and lower left images indicate the location of the field magnified in subsequent images.

baseline conditions, and no changes in *Gusb* expression were detected by microarray analysis of joint tissue from naïve and infected C3H and B6 mice (Crandall et al., 2006; Bramwell et al., 2014). Thus, *Gusb* and other similar genes associated with LSD are not likely to be picked up by a microarray or RNA-Seq study in Lyme arthritis patients. *Gusb* was also not included in the ImmunoChip used in human RA and juvenile RA studies, because it had not yet been identified as a potential regulator (Eyre et al., 2012; Hinks et al., 2013). As mentioned earlier, recent development of an expanded group of recombinant inbred strains incorporating 8 strains of laboratory and wild mice would appear to be a powerful resource for investigating regulators of Lyme arthritis severity. However, none of the three inbred strains carrying the *Gusb*^{h} polymorphism were included in the Collaborative Cross, so it could not have been identified through this approach. Against all odds, the identification of *Gusb* is a prime example of how relentlessly following the phenotype throughout a process of unbiased genetic refinement can overcome preconception and bias to lead the way to truly novel and unexpected discoveries.

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Emergence of *Ixodes scapularis* and *Borrelia burgdorferi*, the Lyme disease vector and agent, in Ohio

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Lyme disease, the most common vector-borne disease in the United States, is caused by a tick-borne infection with *Borrelia burgdorferi*. Currently, Ohio is considered by the Centers for Disease Control and Prevention (CDC) to be non-endemic for Lyme disease. The low incidence of Lyme disease in this state was largely attributed to the absence of the transmitting vector, *Ixodes scapularis*, commonly known as the blacklegged tick. However, a tick surveillance program established by Ohio Department of Health indicated that the number of *I. scapularis* in Ohio had increased sharply in recent years, from 0 - 5 ticks per year during 1983–2008 to 15 in 2009, 40 in 2010, and 184 in 2011. During the fall deer hunting season, examination of deer heads submitted to Ohio Department of Agriculture found 29 *I. scapularis* from 7 counties in 2010 and 1,830 from 25 counties in 2011. As of 2012, the tick had been found in 57 of the 88 counties of Ohio. In addition, all three active stages (larva, nymph, and adult) of *I. scapularis* were found in Tiverton Township of Coshocton County, demonstrating the presence of established tick populations at this central Ohio location. Of 530 nymphal or adult *I. scapularis* analyzed by quantitative polymerase chain reaction (qPCR), 32 (6.1%) tested positive for the *B. burgdorferi flaB* gene, ranging from 36 to 390,000 copies per tick. Antibodies to *B. burgdorferi* antigens were detected in 2 of 10 (20%) field-captured *Peromyscus leucopus* from Tiverton Township, and in 41 of 355 (11.5%) dogs residing in Ohio. Collectively, these data suggest that the enzootic life cycle of *B. burgdorferi* has become established in Ohio, which poses risk of Lyme disease to people and animals in the area.

Keywords: Lyme disease, Ohio, *Borrelia burgdorferi*, *Ixodes scapularis*, *Peromyscus leucopus*

INTRODUCTION

Since its initial description in late 1970's (Steere et al., 1977), Lyme disease has been recognized as the most common vector-borne disease in temperate regions of the northern hemisphere (Steere, 2001; Stanek et al., 2012). The disease is caused by spirochetes belonging to the *Borrelia burgdorferi* sensu lato complex, which is maintained by an enzootic life cycle typically involving *Ixodes* species ticks and small vertebrate hosts (Piesman and Gern, 2004). In the United States, Lyme disease is highly endemic in two distinct regions, one in the Northeastern states and the other in the Upper Midwestern states (Orloski et al., 2000; Bacon et al., 2008). In these regions, the blacklegged tick *Ixodes scapularis* is the transmitting vector, and the white-footed mouse *Peromyscus leucopus* serves as a common reservoir host for the spirochete (Piesman and Gern, 2004). Lyme disease is also reported in the Western United States, where the western blacklegged tick *I. pacificus* is the transmitting vector and the dusky-footed wood rat *Neotoma fuscipes* and California kangaroo rat *Dipodomys californicus* are the main reservoir hosts (Brown and Lane, 1992).

Ohio is situated between the Northeastern and the Upper Midwestern Lyme disease-endemic regions of the US, and it historically had a low incidence of diagnosed Lyme disease. According to CDC data from 2007 to 2012, the annual incidences of Lyme disease in Ohio and the three surrounding states to its west, Michigan, Indiana, and Kentucky, were all <1 case per 100,000 people; the annual incidences in the two states to the east of Ohio were higher, ranging 4–8 cases per 100,000 people in West Virginia and 26–37 cases per 100,000 people in Pennsylvania, a highly endemic area. The low incidence of Lyme disease in Ohio was largely attributed to the absence of *I. scapularis* in the area (Dennis et al., 1998; Hoen et al., 2009; Rollend et al., 2013). Here, we showed that since 2009, there had been an exponential increase in the number of *I. scapularis* found in Ohio. To further assess the risk of Lyme disease in Ohio, we investigated if there were established *I. scapularis* populations in Ohio, if the ticks were infected with *B. burgdorferi*, and if white-footed mice and dogs in Ohio had been exposed to *B. burgdorferi*. Our data suggest that the enzootic life cycle of *B. burgdorferi* has become established in Ohio.

MATERIALS AND METHODS

OHIO DEPARTMENT OF HEALTH TICK SURVEILLANCE PROGRAM

In 1983, Ohio Department of Health (ODH) began soliciting ticks from the general public, hospitals, physicians, and local health departments in an effort to determine the distribution and dynamics of pathogens in Ohio tick populations. This program of passive tick surveillance for all tick species continued through 2012 and was promoted through media, university extension fact sheets, health department newsletters, presentations and select mailings (Pretzman et al., 1990). Active surveillance to specifically search for *I. scapularis* began in 1986, which included examination of trapped rodents, flagging vegetation at suspect Lyme disease locations, and examination of deer brought to Ohio Department of Natural Resources (ODNR) deer check stations. Tick collection records and specimens were maintained at ODH. Due to a loss of funding, the ODH tick surveillance program was discontinued in 2013.

EXAMINATION OF DEER HEADS FOR TICKS

From 2002 to 2011, Ohio Department of Agriculture (ODA) worked with ODNR and ODH to conduct surveillance of chronic wasting disease (CWD) during the fall deer hunting season by examining hunter-harvested deer heads. These deer heads were also examined for ticks. This active surveillance for CWD was discontinued in 2012.

TICK SURVEY IN TIVERTON TOWNSHIP

From March to November of 2010, ticks were collected from Tiverton Township in Coshocton County, Ohio by two different methods, flagging ticks from vegetation and soliciting ticks from local residents who found and removed them from people or domestic animals. All ticks collected in the area were identified by Glen R. Needham to be either *I. scapularis* or *Dermacentor variabilis* based on morphology.

QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR) FOR DETECTION OF *B. BURGDORFERI* DNA

DNA was purified from individual or pooled ticks using the DNeasy Blood and Tissue Kit (QIAGEN) following the manufacturer's protocol, and qPCR reactions (25-μl each) were set up in 96-well plates using TaqMan Universal PCR Master Mix (Applied Bioscience) and a pair of primers and a TaqMan probe specific for the *B. burgdorferi flaB* gene (Li et al., 2007). The usage of a sequence-specific fluorescent probe in qPCR analysis not only provides a higher level of specificity than conventional PCR but also allows real-time detection of signals, circumventing the need of analyzing samples by gel electrophoresis. Reactions were run on a MX3005P QPCR system (Agilent Technologies) and data were analyzed using the MxPro QPCR software (Agilent Technologies). For determination of gene copy number, reactions containing 10-fold serial dilutions of DNA with known concentration were included on each plate. This generated a standard curve to extrapolate gene copy numbers from the threshold cycle (Ct) numbers. Given that only 5% of the total DNA from each tick was analyzed in a reaction, the theoretic limit of detection was 20 copies of *B. burgdorferi* genome per tick.

RODENT TRAPPING IN TIVERTON TOWNSHIP

In September of 2010, rodent trapping was carried out in Tiverton Township for two consecutive nights. Sherman live traps (150 at the first night and 180 at the second night) were set using sunflower seeds and peanut butter sandwiches as bait. Captured rodents (3 from the first night and 7 from the second night) were euthanized by barbiturate overdose followed by heart exsanguination, and then checked for the presence of ticks. All 10 captured rodents were morphologically identified to be *P. leucopus* by John Harder, a mammalogist at The Ohio State University. Wildlife procedures were carried out in accordance with the Animal Welfare Act and were approved by ODH as part of disease surveillance investigation.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Serum samples were tested for IgM and IgG antibodies against *B. burgdorferi* antigens by enzyme-linked immunosorbent assay (ELISA). ELISA was performed in 96-well flat-bottom Immulon 1B plates (Thermo Scientific) according to a standard protocol (Hornbeck et al., 2001). Briefly, the plates were coated with 100 ng/well whole-cell lysate of *B. burgdorferi* type strain B31. Serum samples were assayed at a 1:400 dilution. The secondary antibodies, either goat anti-mouse IgM or IgG or goat anti-dog IgG, were conjugated with horseradish peroxidase (HRP) (Kirkegaard & Perry Laboratories) and used at a 1:1000 dilution. Signals were developed using the TMB Microwell Peroxidase Substrate System (Kirkegaard & Perry Laboratories) and measured using a SpectraMax M2 plate reader (Molecular Devices, LLC). For the study of *B. burgdorferi* seroprevalence in Ohio dogs, the titer of a highly positive serum sample was determined using the serial dilution method. Eight two-fold serial dilutions of this sample as well as eight blank wells were included on each ELISA plate, which were used for normalization to minimize plate-to-plate variation and for generating standard curves to extrapolate titers of unknown samples from the absorbance values.

IMMUNOBLOT ANALYSIS

Borrelia burgdorferi whole cell lysate was separated on a sodium dodecyl sulfate-12% polyacrylamide gel prepared using the Mini-Protean Tetra hand cast system and a prep/2-D well comb (Bio-Rad Laboratories), and then transferred to a nitrocellulose membrane. Mouse serum samples were incubated at a 1:100 dilution with the membrane in a Mini-Protean II multiscreen apparatus (Bio-Rad Laboratories). After incubation with HRP-conjugated secondary antibodies, goat anti-mouse IgM or IgG, signals were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) following manufacture's protocol, and captured on X-ray films.

ViraStripe® TEST

ViraStripe® Lineblot test kits (Viramed) received clearance from the Food and Drug Administration (FDA) in December 2009 for use in clinical diagnosis of human Lyme disease. The IgG Lineblot uses all 10 native *B. burgdorferi* B31 proteins recommended by the CDC for serological evaluation of human exposure to the Lyme disease spirochete. Serum samples from dogs

and rodents were analyzed according to the manufacturer's protocol with only one modification—the alkaline phosphatase (AP)-conjugated secondary antibodies were goat anti-mouse IgM for the rodent samples and rabbit anti-dog IgG for the dog samples. Both of these conjugates were obtained from the Jackson ImmunoResearch Laboratories. The positive, cut-off, and negative controls were processed as instructed using the anti-human IgG-AP conjugate provided in the kit. Signals were developed using the chromogen/substrate solution provided in the kit, and reactions were stopped when the cut-off control was clearly visible. Based on the CDC recommendations, serum samples that reacted with at least 5 of the 10 proteins above the cut-off intensity were interpreted as positive.

STATISTICAL ANALYSIS

All statistical and graphic analyses were performed using the software GraphPad Prism 5 for Windows (version 5.0.1). All reported P values are 2-tailed. P values < 0.05 are considered statistically significant. The statistical test for each P value is indicated in the text.

RESULTS

EVIDENCE FOR THE EMERGENCE OF *I. SCAPULARIS* IN OHIO

In 1989, a nymph collected in Butler County became the first *I. scapularis* confirmed in Ohio by the ODH tick surveillance program. The number of *I. scapularis* collected annually remained low from 1989 to 2008, averaging 1.75 (range, 0–5), accounting for well below 1% of ticks collected. Numbers of *I. scapularis* began to increase after 2008, with 15 in 2009, 40 in 2010, 184 in 2011, and 182 in 2012 (**Figure 1**). By 2012, *I. scapularis* ticks accounted for 24.8% of ticks collected by ODH tick surveillance program. Of the 456 *I. scapularis* collected 1989–2012, there were 315 (69%) female, 127 (28%) male, 13 (3%) nymphs, and 1 larva.

Between 2002 and 2008, no *I. scapularis* were found from examination of approximately 200–500 deer heads every year during the fall deer hunting season as part of the multi-agency surveillance of chronic waste disease (CWD) in Ohio. Almost all ticks found on deer heads during this period were *Dermacentor albipictus*, the one-host winter tick. In 2009, deer heads examined for CWD were not examined for ticks. In 2010, 29 *I. scapularis* were recovered from 12 (∼6%) of approximately 200 deer heads examined. In 2011, 1,830 *I. scapurlaris* were recovered from 96 (17%) of 560 deer heads examined (**Table 1**). All 1,859 *I. scapularis* collected from deer heads were adults, 59% male and 41% female. The 29 ticks recovered in 2010 were from 7 counties, whereas the 1,830 ticks recovered in 2011 were from 25 counties (**Figure 2**). There was no noticeable increase in the number of *D. albipictus* found on deer heads in 2010 and 2011.

Taken together, as of 2012, *I. scapularis* ticks were found in 57 of the 88 counties in Ohio (**Figures 1**, **2**). The fact that these surveillance activities had been established for many years, and the increase in *I. scapularis* (both in numbers and in geographical ranges) was not observed until 2009 provides strong evidence that *I. scapularis* is an emerging vector in Ohio.

EVIDENCE FOR ESTABLISHED *I. SCAPULARIS* COLONIES IN TIVERTON TOWNSHIP

On March 8, 2010, 10 adult *I. scapularis* were collected by flagging in less than 30 min. Between March and November of 2010, 207 adult and >200 larval *I. scapularis* were flagged from vegetation, and 52 nymphal and 14 adult *I. scapularis* were submitted by local residents who found and removed the ticks from people

Table 1 | *Ixodes scapularis* found on deer heads during the fall deer hunting season.

Year	Number of deer heads examined for ticks	Number of deer heads with *I. scapularis*	Number of *I. scapularis* (female/male)	Number of counties where *I. scapularis* found
2010	∼200	12	12/17	7
2011	560	96	747/1,083	25

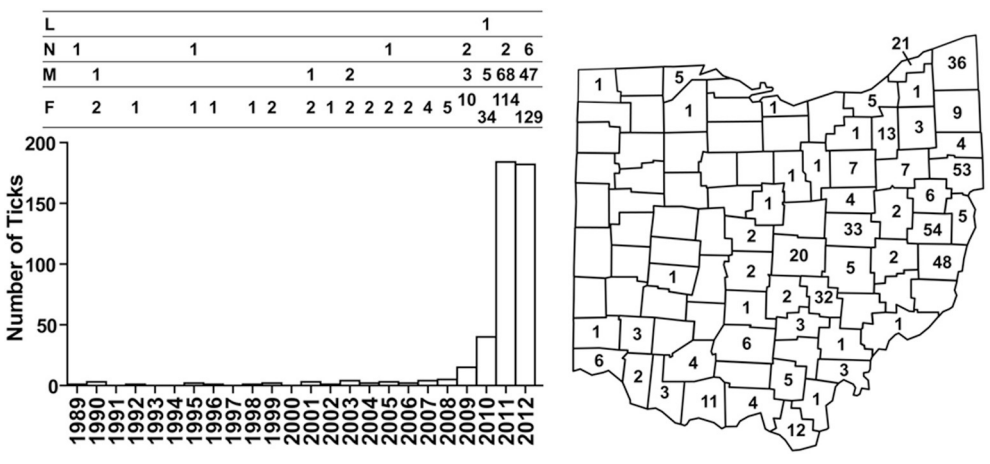

FIGURE 1 | Numbers and distribution of *I. scapularis* collected through the ODH tick surveillance program from 1989 to 2012. Numbers of *I. scapularis* ticks are plotted according to year and the development stages of the ticks are indicated above the bar graph: L, larva; N, nymph; M, male, F, female. Cumulative numbers of *I. scapularis* found in Ohio counties are indicated on the map.

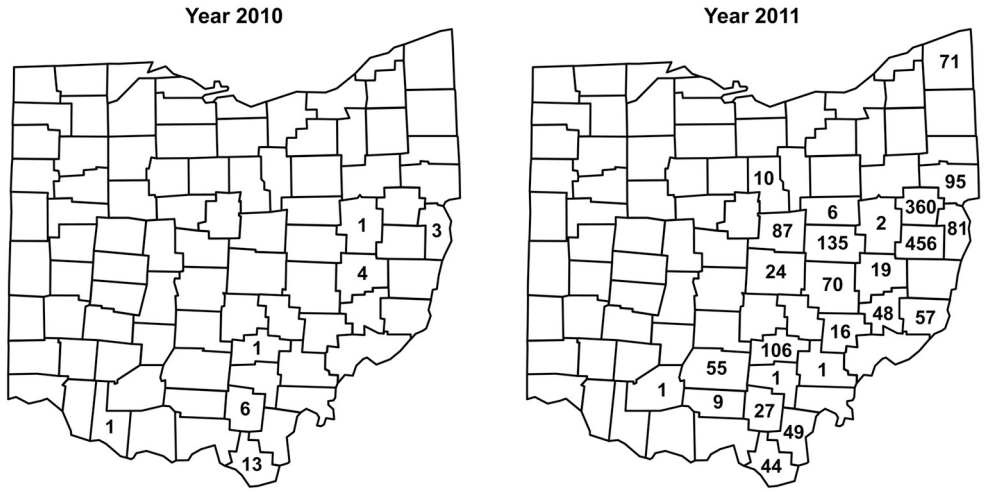

FIGURE 2 | Numbers and distribution of *I. scapularis* found on deer heads through the CWD surveillance program in 2010 and 2011.

Table 2 | Tick survey at Tiverton Township in 2010.

Month	Number of *I. scapularis*			
	Larvae	Nymphs	Adults	
	Vegetation	Host	Vegetation	Host
March	–	–	27	–
April	–	–	70	–
May	–	6	43	3
June	–	46	–	4
July	>200	–	–	–
August	–	–	–	–
September	–	–	–	–
October	–	–	15	6
November	–	–	52	1

or domestic animals (**Table 2**). Therefore, all 3 active stages of *I. scapularis* have been found in Tiverton Township.

PREVALENCE OF *B. BURGDORFERI* INFECTION IN *I. SCAPULARIS* COLLECTED IN OHIO

Nymphal and adult *I. scapularis* collected from Tiverton Township were analyzed by qPCR to determine the prevalence of *B. burgdorferi* infection. Larval *I. scapularis* ticks flagged from vegetation were not analyzed because transovarial transmission of *B. burgdorferi* is rare or nonexistent (Rollend et al., 2013). Initially, the *I. scapularis* ticks were analyzed individually. However, of the 27 adult *I. scapularis* flagged from vegetation in March 2010, none tested positive, indicating a low prevalence of *B. burgdorferi* infection. Thereafter, some *I. scapularis* ticks were tested in batches of 2–10 each. Overall, of the 273 *I. scapularis* tested, 138 were assayed individually and 135 were assayed in batches. Of the 15 *I. scapularis* samples tested positive for *B. burgdorferi flaB* DNA, one was a batch of 4 nymphs, one was a batch of 3 nymphs, and all the others were of single *I. scapularis*. To be conservative in our estimation of the infection rate, in both of these cases where a batch of multiple *I. scapularis* ticks tested positive, only one was considered positive.

Of the 207 adult *I. scapularis* flagged from vegetation, only 5 (2.4%) tested positive for *B. burgdorferi flaB* gene (**Figure 3**). Notably, none of the 140 adult *I. scapularis* collected from vegetation in spring tested positive, whereas 5 of the 67 (7.5%) adults collected in fall tested positive ($P = 0.003$, Fisher's exact test). Of the 66 *I. scapularis* collected from people or domestic animals, a total of 10 (15.2%) tested positive. The infection rate was 13.5% (7/52) for the nymphal *I. scapularis* and 21.4% (3/14) for the adult. In comparison, the infection rate in adult *I. scapularis* flagged from vegetation was significantly lower than that in adults collected from hosts ($P = 0.0095$, Fisher's exact test). Overall, 15 of 273 (5.5%) *I. scapularis* ticks were infected with *B. burgdorferi*, and the spirochete burden of infected *I. scapularis* ranged from 36 to 390,000 per specimen, with a median value of 80. The tick that had the highest burden was a male that was found crawling off a dog shortly after the removal of a partially engorged female from the animal. Similarly high levels of *Borrelia* burden in *Ixodes* species were previously reported by others (Wang et al., 2003; Wilhelmsson et al., 2010).

Although it is not a competent vector for transmitting *B. burgdorferi*, the dog tick *D. variabilis* can acquire the spirochete from infected rodents (Soares et al., 2006). Therefore, we also tested 67 adult *D. variabilis* flagged from vegetation in Tiverton Township, and 3 (4.5%) of them tested positive for *B. burgdorferi flaB* DNA. This indicated that the infection rate was similarly low in both *I. scapularis* and *D. variabilis* found in this area.

Ixodes scapularis from the ODH collections were also tested individually for the presence of *B. burgdorferi flaB* gene. Of the 184 *I. scapularis* ticks collected through ODH surveillance in 2011, only 27 were available for DNA testing. Among them, 2 (7.4%) tested positive, one with 220 and the other with 580 copies of *B. burgdorferi* genome per tick. Of the 1,830 *I. scapularis* recovered from CWD deer head surveillance in 2011, 220 (no more than 3 from each deer head) were selected for testing, and 15

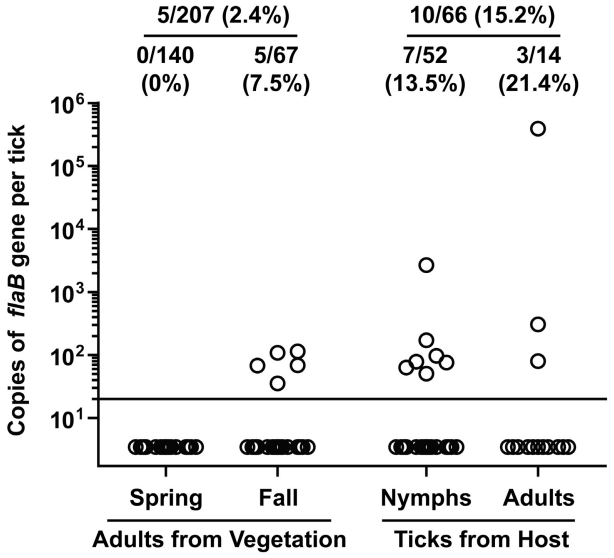

FIGURE 3 | *Borrelia burgdorferi* DNA burden in *I. scapularis* collected from Tiverton Township. Circles represent copies of the *B. burgdorferi flaB* gene in individual ticks. The ratio and percentage of samples tested positive were shown at the top of each group. The adult ticks collected from vegetation were divided into 2 groups depending on when they were collected, and the ticks collected from a host were divided into 2 groups according to their developmental stage.

(6.8%) tested positive, with the spirochete burden ranging from 111 to 11,800 per ticks. Therefore, the prevalence of *B. burgdorferi* infection in *I. scapularis* collected from Ohio statewide is similar to that of *I. scapularis* collected from Tiverton Township.

BORRELIA BURGDORFERI SEROPREVALENCE IN FIELD-CAPTURED *P. LEUCOPUS*

We also investigated if the wild rodent population in Tiverton Township had been exposed to *B. burgdorferi*. In September of 2010, a total of 10 *P. leucopus* were captured during 2 consecutive nights. Serum samples were tested by ELISA and immunoblot analysis for IgG and IgM antibodies against *B. burgdorferi* antigens. For comparison, serum samples from laboratory C3H mice infested by naïve or *B. burgdorferi*-infected nymphs were included as negative and positive controls, respectively. All 10 *P. leucopus* serum samples tested negative for IgG antibodies to *B. burgdorferi*, but 2 of them (no. 3 and no. 8) had a positive IgM response by both ELISA and immunoblot analysis (**Figure 4**). It is intriguing that the no. 8 *P. leucopus* serum sample had much higher reactivity than the no. 3 *P. leucopus* serum sample in the ELISA assay, but in the immunoblot assay, the results appeared to be reversed, the no. 8 sample yielded much weaker signals than did the no. 3 sample. We repeated the IgM ELISA and immunoblot analyses for all samples to rule out the possibility that we inadvertently switched the samples. One possible explanation for this discrepancy may be that the *B. burgdorferi* component that reacted strongly with the no.8 serum sample in ELISA somehow was not efficiently separated by SDS-PAGE and transferred to the membrane in the immunoblot analysis. Nevertheless, serum samples from infected laboratory mice and the two positive *P. leucopus* serum samples all reacted very strongly with an approximately 20-kDa *Borrelia* antigen, which may be the outer surface protein C (OspC) that is highly expressed during mammalian infection. Thus, we further analyzed these serum samples using the ViraStripe IgG Lineblot, which contains 10 purified native proteins from *B. burgdorferi* B31 strain, including the 23-kDa OspC. The result indicated that the common band that reacted with both the laboratory positive controls and the two *P. leucopus* serum samples was indeed OspC (**Figure 4**).

The 10 captured *P. leucopus* were examined for ticks. Six mice had a total of 28 ticks attached, which included 23 larval *I. scapularis* as well as 2 nymphal and 3 larval *D. variabilis*. Only 1 of the 2 *D. variabilis* nymphs tested positive, which had 267 copies of *flaB* gene. The *D. variabilis* nymph that tested positive for *B. burgdorferi flaB* gene was collected from a rodent that tested negative for antibodies against *B. burgdorferi* antigen, suggesting that this dog tick may have acquired the spirochete when taking its first blood meal at the larval stage. One nymphal and 1 larval *D. variabilis* and 12 larval *I. scapularis* were found co-feeding on one of the rodents that tested positive for IgM antibodies to *B. burgdorferi*, but none of these ticks tested positive for *B. burgdorferi* DNA.

BORRELIA BURGDORFERI SEROPREVALENCE IN OHIO DOGS

It has been proposed that seroprevalence in dogs is a good indicator for Lyme disease risk (Bowman et al., 2009; Mead et al., 2011). Therefore, we investigated if there was evidence for *B. burgdorferi* exposure in Ohio dogs. From June to August of 2011, leftover plasma samples from a total of 355 Ohio dogs that had blood work done at the OSU Veterinary Medical Center were tested by ELISA for IgG antibodies to *B. burgdorferi* whole-cell lysate. Among them, 197 (55.4%) were from Franklin County, none were from Coshocton County, and the remaining 158 dogs were from 44 of the other 86 counties in Ohio. Serum samples from 76 healthy greyhound blood donors that had been screened to be free of common vector-borne diseases prior to enrollment and have since been treated with Frontline to prevent vector-borne diseases were included as a control group. The ELISA result indicated that the medium IgG titer was significantly higher in the patient group than in the control group ($P < 0.0001$, Mann-Whitney test) (**Figure 5**). With an arbitrary cut-off set at the titer of 400, the seroprevalence was 11.5% (41/355) for the patient group, significantly higher than the 1.3% (1/76) for the control group ($P = 0.0045$, Fisher's exact test). Of the 197 dogs from Franklin County, 26 (13.2%) tested positive, which is not significantly different from the 9.5% (15/158) for dogs outside of Franklin County ($P = 0.4$, Fisher's exact test).

We next compared the ELISA result with the ViraStripe Lineblot test, an FDA-approved test for diagnosis of human Lyme disease. A total of 71 samples were tested using the ViraStripe IgG Lineblot, which contains 10 purified native *B. burgdorferi* B31 proteins. Criteria for a positive ViraStripe result are based on the CDC recommendation—reactivity with no less than five of the ten proteins, and each at no less than the cut-off intensity. Notably, these criteria are more stringent and more specific than the criteria that we set for a positive ELISA result—reactivity with *B. burgdorferi* whole-cell lysate at a titer above 400. The 71 samples subjected to the ViraStripe test included all 42 ELISA-positive

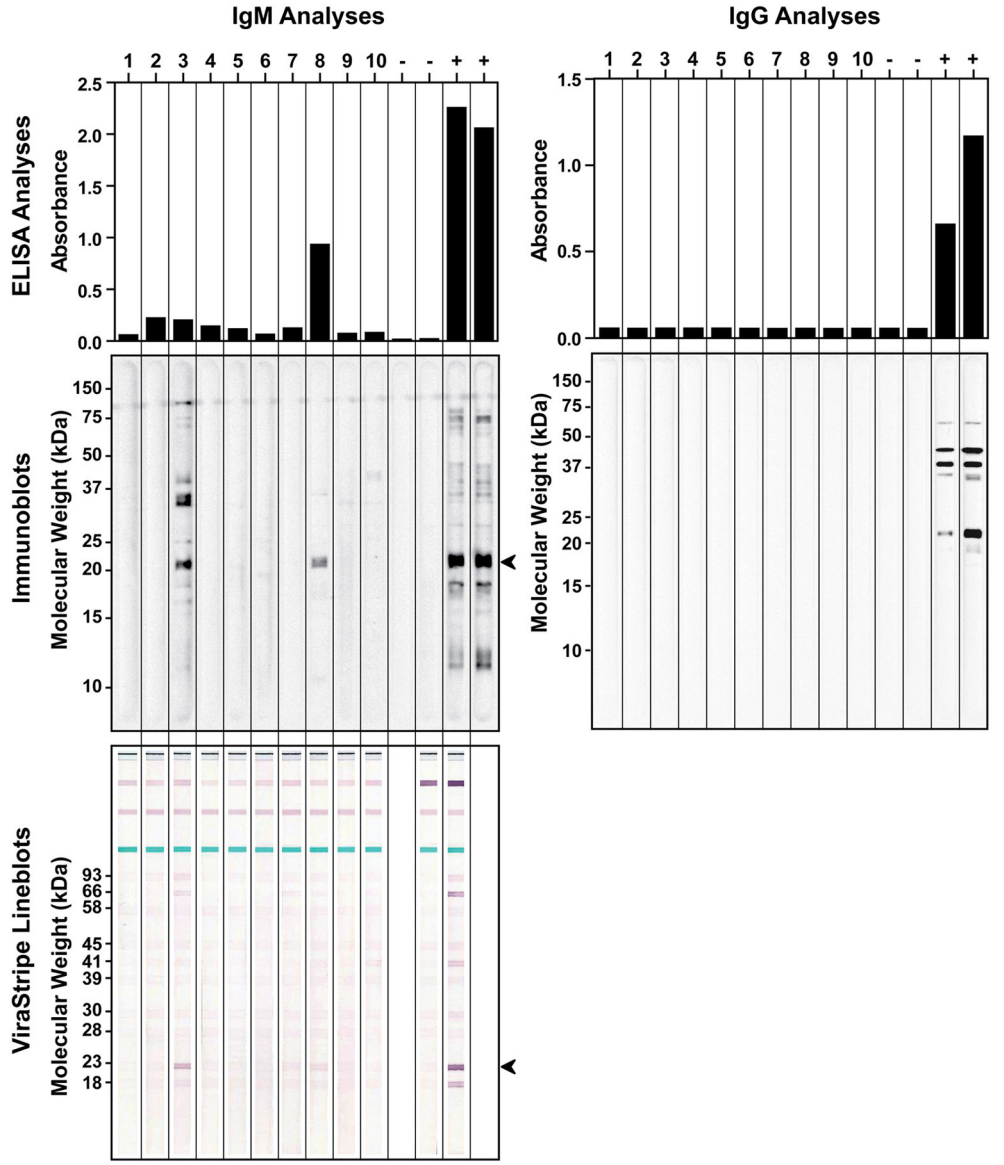

FIGURE 4 | Detection of antibodies to *B. burgdorferi* antigens in field-captured *P. leucopus* from Tiverton Township. ELISA (top panels) and immunoblot (middle panels) analyses of IgM (left panels) and IgG (right panels) responses to *B. burgdorferi* whole-cell lysate were shown for serum samples from 10 captured *P. leucopus* and 4 laboratory mice that had been infested with naïve (–) or *B. burgdorferi*-infected (+) nymphs. The identity of the ~20-kDa protein, indicated by an arrowhead in the IgM immunoblot, was confirmed to be the 23-kDa OspC by the ViraStripe Lineblot analysis (bottom left panel).

samples (41 patients and 1 greyhound donor) and 29 randomly selected ELISA-negative samples (20 patients and 9 greyhound blood donors). Results are shown in **Table 3**, and representative blots are shown in **Figure 5**. Twenty-seven (64%) of the 42 ELISA-positive samples tested positive by ViraStripe, whereas 2 (7%) of the 29 ELISA-negative samples tested positive by ViraStripe ($P < 0.0001$, Fisher's exact test). Moreover, of the ELISA-positive samples, the ones with a higher titer are more likely tested positive by ViraStripe than those with a lower titer. All of the 23 samples that had a titer ≥3,122 tested positive by ViraStripe whereas only 4 of the 19 samples with a titer between 400 and 3104 tested positive by ViraStripe ($P < 0.0001$, Fisher's exact test). Therefore, there is a strong correlation between the ELISA and the ViraStripe results (**Table 3**).

DISCUSSION

Ohio had seen a low incidence of human Lyme disease, and this was largely attributed to the absence of the transmitting vector, *I. scapularis*. However, evidences presented in this study suggest that the blacklegged tick is becoming established in Ohio.

The most compelling evidences are from the tick surveillance programs that had been in place in Ohio for many years. First, the ODH tick surveillance program that was active 1983–2012 indicated that the number of *I. scapularis* found in Ohio had

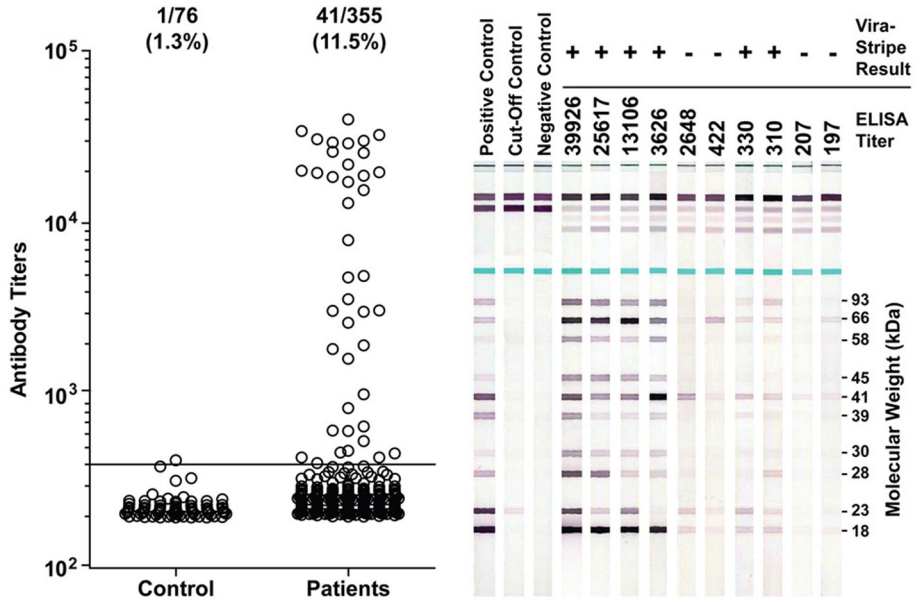

FIGURE 5 | Detection of antibodies to *B. burgdoferi* antigens in Ohio dogs. Antibody titers as determined by ELISA are plotted in the left graph. The ratio and percentage of samples tested positive (titer >400) were shown above the graph for each group. The serum samples from 76 healthy greyhound blood donors were included as a control group for the 355 serum samples from canine patients who had blood work done at the OSU Veterinary Medical Center in summer of 2011. Representative ViraStripe Lineblots were shown on the right. The ELISA titer and the ViraStripe result for each test sample are indicated on the top.

remained low until 2009, and had gone through an exponential increase in the following years. Second, the multi-agency CWD surveillance program that was active 2002–2011 also showed a sharp increase in the number of *I. scapularis* found on deer heads, from 29 in 2010 to 1830 in 2011. It is important to note that this increase is not due to invigorated surveillance. To the contrary, these data were recorded when both programs were being considered for termination due to decreasing budgets. The CWD monitoring program was terminated after the 2011 season. The ODH tick surveillance program, although active until the end of 2012, was scaled back in that final year, which may explain the plateau in the number of *I. scapularis* found. It is also important to point out the consistency between these data sets. During 2002–2008, while the ODH tick surveillance program recorded steady and low numbers of *I. scapularis*, no such ticks were recovered from deer heads. These data are also consistent with the result from a 2004–2007 survey by others that no nymphs were collected in and around Ohio (Diuk-Wasser et al., 2012). Therefore, the emergence of *I. scapularis* in Ohio appears to be relatively recent.

Of the 15 *I. scapularis* that were submitted to ODH in 2009, 3 (20%) were from Coshocton County. In addition, an Amish family in Tiverton Township of Coshocton County contacted Glen R. Needham regarding a case of un-reported human Lyme disease and sightings of *I. scapularis* on their property. Glen R. Needham thus surveyed this area in Coshocton County to determine if *I. scapularis* populations were established. The seasonal activities of larval, nymphal, and adult *I. scapularis* in Tiverton Township as shown in **Table 2** were consistent with those described for other endemic areas in the US (Fish, 1993), with adults being active in spring and fall, nymphs being active in early summer, and larvae being active in summer. Finding all 3 active stages of *I. scapularis* in Tiverton Township demonstrated the presence of established tick populations in this area, although it did not provide a timeline for the establishment. The low rate of *B. burgdorferi* infection in the *I. scapularis* and *D. variabilis* ticks found in this area and in the field-captured *P. leucopus*, however, is more consistent with a recent emergence rather than a long-term establishment of the enzootic life cycle in the area.

Given that enzootic life cycle *B. burgdorferi* in Ohio appears to be nascent, we expect that the number of *I. scapularis* and the percentage of *B. burgdorferi*-infected *I. scapularis* found in this state will continue to increase. However, we do not expect to see further expansion of the range of this tick, because the distribution of *I. scapularis* found in Ohio so far is, by and large, consistent with the state's deciduous forest range. Given the proximity of these Ohio counties to highly endemic Pennsylvania, one possibility is that the emergence of this tick vector in Ohio simply reflects the continuing expansion of the Northeast Lyme disease-endemic region in the US. Migrating birds and/or deer as well as human activities could play a role in the spreading of the Lyme disease vector and agent. For example, transporting harvested deer from tick-infested areas of nearby Pennsylvania may be a factor because hides are often composted in wooded areas where ticks could detach and begin a "hot-spot." Hunters should be alerted to this possibility so that hides can be properly disposed.

The apparently recent emergence of *I. scapularis* in Ohio is expected to lead to higher incidences of Lyme disease in people and in domestic animals in the future. Of 355 Ohio dog serum samples that were tested by ELISA, 41 (11.5%) had a titer above 400, and 27 (66%) of these 41 ELISA-positive samples tested positive by an immunoblot analysis using the CDC criteria set for human exposure to the Lyme disease spirochete. Given that

Table 3 | Comparison of ELISA and ViraStripe analyses of dog serum samples.

Sample ID[a]	ELISA Titer	ViraStripe Interpretation	Sample ID[a]	ELISA Titer	ViraStripe Interpretation	Sample ID[a]	ELISA Titer	ViraStripe Interpretation
POS001	39926	+	POS025	3064	+	NEG007	331	–
POS002	34205	+	POS026	2648	–	NEG008	330	+
POS003	32467	+	POS027	1960	+	NEG009	328	–
POS004	30652	+	POS028	1865	–	NEG010	325	–
POS005	30106	+	POS029	1640	–	NEG011	321	–
POS006	29401	+	POS030	1019	–	NEG012	313	–
POS007	29075	+	POS031	848	–	NEG013	310	+
POS008	25998	+	POS032	664	–	NEG014	305	–
POS009	25617	+	POS033	628	+	NEG015	258	–
POS010	21927	+	POS034	625	–	NEG016	229	–
POS011	20182	+	POS035	545	–	NEG017	207	–
POS012	19732	+	POS036	480	–	NEG018	205	–
POS013	19556	+	POS037	469	–	NEG019	204	–
POS014	18888	+	POS038	463	–	NEG020	204	–
POS015	18542	+	POS039	438	–	NEG021	203	–
POS016	17377	+	POS040	438	–	NEG022	201	–
POS017	15535	+	POS041	422	–	NEG023	200	–
POS018	13106	+	POS042	407	+	NEG024	199	–
POS019	7989	+	NEG001	389	–	NEG025	199	–
POS020	4950	+	NEG002	384	–	NEG026	197	–
POS021	4845	+	NEG003	350	–	NEG027	197	–
POS022	3626	+	NEG004	348	–	NEG028	197	–
POS023	3122	+	NEG005	336	–	NEG029	197	–
POS024	3104	–	NEG006	333	–			

[a] *Of the 71 dog serum samples tested by ViraStripe, the 42 that had an ELISA titer above 400 were designated POS001-042, and the 29 that had an ELISA titer below 400 were designated NEG001-029.*

the SNAP 4Dx Plus Test (IDEXX) employs a more stringent criterion for exposure to the Lyme disease spirochete—the presence of antibodies to a specific 26-amino acid residue peptide known as C6 (Liang et al., 1999), not all of the 27 ELISA-positive and ViraStripe-positive samples are expected to test positive by the SNAP 4Dx Plus Test. Also, given that these dogs were seen at a major medical center, the rate of seroprevalence in this group of patients could be higher than that in the general population. According to data published by the Companion Animal Parasite Council website (www.capcvet.org), the percentage of Ohio dogs tested positive for exposure to the Lyme disease spirochete by the SNAP 4Dx Plus Test was 0.39% (218 of 54,963) for 2011, 0.55% (250 of 45,376) for 2012, 0.56% for 2013 (543 of 96,016), and 0.62% (102 of 16,343) for 2014 as of April 23, 2014, showing a significant trend of increase ($P < 0.0001$, Chi-square test for trend). According to data available from the CDC, the number of confirmed human Lyme disease cases in Ohio was 21 (0.2 per 100,000 persons) for 2010, 36 (0.3 per 100,000 persons) for 2011, 49 (0.4 per 100,000 persons) for 2012, and 74 (0.6 per 100,000 persons) for 2013. However, preliminary estimates presented by CDC at the 2013 International Conference on Lyme Borreliosis and Other Tick-Borne Diseases suggest that the actual number of Lyme disease cases may be 10 times higher than the number of reported cases. Given that Ohio was not previously considered endemic for Lyme disease, it is likely that many cases in Ohio might have gone unreported or even undetected. Nevertheless, these numbers of confirmed Lyme disease cases in Ohio, again, indicate a significant trend of increase ($P < 0.0001$, Chi-square test for trend). The increased exposure to the Lyme disease agent seen in people and dogs in Ohio further corroborate with our data, and is consistent with a recent emergence of *I. scapularis* in this state. Healthcare professionals (e.g., physicians, nurse practitioners, and veterinarians) and residents in Ohio should become aware of this emerging tick and its associated risk of disease.

AUTHOR CONTRIBUTIONS

Glen R. Needham, Kathleen A. Smith, Richard E. Gary, and Xin Li. designed the experiments, Peng Wang and Meaghan N. Glowacki performed laboratory analysis, and all authors contributed to data analysis and interpretation. Peng Wang, Meaghan N. Glowacki and Xin Li wrote the manuscript, and Armando E. Hoet, Glen R. Needham, Kathleen A. Smith, and Richard E. Gary critically revised the manuscript. All authors approved the final manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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The role of eicosanoids in experimental Lyme arthritis

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Experimental Lyme arthritis is an inflammatory arthritis caused by infection of mice with the spirochete, *Borrelia burgdorferi*. It recapitulates many of the disease parameters seen in human patients with Lyme arthritis, and thus serves as a model system for the investigation of disease pathogenesis. While much progress has been made in defining components of the immune response to *Borrelia* infection, an overall understanding of the host response leading to arthritis resistance or susceptibility remains elusive. In this review, we will focus on recent advancements of our understanding of the roles of eicosanoids as inflammatory mediators in the regulation of experimental Lyme arthritis. Eicosanoids, such as PGE_2 and LTB_4, are powerful regulators of inflammatory responses and thus may be important mediators of Lyme arthritis.

Keywords: *Borrelia burgdorferi*, Lyme arthritis, eicosanoids, resolution of inflammation, inflammation

INTRODUCTION

Lyme disease is a major source of morbidity due to the high incidence of rheumatic, cardiovascular, and neurologic complications that follow infection with the etiologic agent, *Borrelia burgdorferi* (Barbour and Fish, 1993). *B. burgdorferi* is transmitted to the mammalian host through the bite of an infected *Ixodes* tick (Burgdorfer et al., 1982). Acute disease is characterized by a classic enlarging bulls-eye rash called erythema migrans, which resolves on its own if left untreated (Steere et al., 2004). Twenty percent of individuals, despite being infected, will remain disease free following resolution of their skin rash. If not treated with antibiotics at this stage, however, most infected individuals will go on to develop secondary complications including carditis, arthritis, or neurological disease (Steere et al., 2004). Subsets of individuals who receive appropriate antibiotic therapy still develop recurrent episodes of chronic joint inflammation up to years after receiving appropriate treatment (Steere et al., 2004; Iliopoulou and Huber, 2010). The genetic components and/or immune parameters that predispose individuals to develop chronic symptoms associated with Lyme disease or to remain disease free are unclear and the subject of ongoing research (Steere et al., 2004).

Experimental Lyme arthritis is the murine model system of Lyme arthritis and recapitulates many of the disease parameters seen in patients with Lyme arthritis. The murine model is an inflammatory arthritis and requires the presence of live spirochetes within the joint for disease development. Arthritis development, however, is genetically controlled resulting in Lyme arthritis-resistant and -susceptible mouse strains (Barthold et al., 1990). C57BL/6 (B6) mice are the most commonly used Lyme arthritis-resistant strain, while C3H/He (C3H) mice are the most commonly used Lyme arthritis-susceptible strain. Infection of susceptible mouse strains with *B. burgdorferi* results in the development of arthritis which peaks around 3–4 weeks post-infection, and then spontaneously resolves over the next few weeks (Barthold et al., 1996). While live spirochetes are required for disease development, their absolute numbers within the joint do not correlate with arthritis severity. Lyme arthritis-resistant and -susceptible mouse strains can harbor equivalent numbers of spirochetes within their joints, yet maintain their distinct disease phenotypes (Brown and Reiner, 1998; Ma et al., 1998). This defines experimental Lyme arthritis as an immunopathology. Disease development in mice is driven primarily by innate immunity, since arthritis-susceptible mice devoid of T and B cells retain their disease susceptibility (Schaible et al., 1989; Brown and Reiner, 1999). Arthritis resolution, on the other hand, appears to be mediated by the production of anti-*Borrelia* antibodies and spirochete clearance from the joints (Barthold et al., 1996). While infection of mice with *B. burgdorferi* is a useful model for studying disease pathogenesis, it is currently unclear if similar disease mechanisms are operational during the immune response to *B. burgdorferi* infection in humans.

EICOSANOIDS IN LYME ARTHRITIS

Eicosanoids are 20-carbon fatty acids derived from the metabolism of arachidonic acid (AA) and are powerful mediators of inflammation (Stables and Gilroy, 2011). Upon activation of immune cells, AA is released from cellular membrane stores primarily via the activity of cytosolic phospholipase A2 ($cPLA_2$). The released AA is then metabolized to various biological mediators via three primary enzymatic pathways: cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYTP) (see **Figure 1**). Each pathway contains additional metabolite-specific enzymatic steps resulting in a wide variety of bioactive compounds (e.g., prostaglandins, leukotrienes, etc). Not all inflammatory cells express all three pathways and there is considerable variation in the production of specific metabolites. In addition, there appears to be a predisposition for certain cells to produce specific metabolites, e.g., macrophages tend to make high levels of prostaglandin (PG)E_2 while neutrophils tend to produce high levels of leukotriene (LT)B_4, although they are each

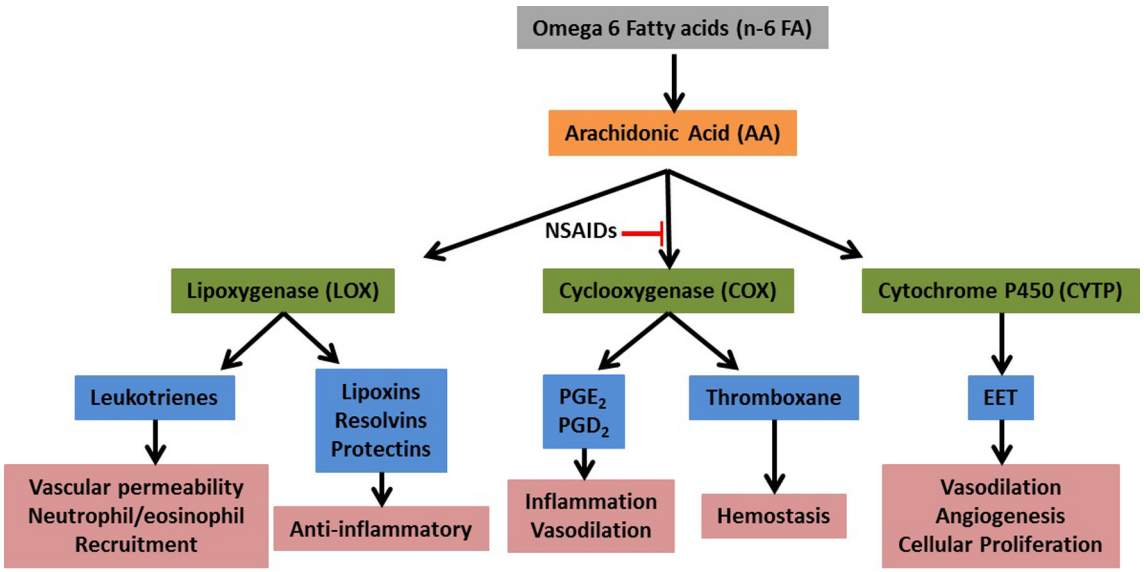

FIGURE 1 | Simplified eicosanoid metabolic pathway. Upon tissue damage or infection arachidonic acid (AA) is released from membrane stores by the activity of cytosolic phospholipase 2. The free AA is then acted upon by the primary metabolic enzymes (green) and converted to numerous bioactive compounds (blue).

capable of making both of these metabolites when stimulated under certain conditions *in vitro* (Kihara et al., 2014). Although eicosanoids are powerful regulators of inflammation, in general their role in mediating an immune response to infection is incompletely understood.

Since experimental Lyme arthritis is an inflammatory arthritis that develops and then spontaneously resolves, it is an ideal model to study how eicosanoids regulate the induction and resolution of an inflammatory response. COX-2 is an inducible gene expressed primarily in immune cells and is responsible for the production of PG during an inflammatory response (Mitchell et al., 1993). Non-steroidal anti-inflammatory drugs (NSAIDs, e.g., aspirin, ibuprofen) are powerful inhibitors of the COX enzymes, with newer drugs (e.g., Celebrex) preferentially inhibiting COX-2. In an initial study of the role of COX-2 in the host immune response to *B. burgdorferi* infection, the expression of COX-2 was found to increase in the joints of infected C3H mice by day 14 post-infection and remain elevated through day 60 (Anguita et al., 2002). Furthermore, treatment of infected mice with a COX-2-specific inhibitor, MF-tricyclic, decreased arthritis severity scores compared to untreated controls at day 14 post-infection without altering T or B cell responses. These results suggested that NSAIDs or other COX-2-specific inhibitors might be effective treatments for Lyme arthritis, possibly without compromising spirochete clearance. We followed up on these results and conducted experiments using two commercially available COX-2-specific inhibitors, rofecoxib (Vioxx) and celecoxib (Celebrex), as well as C3H COX-2 knockout mice (Blaho et al., 2008). In contrast to the previous study, we found no effect of COX-2 deletion or inhibition on the development of experimental Lyme arthritis at a number of time-points throughout the infection. We did, however, find a significant delay or inhibition of arthritis resolution in mice devoid of COX-2 activity. In agreement with the previous study, we found that COX-2 inhibition or deletion had little effect on *Borrelia*-specific antibody production, and clearance of spirochetes from tissues appeared to occur at the same rate in COX-2 deficient and control mice. This demonstrated that arthritis resolution could be uncoupled from spirochete clearance from the joint, and may have important implications for patients with persistent arthritis despite seemingly effective antibiotic therapy. In addition, NSAID therapy to counteract joint pain and swelling during acute Lyme arthritis may be contraindicated and lead to a prolongation of arthritis symptoms. Only a single time point (14 days post-infection) was reported by Anguita et al. which suggested a delay in arthritis development. We did not see a delay in arthritis development in our study, but this may be due to differences in intradermal vs. footpad routes of infection. Further work in this area is required to define the roles of COX-2 metabolites on the development and resolution of experimental Lyme arthritis.

COX-2 is an inducible enzyme that when activated, produces metabolites that are responsible for inciting inflammation and vasodilation (Stables and Gilroy, 2011). The most prominent of these metabolites are PGE_2 and PGD_2. Patients diagnosed with arthritis, infectious or immune mediated, are frequently treated with NSAIDs. These drugs are designed to be COX-2 specific to combat the inflammation associated with the activation of the COX pathway. In the experimental Lyme arthritis model, inhibition of COX-2 through gene deletion resulted in milder ankle swelling and arthritis that failed to resolve (Blaho et al., 2008). Histologically, neutrophilic infiltration was still present to the same degree in the COX-$2^{-/-}$ mice as in their wild-type counterparts. PGE_2 and PGD_2 levels in the tissue of uninfected and infected COX-$2^{-/-}$ mice were decreased, as expected. Since PGs are responsible for vascular tone, this could explain the attenuation of swelling resulting from their failure to vasodilate to the

same degree as wild-type mice. Infected COX-$2^{-/-}$ mice also had unexpected decreased levels of 5-LOX metabolites, potentially contributing to the prolonged inflammatory response seen in these mice (as discussed in the following paragraph).

We have also completed a study of the role of 5-LOX in the development of experimental Lyme arthritis (Blaho et al., 2011). This enzyme is primarily expressed in neutrophils and catalyzes the conversion of AA to LTB_4 and the cysteinyl leukotrienes (LTC_4, D_4, and E_4). LTB_4 is a powerful neutrophil chemoattractant and plays an important role in the initiation of arthritis in the K/BxN serum transfer model (Kim et al., 2006), and in collagen-induced arthritis (CIA) (Shao et al., 2006). Both K/BxN serum transfer and CIA arthritis models are experimental autoimmune models of rheumatoid arthritis. Serum from K/BxN mice contain autoantibodies against glucose 6-phosphate isomerase, while the CIA model involves the induction of cross-reactive antibodies to mouse collagen. Both models develop polyarthritis and can be induced in certain mouse strains by the passive transfer of autoimmune serum. Inhibition of 5-LOX activity or inhibition of LTB_4 signaling through its high-affinity receptor, BLT1, can inhibit the development of arthritis in both of these models. Following infection of C3H mice with *B. burgdorferi,* we found increased expression of mRNA for 5-LOX and its accessory protein, five lipoxygenase activating protein in joint tissue, as well as increased production of LTB_4 (Blaho et al., 2011). Infection of C3H 5-LOX knockout mice, in contrast to the K/BxN or CIA arthritis models, led to an earlier development of Lyme arthritis. In addition, similar to what was seen in the COX-2 deficient mice, arthritis resolution was delayed or inhibited in the 5-LOX deficient mice as compared to wild type C3H controls. Production of *Borrelia*-specific IgG was decreased in the C3H 5-LOX knockout mice, but spirochete clearance from tissues appeared to be similar to controls, indicating the amount of antibody produced was adequate to mediate spirochete clearance.

Products from the 5-LOX metabolic pathway, especially LTB_4, have been shown to have a significant impact on macrophage phagocytosis and killing of various pathogens (Serezani et al., 2005). **Figure 2** depicts how a failure in LTB_4 production by neutrophils might influence arthritis resolution. *In vitro*, we demonstrated that spirochete uptake and killing by 5-LOX-deficient neutrophils was mostly intact, while 5-LOX-deficient macrophage uptake of *B. burgdorferi* and apoptotic neutrophils was significantly impaired (Blaho et al., 2011). These results suggested that neutrophils may be the primary cells responsible for spirochete clearance, but that macrophage clearance of apoptotic neutrophils may be important for the timely and efficient induction of arthritis resolution and return of the tissue to homeostasis. Further work in this area is required to identify the mechanisms involved, but macrophage uptake of apoptotic cells has been shown to induce their phenotypic change from pro-inflammatory to anti-inflammatory and promote resolution of inflammation (Freire-de-Lima et al., 2006).

EICOSANOID PRODUCTION DURING INFECTION

To gain a more complete understanding of the production of eicosanoids in the arthritic joint during *B. burgdorferi* infection, we conducted the first published comprehensive lipidomic study of infected tissue (Blaho et al., 2009a). Additional lipidomic studies using other infectious disease models have since been published, providing further insight into the impact of eicosanoids on immune function and as targets for future therapeutics (Tam, 2013). In our study, joint tissue from arthritis-resistant DBA/2, and -susceptible C3H mice were analyzed for the production of 104 unique lipid species in the eicosanoid metabolome during the development and resolution phases of experimental Lyme arthritis. Several of the eicosanoid pathways between an arthritis-resistant and susceptible strain were different at baseline as well as during *B. burgdorferi* infection, and these differences may drive genetic predispositions to arthritis resistance or susceptibility. Upon infection with *B. burgdorferi*, alterations in the production of metabolites from all three eicosanoid pathways became evident. Specific strain differences noted were elevation in 5,6 epoxyeicosatrienoic acid (EET), and lower $PGD_2$and protectin D1 (PD1) levels in DBA/2 mice as compared to C3H mice. Both strains exhibited elevations in PGE_2 and a decrease in 11,12 EET. PGE_2 and PGD_2 are metabolites derived from the COX pathway and are considered primarily pro-inflammatory. Although PGE_2 becomes elevated in both strains, PGD_2 did not significantly increase from basal levels in DBA/2 mice. The lack of elevation of PGD_2 in arthritis-resistant DBA/2 mice may be one potential factor contributing to the observed strain susceptibility differences.

The CYTP pathway is responsible for producing EETs. The overall function of EETs is vasodilation, angiogenesis, and cellular proliferation (Fang et al., 1996; Pozzi et al., 2005; Blaho et al., 2009a; Stables and Gilroy, 2011). The ratio of 5,6 EET was higher in DBA/2 mice as compared to C3H mice at basal levels and after *B. burgdorferi* infection (Blaho et al., 2009a). 5,6 EET specifically causes vasodilation and is a potent neo-vascular agent (Pozzi et al., 2005). An increase in vessel formation and blood flow at the site of inflammation due to 5,6 EET could improve the clearance of *Borrelia* bacteria in DBA/2 mice as compared to C3H mice, although no differences in bacterial tissue loads were detected. The most significant change within the CYTP pathway in both strains was a decrease in 11,12 EET as compared to other EETs and dihydroxyeicosatrienoic acid (DHET) molecules (Blaho et al., 2009a). Vascular cell adhesion molecule expression is down-regulated in the presence of 11,12 EET in experimentally inflammed murine carotid arteries, which prevented the recruitment of leukocytes to the site of inflammation (Node et al., 1999). It is hypothesized that 11,12 EET acts as an anti-inflammatory agent in a similar fashion to counter balance the other pro-inflammatory mediators in Lyme arthritis, however, more work is needed in this area.

Protectin D1 (PD1) is a product of the metabolism of the omega-3 fatty acid, docosahexaenoic acid (DHA), via the 12/15-LOX pathway. It is associated with anti-inflammatory and pro-resolution properties, including the inhibition of neutrophil influx (Levy et al., 2007; Stables and Gilroy, 2011). In Lyme arthritis, PD1 was significantly elevated in C3H mice, but not in DBA/2 mice (Blaho et al., 2009a). The stable metabolite of PD1 and resolvins, 17-HDoHE, was elevated earlier in infection (day 14) in C3H mice as compared to DBA/2 mice (day 28). Based on this experimental finding, we hypothesize that C3H

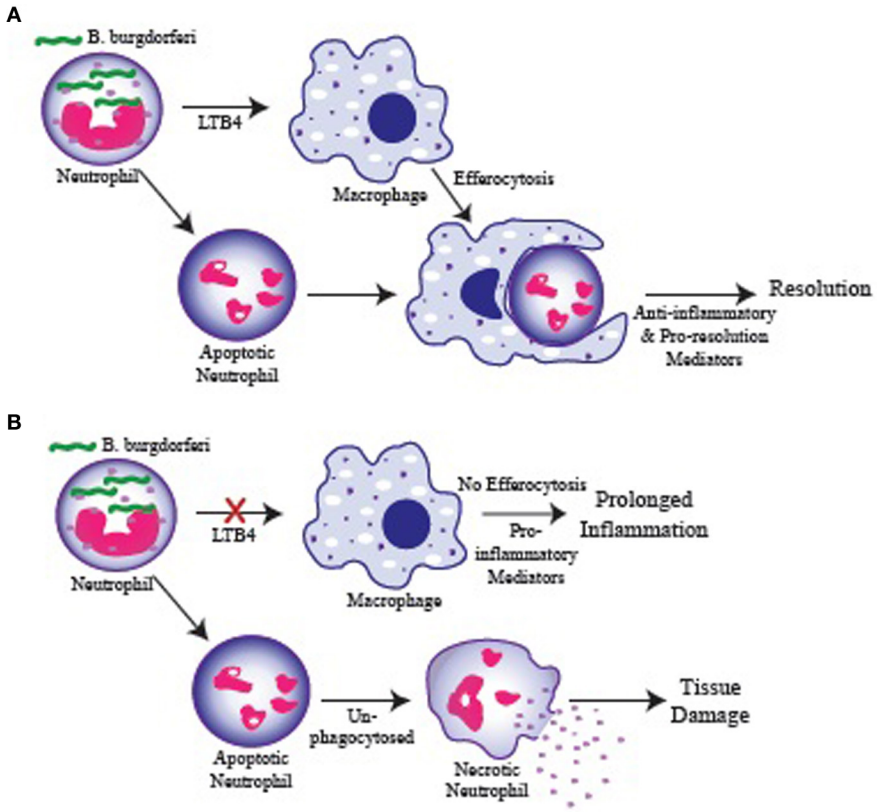

FIGURE 2 | Possible role for LTB_4 in regulating resolution of inflammation. (A) Neutrophils are recruited to the infected joint and engulf *B. burgdorferi*. Neutrophils produce LTB_4 which stimulate macrophage phagocytosis of apoptotic neutrophils (efferocytosis) and their switch to an anti-inflammatory/pro-resolution phenotype promoting a return to homeostasis. **(B)** Failure of neutrophils to produce LTB_4 results in inefficient macrophage phagocytosis and failure to clear apoptotic neutrophils in a timely manner. Apoptotic neutrophils in the tissue proceed to necrosis and release their cytoplasmic contents causing tissue damage and promoting continued inflammation. Similarly, macrophages that fail to engulf apoptotic cells remain in a pro-inflammatory state and promote prolonged inflammation.

mice may have a higher threshold of tolerance for PD1, requiring higher levels of PD1 to produce the same effects as in DBA/2 mice. In addition, the susceptibility of C3H mice to Lyme arthritis could be explained by the altered metabolism of up-stream products supported by the appearance of 17-HDoHE earlier in C3H as compared to DBA/2 mice. Further studies are required to determine the exact role these metabolites play in Lyme arthritis resolution.

MANIPULATING THE EICOSANOID PROFILE

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) have received attention for their potential beneficial effects in a vast number of diseases including dermatitis, rheumatoid arthritis, osteoarthritis, neoplasia, cardiovascular disease, and inflammatory bowel disease (Dumlao et al., 2012; Shek et al., 2012). The prominent feature of n-3 PUFAs in these diseases is their anti-inflammatory effects. A diet rich in n-6 PUFAs (e.g., AA) has been linked to the development and persistence of various diseases (Shek et al., 2012). Dietary n-3 PUFAs can compete with AA for incorporation into cellular membranes and for use as substrates for COX and LOX enzymes (Lands et al., 1990). Use of n-3 PUFAs as substrates results in the generation of (n-3) eicosanoids that are generally less potent than analogous (n-6) eicosanoids (Wada et al., 2007). However, the role n-6 and n-3 PUFAs play in the face of infection and how they alter the production of eicosanoids is unclear. There are conflicting reports regarding the benefits of dietary n-3 PUFAs modulating the immune system to aid in the recovery from bacterial pneumonia, while other reports find a detrimental effect during influenza infection (Shek et al., 2012; Sharma et al., 2013). We investigated the effect of dietary n-3 and n-6 PUFAs in murine Lyme arthritis (Dumlao et al., 2012). Feeding *B. burgdorferi*-infected C3H mice a fish oil diet, rich in n-3 PUFAs, shifted the eicosanoid profile toward an anti-inflammatory one, while a soy oil diet, rich in n-6 PUFAs, shifted it toward a pro-inflammatory profile. Again using a lipidomics approach, we found more fatty acid (FA) metabolites identified in fish oil fed mice as compared to soy oil fed mice, with most metabolites being EPA and DHA derived. Based on previous reports, it would be expected that a diet rich in n-3 PUFAs would produce an anti-inflammatory eicosanoid profile. Despite a shift toward anti-inflammatory eicosanoids in mice fed a n-3 PUFA rich diet in Lyme arthritis, there was no effect on the clinical observations

or histological findings between the two different diets (Dumlao et al., 2012). It is likely there are several factors involved in the development of arthritis, in addition to the eicosanoid profile. Also, one caveat to this study was that we did not examine the resolution phase of the disease process and thus may have missed the effects of dietary fish oil on arthritis resolution and return to homeostasis.

EICOSANOID INFLUENCE ON ANTIBODY PRODUCTION

Production of *Borrelia*-specific antibodies and clearance of spirochetes from the inflamed joint is thought to mediate the resolution of Lyme arthritis (Barthold et al., 1996). However, as discussed above, C3H mice deficient in COX-2 or 5-LOX developed Lyme arthritis, but failed to resolve their inflammation despite a seemingly effective antibody response and spirochete clearance. Thus, we were interested in taking a closer look at the role eicosanoids might play in antibody responses. Historically the inhibition of prostaglandin production has been linked to decreased antibody responses (Roper et al., 2002). The roles of COX enzymes in antibody production, however, appear to vary depending on the underlying pathology and mode of stimulation. Deletion of the COX-2 gene in a CIA model resulted in decreased production of anti-collagen antibodies and markedly attenuated arthritis development as compared to wild-type and COX-1 knockout mice (Myers et al., 2000). The effect of COX inhibition on antibody production in an infectious scenario has revealed conflicting results. During vaccinia virus infection, deletion of COX-2 resulted in decreased levels of several subclasses of IgG due to failure of immunoglobulin class-switching (Bernard et al., 2010). Similarly, pathogen-specific antibody production was decreased by COX-2 inhibition during *Mycobacterium bovis*-induced arthritis (Turull and Queralt, 2000). More recently, our lab has investigated the effect of COX-2 and COX-1 gene deletion using *B. burgdorferi*. In this infectious model, COX-$2^{-/-}$ mice produced comparable antibody levels to infected wild-type mice. However, infected COX-$1^{-/-}$ mice revealed an inhibition of immunoglobulin class-switching supported by elevated IgM and decreased total IgG levels (Blaho et al., 2009b). In addition, histology revealed defective germinal center formation in COX-1 deficient mice. The prostaglandin, PGE_2, has been shown to influence the activation and proliferation of lymphocytes, and assist in B cell antibody class-switching (Ryan et al., 2005). We investigated the effect of eicosanoid production when COX-1 was inhibited or deleted *in vitro* and *in vivo*. Deletion of COX-1 decreased the production of PGE_2 and PGD_2, although not significantly so as compared to infected wild-type mice in splenic tissue. However, additional eicosanoids that were significantly decreased were TXB_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$, and PGJ_2. Inhibition of COX-1 or FP receptor, the receptor for $PGF_{2\alpha}$, in cultures of *B. burgdorferi* infected splenic B cells caused both IgM and IgG production to become significantly decreased as compared to infected controls. This provided evidence that other prostaglandins in addition to PGE_2 may also be responsible for assisting in antibody production and class-switching. Experiments evaluating the maturation and function of B cells in COX-1 inhibited or deleted mice are needed to further elucidate the mechanism for the failure of immunoglobulin class-switching identified in *B. burgdorferi*-infected mice.

CONCLUSION

Lyme arthritis caused by *B. burgdorferi* remains a significant disease in human medicine. The cause for individual susceptibility to chronic arthritis associated with Lyme disease has yet to be explained. The use of murine models of Lyme arthritis has provided significant insight into the pathogenesis and clues into individual susceptibility to chronic disease. It is clear that the immune response and development of Lyme arthritis is complex and multifaceted. Dysregulation of inflammatory and anti-inflammatory mediators, in addition to genetics, add to the factors that determine the ultimate clinical course of disease. Understanding the interaction of cytokines, chemokines, eicosanoids, and genetic influences during *B. burgdorferi* infection will allow improved treatment for individuals with Lyme arthritis.

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Transposon mutagenesis as an approach to improved understanding of *Borrelia* pathogenesis and biology

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Transposon insertion provides a method for near-random mutation of bacterial genomes, and has been utilized extensively for the study of bacterial pathogenesis and biology. This approach is particularly useful for organisms that are relatively refractory to genetic manipulation, including Lyme disease *Borrelia*. In this review, progress to date in the application of transposon mutagenesis to the study of *Borrelia burgdorferi* is reported. An effective *Himar1*-based transposon vector has been developed and used to acquire a sequence-defined library of nearly 4500 mutants in the infectious, moderately transformable *B. burgdorferi* B31 derivative 5A18NP1. Analysis of these transposon mutants using signature-tagged mutagenesis (STM) and Tn-seq approaches has begun to yield valuable information regarding the genes important in the pathogenesis and biology of this organism.

Keywords: *Borrelia burgdorferi*, Lyme disease, transposon mutagenesis, microbial pathogenesis, bacterial physiology, mouse models

Lyme disease is a tick-transmitted, multi-stage bacterial infection caused by several species of the genus *Borrelia*, including the primary agents *B. burgdorferi*, *B. garinii*, and *B. afzelii* (Steere et al., 2004). These spirochetes are maintained in nature through a cycle involving transmission between small mammals and ticks of the *Ixodes ricinus* group. Large mammals such as deer are also required for the feeding of the adult ticks and hence egg production. Humans are accidental hosts that are infected by the bite of *Borrelia*-infested ticks, usually of the nymphal stage. Most infected humans develop mild fever, malaise, and a localized skin lesion called erythema migrans days to weeks after the tick bite, followed by dissemination of propagating organisms through the blood and lymphatics to other tissues. Later manifestations may include neurologic effects, joint involvement, cardiac block, and skin lesions called acrodermatitis chronica atrophicans. If untreated, infection can last for months to years; in mouse studies, organisms can be reisolated from almost any tissue throughout the lifetime of the animal. Despite this pattern of pathogenesis, *B. burgdorferi* produces no known toxins. Lyme disease *Borrelia* thus appear to represent non-toxigenic, invasive, persistent pathogens that cause disease through the induction of host inflammatory reactions, similar to *Treponema pallidum* (syphilis) and *Mycobacterium tuberculosis* (Norris et al., 2010).

Since its initial discovery and culture in 1981, *B. burgdorferi* has been the subject of intensive study in an attempt to better understand the biology of this organism and thereby identify properties useful in the diagnosis, treatment, or prevention of Lyme disease. This work has resulted in tremendous progress (Rosa et al., 2005; Samuels and Radolf, 2010), particularly in terms of understanding the spirochete's molecular biology and the massive gene regulation that accompanies the transition between the disparate mammalian and tick host environments. Despite these advances, several barriers (**Table 1**) have hampered the ability to fulfill molecular Koch's postulates regarding the role of borrelial genes in biological processes and pathogenesis. While some of these barriers have been at least partially overcome, transformation of low-passage, infectious *B. burgdorferi* remains a challenge. Thus, site-directed mutagenesis of a particular gene may require 3–6 person-months for the transformation process, outgrowth of transformants, screening for mutants with appropriate insertions, and plasmid analysis. As a result, fewer than 100 of the 1739 open reading frames (ORFs) in infectious *B. burgdorferi* have been subjected to site-directed mutagenesis despite intensive efforts by several laboratories.

TRANSPOSON MUTAGENESIS OF *B. BURGDORFERI*

Transposon mutagenesis is a powerful means of producing randomized gene mutations in bacterial genomes (Beaurepaire and Chaconas, 2007). In 2004, Stewart et al. (2004) reported the development of a *Himar1*-based transposon suicide vector called pMarGent for use in *Borrelia* organisms. *Himar1* is a transposon of the *mariner* family that was originally isolated from the blowfly, *Drosophila mauritiana*. Lampe et al. (1999) selected point mutants of the *Himar1* transposase (included so-called C9 and A7 derivatives) that exhibited increased transposition rates; the C9 variant was used in pMarGent. A modified version of pMarGent called pGKT (**Figure 1**) was later developed to include a second selectable marker (Kan^R) in the non-transposed "backbone" in addition to the gentamycin resistance gene present in the transposable element (Stewart and Rosa, 2008). This modification greatly increases the stability of the vector in *E. coli* and facilitates additional alterations (such as the addition of signature tags). In both pMarGent and pGKT, the transposable element consists of the *B. burgdorferi* constitutive promoter *flgB*$_P$ coupled with the gentamicin resistance cassette *aacC1* and the ColE1 origin of replication flanked by two *Himar1* inverted

Table 1 | Challenges to genetic manipulation of *Borrelia* species.

Barrier	Solution(s)
Fragility of organisms	Specialized methods for preparation of *Borrelia* for electroporation (Samuels, 1995)
Slow growth rate; inefficient isolation of mutants	Utilization of either plating or limiting dilution approaches
Plasmid loss during *in vitro* culture	Minimization of *in vitro* passages (Barbour, 1988) Careful monitoring of plasmid content Use of shuttle vectors to replace virulence-associated plasmid-encoded genes (e.g., *pncA*)
Restriction-modification (R-M) systems	Disruption of R-M genes Use of strains lacking plasmids (lp25, lp56) that encode R-M systems DNA methylation prior to transformation

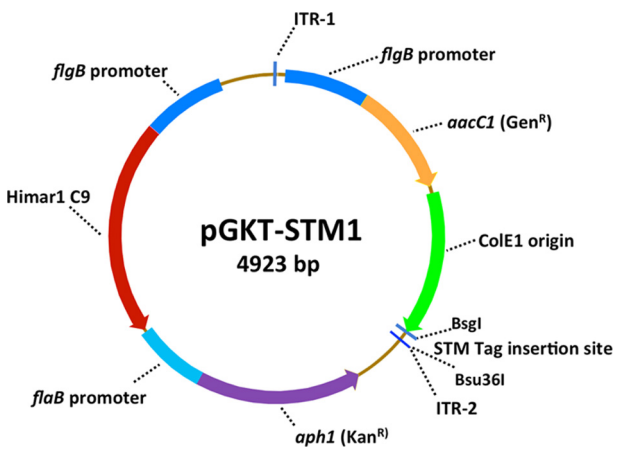

FIGURE 1 | Diagram of the transposon vector pGKT-STM1, modified from the *Borrelia*-enhanced transposon deliver suicide vector pGKT of Stewart et al. (Stewart and Rosa, 2008). Features of pGKT include the use of *B. burgdorferi flgB* and *flaA* promoters to increase the efficiency of expression of the Himar1 c9 transposase and the gentamicin and kanamycin antibiotic resistance markers. The transposable element is flanked by the inverted tandem repeats ITR-1 and ITR-2, and contains the *aacC1* gentamicin resistance cassette and the ColE1 origin of replication (to permit plasmid replication in *E. coli* and transposon insertion site rescue) (Stewart et al., 2004). The modification in pGKT-STM1 and other STM derivatives is the addition of a unique 7-bp signature tag close to ITR2.

tandem repeat sequences (**Figure 1**). The non-transposed region of pGKT includes $flgB_P$::C9 transposase gene and the *B. burgdorferi flaB* promoter with the *aph* kanamycin resistance cassette. *mariner*-based transposons insert at any 5′-TA-3′ sequence with no apparent sequence bias, and result in duplication of the TA sequence at the other end of the transposon. Stewart et al. (2004) demonstrated the apparent random insertion of the pMarGent transposable element in both the chromosome and plasmids with high transformation efficiency ($>3 \times 10^{-5}$) in the non-infectious *B. burgdorferi* clones B31-A*chb*C72 and A3-89. However, these strains lack both lp25 and lp56, which contain the restriction-modification genes *bbe02* and *bbq67* (Lawrenz et al., 2002); lp25 also contains the *pncA* nicotinamidase gene that is required for mammalian and tick infection (Purser et al., 2003; Deneke and Chaconas, 2008). Attempts to transform the low-passage, infectious *B. burgdorferi* A3 and N40 strains (which contain lp25) with pMarGent were unsuccessful (Stewart et al., 2004).

In 2004, Kawabata et al. (2004) introduced the infectious, transformable *B. burgdorferi* strains 5A4NP1 and 5A18NP1. Both strains have a partial deletion and insertion of an *aph* Kan^R cassette in *bbe02*; 5A18NP1 also lacks the plasmid lp56 and hence *bbq67*. In electroporation studies with a shuttle vector, 5A4NP1 and 5A18NP1 yielded 10 colonies/μg DNA and 600 colonies/μg DNA, respectively; in contrast, the parental strains 5A4 and 5A18 (which contain functional *bbe02*) had <1 and 14 colonies per μg DNA. These strains thus reduce the transformation barrier while retaining full infectivity in the mouse model (Kawabata et al., 2004).

Utilization of pMarGent in combination with strain 5A18NP1 resulted in the first successful transposon mutagenesis of infectious *B. burgdorferi* (Botkin et al., 2006). A small library of 33 mutants was examined for transposon insertion site by *E. coli* rescue (Stewart et al., 2004), plasmid content, and infectivity in C3H/HeN mice. Mutations in the genes encoding IMP dehydrogenase (GuaB, involved in inosine-guanine interconversion) and the flagellar switch protein FlaG-1 were found to render *B. burgdorferi* non-infectious, but complementation was not attempted in these experiments. This study, although limited, indicated the feasibility of larger scale transposon mutagenesis studies.

ORDERED TRANSPOSON MUTANT LIBRARY

In 2007, Lin et al. (2012) began the process of accumulating a comprehensive transposon mutagenesis library utilizing signature-tagged versions of pKGT and the *B. burgdorferi* B31 derivative 5A18NP1. pKGT was modified to contain 12 different 7 bp "signature tags" that could be utilized to distinguish between co-infecting strains. This approach was based on earlier STM studies originated by Holden and colleagues (Hensel et al., 1995), as has been widely used for the study of bacterial-host interactions and pathogenesis. Relatively few signature tags were utilized in the construction of the *B. burgdorferi* library because of the inherent difficulties in producing large numbers of mutants in this organism. Each transformation typically yielded between 200 and 300 transformants, so several years was required to accumulate and characterize the number of clones estimated to yield a near saturation library. Overall, 6325 clones were isolated, and the insertion sites for 4479 clones were mapped by *E. coli* rescue and sequencing; the insertion sites for some transformations were not determined because of a high proportion of sibling clones. The plasmid content of the sequence-defined clones was determined using a novel, multiplex Luminex-based approach in which the samples can be analyzed in a high-throughput, 96-well format (Norris et al., 2011).

The large number of transposon mutant clones isolated precluded the use of re-cloning to assure clone purity. Although

only well-isolated colonies were selected, it has been found that a fair number of the preparations contain two co-isolated clones. Colonies of *B. burgdorferi* are diffuse and transluscent, so this co-isolation is most likely due to the presence of overlapping colonies. It is recommended that the transposon mutants be re-cloned prior to detailed analysis and examined for the presence of other clones. The method currently in use involves PCR using primers flanking the transposon insertion site. A "wild type" PCR product the same size as obtained with 5A18NP1 parental strain in addition to a larger product (consistent with the expected transposon insertion) indicates the presence of clone(s) with insertions at other sites.

Each transposition event represents a mini-experiment, in that clones containing transposon insertions that significantly reduce fitness for *in vitro* growth will not survive. The distribution of transposon insertion sites thus provides a map of the genes and other elements essential for *in vitro* culture or replicon maintenance. The map is incomplete in the case of the existing *B. burgdorferi* transposon mutagenesis library, in that the library is not saturating. However, patterns can be discerned based on the lack of crippling mutations in groups of required genes. For example, the only ribosomal protein gene mutation isolated was in the last 10% of the ORF for the S21 protein, consistent with this mutation resulting a truncated but functional product. The 22 clones recovered with mutations in one of the two 23S ribosomal RNA genes had no obvious growth defect, indicating that cells are functional with only a single copy of this redundant gene. Only 34% of predicted protein-encoding genes in the chromosome had insertions, whereas 57% of predicted plasmid genes had insertions, most likely because of the higher proportion of essential genes required for *in vitro* culture in the chromosome. Variability occurred in the "hit rate" of both genes and replicons, with some genes and plasmids having as high as 23.06 (conserved hypothetical gene *bb0017*) and 18.01 (circular plasmid cp9) unique insertions per kb of DNA, respectively. The reason for this variability is not known, but conceivably may be related to improved *in vitro* growth characteristics in some mutants. Core gene classes that did not have debilitating mutations included those involved in DNA synthesis, transcription, translation, protein translocation, peptidoglycan synthesis, glycolysis, and pentose phosphate pathway, lipid interconversion, the V-type ATPase (presumably involved in maintaining the proton gradient across the cytoplasmic membrane), and the ubiquitous chaperones GroE and HP70 (Lin et al., 2012). Gene mutations that were permissive for *in vitro* growth included most of the numerous lipoprotein genes and several genes involved in chemotaxis. Whereas an intact chemotaxis apparatus is not required for culture, relatively few mutants were obtained in flagellar genes. Those mutants that were obtained in flagellar genes often produced elongated cells, indicative of defective cell division. Thus, spirochetal motility may facilitate the cell division process.

DETECTION OF VIRULENCE DETERMINANTS IN FUNCTIONAL GROUPS

Screening of mutants for mouse infectivity by needle inoculation has been initiated using a Luminex-based STM analysis approach (Lin et al., 2012). In a typical STM experiment, 11 mutants, each with a different signature tag, were inoculated subcutaneously at 10^5 of each mutant into six C3H/HeN mice. Groups of three mice were analyzed at 2 and 4 weeks post-inoculation (PI), and up to five tissues (joint, heart, bladder, inoculation site, and a distal skin site) were examined from each mouse. In many experiments, the tissue specimens were each examined by both direct extraction of DNA from the tissue and by culture of the *B. burgdorferi* from the tissue followed by DNA extraction from the culture. A semi-quantitative, high-throughput Luminex©FlexMap™ procedure was developed for detection of *B. burgdorferi* STM mutants in mammalian hosts. The Luminex technology allows the assessment of up to 200 signals in a single well of a 96-well plate, thus providing a high-throughput method. Five tissues per mouse (joint, heart, bladder, inoculation site, and a distal skin site) were examined at two time points (2 and 4 weeks) with three mice each. A relatively small number of signature-tagged mutants were utilized in each STM infection experiment because of the challenges of constructing *B. burgdorferi* mutant libraries with multiple sequence tags, as well as the potential of a bottleneck effect. By combining the multiple mice, time points, tissues, and DNA preparation procedures, up to 60 data points per clone were obtained in a typical STM experiment (Lin et al., 2012). The data consisted of the Median Fluorescence Intensity (MFI) values obtained by Luminex analysis for each clone. Results obtained with representative infectious and non-infectious clones indicated that MFI values <100 (arbitrary units) were consistent with low infectivity. Variability in the numbers of organisms in individual tissue specimens occurs because of the paucibacillary nature of infection, and averaging of the multiple data points was found to yield more reliable results.

Functional genomics and pathogenomics approaches were applied to select mutants for the analysis of the roles of *B. burgdorferi* genes in metabolism, structure, pathogenesis, and other biological functions. The infectivity of 502 mutants in 422 genes has been tested (Lin et al., 2012), and the roles of some of these genes in pathogenesis and biological activities are being characterized more fully. Transposon mutations in genes associated with DNA recombination and repair, chemotaxis, motility, transport systems, plasmid maintenance, genes involved in gene regulation, sRNA genes, metabolic pathways, proteases, complement regulator-acquiring surface proteins (CRASPs), predicted lipoproteins, conserved hypothetical proteins (CHPs), and hypothetical proteins (HPs) of *B. burgdorferi* were selected for STM screening. A representative number of mutants in intergenic regions were also tested for infectivity to evaluate potential polar effects, small RNA genes, and regulatory regions (Lin et al., 2012). Some of the mutants exhibiting reduced infectivity in the STM screen are listed in **Table 2**. As an example, the effects of mutations in 23 genes involved in DNA recombination and repair on *vlsE* recombination were determined, and the results indicated that transposon mutants in *ruvA*, *ruvB*, and *mutS* exhibited reduced infectivity in mice and diminished *vlsE* recombination. Using this approach and site-directed mutagenesis, RuvA, RuvB, and MutS were the first trans-acting factors identified as necessary for *vlsE* recombination and antigenic variation (Dresser et al., 2009; Lin et al., 2009).

Table 2 | *B. burgdorferi* B31 virulence determinant candidates as indicated by STM analysis[a].

Functional categories and gene products	Genetic locus	STM mouse infectivity phenotype	Verification by needle (N) or tick (T) inoculation (References)
DNA RECOMBINATION AND REPAIR			
RuvA	BB0023	Intermediate infectivity	N (Lin et al., 2009); T (Lin et al., 2009)
RuvB	BB0022	Intermediate infectivity	N (Lin et al., 2009); T (Lin et al., 2009)
MutS	BB0797	Intermediate infectivity	N (Lin et al., 2009)
CHEMOTAXIS			
CheA1	BB0567	Non-infectious	T (Lin et al., 2012)
CheA2	BB0669	Non-infectious	T (Lin et al., 2012)
CheB1	BB0415	Intermediate infectivity	
CheB2	BB0568	Non-infectious	T (Lin et al., 2012)
CheR2	BB0414	Intermediate infectivity	
CheW2	BB0565	Non-infectious	
CheW3	BB0670	Non-infectious	
CheX	BB0671	Non-infectious	
CheY2	BB0570	Non-infectious	
SulP	BB0566	Non-infectious	
Mcp1	BB0578	Non-infectious	T (Lin et al., 2012) (intermediate)
Mcp3	BB0597	Non-infectious	
Mcp4	BB0680	Non-infectious	T (Lin et al., 2012)
Mcp5	BB0681	Non-infectious	T (Lin et al., 2012)
FLAGELLAR STRUCTURE AND ASSEMBLY			
FliG1	BB0221	Non-infectious	
FliZ	BB0276	Non-infectious	
FlbA	BB0287	Non-infectious	T (Lin et al., 2012)
FliI	BB0288	Non-infectious	T (Lin et al., 2012)
FliH	BB0289	Non-infectious	T (Lin et al., 2012)
FlaA	BB0668	Non-infectious	T (Lin et al., 2012)
FlgI	BB0772	Non-infectious	
PHOSPHOTRANSFERASE SYSTEM			
ptsG	BB0645	Non-infectious	
FruA1	BB0408	Non-infectious	
FruA2	BB0629	Variable infectivity	
MalX1	BB0116	Intermediate infectivity	
MalX2	BBB29	Intermediate infectivity	
ChbB	BBB06	Non-infectious	
ABC TRANSPORT PROTEINS, *BORRELIA* EFFLUX SYSTEM (Bes), AND OTHER TRANSPORT SYSTEMS			
ProX	BB0144	Intermediate infectivity	
MglA	BB0318	Non-infectious	
ABC transporter ATP-binding protein	BB0573	Non-infectious	
OppA-2	BB0329	Intermediate infectivity	
OppA-3	BB0330	Intermediate infectivity	
OppA-4	BBB16	Variable infectivity	
OppA-5	BBA34	Variable infectivity	
BesA	BB0141	Intermediate infectivity	
BesC	BB0142	Intermediate infectivity	
LctP	BB0604	Non-infectious	
NhaC-1	BB0637	Non-infectious	
NhaC-2	BB0638	Non-infectious	
Na^+/Ca^+ exchange protein	BB0164	Intermediate infectivity	
GlpF	BB0240	Intermediate infectivity	
GltP	BB0729	Intermediate infectivity	
purine permease P1	BBB22	Intermediate infectivity	

(Continued)

Table 2 | Continued

[a] Transposon mutants lacking infectivity-related plasmids (lp25, lp28-1, lp36) or with insertions in the last 10% of the gene were excluded from this analysis. Non-infectious, mean MFI <100 and <20% of tissue sites with MFI >100. Intermediate infectivity, mean MFI between 100–500, and 20–50 percent of tissue sites with MFI >100. Variable infectivity, independent transposon mutants yielded different infectivity results. Transposon mutant STM infectivity results that have been verified by needle or tick inoculation with individual clones are shown with the pertinent reference(s).

Motility and chemotaxis have long been thought to be key factors in borrelial infectivity and pathogenesis, but genetic evidence for that role has been obtained only recently (Li et al., 2010; Sze et al., 2012; Guyard et al., 2013; Sultan et al., 2013). In the STM transposon mutant analysis, 10 of 14 mutations in chemotaxis genes resulted in a severe loss of infectivity, indicating that the chemotaxis pathway is critical to infectivity (Lin et al., 2012). The genes involved in flagellar structure and assembly are also required for mouse infection. Some of these mutants, such as *fliH*, *fliI*, and *flbA*, had reduced motility, division defects, and structural changes in the flagellar motor. The cell division, motility, and structural defects of these mutants may all play a role in the observed low infectivity phenotype.

Transport is another key function in pathogenesis. The phosphoenolpyruvate phosphotransferase system (PEP-PTS) plays an important role in carbohydrate transportation and phosphorylation, also a potential role in gene regulation. STM results consistent with non-infectivity were observed for a transposon mutant in the gene encoding the glucose-glucoside specific IIBC component PtsG (**Table 2**); reduced infectivity was observed with mutants in the genes for the maltose specific PTS transporters

MalX-1 and MalX-2 and the fructose-mannose transporter FruA-1. Disparate infectivity results were obtained with two mutants in the gene encoding the fructose-mannose specific IIB component FruA-2, so further analysis is needed. The Chb system is thought to play important roles in the utilization of chitobiose during infection of ticks (Tilly et al., 2001; Rhodes et al., 2010; Sze et al., 2013). Not surprisingly, inactivation of the genes encoding the chitobiose-specific components ChbA and ChbC did not affect mouse infection by needle inoculation in our system. However, the chitobiose-specific IIBC component ChbB mutant tested exhibited a no-infectivity phenotype (Lin et al., 2012); this finding will require further verification and investigation. In addition, the ABC transporter genes encoding the ATP-binding protein of the galactose transporter MglA, ProX, and an uncharacterized ATP-binding protein (*bb0573*) exhibited a low infectivity phenotype in the STM system. The five OppA oligopeptide ABC periplasmic binding proteins of *B. burgdorferi* exhibit varied infectivity phenotypes, but obviously have required specific binding properties. Genes encoding the lactose permease (LctP), Na^+/H^+ antiporter proteins (NhaC-1 and NhaC-2) also appear to required for mouse infection. Mutation of *Borrelia* efflux system genes *besC* and *besA*, Na^+/Ca^+ exchange protein (*bb0164*), glycerol uptake facilitator GlpF (*bb0240*), glutamate transporter (*bb0729*), and purine permease P1 (*bbB22*) resulted an intermediate infectivity phenotype (**Table 2**).

SCREENING OF VIRULENCE GENES IN INFECTIVITY-RELATED PLASMIDS

Lyme disease *Borrelia* species typically contain over 20 linear and circular plasmids. These replicons have been called "minichromosomes" because several are required for the complex life cycle of these organisms. *Himar1*-based transposon mutagenesis resulted in over 2500 sequence-defined unique insertion sites in the *B. burgdorferi* plasmids, disrupting 503 of 790 ORFs (68%); the proportion of ORFs disrupted in each plasmid ranged from 29 (in lp5) to 91% (cp9). A high proportion of borrelial plasmid genes do not have orthologs in other organisms, so many have no predicted function. There are also many paralogous families among the plasmid genes. Although over 30 *B. burgdorferi* plasmid genes have been successfully inactivated by site-directed mutagenesis (Rosa et al., 2005), the availability of the transposon mutant library will yield valuable information about these enigmatic replicons and their genes.

To date, representative mutants in genes associated with the infectivity-related plasmids cp26, lp25, lp28-1, lp36, and lp54 were analyzed using the STM process and Luminex-based technology (Lin et al., 2012). A summary of the results obtained for cp26, lp36, and lp54 are displayed in **Figure 2**. The percentage of positive tissue sites and mean MFI value were found to be useful parameters in summarizing the data from many animals, time points, tissue sites, and sampling methods, resulting in up to 60 data points. These two criteria yielded comparable patterns (**Figure 2**). The contiguous range of values sometimes does not permit a clear "±" result, resulting in an intermediate (or indeterminate) infectivity category in some instances. Nevertheless, the results obtained with mutants in these plasmids provide evidence that a high number of genes encoded in these plasmids appear to be required for full infectivity in mice. STM analysis is considered a screening procedure, and all results obtained by this method need to be confirmed by single clone inoculation and genetic complementation.

Transposon insertions were obtained in 26 of the 29 genes in cp26. Inactivation of 11 genes appeared to render a non-infectious phenotype based on the STM analysis, including those encoding chitobiose transporter ChbB, the PF32 and PF49 plasmid maintenance proteins, CHP BBB14, GMP synthase GuaA, and OspC (**Figure 2**). In addition, mutations in the genes encoding the CHP BBB02, HP gene BBB09, GuaB (inosine-5-monophosphate dehydrogenase, and purine permeases PbuG1 and PbuG2) yielded reduced STM parameter values (**Figure 2**) (Lin et al., 2012). Mutants in the genes encoding OspC, GuaA, GuaB, PbuG1, and PbuG2 had all been identified as virulence determinants previously (Byram et al., 2004; Grimm et al., 2004; Pal et al., 2004; Stewart et al., 2006; Tilly et al., 2006, 2009; Jewett et al., 2007a).

Of the 60 annotated genes in lp36, 38 protein-encoding genes have been disrupted. Transposon insertions were also found in one small RNA gene and two intergenic spacers. A total of 41 unique mutants have been examined for their infectivity in STM system (**Figure 2**). Consistent with previously published report by Jewett et al. (2007b), our result indicates that inactivation of the gene encoding adenine deaminase AdeC resulted in reduced mouse infection. Disruption of the BBK32 fibronectin-binding protein gene did not have an apparent effect on infectivity. BBK32 is involved in vascular endothelium adherence and dissemination, but is not absolutely required for infection (Li et al., 2006; Seshu et al., 2006; Norman et al., 2008; Moriarty et al., 2012); it is possible that the high infectious dose (10^5) used for each mutant in the STM analysis was sufficient to establish disseminated infection. Mutations in a region encompassing *bbk02.1* to *bbk07* (a poorly defined region in which some of the genes are no longer annotated) resulted in decreased infectivity. Mutations in several other CHP genes appear to result in reduced infectivity, including those in *bbk15*, and putative lipoprotein genes *bbk13*, *bbk36*, *bbk37*, and *bbk45* through *bbk50* (**Figure 2**) (Lin et al., 2012). *In vivo* expression technology (IVET) studies by Ellis et al. (2013) indicated that *bbk46* is highly expressed during mouse infection, and further analysis showed that a *bbk46* mutant was defective in persistent infection of mice.

Linear plasmid lp54 is one of the most highly conserved elements in the *Borrelia* genome. A total of 75 protein-encoding genes are encoded on lp54, and some of them are differentially expressed in tick vector and mammalian hosts. Most proteins encoded on lp54 do not have homologs in other organisms and are of unknown function (Casjens et al., 2000; Jewett et al., 2007a). Transposon mutants were obtained in 58 genes on lp54, and a high proportion of these mutants had decreased infectivity (**Figure 2**). Most of these are *Borrelia* species-specific proteins with unknown functions and several are *B. burgdorferi*-specific proteins. Transposon insertions in BBA03 (outer surface protein), BBA14 (OrfD paralog), BBA21 (PF49), BBA24 (DbpA), BBA30 (CHP), BBA31 (CHP), BBA35 (HP), BBA40 (HP), BBA43 (CHP), BBA44 (CHP), BBA55 (CHP), BBA57 (CHP), and BBA59

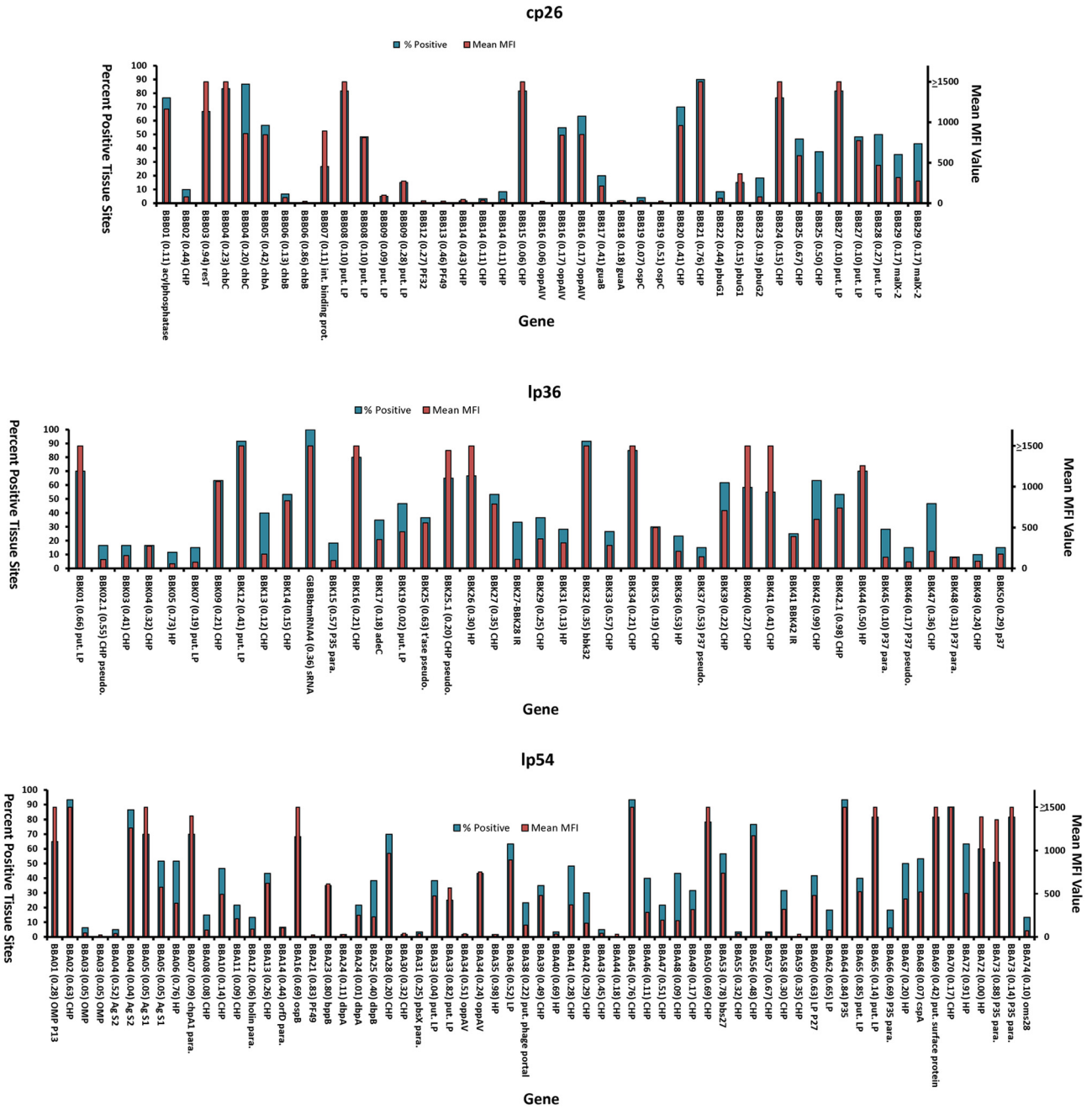

FIGURE 2 | Mouse infectivity of transposon mutants in plasmid-encoded genes, as determined by Luminex-based STM analysis. Results obtained for genes in plasmids cp26, lp36, and lp54 are shown. For each mutant, the data represent the percent of positive tissue sites (MFI >100; blue bars) and mean MFI value (red bars) for 30–60 data points. Only clones that have all plasmids known to be required for mouse infection (cp26, lp25, lp28–1, lp36, and lp54) were analyzed. The gene designation, insertion ratio (bp from 5′ end to insertion/total bp), and description are provided. Abbreviations: HP, hypothetical protein; CHP, conserved hypothetical protein; para., paralog; pseudo., pseudogene; put. LP, putative lipoprotein; t'ase, transposase.

(CHP) resulted in a non-infectious phenotype by STM analysis. Additional mutants exhibited reduced STM analysis parameters, and may also have attenuated phenotypes. Disparate results were obtained for *bba04*, with two clones having high and low infectivity phenotypes (**Figure 2**) (Lin et al., 2012). Bestor et al. (2012) determined previously that a *bba03* deletion mutant had a competitive disadvantage in tick-mediated infection of mice in comparison to the wild type parental strain; a deletion mutant lacking *bba01-bba07* was further attenuated, indicating that other genes in this region may be required for full infectivity in mice. In other studies, site-directed disruption of the decorin binding protein DbpA was found to reduce mouse infectivity by

needle inoculation but not by tick-mediated infection (Hagman et al., 2000); the transposon mutant results are consistent with the prior needle inoculation findings. Mutation in gene of *ospB* had little apparent effect on mouse infection, although this surface lipoprotein expressed predominantly in the tick infection and play the important role in tick midgut colonization. BBA64 is a member of the P35 paralogous protein family, and previous studies indicated that BBA64 is required for tick transmission (Gilmore et al., 2010), but mutants in this gene had only minor effects on infection by needle inoculation (Maruskova and Seshu, 2008; Maruskova et al., 2008). The STM analysis was consistent with the latter result, in that the available *bba64* mutant (which had a transposon insertion in last 16% of reading frame) was fully infectious (**Figure 2**); in this case, however, it is possible that the mutant expresses a truncated but functional protein. Further studies with BBA64 immunization indicate that antibodies against this protein do not protect against needle or tick infection (Brandt et al., 2014). STM analysis indicated that mutation of lp54-encoded protein Oms28 (*bba74*) resulted in decreased infectivity (Lin et al., 2012), but these results are not consistent with prior studies with B31 A3, which apparently expresses a truncated BBA74 product (Mulay et al., 2009).

BptA, PncA, and Bbe31 encoded on lp25 had been identified as virulence determinants previously (Purser et al., 2003; Revel et al., 2005; Strother et al., 2005; Zhang et al., 2012). The transposon mutant library contains insertions in the genes encoding BptA and PncA, and these mutants exhibited reduced or non-infectivity as reported previously. In addition, mutations in genes of *bbe04.1*, *bbe09*, *bbe18*, *bbe19*, *bbe24*, and *bbe29.1* substantially reduced infectivity, and intermediate infectivity was observed in mutants in genes of *bbe07* and *bbe18* (Lin et al., 2012).

In the STM studies, low infectivity was observed among lp28-1 mutants of genes encoding BBF03, BBF05, BBF10, BBF18, and BBF25. Mutation of genes of BBF04, BBF07, BBF08, BBF12, BBF19 appear to cause reduce infectivity. Interestingly, transposon insertions in intergenic spacers between *bbf25-26* and *bbf28-29* causes severe defect in spirochete infectivity (Lin et al., 2012). The *vls* locus involved in VlsE antigenic variation and immune evasion is located at one end of lp28-1 (Zhang et al., 1997). Only one transposon insertion was obtained in the *vls* silent cassettes, and none were obtained in the *vlsE* expression site (Lin et al., 2012). The silent cassette mutant was infectious, indicating that interruption of the silent cassette region is not sufficient to inhibit the *vlsE* recombination process.

Tn-SEQ

Transposon insertion site sequencing approaches such as Tn-seq is a powerful genetic screening technique in which high-throughput sequencing is used as a detection method in transposon mutagenesis screens to identify the contribution of individual genes to bacterial fitness under specific growth conditions (van Opijnen and Camilli, 2013). A library of transposon mutants (input pool) can be utilized to infect animals or subjected to *in vitro* selection conditions, and the resulting organisms (output pool) collected. Amplification of the transposon insertion site region and massively parallel sequencing provides a high-throughput comparison of the input pool and output pool. In comparison with STM approaches, Tn-seq has several advantages (**Table 3**), including the ability to examine an entire library in one set of replicate experiments without prior characterization of the insertion sites. For *Borrelia* studies, a disadvantage is that the plasmid content of each clone is not known, so the low infectivity of a particular mutant may be due to concomitant plasmid loss. Thus, presence of more than one clone with a mutation in a particular gene in the input pool and the underrepresentation of those clones in the output pool may be necessary to establish the gene product as a potential virulence determinant.

In 2013, Troy et al. adapted Tn-seq for use in studies of *B. burgdorferi* using the STM transposon library (Troy et al., 2013). In a Tn-seq experiment, the transposon library is subjected to growth under a test condition. Genomic DNA is then isolated from the *B. burgdorferi* population before and after selection. The DNA is fragmented and cytosine tails (C-tails) are added using terminal deoxynucleotidyl transferase. The addition of a C-tail allows for the amplification of the transposon-genomic

Table 3 | Advantages and disadvantages of ordered library, signature-tagged mutagenesis (STM) and Tn-seq approaches.

Approach	Advantages	Disadvantages
STM analysis	Does not require availability of high-throughput sequencing	Requires sequence analysis of the Tn insertion points of individual clones of interest either before (ordered library) or after STM screening
	Tn mutant clones available for further analysis after STM screening	Relatively low throughput method that requires multiple animal experiments for infectivity screening
	Relatively small number of clones used per experiment decreases possibility of bottleneck effects	Potential cross-contamination during clone isolation can complicate interpretation
Tn-seq	Capacity to screen an entire library in a single infectivity experiment	Lack of plasmid content information; low infectivity may be related to plasmid loss
	Does not require isolation and characterization of individual Tn mutant clones	Tn mutants of interest would have to be re-isolated for further study (if not from an ordered library)
	Easier application to *in vitro* screening methods (e.g., metabolic, adherence, or cell co-culture experiments)	Possible bottleneck effects (non-uniform recovery of organisms) may necessitate use of large numbers of animals or cultures
		Relatively high minimum analysis cost

DNA junction using primers specific to the ColE1 site on the end of the transposon and the C-tail. The relative abundance of each mutant is determined by sequencing the chromosomal DNA flanking the transposon *en masse* using a primer that anneals to the end of the transposon. In this way, the exact location of every transposon insertion present in the library is revealed. The frequency of the insertion sequence in the library corresponds to the frequency of the transposon mutant containing that insertion in the original bacterial populations. The fitness of each insertion mutant in the test condition is determined by comparing the relative frequency of each mutant before and after growth under the selective pressure. Sequences that decrease in frequency in the library after selection have insertions in genes contributing to bacterial growth in the test condition. The quantitative nature of the high-throughput sequencing enables the discovery of unknown growth determinants including the differentiation of factors with partial phenotypes and/or those acting in redundant pathways. Furthermore, due to the non-specific nature of the genome library preparation, this Tn-seq technique can be used with any transposon as long as the sequence of the 3′ end of the transposon is known.

Tn-seq is particularly amenable to *in vitro* screens as unlike STM experiments that are limited to a small subset of mutants in each experiment, the entire transposon library can be tested in a single competition assay. Tn-seq has also been used to screen *in vivo* fitness. However, a potential complication of animal screens is a population bottleneck at the site of infection that results in the stochastic loss of a significant portion of the injected *B. burgdorferi* by 3 days post-infection. If all transposon mutants containing a particular insertion are lost at the inoculation site due to the bottleneck rather than decreased fitness, the disrupted gene may be misidentified as contributing to bacterial survival during infection. However, experiments by Troy et al. have demonstrated that the effects of this bottleneck can be circumvented, at least in part, by combining results from the *B. burgdorferi* populations recovered from multiple mice infected with the same inoculum (Troy et al., 2013). Part of the bottleneck effect is due to innate immune mechanisms (resulting in a non-specific reduction of the inoculated population), as demonstrated by the reduced magnitude of the bottleneck in MyD88$^{-/-}$ mice in comparison with wild type mice.

CONCLUSIONS

Results obtained to date with *B. burgdorferi* transposon mutants indicate that a high proportion of genes are required for mouse infection. The assessment of multiple tissue sites at two different time points using Luminex FlexMap technology provides a robust data set. Tn-seq has multiple advantages and is expected to greatly accelerate the application of transposon mutant libraries to studies of infectivity and other biological processes. Both STM and Tn-seq are considered screening methods, so the results must be confirmed by studies with isolated transposon mutants (or independently derived mutants) and genetic complemention. In addition, polar effects on downstream genes should be verified for observed reduced infectivity phenotype. All mutants should be re-cloned prior to more thorough studies due to the potential occurrence of co-isolation of two or more clones in a single colony. Finally, cross-complementation may occur between clones in the transposon library. We believe that the continued analysis of the mutant library will lead to a more comprehensive delineation of the virulence determinants of *B. burgdorferi* and will improve our understanding of the novel pathogenic and biologic properties of Lyme *Borrelia* organisms and other invasive, non-toxigenic, persistent pathogens.

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Phagosomal TLR signaling upon *Borrelia burgdorferi* infection

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Internalization and degradation of live Bb within phagosomal compartments of monocytes, macrophages and dendritic cells (DCs), allows for the release of lipoproteins, nucleic acids and other microbial products, triggering a broad and robust inflammatory response. Toll-like receptors (TLRs) are key players in the recognition of spirochetal ligands from whole viable organisms (i.e., vita-PAMPs). Herein we will review the role of endosomal TLRs in the response to the Lyme disease spirochete.

Keywords: *Borrelia burgdorferi*, toll-like receptors, innate immunity, phagosomal signaling, Lyme disease

INTRODUCTION

Borrelia burgdorferi (Bb), the causative agent of Lyme disease (Radolf et al., 2012) is the most prevalent vector-borne disease in North America (Levi et al., 2012). In nature, Bb persists asymptomatically in rodent reservoirs, such as the white-footed mouse *Peromyscus leucopus* (Barthold and Philip, 2010). Infection in humans is incidental, and probably represents a dead-end for the bacterium, as the spirochete is not well adapted to the human host.

Bb contains a complex bacterial genome (Fraser et al., 1997), and is composed of different strains and genospecies with differences in infectivity, host range, and tissue tropism (Schutzer et al., 2011; Yang et al., 2013). Different from gram-negative bacteria, Bb does not contain LPS (Takayama et al., 1987), and instead it has a number and variety of lipoproteins, many of them embedded on the spirochete's outer membrane (Bergstrom and Zückert, 2010). A number of well-known borrelial lipoproteins (e.g., OspA) are preferentially expressed at particular stages of the enzootic cycle (Radolf et al., 2012).

Bb also lacks any toxigenic molecules, thus, the clinical manifestations associated with Lyme disease are thought to result from the host's innate and adaptive immune response to the invading spirochete (Radolf et al., 2012). A great amount of research has been done trying to elucidate the mechanism by which Bb causes inflammation, which in some Lyme Disease patients manifests as severe arthritis, carditis, and/or central nervous system disorders (Radolf and Samuels, 2010). Initial studies focused on the role of *Borrelia*'s abundant lipoproteins, which are capable of binding CD14 and Toll-like receptor (TLR) 2/TLR1 heterodimers on the surface of phagocytic cells to induce the production of inflammatory cytokines (Weiss, 2010; Radolf et al., 2012). However, we and others have shown that internalization and degradation of live Bb by monocytes and macrophages within phagosomal compartments, allows for the release of lipoproteins and other microbial products, including RNA and peptidoglycan, eliciting a broader and more complex inflammatory response that can possibly take place on the cell surface of innate immune cells (Salazar et al., 2009; Cervantes et al., 2011). We have called this process "phagosomal signaling."

TLRs are transmembrane receptors which can recognize various pathogen-associated molecular patterns (PAMPs), including spirochetal lipoproteins and nucleic acids. The interaction between spirochetal PAMPs and the TLRs triggers a variety of intracellular signaling pathways leading to the production of various cytokines, including type I interferons (Salazar et al., 2009; Cervantes et al., 2011). This review focuses on the role of endosomal TLRs in Bb-mediated phagosomal signaling.

B. BURGDORFERI INFECTION AND THE INNATE IMMUNE RESPONSE

While studies have shown that neutralizing antibodies are important in host defense against Bb infection, the innate immune system is now known to have a critical role in spirochetal recognition and clearance from infected blood and tissues (Barthold et al., 1992). Monocytes, dendritic cells, macrophages, Natural Killer cells (NK-cells), NK-T cells, and polymorphonuclear cells (PMNs), all contribute to generate a coordinated and robust response to Bb infection (Salazar et al., 2003; Moore et al., 2007). We have proposed a model, where this response is initiated through recognition of specific Bb ligands during phagocytosis, primarily by the activation of endosomal TLRs (Moore et al., 2007; Petzke et al., 2009; Salazar et al., 2009; Cervantes et al.,

2011). In this section we will briefly describe the role of NKT cells, PMNs, monocytes/macrophages and DCs in spirochetal recognition.

NATURAL KILLER T (NKT) CELLS

NKT cells play an important role in the regulation of the inflammatory response during Bb infection (Lee et al., 2010). In one study, Bb-infected NKT-depleted-mice had greater inflammation in infected joint tissues, as well as decreased pathogen clearance (Tupin et al., 2008). This finding is not surprising, given that Antigen-presenting cells (APCs) activate NKT cells through presentation of a diacylglycerol glycolipid (Olson et al., 2009). Upon activation with Bb, NKT cells upregulate of IFNγ to activate and differentiate macrophages (Olson et al., 2009). It is not know whether NKT cells have a role in the pathogenesis of Lyme arthritis in humans.

POLYMORPHONUCLEAR CELLS (PMNs)

The neutrophil is an essential element of the inflammatory response to the Lyme disease spirochete in both skin and joints (Salazar et al., 2003; Radolf and Samuels, 2010). Indeed, PMNs have been shown to play a significant role in the development of Lyme arthritis in experimentally infected mice (Nardelli et al., 2008; Codolo et al., 2013). In humans, PMNs are the primary cell type present in joint fluids from LD patients diagnosed with acute arthritis. PMNs are responsible for the production of several inflammatory cytokines, various chemokines, and stimulating factors that are likely to contribute to inflammation in the joints by inducing macrophage migration and differentiation and T-cell activation (Georgilis et al., 1991; Brown et al., 2003; Mantovani et al., 2011).

MONOCYTES

Monocytes are thought to have an important role in the production of proinflammatory cytokines during Bb infection (Salazar et al., 2009). Phagocytosis of Bb by human monocytes activates signaling cascades, which induce transcription of proinflammatory cytokines, including IL-6, TNF-α, and IL-12 (Salazar et al., 2003; Cruz et al., 2008; Salazar et al., 2009)

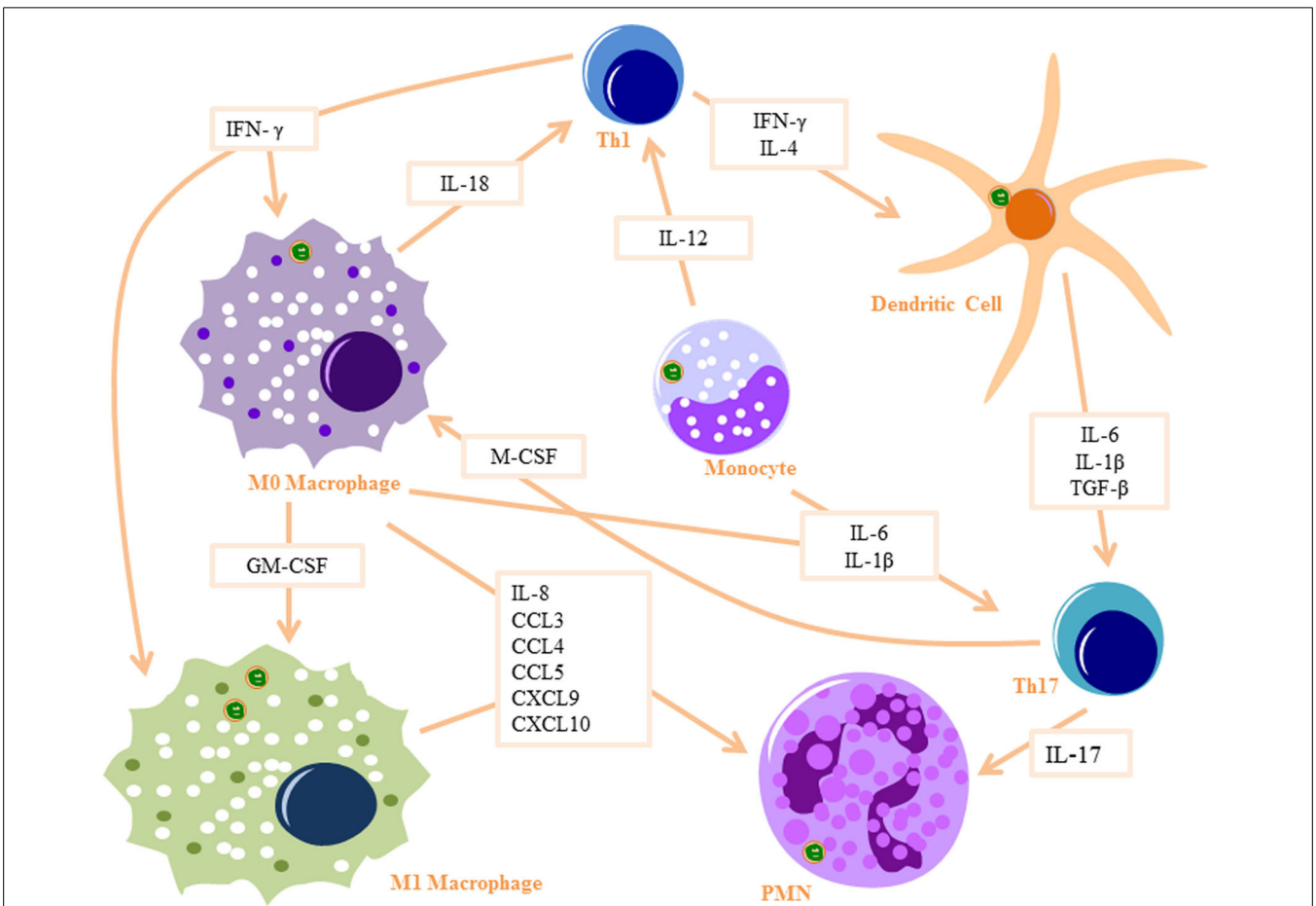

FIGURE 1 | ***Innate signaling cascade in response to Bb Phagocytosis*****: Monocytes produce IL-12 to activate Th1 cells and IL6 and IL-1β to activate Th17 cells.** Monocytes differentiate into macrophages when stimulated with IFN-γ (produced by Th1 cells) and M-CSF (produced by Th17 cells) and differentiate into dendritic cells when stimulated with IL-4 and IFN-γ. M0 macrophages produce IL-18 to activate Th1 cells and IL-6 and IL-1β to activate Th17 cells, as well as several PMN recruitment chemokines. They also differentiate into M1 macrophages when stimulated with GM-CSF and IFN-γ. Dendritic cells produce IL-6, IL-1β, and TGF-β to activate Th17 cells. Th17 cells produce IL-17, which is a strong PMN attractant. Part of images from Motifolio drawing toolkit (www.motifolio.com) were utilized in the figure preparation.

(**Figure 1**). IL-6 activates T helper cells and induces Th17 differentiation, which in turn can recruit PMNs through IL-17 production (Burchill et al., 2003). TNF-α has the ability to increase vascular permeability at the infection site, resulting in increased infiltration of PMNs and other innate immune cells. IL-12 production by the monocytes is likely to induce Th1 differentiation and increased secretion of IFNγ (Biswas and Mantovani, 2010). IFNγ plays an important role in M1 macrophage differentiation at infection sites (Mosser and Edwards, 2008). Monocytes also upregulate pro-IL-1β in response to Bb (Cruz et al., 2008), which when cleaved into IL-1β by caspase-1 can also induce Th17 differentiation (Chung et al., 2009). Bb is also known to induce monocyte inflammatory cell death through an intrinsic signaling pathway (Cruz et al., 2008), a mechanism which possibly leads to the recruitment of other immune cells to Bb-infected tissues.

MACROPHAGES

Bb phagocytosis by macrophages results in increased transcription of IL-1β, IL-6, TNF-α, and type I IFNs (Strle et al., 2009). Bb-infected macrophages also produce a number of PMNs chemoattractants, including CCL3, CCL4, CCL5, CXCL9, and CXCL10 (Gautam et al., 2012). Macrophages also produce IL-18 in response to Bb infection (Dennis et al., 2006; Oosting et al., 2011), which in turn induces IFNγ production by Th1 cells, driving M1 macrophage polarization (Mosser and Edwards, 2008). M1 macrophages upregulate iNOS and reactive oxygen species (Biswas and Mantovani, 2010), which are essential in clearance of Bb (Boylan et al., 2008). Phagocytosis of Bb by macrophages results in significant production of the anti-inflammatory cytokine IL-10, to mediate resolution of the already initiated cytokine response, a phenomenon which may play a critical role in Lyme disease severity and arthritis development (Lazarus et al., 2008; Gautam et al., 2012; Chung et al., 2013).

DENDRITIC CELLS (DCs)

DCs are enriched in early Lyme disease skin lesions (*erythema migrans*) (Salazar et al., 2003) and are amongst the first immune cells to come into contact with Bb in the skin (Mason et al., 2014). Phagocytosis of Bb activates DCs by inducing expression of CD83 and upregulating expression of CD40, CD80, CD86, and HLA-DR (Suhonen et al., 2003) and lead to increased transcription of proinflammatory cytokines, including IL-6, IL-1β, and TNF-α (Petzke et al., 2009). TGF-β is also produced by DCs after Bb infection, a cytokine which in turn induces Th17 differentiation (Chung et al., 2009). The PMN chemotactic factor IL-8 (Dennis et al., 2006), as well as a number of other PMN chemoattractants (Hartiala et al., 2007) are produced by DCs following Bb infection. Upon contact with Bb in the skin, DCs migrate to the lymph nodes where they present antigens to T cells and induce adaptive immune responses (Mason et al., 2014). Similarly to Bb-stimulated macrophages, IL-10 is also elicited from Bb-infected DCs (Chung et al., 2013). IL-10 down-regulates macrophage activation, decreases the production of proinflammatory mediators, and suppresses phagocytosis-associated events that are important for mediating both innate and adaptive immune responses by APCs (Chung et al., 2013).

MURINE vs. HUMAN

The development of a murine model to study Lyme disease has greatly advanced our understanding of the cellular and humoral responses to Bb. All inbred stains of laboratory mice are susceptible to Bb infection, although each strain differs in their disease severity. C3H/He and Balb/c mice are more susceptible while C57BL/6 and SJL mice tend to be more resistant to Bb infection (Barthold and Philip, 2010). Differences in the arthritis severity of these two strains might not involve discrepancies in bacterial clearance mechanisms, as both harbor similar numbers of spirochetes within their ankle joints (Ma et al., 1998). C3H cells have been reported to produce higher levels of NFκ-B cytokines than C57BL/6 upon stimulation with purified borrelial lipoproteins (Ganapamo et al., 2003). However, differences in acquired immune responsiveness after whole Bb infection are still not well understood. Furthermore, studies on the role of NFκB-dependent cytokines in arthritis development have yielded conflicting results (Wooten and Weis, 2001). The susceptible strains produce higher IgG titers and tend to have more severe arthritis and carditis with similar manifestations to human infection (Barthold and Philip, 2010; Weiss, 2010). Importantly, no mouse strain develops the hallmark skin lesion *erythema migrans* caused by the inflammatory response elicited by the spirochetes, nor neurological disease, making the murine Lyme model an imperfect model for human Lyme disease. Rhesus monkeys infected with Bb develop neuroborreliosis and *erythema migrans* in addition to arthritis, thus making the disease most similar to human infection. Costs and difficulties in genetic manipulation make the murine model more commonly used over the primate model (Barthold and Philip, 2010; Radolf et al., 2012).

Lyme carditis is caused primarily by the infiltration of monocytes and macrophages in Bb infected mice (Weiss, 2010). The genetic background of the experimentally infected murine model influences the susceptibility to the development of carditis, although the root of the diversity remains unknown. IFNγ has been shown to locally modulate Lyme carditis and enhance the phagocytic capacity of macrophages (Olson et al., 2009). Macrophages are a critical part of the response to Bb, especially for the control of spirochetal numbers in the heart (Behera et al., 2006; Olson et al., 2009). In humans, symptomatic Lyme carditis is rare and usually resolves with antibiotic therapies (Krause and Bockenstedt, 2013), although recently it has been shown to be associated with mortality rates higher than previously reported (CDC, 2013).

The field of Lyme arthritis has gained much insight from the use of C3H mouse models due to the robust phenotype that mimics much of human Lyme disease arthritis complications (Barthold and Philip, 2010). However the spectrum of pathology observed in humans is not observed in the murine model (Steere et al., 1987). It has also been reported that there is a lack of T cell involvement in mice, which is contradictory to the observations of some human patient reports, suggesting the importance of Th1 and γ/δ T cells (Duray, 1989; Vincent et al., 1996; Gross et al., 1998; Roessner et al., 2003). A select

group of Lyme arthritis patients experience persistent arthritis even following an appropriate course of antibiotic therapy (Shin et al., 2007). Investigation of this cohort of patients' synovial fluid suggests there is a possible dysregulated inflammatory response that presents with increased IL-1β, IL-6, and IFNγ (Shin et al., 2007) and chemokines CXCL9 and CXCL10 (Shin et al., 2007). IFN-responsive genes have been reported to be strongly up-regulated within the joints of Bb-infected C3H mice, but not in mildly arthritic C57BL/6 mice (Crandall et al., 2006). Nevertheless, bone marrow-derived macrophages from both C3H and C57BL/6 mice induce IFN-responsive genes following Bb stimulation (Miller et al., 2008a), and this expression appears to require a functional type I IFN receptor (Miller et al., 2008b). The arthritis observed in C57BL/6 mice is modulated by the production of IL-10 from $CD4^+$ T cells and macrophages (Lazarus et al., 2008). IL-10 regulates the expression of IFNγ and production of CXCL9 and CXCL10 (Lazarus et al., 2008; Sonderegger et al., 2012), and produce a similar phenotype of persistent arthritis patients (Shin et al., 2007).

TLR expression varies slightly between humans and mice, with ten human TLRs, and twelve murine functional TLRs identified to date (Gosu et al., 2012). TLR1-9 are conserved between the two species (Kawai and Akira, 2010). TLR8 is non-functional in mice because it lacks five amino acids (Liu et al., 2010). However, recently, a murine model expressing functional human TLR8 on a C57BL/6 background has been generated (Guiducci et al., 2013). TLR10 is also non-functional in mice due to a retrovirus insertion (Gosu et al., 2012). TLR11-13 are present in mice, but absent in humans (Kawai and Akira, 2010). In humans, TLR7 is mainly co-expressed with TLR9 on B cells and plasmacytoid DCs and TLR8 is nearly absent in this cell line; whereas TLR8 is highly expressed on monocytes/macrophages and myeloid DCs (Hornung et al., 2002; Cervantes et al., 2012). Differences in the inflammatory responses to Bb observed between human and mice, could be explained by variations in TLR- signaling (Petnicki-Ocwieja et al., 2013). Newly generated humanized mouse models would allow for future studies regarding the role of human TLRs in Lyme disease clinical manifestations and severity of disease.

PHAGOCYTOSIS OF *B. BURGDORFERI*

Phagocytosis is an important component of innate immunity to the Lyme disease spirochete. Uptake and degradation of the bacterium results in the induction of intracellular signals leading to the generation of cytokines, antigen processing and presentation, which ultimately leads to the development of acquired immunity (Greenberg and Grinstein, 2002; Moore et al., 2007).

Delivering individual PAMPs in experimental systems fails to mimic the natural processes of innate immune activation by TLRs in response to a live organism. In the case of endosomal TLRs, ligands are usually delivered in a complex with a cationic polymer such as polyethylenimine or DOTAP (Cervantes et al., 2013; Love et al., 2014). The natural agonists for endosomal TLRs are, however, an integral part of live pathogens and as such, are not directly accessible to receptors but after the whole organism has been degraded in the endolysosomal compartment (Vance et al., 2009). The immune system responds more robustly to viable microorganisms than it does to dead organisms. Blander and colleagues found that viable bacteria, but not killed ones, contain a class of PAMPs which they coined as vita-PAMPs (Sander et al., 2011). These vita-PAMPs, such as mRNA, signify microbe viability to the innate immune system (Sander et al., 2011). Pathogen and host cell-derived material associated with pathogen nucleic acids have to be taken up into the endolysosomal compartment, where degradation allows the nucleic acids to become available for TLR binding. The detection of vita-PAMPs and conventional PAMPs could interact with multiple PRRs within the phagosome and/or the cytosol and have a crucial role in antimicrobial immunity (Sander et al., 2011).

Professional immune phagocytes, such as monocytes, macrophages, DCs (Benach et al., 1984a; Filgueira et al., 1996; Moore et al., 2007) as well as other various cell types (ex. murine microglia, chondrocytes, synovial, and L929 fibroblast) have been demonstrated to internalize Bb (Franz et al., 2001; Kuhlow et al., 2005; Behera et al., 2008; Chmielewski and Tylewska-Wierzbanowska, 2010). The initiation of phagocytosis requires interaction of phagocytic receptors (other than CD14 and TLRs) located on the surface of innate immune cells with surface molecules of Bb innate immune cells (Shin et al., 2008; Sahay et al., 2009), and internalization of Bb by murine, rat and rabbit macrophages can occur in the presence (Fc-mediated phagocytosis) (**Figure 2A**) or absence of opsonic antibodies (**Figure 2B**) (Benach et al., 1984b; Montgomery et al., 1994). Bb is able to activate both the classical and alternative complement pathways (Kochi and Johnson, 1988). C3b can either bind to the surface of bacteria and facilitate internalization of the spirochete by opsonization or C3b or proceed to membrane attack complex formation and lysis of the bacteria by depositing downstream components into the cell wall. The spirochete has evolved mechanisms that enable them to evade complemented-mediated lysis (Fraser et al., 1997), such as expression of complement regulator-acquiring surface proteins (CRASPs). CRASPs act as binding sites for the complement inhibitor factor H and factor H-like protein (Hellwage et al., 2001; Kraiczy et al., 2001, 2004) and cleave bound serum complement protein C3b into the serum opsonin inactivated C3b (iC3b) (**Figure 2A**).

Complement Receptor 3 (CR3) (integrin $\alpha_M\beta_2$, CD11b/CD18) was demonstrated to directly bind Bb and the presence of complement enhanced spirochetal binding (Cinco et al., 1997; Garcia et al., 2005). ICAM-1, iC3b and fibrinogen, known CR3 protein ligands, have been shown to interact with the I-domain of CR3 (Humphries, 2000). Only recently, Hawley et al. identified CR3 to be a phagocytic receptor in murine macrophage and human monocyte (**Figure 2B**). This study revealed that CR3 requires cooperation of the GPI-anchored protein, CD14 for the internalization of unopsonized Bb and that CR3-mediated phagocytosis of Bb occurs in a Myeloid differentiation primary response gene 88 (MyD88)-independent manner, thus suggesting the involvement of additional receptors in MyD88-dependent Bb phagocytosis (Hawley et al., 2012). CD14 is involved in translocation of CR3 to the lipid rich microenvironments known as lipid rafts following interaction with live Bb (Hawley et al., 2013). The findings suggest that CD14 interacts with the C-lectin domain of the integrin to induce crosslinking of the integrin and

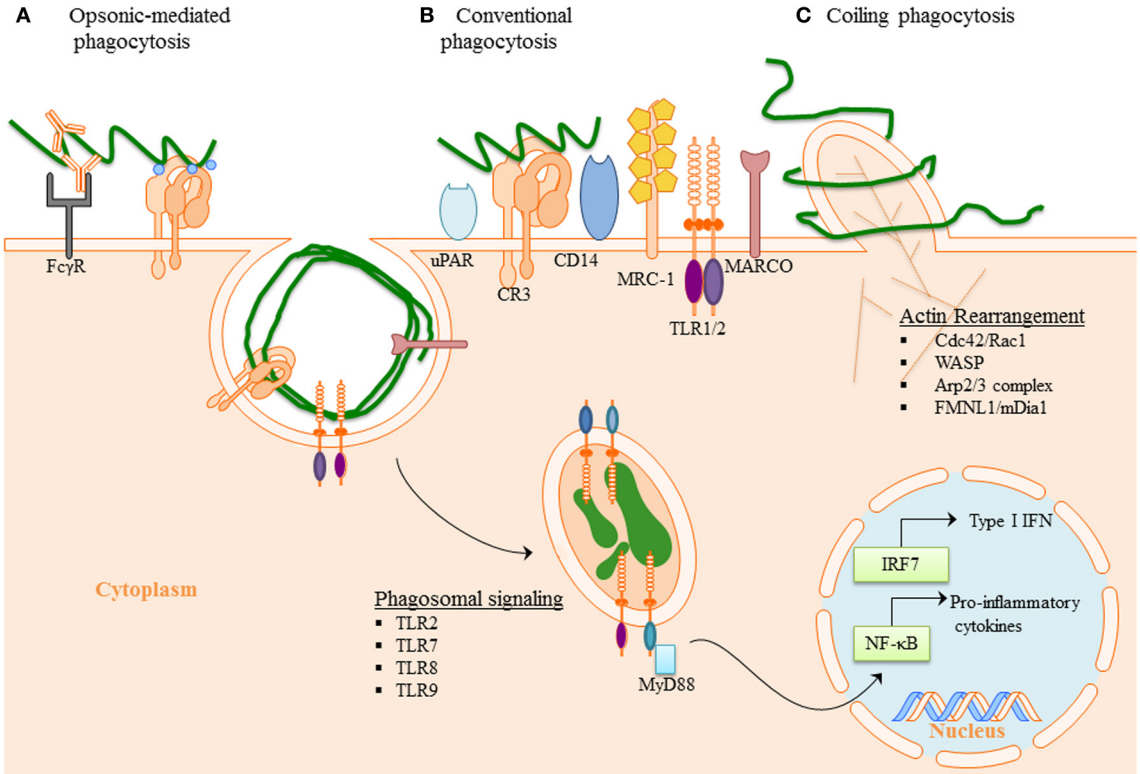

FIGURE 2 | Phagocytosis of *B. burgdorferi*: (A) Opsonic-mediated phagocytosis—complement factors, such as C3b and iC3b, bound to the surface of Bb can interact with complement receptors and mediate phagocytosis. Additionally, the immune cells Fc-receptors have the ability to bind to the opsonic antibodies that coat Bb and internalize the pathogen. **(B)** Conventional phagocytosis—the direct interaction of surface receptors with Bb, such as integrins and C-type lectins, allows for tether of the spirochete to the cell surface. Various PRRs induced signal cascade initiates formation of the phagocytic cup and spirochete engulfment. **(C)** Coiling phagocytosis—the preferred mechanism of spirochete internalization in which the phagocytic cell uses filopodial protrusions that capture Bb. The filopodial enwrap the spirochete and convert into coiling pseudopods. During this dynamic process FMNL1, mDai1 Arp2/3 complex, and WASP are involved in actin rearrangement of the cell, which then facilitates subsequent phagocytosis of Bb. Following internalization of Bb, the spirochete is degraded within the phagosome thus exposing additional PAMPs to PRR with in the phagosome. The phagosomal signals initiated by Bb generates a robust inflammatory response, including the induction of pro-inflammatory genes and Type I IFNs.

efficient internalization of Bb. Additionally, MyD88-independent inflammatory pathways have been reported following interaction of the spirochete with the integrin $\alpha_3\beta_1$ on primary human chrondrocytes and may be a mechanism directly relevant to the development of arthritis (Behera et al., 2008). Moreover, urokinase receptor (uPAR, CD87) has been shown to facilitate clearance of Bb (**Figure 2B**), and provides an area for additional investigation as to the mechanistic involvement in bacterial clearance (Hovius et al., 2009).

Scavenger receptors comprise a group of unrelated transmembrane surface molecules with relatively promiscuous ligand binding such as scavenger receptor A (SR-A), MARCO (Macrophage receptor with Collagenouse structure) and CD36 (Areschoug and Gordon, 2009). This promiscuity allows scavenger receptors to mediate uptake of a wide range of pathogens including bacteria (Peiser et al., 2000; Thelen et al., 2010), yeast, viruses and parasites (Mukhopadhyay and Gordon, 2004), as well as removal of dead cell material by both macrophages and DCs (Brencicova and Diebold, 2013). Little is known about their natural ligands and the structural basis for ligand binding of bacteria. CD36 and MARCO can interact with TLR2 and CD14, regulating NFκ-B cytokine responses (Bowdish et al., 2009; Jordo et al., 2011). MARCO expression appears to be MyD88 dependent in mice, and MARCO mediated phagocytosis of Bb seems an important mechanism for TRIF signaling (Petnicki-Ocwieja et al., 2013). The mannose receptor has been demonstrated to bind to Bb (Cinco et al., 2001), but its role in Bb phagocytosis has not yet been investigated. Unlike the mannose receptor or CR3 that reconstitute phagocytosis in non-phagocytic cells, expression of SRA or MARCO confers only binding, without significant internalization of Gram positive or Gram negative bacteria (Underhill and Ozinsky, 2002). In macrophages, the scavenger receptors MARCO and SR-A are involved in uptake of CpG ODN and influence TLR9-mediated IL-12 induction with MARCO enhancing its production (Jozefowski et al., 2006).

Coiling phagocytosis is the preferred mechanism of Bb uptake, accounting for approximately 60 to 70% of phagocytosis (Rittig et al., 1992, 1998; Naj et al., 2013). Coiling phagocytosis was first described as the phagocytic mechanism used to internalized *Legionella pneumophila* (Horwitz, 1984) where a unilateral pseudopod bends around the bacteria in a hook like fashion. Phagocytosis is a complex mechanism where F-actin

polymerization occurs to reorganize the membrane to internalize Bb (Chimini and Chavrier, 2000; Cruz et al., 2008). The Rho GTPases, Cdc42 and Rac1 regulate the actin dynamics (Chimini and Chavrier, 2000; Linder et al., 2001). WASP (Wiskott-Aldrich syndrome protein) and the Arp2/3 complex are mostly involved in branched actin network, to reorganize to the pseudopod intertwining Bb (Amann and Pollard, 2001; Higgs and Pollard, 2001; Linder et al., 2001) (**Figure 2C**). The formins, FMNL1 and mDia1, are actin nucleating proteins that influence formation of unbranched actin filaments and regulate coiling phagocytosis of Bb by primary human macrophages (Naj et al., 2013). In neutrophils, mDia1 is required for both CR3-mediated and FcγR-mediated phagocytosis (Shi et al., 2009).

Much focus has been directed to the downstream signals initiated by TLRs following internalization of Bb, especially those involving the adaptor molecule MyD88. Mice deficient in MyD88 have increased bacterial burdens but normal antibody production in comparison to wild-type (WT) mice infected with Bb (Liu et al., 2004). MyD88 deficient macrophages have a significant defect in their phagocytic ability of Bb, roughly 50% reduction compared to the WT macrophages, supporting observations with phagocytosis and killing of bacteria, rather than a previously suggested defect in degradation in phagolysosome (Blander and Medzhitov, 2004; Yates and Russell, 2005; Shin et al., 2009).

PHAGOSOMAL RECOGNITION OF *B. BURGDORFERI* LIGANDS

Although there is plenty of evidence showing that formerly "outer membrane-associated TLRs" such as TLR2 and TLR4 are also recruited to the endosome (McGettrick and O'Neill, 2010; Gangloff, 2012; Brandt et al., 2013), the classic "endosomal nucleic acid-sensing TLRs" comprises TLR3, TLR7, TLR8, and TLR9 (Brencicova and Diebold, 2013). TLR 7, TLR8, and TLR9 depend on the endoplasmic reticulum (ER)-resident protein UNC93B1 for trafficking from the ER via the Golgi to the endolysosomal compartment (Brinkmann et al., 2007; Kim et al., 2008; Lee et al., 2013). UNC93B1 physically associates with the endosomal TLR (McGettrick and O'Neill, 2010; Itoh et al., 2011) and it appears to be essential for TLR8-mediated signaling (Itoh et al., 2011). Other factors involved in endosomal TLR trafficking are the ER chaperone GP96 and PRAT4A (McGettrick and O'Neill, 2010; Lee et al., 2013). Furthermore, trafficking of endosomal TLRs is also affected by recruitment of adaptor protein complexes, which are not only TLR-specific (Lee et al., 2013) but cell specific as well (Sasai et al., 2010; Henault et al., 2012).

Although originally associated with the recognition viral pathogens, endosomal TLRs are also able to sense bacterial nucleic acids. The earliest evidence for such recognition was done using TLR7 and TLR8-stably transfected HEK cell lines, showing that *Escherichia coli* total RNA induced activation of TLR7 and TLR8 (Kariko et al., 2005). More recent experimental evidence confirms that TLR7 is capable of sensing bacterial RNA in both human and murine DCs, inducing the production of several NFκ-B cytokines (Eberle et al., 2009; Mancuso et al., 2009). A similar role for TLR8 activation, triggered by recognition of borrelial RNA delivered to endosomal vacuoles in human monocytes, was recently demonstrated by our group (Cervantes et al., 2013). The role of TLR8 in nucleic acid sensing was initially suggested by results from two previous studies reporting TLR8 upregulation after phagocytosis of *Mycobacterium bovis* (Davila et al., 2008), and *Helicobacter pylori* by THP-1 cells (Gantier et al., 2010). In our own studies we first showed that phagocytosis of live *Borrelia burgdorferi* by human monocytes (**Figure 3**) induces transcription of IFN-β (Salazar et al., 2009), and subsequently confirmed that this phenomenon was entirely dependent on TLR8 (Cervantes et al., 2011), through IRF-7; a signaling pathway traditionally associated with recognition of viral RNA (Boo and Yang, 2010).

Type I interferon responses following phagocytosis of live Bb are not restricted to IFN-β transcription by monocytes. In pDCs, production of IFN-α involves recognition of Bb ligands by TLR7 and TLR9 (Petzke et al., 2009; Love et al., 2014). In this cell type, IFN-responsive genes seem to be induced by Bb RNA through TLR7 recognition (Love et al., 2014).

Bacterial ribosomal RNA appears to be the major PAMP responsible for the production of IFN-α by human PBMCs (Eberle et al., 2009). Transfer RNA from some bacteria may also induce production of IFN-α through TLR7 activation (Jockel et al., 2012). In the case of Gram positive bacteria, such as Group

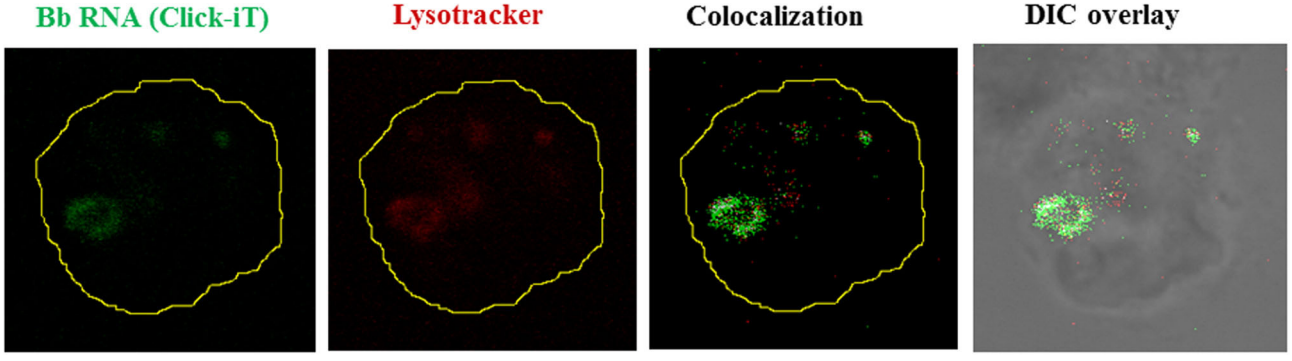

FIGURE 3 | Borrelial RNA is confined to the phagolysosome. Live Bb whose nascent RNA has been stained with Click-iT (5 Uridine) (Green), seen internalized by a human monocyte. Lysosome stained with Lysotracker Red (Red). Colocalization shown as white pixels of the green channel colocacalizing with the red channel.

B Stretptococci, bacterial ssRNA is recognized in macrophages by a TLR-MyD88-UNC93B1 complex (Deshmukh et al., 2011).

ENDOSOMAL TLR INVOLVEMENT IN AUTOIMMUNITY AND Bb NUCLEIC ACID PERSISTENCE

Although the immune system has evolved mechanisms to prevent stimulation by self-nucleic acids, nucleic acid-sensing TLRs can trigger innate immune activation resulting in induction of autoimmunity (Saitoh and Miyake, 2009; Brencicova and Diebold, 2013). In fact TLR7, TLR8, and TLR9 are unable to distinguish between pathogen and self-nucleic acids on the basis of their molecular structures (Diebold et al., 2004; Heil et al., 2004; Barbalat et al., 2011). Mouse TLR7, and human TLR7 and TLR8 serve as PRR for single-stranded RNA (ssRNA), whereas the functionality of mouse TLR8 is still somewhat obscure (Cervantes et al., 2012). Presence of methylated nucleosides or pseudouridines in mammalian tRNA may also prevent TLR7 and TLR8 activation (Kariko et al., 2005). However, these nucleotide modifications are less frequent in mammalian mRNA (Maden and Hughes, 1997), which can become immunostimulatory when delivered to the endosome in form of complexes with polycations such as polyethylenimine (Koski et al., 2004; Kariko et al., 2005; Diebold et al., 2006).

TLR7 and TLR8 have already been shown to play a central role for the recognition of self RNA in the immunopathogenesis of autoimmune diseases such as systemic lupus erythematosus (SLE), psoriasis, rheumatoid arthritis, Sjögren's syndrome and others (Demaria et al., 2010; Zheng et al., 2010; Theofilopoulos et al., 2011). Human TLR8 inhibits TLR7 and TLR9 activation (Guiducci et al., 2013), and murine TLR8 also inhibits TLR7 activation (Wang et al., 2006). TLR8 deficiency leads to overexpression of TLR7 in murine DCs with increased NFκ-B activation and development of autoimmunity (Demaria et al., 2010). In humans, TLR7 and TLR9 are upregulated in patients with Sjogren's syndrome (Zheng et al., 2010). Similarly, genetic modifications that lead to a duplication of the TLR7 gene or over-expression of transgenic TLR7 are associated with exacerbated lupus-like symptoms in murine models (Pisitkun et al., 2006; Deane et al., 2007). It is worth noting that TLR7 is located on the X chromosome and that females induce higher levels of IFN-α in response to TLR7 agonists (Berghofer et al., 2006), which could represent a major factor responsible for the higher prevalence of SLE in women.

One of the more puzzling aspects of Lyme disease is the persistence, in some patients, of musculoskeletal symptoms following Bb infection, and their refractoriness to rapid improvement despite proper antibiotic treatment (Bockenstedt et al., 2012). While *erythema migrans* and Lyme carditis often present within the first few weeks of intection, Lyme arthritis more often presents several weeks after the initial infection in untreated patients and can persist even after antibiotic treatment (Kean and Irvine, 2013). Since initial studies failed to detect spirochetal DNA in human synovial fluid following antibiotic treatment in LD with persistent arthritis, also called antibiotic-resistant Lyme arthritis, it was considered an autoimmune disease (Benoist and Mathis, 2001; Steere, 2012), possibly mediated by shedding of borrelial outer surface lipoproteins (Osps) within the synovial fluid (Batsford et al., 2004). However, in recent studies, investigators were able to detect Bb DNA in joint fluid from Lyme arthritis patients who received appropriate antibiotic therapy (Picha et al., 2008; Li et al., 2011; Picha et al., 2014). Bb positive PCR results have been reported to persist for as long as 11 months in patients with antibiotic-refractory arthritis, although detection of Bb DNA did not translate into active joint disease (Li et al., 2011). DNA and antigen deposits have been shown to persist after antibiotic treatment in cartilage of mice deficient in MyD88 (Bockenstedt et al., 2012). This mouse strain exhibits more severe arthritis than WT (Bolz et al., 2004; Liu et al., 2004), and presents higher levels of IFN-β in joint tissue after infection (Petnicki-Ocwieja et al., 2013).

Naked pathogen-derived nucleic acids present in the extracellular space upon release from damaged or disintegrated microbes or the infected host cells, may be ultimately degraded by extracellular DNases and RNases before they can access the endolysosomal compartment of other immune cells (Brencicova and Diebold, 2013). If such degradation fails to occur, the presence of this remaining nucleic acid could potentially trigger an autoimmune response. This has been shown to occur with released self-DNA in SLE patients carrying mutations in DNase I (Yasutomo et al., 2001), and in DNase I-deficient mice, which develop a lupus-like disease (Napirei et al., 2000). It has been hypothesized that the sensing of naked ssRNA and DNA, which is not associated with pathogen-derived material including non-nucleic acid PAMP, doesn't allow for the discrimination between pathogen-associated vs. self-nucleic acids and, therefore, has the potential to lead to autoimmunity (Brencicova and Diebold, 2013). Mechanisms that aid in the discrimination between foreign (pathogenic) and self (cellular) nucleic acids, aim to inhibit endosomal TLR activation, or prevent cellular nucleic acid to bind to endosomal TLRs. These mechanisms include the presence of modified RNA species such as tRNA and rRNA in total cellular nucleic acids (Kariko et al., 2005), sequestration of cellular nucleic acids through binding to cellular components, and/or recruitment of nucleic acid-sensing TLR to the endolysosomal compartment or their functional activation by cleavage, a process that may be regulated by gatekeeper receptors with the ability to detect PAMP and/or DAMP absent from uninfected cells (Brencicova and Diebold, 2013). Hence, any mechanisms that allow for or promote the recognition of naked ssRNA and DNA such as in the form of immune complexes should be regarded as non-physiological events.

TLR9 is another endosomal TLR that has been linked to autoimmunity (Theofilopoulos et al., 2011), and a potential role for TLR9 in recognition of Bb DNA may exist (data not shown). Initially, it was thought that TLR9 is located in the ER in unstimulated cells and is recruited to the endolysosomal compartment only after uptake of TLR9 agonist (Latz et al., 2004). However, there is evidence of steady-state low level of trafficking of nucleic acid-sensing TLR via the Golgi to the endolysosomal compartment which may initiate TLR recruitment upon stimulation with nucleic acids (Brencicova and Diebold, 2013).

POTENTIAL ROLE OF TLR IN CLINICAL DISEASE SEVERITY

While TLRs are capable of sensing pathogenic materials, defective TLR signaling can hinder activation of the adaptive response.

On the other hand, excessive response of TLRs and production of cytokines may increase the disease state (Kean and Irvine, 2013).

Bb produces many symptoms within the human host, including *erythema migrans*, systemic inflammation, Lyme arthritis, Lyme carditis, and neuroborreliosis (Radolf et al., 2012; Kean and Irvine, 2013). It is now known that lipoproteins can serve as potent ligands for TLRs. Bb has many Osps that are capable of triggering the innate immune system through activation of TLRs (Gondolf et al., 1994; Radolf et al., 2012). The three major Osps of Bb include OspA, OspB, and OspC. Moreover, the CD14/TLR2 complex be activated by these lipid moieties. Using a rat model Batsford et al. showed that polymerized peptide OspA produced a short lasting arthritis and that lipidated OspA and OspA elicited severe arthritis (Batsford et al., 2004). Other studies have demonstrated that macrophages play a direct role in the induction of Lyme arthritis in hamsters (DuChateau et al., 1999). These facts implicate TLRs as key players in the development of Lyme arthritis and critical receptors for recognition of vita-PAMPs.

Borrelia infection can also lead to Lyme carditis, a dangerous condition that can in rare instances can lead to sudden death (CDC, 2013). The mechanism of Lyme carditis has been shown to involve invariant NKT cells (iNKT cells) (Olson et al., 2009). This type of cell can be activated by bacterial infection through TLR4, TLR7 and TLR9-driven maturation of dendritic cells (Brigl et al., 2003). Mice deficient in iNKT cells developed significantly worse inflammation in the heart following during Bb infection (Olson et al., 2009). iNKT cells localize to the inflamed heart, enhancing macrophage phagocytosis through IFNγ leading to control of infection (Olson et al., 2009).

With recent progress in DNA analysis, the extremely polymorphic genes of TLRs can finally be understood. The genetic variability in TLRs can result in functional deficiency, which ultimately leads to immunodeficiency syndromes. The TLR1 1602S polymorphism, found predominantly in European-Caucasian populations, has been correlated with low expression of TLR1 at the surface membrane (Kean and Irvine, 2013). This mutation results in a diminished response to OspA due to the decreased capability of TLR2 to couple with TLR1, thus leading to an increased susceptibility to Lyme disease (Kean and Irvine, 2013). Individuals with mutations in the *TLR2* gene, specifically Arg753Gln, are less responsive to PAMPs derived from Bb (Schwartz and Cook, 2005).

TLR signaling alterations have been linked to more severe clinical manifestations in response to bacterial, fungal and viral infections (Frazao et al., 2013). For instance, single nucleotide polymorphisms (SNPs) found within *TLR7* have been associated with more severe Hepatits C viral infection (Lin et al., 2012). Mutations in the *TLR8* gene have been linked to increased susceptibility to bacterial infections (Davila et al., 2008). Other mutations can occur downstream of TLR-signaling. For example, deficiencies in MyD88 and IRAK4 result in impaired production of pro-inflammatory cytokines following TLR stimulation (Cervantes et al., 2013), and increased susceptibility to bacterial infections (Kenny et al., 2009; Netea et al., 2012).

It is important to note that despite the large number of TLR gene mutations found in the general population, most affected individuals will not suffer life threatening complications or even more severe infections than their unaffected counterparts (Netea et al., 2012). One explanation for the high rate of TLR polymorphism without increase in pathogen susceptibility is the redundancy of the innate and adaptive immune system. The high degree of nucleotide polymorphism seen in TLRs is consistent with the constant "arms race" driven by the rapidly evolving pathogens. Additionally, the lack of reproducibility among many experiments, in conjunction with small sample sizes of people with such phenotypes hints at the large genetic diversity among TLRs (Lin et al., 2012).

Future studies will attempt to elucidate the relationship between TLR mutations and the short and long-term outcome of human Lyme disease, particularly as to changes in these key innate immune receptors have a role in patients with prolonged, antibiotic refractory Lyme disease.

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The cyclic-di-GMP signaling pathway in the Lyme disease spirochete, *Borrelia burgdorferi*

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In nature, the Lyme disease spirochete *Borrelia burgdorferi* cycles between the unrelated environments of the *Ixodes* tick vector and mammalian host. In order to survive transmission between hosts, *B. burgdorferi* must be able to not only detect changes in its environment, but also rapidly and appropriately respond to these changes. One manner in which this obligate parasite regulates and adapts to its changing environment is through cyclic-di-GMP (c-di-GMP) signaling. c-di-GMP has been shown to be instrumental in orchestrating the adaptation of *B. burgdorferi* to the tick environment. *B. burgdorferi* possesses only one set of c-di-GMP-metabolizing genes (one diguanylate cyclase and two distinct phosphodiesterases) and one c-di-GMP-binding PilZ-domain protein designated as PlzA. While studies in the realm of c-di-GMP signaling in *B. burgdorferi* have exploded in the last few years, there are still many more questions than answers. Elucidation of the importance of c-di-GMP signaling to *B. burgdorferi* may lead to the identification of mechanisms that are critical for the survival of *B. burgdorferi* in the tick phase of the enzootic cycle as well as potentially delineate a role (if any) c-di-GMP may play in the transmission and virulence of *B. burgdorferi* during the enzootic cycle, thereby enabling the development of effective drugs for the prevention and/or treatment of Lyme disease.

Keywords: c-di-GMP, *Borrelia burgdorferi*, Lyme disease, motility, chemotaxis, virulence

INTRODUCTION

Borrelia burgdorferi is the causative agent of Lyme disease or Lyme borreliosis—the most common arthropod-borne disease in the United States and Europe (Adams et al., 2012, 2013). Although the total number of reported cases of Lyme disease each year in the United States averages at about 30,000 (Adams et al., 2013), the Centers for Disease Control and Prevention (CDC) estimates that the actual number of people diagnosed with Lyme disease each year is approximately 300,000 (Kuehn, 2013). However, while this disease has been reported in every state, it is substantially concentrated in the states of the upper Midwest and Northeast (Adams et al., 2013). The concentration of Lyme disease in these areas is directly related to the large population of the white-footed mouse (*Peromyscus leucopus*), which is thought to be the main reservoir of the Lyme disease spirochete (Levine et al., 1985; Mather et al., 1989; Ostfeld, 1997; Schmidt and Ostfeld, 2001; Tsao, 2009). Despite tremendous amounts of research and many promising results, there is no current vaccine available which protects humans against Lyme disease. Prevention—reducing exposure to ticks as well as to areas where ticks and their reservoir hosts may congregate—is currently the best defense against Lyme disease (Barrett and Portsmouth, 2013). However, the incidence and geographical distribution of Lyme disease is increasing, and is predicted to continue, in conjunction with the increasing overlap between humans, ticks, and their reservoir hosts (Ostfeld, 1997). Additionally, the persistent and debilitating nature of the disease leads to approximately two billion dollars in direct medical expenses and lost productivity each year in the United States (Maes et al., 1998; Zhang et al., 2006). Thus, these circumstances clearly warrant a basic understanding of the disease mechanism, which could lead to the development of a vaccine for the treatment (and potentially prevention) of Lyme disease.

Cyclic di-GMP (c-di-GMP) [bis(3′,5′)-cyclic diguanylic acid] was initially identified as a nucleotide-based, allosteric activator of cellulose synthase activity in *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*) (Ross et al., 1987). Since then, c-di-GMP has evolved as a broadly conserved second messenger unique to bacteria, which operates as a global regulatory molecule capable of altering a plethora of biological processes, including, but not limited to exopolysaccharide matrix components (Jenal and Malone, 2006; Hengge, 2009), virulence of plant and animal pathogens (Cotter and Stibitz, 2007; Tamayo et al., 2007, 2008; Chatterjee et al., 2008; McCarthy et al., 2008; Hammer and Bassler, 2009; Lai et al., 2009), motility and sessility (Wolfe and Visick, 2008; Armitage and Berry, 2010; Boehm et al., 2010; Paul et al., 2010), regulated proteolysis and cell cycle progression (Duerig et al., 2009), photosynthesis (Thomas et al., 2004), heavy metal resistance (Brown et al., 1986; Römling et al., 2005), and phage resistance (Chae and Yoo, 1986; Römling et al., 2005). These physiological functions regulated by c-di-GMP are controlled at the transcriptional, translational, and posttranslational levels (Ryjenkov et al., 2006; Weber et al., 2006; Lee et al., 2007; Merighi et al., 2007; Monds et al., 2007; Hickman and Harwood, 2008; Pesavento et al., 2008; Sudarsan et al., 2008; Duerig et al., 2009; Boehm et al., 2010; Fang and Gomelsky, 2010; Paul et al., 2010). This is in contrast to canonical two-component signal transduction systems, where a signal is detected by a sensor kinase, which phosphorylates its cognate response regulator, leading to

the modulation of a limited number of genes (Albright et al., 1989). There are at least four components needed for functional activity of a c-di-GMP "control module": a diguanylate cyclase (DGC) that synthesizes c-di-GMP; a phosphodiesterase (PDE) which hydrolyzes c-di-GMP; a receptor component that senses c-di-GMP by directly binding to it; and a target that directly contacts and is controlled by the effector, resulting in an output that is determined by the interplay of all of these components.

In nature, *B. burgdorferi* cycles between the disparate environments of the *Ixodes* tick vector and mammalian host (Burgdorfer et al., 1982; Levine et al., 1985; Lane et al., 1991; Tsao, 2009; Brisson et al., 2012). During transmission between hosts, *B. burgdorferi* detects changes in its environment, such as pH, CO_2, nutrient availability, and temperature, and responds appropriately by modulating its gene expression (Carroll et al., 1999, 2001; Revel et al., 2002; Brooks et al., 2003; Ojaimi et al., 2003; Tokarz et al., 2004; Tilly et al., 2008; Samuels and Radolf, 2009; Samuels, 2011). Differential gene expression is essential for the Lyme disease spirochete to endure the diverse and fluctuating environments encountered over the course of the enzootic cycle. Similar to other bacterial species (Stock et al., 2000; West and Stock, 2001; Kazmierczak et al., 2005; Beier and Gross, 2006; Tamayo et al., 2007), *B. burgdorferi* utilizes two-component systems (TCS) to modulate its gene expression (Fraser et al., 1997; Casjens et al., 2000; Radolf et al., 2012; Groshong and Blevins, 2014). Unlike many other species of bacteria, *B. burgdorferi* was reported to encode only two TCS—Hk1-Rrp1 and Hk2-Rrp2—that have demonstrated global gene regulatory capabilities (Yang et al., 2003a; Rogers et al., 2009; Samuels, 2011; Radolf et al., 2012; Groshong and Blevins, 2014). The Hk2-Rrp2 TCS activates the expression of the stationary phase sigma factor RpoS synergistically with RpoN (Burtnick et al., 2007; Ouyang et al., 2008; Blevins et al., 2009), which, in turn, chiefly regulates plasmid-borne genes (Yang et al., 2003a,b; Caimano et al., 2007) and induces the expression of genes, such as *ospC* (Hübner et al., 2001), which are known to be important for mammalian infection (Caimano et al., 2004; Fisher et al., 2005; Caimano et al., 2007; Boardman et al., 2008; Ouyang et al., 2008; Dunham-Ems et al., 2012; Ouyang et al., 2012) as well as genes involved in chitobiose utilization, which has been shown to be important for colonization of the tick (Sze et al., 2013). The Hk1-Rrp1 TCS converges with the Hk2-Rrp2 TCS through the regulator, BosR—a Fur/Per-like transcription factor that has been demonstrated to be essential for expression of *rpoS* (Boylan et al., 2003; Katona et al., 2004; Seshu et al., 2004; Hyde et al., 2009; Ouyang et al., 2009, 2011; Hyde et al., 2010)—which primarily regulates core chromosome-encoded genes (Rogers et al., 2009; He et al., 2011, 2014) and is required for tick colonization (Caimano et al., 2011; He et al., 2011; Kostick et al., 2011). Interestingly, Rrp1, the response regulator, lacks a DNA-binding domain, but instead contains a GGDEF domain, which has been associated with diguanylate cyclase activity (cyclic di-GMP synthase) in *B. burgdorferi* (Ryjenkov et al., 2005). Furthermore, the diguanylate cyclase, Rrp1, is active only when it is phosphorylated, presumably by the histidine sensor kinase, Hk1 (Caimano et al., 2011) (**Figure 1**). While Hk2-Rrp2 is primarily involved in mammalian host adaptation (Groshong and Blevins, 2014), recent studies suggest that c-di-GMP is a key regulator in the adaptive responses of *B. burgdorferi* to the tick environment (Caimano et al., 2011; He et al., 2011, 2014; Kostick et al., 2011; Pitzer et al., 2011; Sultan et al., 2011). While the genomes of several species of bacteria were reported to encode multiple c-di-GMP-metabolizing enzymes (Galperin et al., 1999, 2001; Galperin, 2004), both bioinformatics and experimental analysis indicate that *B. burgdorferi* possesses only a limited number of genes responsible for regulating c-di-GMP levels—one diguanylate cyclase (*bb0419*/*rrp1*), two distinct phosphodiesterases (*bb0363*/*pdeA* and *bb0374*/*pdeB*), and one c-di-GMP-binding PilZ-domain protein, PlzA (*bb0733*) (**Figure 1**) (Ryjenkov et al., 2005; Rogers et al., 2009; Freedman et al., 2010; Sultan et al., 2010, 2011; Caimano et al., 2011; He et al., 2011; Kostick et al., 2011; Pitzer et al., 2011).

THE DIGUANYLATE CYCLASE IN *B. BURGDORFERI*

Hk1 (BB0420), the histidine sensor kinase of the Hk1-Rrp1 TCS in *B. burgdorferi*, is comprised of two periplasmic sensor domains (D1 and D2), a conserved cytoplasmic histidine kinase core, REC, and histidine-containing phosphotransfer (Hpt) domains. *hk1* is located on the chromosome next to a response regulatory protein (*rrp1*; *bb0419*) (Fraser et al., 1997), and although it has not been experimentally confirmed that Hk1 is capable of autophosphorylation or that Hk1 is capable of phosphorylating Rrp1, the operon structure of Hk1-Rrp1, as well as genetic analysis, suggests that Hk1-Rrp1 form a TCS (Ryjenkov et al., 2005; Rogers et al., 2009; Caimano et al., 2011; He et al., 2011). Studies by Caimano et al. have shown that while Hk1-deficient spirochetes are able to infect mice normally, they are cleared from the tick within 48 h of the tick feeding. It is hypothesized that the phosphorelay between Hk1 and Rrp1 results in the production of c-di-GMP, which in turn, modulates the downstream gene expression required for survival within feeding ticks (Caimano et al., 2011). Rrp1 is the sole protein in *B. burgdorferi* that harbors a GGDEF domain, and thus, appears to be the only protein capable of producing c-di-GMP. Additionally, the diguanylate cyclase activity of Rrp1 is strictly dependent on the phosphorylation state of the REC domain of Rrp1 (Ryjenkov et al., 2005).

rrp1 is constitutively expressed *in vivo*, regardless of growth temperature and is increased in the feeding tick relative to the unfed tick (Rogers et al., 2009). Similar to the *hk1* mutant, the *rrp1* mutant was shown to be infectious in mice by needle inoculation, but unable to survive in feeding ticks (He et al., 2011; Kostick et al., 2011). To demonstrate molecular mechanisms underlying the requirement of c-di-GMP for the survival of spirochetes in the fed ticks, microarray analyses of the *rrp1* mutants demonstrated that disruption of c-di-GMP production causes global alteration in gene expression (Rogers et al., 2009; He et al., 2011). Both reports indicated that Rrp1 governs many genes, including the genes required for glycerol transport and metabolism (*glp* genes) (Rogers et al., 2009; He et al., 2011), and mutants defective in glycerol metabolism are attenuated in overall survival in the tick vector (He et al., 2011; Pappas et al., 2011). Interestingly, constitutive expression of the *glp* operon in the *rrp1* mutant only partially rescued survival of the mutant spirochetes in ticks, suggesting that, in addition to *glp*, there are other unidentified factors likely to be critical for the viability

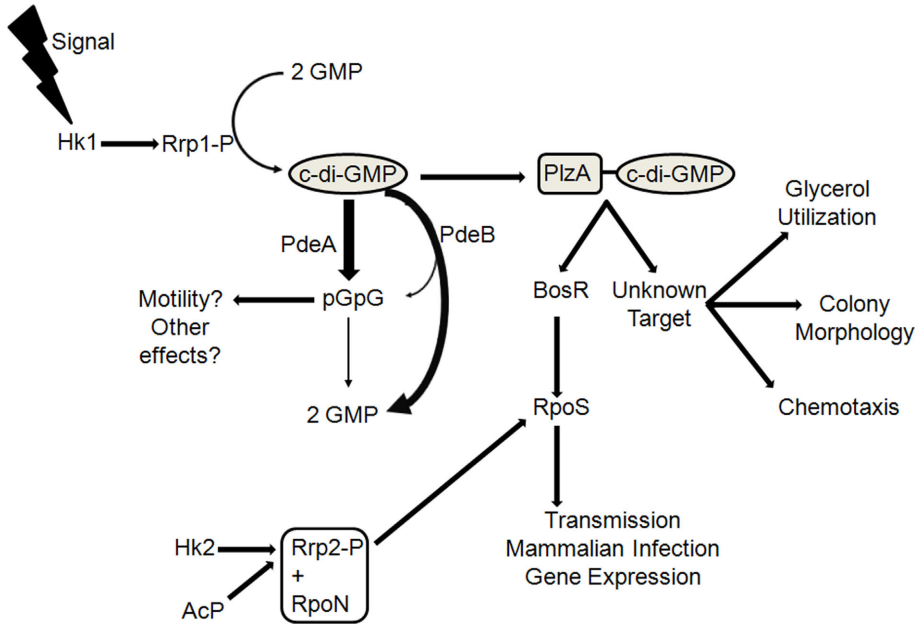

FIGURE 1 | A simplified working model of the c-di-GMP signaling pathway in *B. burgdorferi*. Hk1 and Rrp1 are present in the same operon and are thought to comprise a TCS. The diguanylate cyclase activity of Rrp1 is dependent upon its phosphorylation state, which presumably occurs in the cell by its cognate sensor kinase, Hk1. PlzA, a c-di-GMP-binding protein, is hypothesized to affect multiple virulence characteristics in *B. burgdorferi*. PlzA has been shown to positively regulate BosR protein levels, which regulates *rpoS* expression. RpoS, in turn, induces expression of genes, such as *ospC*, which are known to be important for mammalian infection as well as for genes involved in chitobiose utilization, which was shown to be important for colonization in the tick. *rpoS* expression is also governed by the Hk2-Rrp2 TCS; thus, PlzA serves as the connector between both TCS in *B. burgdorferi*. There is also a possibility that the PlzA-c-di-GMP complex interacts with another target(s), mediating glycerol utilization, colony morphology, and chemotaxis. The levels of c-di-GMP are controlled by the opposing activities of Rrp1 and PdeA and PdeB, the phosphodiesterases in *B. burgdorferi*. PdeA is an EAL-domain PDE, which hydrolyzes c-di-GMP to pGpG as its major product, which is then subsequently hydrolyzed to GMP by nonspecific PDEs. PdeB contains a HD-GYP domain, which hydrolyzes c-di-GMP into pGpG transiently en route to 2 GMPs as the predominant end product. It is unknown if pGpG plays a role in regulating motility or any cellular function in *B. burgdorferi*. Abbreviations: AcP, acetyl phosphate. Thicker arrow next to PdeA indicates pGpG is the major hydrolysis product; thicker arrow next to PdeB indicates the major product of PdeB hydrolysis is 2 GMPs.

of *B. burgdorferi* in its arthropod host (He et al., 2011). The *rrp1* mutant was also reported to exhibit a reduced chemotaxis phenotype and failed to reverse its swimming direction (Kostick et al., 2011). Kostick et al. proposed that this chemotaxis and motility phenotype is likely linked to the fact that the *rrp1* mutant had altered expression of chemotaxis and motility genes (Kostick et al., 2011). Rrp1 was also shown to regulate chitobiose utilization *in vitro* in *B. burgdorferi*. Furthermore, these mutants were unable to transmit to mice via tick bite unless the ticks were supplemented with N-acetylglucosamine (Sze et al., 2013).

The signal that activates Hk1 is not known; however, bioinformatics analysis proposes that Hk1 contains a periplasmic-located sensor domain homologous to the family 3 substrate-binding proteins (SBP_3) (Caimano et al., 2011). SBP_3 family proteins have been shown to bind amino acids or opine molecules (Tam and Saier, 1999). Because of its periplasmic location, the signal is likely to be small enough to permeate the outer membrane of the spirochete and engage the D1 and D2 periplasmic sensor domains of HK1 (Caimano et al., 2011). Based on the phenotypes of the *hk1* mutant, Caimano et al. proposed that the host- and/or tick-derived molecules generated as part of the tick feeding process (either at the bite site or in the tick midgut) may induce the signaling cascade via Hk1, activating the c-di-GMP signal transduction pathway and resulting in the adaptation of *B. burgdorferi* to the callous environment of the tick (Caimano et al., 2011). Although further research is required to establish a definitive biochemical relationship between Hk1 and Rrp1, the similar phenotype exhibited by both the *hk1* mutant and the *rrp1* mutant provides convincing evidence that these two proteins work together to support the production of c-di-GMP. While Rrp2 was thought to be only phosphorylated by its cognate histidine sensor kinase, Hk2 (Burtnick et al., 2007), Xu et al. demonstrated that the high-energy phosphate donor acetyl phosphate (AcP) is also capable of mediating phosphorylation of Rrp2 (Xu et al., 2010). However, this does not appear to be the case for Rrp1 as the phenotype associated with the *hk1* mutant within feeding nymphs suggests that AcP is unable to promote phosphorylation of Rrp1 (Caimano et al., 2011). Together, all of these data suggest that Hk1-Rrp1 is essential for the viability of *B. burgdorferi* in the tick phase of the enzootic cycle, aiding in basic metabolic functions by governing carbohydrate utilization as well as in potential protective functions by shielding the spirochetes from detrimental host factors.

PHOSPHODIESTERASES IN *B. BURGDORFERI*

Once c-di-GMP is synthesized (by Hk1-Rrp1), disposal of the second messenger is critical for modulating the effector protein

activity. Degradation of c-di-GMP is achieved by phosphodiesterases, of which there are two domain families: EAL and HD-GYP. EAL-domain-containing PDEs degrade c-di-GMP into 5′-phosphoguanylyl-(3′-5′)-guanosine (pGpG), which is then further degraded by nonspecific cellular PDEs into GMP at a slower rate (Chang et al., 2001; Paul et al., 2004; Christen et al., 2005; Hickman et al., 2005; Schmidt et al., 2005; Tamayo et al., 2005; Schirmer and Jenal, 2009); and HD-GYP-domain PDEs degrade c-di-GMP into pGpG as an transient intermediate en route to producing two GMPs as the major product (Ryan et al., 2006a, 2007, 2009). The amino acid motifs of both proteins (EAL and HD-GYP) are essential for the enzymatic activities of PDEs (Hengge, 2009). In order to reset its c-di-GMP levels, *B. burgdorferi* contains two evolutionary distinct phosphodiesterases: PdeA (BB0363) and PdeB (BB0374) (Sultan et al., 2010, 2011). PdeA contains a functional EAL-domain (Sultan et al., 2010) while PdeB possesses a functional HD-GYP domain (Sultan et al., 2011). Both PDEs were shown to specifically hydrolyze c-di-GMP with high affinity (Sultan et al., 2010, 2011). Furthermore, Sultan et al. measured the phosphodiesterase enzyme activity in the cell lysate of a *pdeApdeB* double mutant, demonstrating that no additional phosphodiesterases are present in *B. burgdorferi*, which is consistent with bioinformatic analysis (Galperin et al., 2001). While both PdeA and PdeB function as phosphodiesterases, the motility and virulence phenotypes of each mutant differs, (Sultan et al., 2010, 2011), suggesting a complex regulatory system that may include differential expression, localization, or regulation of distinct pathways.

Wild-type *B. burgdorferi* cells exhibit the ability to move directionally away from a point of origin in a run-flex/pause-reverse swimming pattern. This movement is decided by the asymmetric rotation of the flagellar motors (Li et al., 2002; Charon et al., 2012). Inactivation of *pdeA* resulted in cells that were unable to reverse their swimming direction (Sultan et al., 2010). The swimming pattern of the *pdeA* mutant is similar to the motility phenotype of the chemotaxis response regulator *cheY3* mutant, which constantly runs and does not flex or reverse (Motaleb et al., 2011). In *Escherichia coli* and *Salmonella enterica*, the CheY protein is a chemotaxis response regulator that binds to the flagellar switch protein, FliM, when phosphorylated, causing the rotation of the flagella to switch from counterclockwise (cells run) to clockwise (cells tumble). Therefore, *cheY* mutants constantly run because they are incapable of switching the rotation of their flagella (Silversmith and Bourret, 1999; Berg, 2003). A motility defect resulting from inactivation of the *B. burgdorferi pdeA* gene is consistent with motility phenotypes reported in similar mutants in other bacteria (Tamayo et al., 2005; Pesavento et al., 2008; Wolfe and Visick, 2008); however, the exact mechanism through which *pdeA* alters motility in *B. burgdorferi* has yet to be determined.

Both *E. coli* and *S. enterica* possess multiple DGCs and PDEs, but only the absence of one specific PDE, YhjH, results in an impairment of motility (Ryjenkov et al., 2006; Girgis et al., 2007; Pesavento et al., 2008). *yhjH* mutants are reported to have elevated c-di-GMP levels, and the inhibition of motility seen in these mutants is dependent on the PilZ protein, YcgR. Thus, when an *ycgR* mutant was constructed in the *yhjH* mutant background, the motility phenotype of the double mutant was reported to be restored back to the wild-type levels (Ryjenkov et al., 2006; Pesavento et al., 2008). YcgR was demonstrated to posttranslationally interact with the flagellar proteins FliG, FliM, or MotA, most strongly in the presence of c-di-GMP, which causes a reduction in the generation of torque and induces a counter-clockwise motor bias (Armitage and Berry, 2010; Boehm et al., 2010; Fang and Gomelsky, 2010; Paul et al., 2010) (see Figure 1 in Armitage and Berry, 2010; and Figure 6 in Fang and Gomelsky, 2010). Thus, the motility defect seen in the *B. burgdorferi pdeA* mutant may be a result of a receptor-c-di-GMP complex interfering with the function of a flagellar protein(s). Furthermore, by constructing a *plzA* mutant in the *pdeA* mutant background, where the cellular c-di-GMP level was 2× higher than that in the wild-type cells, Pitzer et al. demonstrated that PlzA regulates *B. burgdorferi* motility in a different manner from that in *E. coli*, and that elevated c-di-GMP in the *B. burgdorferi pdeA* mutant regulates motility by a mechanism independent of PlzA (Pitzer et al., 2011). Still, it is impossible to ignore the possibility that *B. burgdorferi* may possess another c-di-GMP receptor, which may be responding to the elevated c-di-GMP levels in the *pdeA* mutant and altering its motility. Further studies are needed to confirm these hypotheses.

In contrast to *pdeA*, inactivation of *pdeB* resulted in cells that exhibited a swimming pattern similar to wild-type except they flexed significantly more, suggesting that c-di-GMP may play a role in chemotaxis (Sultan et al., 2011; Kulasekara et al., 2013; Russell et al., 2013). Increased flexing would be expected to be a result of overexpression of the chemotaxis response regulator, CheY3, inhibition of the activity of CheX (the CheY-P phosphatase), or decreased expression of CheX (Motaleb et al., 2005; Pazy et al., 2010; Motaleb et al., 2011). However, Western blot analysis showed that both proteins are expressed at wild-type levels in the *pdeB* mutant, eliminating the possibility of a receptor-c-di-GMP complex directly regulating the expression of chemotaxis proteins (Sultan et al., 2011). However, Sultan et al. demonstrated that a *pdeBplzA* double mutant in *B. burgdorferi* constantly flexed, which is similar to the phenotype of the *cheX* mutant (Motaleb et al., 2005; Sultan et al., 2011), implying that PlzA may control the chemotaxis system either through modulating the activity of CheX or CheY3 (Sultan et al., 2011). Alternatively, the hydrolyzed products of the phosphodiesterases may be responsible for the phenotypic differences observed in the *pdeA* and *pdeB* mutants. A recent study by Stelitano et al. demonstrated that two HD-GYP domain PDEs (PA4108 and PA4781) in *Pseudomonas aeruginosa* are capable of not only hydrolyzing pGpG into two molecules of GMP, but also are capable of using it as a substrate (Stelitano et al., 2013). Moreover, pGpG is reported to be a member of the "nanoRNA" molecules that are known to be involved in regulating gene expression (Goldman et al., 2011; Nickels, 2012; Vvedenskaya et al., 2012; Römling et al., 2013). If this is also true for *B. burgdorferi*, then pGpG may accumulate in the cell under high c-di-GMP concentrations (in the *pdeB* mutant) and potentially exert its role as a signaling molecule; or perhaps the accumulation of pGpG is responsible for some of the phenotypic differences observed in the *pdeA* and *pdeB* mutant.

In addition to their role in regulating motility, PDEs have also been associated in the virulence of some bacteria (Wolfe and Visick, 2008; Römling and Simm, 2009; Ryan et al., 2010).

Sultan et al. demonstrated that spirochetes lacking PdeA were unable to infect mice either by needle or tick bite. This deficiency in infection is most likely a result of the inability of the *pdeA* mutant to reverse its swimming direction (Sultan et al., 2010) because inactivation of a chemotaxis histidine kinase, *cheA2*, in *B. burgdorferi* yielded spirochetes that constantly swim in one direction and were unable to reverse and were also unable to establish infection in mice by either needle or tick (Sze et al., 2012). Additionally, intravital microscopy of *B. burgdorferi* in a live mouse indicated that the "back-and-forth" (run-reverse) swimming pattern of wild-type cells is important in transendothelial migration (Moriarty et al., 2008; Norman et al., 2008). However, while spirochetes deficient in PdeA are unable to establish infection in mice, this mutant is capable of colonizing *Ixodes* ticks normally (Sultan et al., 2010). The prospect that c-di-GMP signaling by PdeA is essential for mammalian infection cannot yet be eliminated. Loss of PdeB, on the other hand, had no significant effect in mouse infection by needle inoculation (Sultan et al., 2011), which suggests that PdeA and PdeB may exert their regulatory effects through different mechanisms. Interestingly though, spirochetes lacking PdeB exhibited a survival defect within fed ticks, and ticks infected with PdeB-deficient spirochetes failed to transmit the infection to naïve mice during feeding (Sultan et al., 2011). Independent studies performed by He et al. (2011), Kostick et al. (2011), and Caimano et al. (2011) have shown that *hk1* and *rrp1* are essential for survival of *B. burgdorferi* within fed tick midguts. These results, coupled with other studies, imply that the regulation of c-di-GMP levels by *B. burgdorferi* is central to survival in the tick phase of the enzootic cycle. Further studies are needed to not only elucidate the role c-di-GMP plays in the tick phase of the enzootic cycle, but also specifically the function of PdeA and PdeB during the adaptation process of *B. burgdorferi*.

The significance of *B. burgdorferi* possessing two types of unrelated PDEs in its genome is still unclear, especially considering that HD-GYP PDEs are widespread but not ubiquitous in bacterial genomes (only over 1000 genes have been found among the whole sequenced bacterial genomes) (Römling et al., 2013). The presence of genes encoding for EAL and HD-GYP domain proteins in the *B. burgdorferi* genome suggests that this spirochete regulates c-di-GMP turnover in a sophisticated manner. Indeed, EAL and HD-GYP-domain proteins in other bacterial species have been reported to be associated with known or hypothetical signal input domains that are putatively involved in detecting a wide range of environmental signals (Tal et al., 1998; Galperin et al., 2001; Jenal and Malone, 2006; Ryan et al., 2006b; Barends et al., 2009; Tuckerman et al., 2009). Nevertheless, neither PdeA nor PdeB have been linked to sensory domains; thus, exactly how these PDEs function not only together but also in conjunction with Rrp1 to produce a coherent output signal is still unknown. There is the possibility that *B. burgdorferi* has (at least) two c-di-GMP circuits—one that signals through Hk1-Rrp1-PdeA and one that signals through Hk1-Rrp1-PdeB—which operate in divergent mechanisms in response to environmental signals through two different receptors: the PlzA receptor and an unidentified c-di-GMP receptor. This may mean that the concentrations and activities of PdeA and PdeB would vary over time in response to the fluctuating environmental or cellular conditions. This manner of temporal sequestration was shown in *E. coli*, where the expression patterns of 28 GGDEF/EAL genes were analyzed and most of them exhibited differential expression patterns at different temperatures and growth phases (Sommerfeldt et al., 2009). The c-di-GMP signal transduction systems may also act through the functional sequestration (or compartmentalization) of the c-di-GMP control module components—multiprotein complexes that are comprised of a specific DGC and/or PDE, which are mediated by specific input signals and influence certain effectors and target components (Jenal and Malone, 2006; Christen et al., 2010; Massie et al., 2012). Research has provided increasing evidence that these DGC-PDE interactions do occur. In *Yersinia pestis*, interactions were detected between HmsT (DGC), HmsP (PDE), HmsR (putative glycosyltransferase), and its accessory factor (HmsS)—all of which are attached to the inner membrane and necessary for biofilm-associated phenotypes (Bobrov et al., 2008). Additionally, direct interactions were detected between the HD-GYP-type PDE domain of RpfG and many different GGDEF proteins in *Xanthomonas axonopodis* (Andrade et al., 2006). With functional sequestration, the close-association of the components may sterically hinder c-di-GMP from diffusing throughout the cell, thus allowing regulation of the localized c-di-GMP concentration in response to specific signals. This may explain the differing virulence and motility phenotypes seen in the *pdeA* and *pdeB* mutants of *B. burgdorferi* (Sultan et al., 2010, 2011). Clearly, further studies are needed to unravel the complicated signaling generated by c-di-GMP-hydrolyzing proteins in *B. burgdorferi*.

PlzA: THE c-di-GMP-BINDING PROTEIN IN *B. BURGDORFERI*

Little knowledge is available concerning the effector mechanisms of c-di-GMP in *B. burgdorferi* or, moreover, any arthropod-borne pathogen. The ability of a second messenger to have numerous effects on cellular behavior lies in the diversity of c-di-GMP receptors. In other species of bacteria, c-di-GMP has been demonstrated to exert its regulatory effects through proteins with cyclic nucleotide monophosphate domains (Tao et al., 2010), ribonucleoprotein complexes (Tuckerman et al., 2011), transcriptional regulators (Hickman and Harwood, 2008; Krasteva et al., 2010; Fazli et al., 2011), GEMM riboswitches (Sudarsan et al., 2008; Smith et al., 2009; Luo et al., 2013), and PilZ domain-containing proteins (Amikam and Galperin, 2006; Ryjenkov et al., 2006; Bian et al., 2013). To date, only one c-di-GMP-binding protein, the PilZ domain-containing protein PlzA, has been identified in *B. burgdorferi* (Freedman et al., 2010; Pitzer et al., 2011). All *Borrelia* species possess PlzA (the PilZ domain-containing protein in the relapsing fever spirochetes is designated as PlzC), and, furthermore, this protein is highly conserved among species of the *B. burgdorferi* sensu lato complex. Interestingly, some *B. garinii* and *B. burgdorferi* isolates possess two PilZ domain-containing proteins—one that is encoded on the chromosome (PlzA) and one that is encoded on linear plasmid 28 (PlzB). Although all three genes encode for a PilZ domain-containing protein, the amino acid identity values of both PlzB and PlzC have diverged enough from PlzA (64% and 65–69%, respectively) to warrant separate gene designations (Freedman et al., 2010). Although PlzA contains the conserved residues of the PilZ domain (RXXXR; DZSXXG; where "X" is any amino acid and "Z" is a hydrophobic

residue) (Freedman et al., 2010; Pitzer et al., 2011), it shares poor homology with the PilZ domain-containing proteins of other bacterial species, and it lacks the "PilZ-N" domain (i.e., N-terminal domain of *E. coli* PilZ protein YcgR, which contains a fold similar to the PilZ domain) (Amikam and Galperin, 2006; Ryjenkov et al., 2006). Since PlzA appears to not harbor other identifiable functional domains aside from the PilZ domain, it is considered a "stand-alone" c-di-GMP-binding protein.

The *pilZ* mutants of several bacterial species have been reported to have diverse phenotypes such as altered motility and translucent, rough, dry, and rugose colony morphology (Römling, 2005; Ryjenkov et al., 2006; Lee et al., 2007; Pratt et al., 2007; Yildiz and Visick, 2009; Zorraquino et al., 2013). Unlike the translucent colony morphology of wild-type, the *plzA* mutant of *B. burgdorferi* exhibits opaque colonies, and this phenotype is a direct result of the *plzA* mutation as the morphology is restored back to wild-type upon complementation (Pitzer et al., 2011). The molecular mechanisms underlying the opaque colony phenotype exhibited by the *plzA* mutant have yet to be determined. Many bacteria undergo phase variation between translucent and opaque or smooth and rugose colony morphology. This change is mainly dependent upon variation in the expression of surface polysaccharides (Fries et al., 1999; Yildiz and Schoolnik, 1999; Chang et al., 2009). Previous studies in other bacteria have associated opaque colonies with an increase in capsule production. For example, inactivation of the cyclic adenosine monophosphate (cAMP) receptor protein (CRP) in *Vibrio vulnificus* yielded a mutant that was defective in capsular production and formed translucent colonies compared to the wild-type strain (Kim et al., 2013). Additionally, analysis of opaque and transparent variants of a *Streptococcus pneumoniae* clinical serotype revealed that opaque variants produced an extracellular matrix, had 100-fold greater *in vitro* adherence, and showed increased virulence *in vivo* compared to the transparent bacteria (Trappetti et al., 2011). Furthermore, the PelD protein of *P. aeruginosa* was shown to be a c-di-GMP receptor which mediates the regulation of Pel polysaccharide—an extracellular adhesion necessary for the formation and maintenance of biofilms—in a c-di-GMP-dependent manner (Lee et al., 2007). Thus, one possibility is that the *plzA* mutant spirochete has increased levels of surface polysaccharides. However, the type of polysaccharide that has increased expression in the mutant, or even if it is a polysaccharide (and not another type of adhesion or surface molecule) is unknown. Moreover, if and how *plzA* regulates polysaccharide production or if they play a role in *B. burgdorferi* pathogenesis is also currently unknown. Clearly, there are more questions than answers concerning polysaccharide production, indicating the need for further research in this area.

While the swimming pattern (run-flex/pause-reverse) of the *plzA* mutant was indistinguishable from its parental wild-type strain, the swarming motility of these mutant cells were attenuated compared to wild-type cells or the isogenic complemented strain (Pitzer et al., 2011). PilZ proteins were reported to control motility in response to the concentration of c-di-GMP in other bacterial species. For example, the c-di-GMP-binding PilZ protein, DgrA, in *Caulobacter crescentus* was shown to diminish synthesis of FliL when complexed to c-di-GMP, resulting in an alteration of motility (Christen et al., 2007). This scenario is unlikely to occur in the *plzA* mutant of *B. burgdorferi*, however, because the levels of major motility and chemotaxis proteins (i.e., FliL, FliG1, FliG2, FliM, MotB, FlaB, and CheY3) were shown not to be altered (Pitzer et al., 2011). Furthermore, PlzA lacks the N-terminal YcgR domain (Freedman et al., 2010; Pitzer et al., 2011), which has been reported to be important to interact with the flagellar switch proteins to control bacterial motility (Fang and Gomelsky, 2010). As discussed earlier, *B. burgdorferi* may possess an additional c-di-GMP-binding protein(s), which controls motility in response to c-di-GMP levels in a mode similar to that reported in *E. coli* or *C. crescentus*. Studies have shown that PilZ-domain proteins that possess two domains (e.g., PilZ and YcgR-N) undergo a conformational change upon binding to c-di-GMP that exposes a new interaction surface. For example, binding of c-di-GMP to the *Vibrio cholera* PilZ-domain protein, VCA0042, induces a conformational change in the loop connecting the C-terminal PilZ domain and the N-terminal YcgR-N domain, bringing the two domains in close proximity with c-di-GMP at the mutual interface to form a new allosteric interaction surface (Benach et al., 2007). Additionally, unlike VCA0042, PP4397 from *Pseudomonas putida* binds two molecules of c-di-GMP in its YcgR-N and PilZ domain junction, which results in a change of its quaternary structure from a dimer to a monomer (Ko et al., 2010). These studies suggest that different PilZ-domain proteins display diverse binding stoichiometries and mechanistic interactions. One could hypothesize that in *B. burgdorferi* PlzA undergoes a similar conformational change upon binding to c-di-GMP, and the generation of this new molecular surface constitutes the readout of this small signaling protein by providing a highly-charged interaction surface for high-affinity regulatory interactions with downstream target proteins. In support of this, the solution structure of the *P. aeruginosa* single-domain PilZ protein, PA4608, in complex with c-di-GMP, was recently solved by NMR spectroscopy (Habazettl et al., 2011). PA4608 is a stand-alone PilZ-domain protein that was shown to undergo conformational changes at both termini of the protein while maintaining a certain degree of flexibility upon binding to c-di-GMP, which resulted in a severe rearrangement of surface charges. The rearranged termini expose a highly negatively-charged surface on one side of the complex to a potential effector protein (Habazettl et al., 2011). Thus, even though PlzA is a stand-alone PilZ-domain protein and lacks the N-terminal YcgR domain (Freedman et al., 2010; Pitzer et al., 2011), it is still plausible that it functions as a c-di-GMP effector protein. If PlzA is the only c-di-GMP-receptor protein in *B. burgdorferi* and it does undergo a conformational change similar to PA4608, then the degree of flexibility maintained in the complex may influence the target specificity (the downstream target) of PlzA, especially if different c-di-GMP circuits are separated spatiotemporally through compartmentalization. Moreover, the target specificity of PlzA may also be influenced by several other potential factors (e.g., c-di-GMP levels, other proteins, co-factors, temperature, etc.), which may directly be indicative of the local environment. Further studies are needed to demonstrate this in addition to the downstream targets of PlzA.

plzA has been shown to be constitutively expressed during *in vitro* culture (Freedman et al., 2010)—similar to previously published microarray data (Ojaimi et al., 2002; Revel et al., 2002; Brooks et al., 2003)—and this expression is not dependent on temperature (Freedman et al., 2010). Transcripts of *plzA* were also detected in murine bladders as late as 13 weeks after infection (which was the last time point analyzed), indicating that expression of *plzA* is maintained throughout the enzootic cycle (Freedman et al., 2010). Furthermore, the *plzA* mutant is significantly less infectious in mice than the wild-type parental strain (Pitzer et al., 2011). This observed decrease in virulence is consistent with mutations in the PilZ proteins of other bacteria. While motility may play a role in the reduced virulence of the *B. burgdorferi plzA* mutant, as wild-type motility is critical for infectivity, future studies are needed in order to precisely delineate this mechanism. Interestingly, expression of *plzA* was reported to be significantly increased in ticks after a bloodmeal (Freedman et al., 2010), and *plzA* mutant cells were shown to be comprised in fed ticks (Pitzer et al., 2011). While the mechanism resulting in this survival defect in fed ticks is unknown, it is most likely that PlzA (and not solely motility) is responsible for the survivability in the fed tick because other mutants defective in motility (e.g., *cheA2*, *pdeA*) are capable of not only surviving, but multiplying before and after a blood meal (Sultan et al., 2010; Sze et al., 2012).

He et al. have recently shown that PlzA-c-di-GMP functions in linking the two global TCS in *B. burgdorferi*—Hk1-Rrp1 and Hk2-Rrp2 (He et al., 2014). Furthermore, He et al. also demonstrated that PlzA modulates expression of *rpoS* through BosR and this occurs in a mechanism independent of Rrp1 (He et al., 2014) (**Figure 1**). This suggests that aside from functioning as a c-di-GMP-binding protein, PlzA also has other roles in *B. burgdorferi*. Thus, it is likely that PlzA either plays a direct role in the survivability of *B. burgdorferi* in fed ticks or regulates a virulence determinant(s), which influences survival in the tick as well as infectivity in the mouse (He et al., 2014). Furthermore, it is also possible that the opaque colony morphology of the *plzA* mutant reflects an alteration in a cell surface membrane structure, which may be related to both the decreased survivability of the mutant cells in the tick and attenuation in the mouse infection model (Pitzer et al., 2011). Recent studies have demonstrated that *glp* genes are important for the survival of *B. burgdorferi* in the tick (Pappas et al., 2011) and are regulated by Rrp1 (Rogers et al., 2009; He et al., 2011). If PlzA plays an important role in mediating responses initiated by the Hk1-Rrp1, it is probable that the defect seen in the *plzA* mutant is related to a defect in glycerol utilization, and constitutive expression of the *glp* genes in the *plzA* mutant would be expected to rescue the survival defect of the mutant in the tick. However, constitutive expression of the *glp* operon in the *rrp1* mutant only partially rescued the survival of the mutant in the tick (He et al., 2011), and as such, it would be expected the same would occur in the *plzA* mutant, especially if PlzA controls other cellular processes that may be important in virulence and/or survival in both hosts in a c-di-GMP-dependent or -independent manner (He et al., 2014). Moreover, it is unknown if tick-derived elements, host-derived products acquired from the tick's blood meal, or salivary gland factors were at fault for the reduced survival of the *plzA* mutant in the tick (Sonenshine et al., 2002; Sonenshine and Hynes, 2008; Hajdušek et al., 2013). Additionally, the mechanism resulting in the decreased infectivity of the *plzA* mutant in the mouse also has yet to be elucidated. It is known that *B. burgdorferi* utilizes different means to survive in its disparate hosts, but the role PlzA plays in that survival has yet to be revealed. Current research has just begun to scratch the surface, and future studies are needed to unravel the extensive effects both PlzA and c-di-GMP are anticipated to have on *B. burgdorferi*.

CONCLUSIONS

Although 25 years has passed since the discovery of c-di-GMP in bacteria (Ross et al., 1987; Römling et al., 2013), the study of c-di-GMP signaling in *B. burgdorferi* has just begun and is still in its infancy. A great deal more research is needed in order to provide a complete inventory of the effectors molecules and targeted processes of this second messenger. While modulation of c-di-GMP represents an attractive target for controlling the progression of disease, the molecular and biological effects of this signaling system within *B. burgdorferi* must be understood before potential mechanisms of interference can be proposed. Thus, understanding the c-di-GMP signaling cascade of *B. burgdorferi* will prove invaluable to learning how to therapeutically exploit this second messenger system in the treatment and/or prevention of Lyme disease.

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That's my story, and I'm sticking to it—an update on *B. burgdorferi* adhesins

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Adhesion is the initial event in the establishment of any infection. Borrelia burgdorferi, the etiological agent of Lyme disease, possesses myriad proteins termed adhesins that facilitate contact with its vertebrate hosts. *B. burgdorferi* adheres to host tissues through interactions with host cells and extracellular matrix, as well as other molecules present in serum and extracellular fluids. These interactions, both general and specific, are critical in the establishment of infection. Modulation of borrelial adhesion to host tissues affects the microorganisms's ability to colonize, disseminate, and persist. In this review, we update the current knowledge on structure, function, and role in pathogenesis of these "sticky" *B. burgdorferi* infection-associated proteins.

Keywords: *Borrelia burgdorferi*, Lyme disease, bacterial adhesins, extracellular matrix, fibronectin, decorin, integrins

INTRODUCTION

Spirochetes are unique among bacteria in their extraordinary ability to invade, disseminate, and persist in their hosts. There is no invasion, dissemination, or persistence, however, without the ability to first adhere to host cells and surfaces. Adhesion is the initial event in the establishment of any infection. Failure to bind with sufficient strength to host tissues results in the bacterial pathogen's clearance by host innate immune mechanisms. Binding too strongly to host tissues can be detrimental to the pathogen, however, by hindering the ability of the bacterium to disseminate throughout the host. To facilitate this dynamic attachment, bacteria possess proteins called adhesins that enable binding to specific host molecules. A major adhesive target of bacterial pathogens is the extracellular matrix (ECM), a complex network of proteins and carbohydrates between and beneath cells. This supportive lattice typically functions in eukaryotic cell movement, development, and growth (Lodish and Darnell, 2000).

Lyme disease is the most common arthropod-borne disease in the northern hemisphere, and is caused by species of the *Borrelia burgdorferi* sensu lato complex, which includes *B. burgdorferi* sensu stricto (henceforth *B. burgdorferi*), *B. garinii*, *B. afzelii*, *B. spielmanii*, and *B. bavariensis* (Margos et al., 2011; Stanek and Reiter, 2011; Stanek et al., 2012). The Lyme disease spirochete can infect immunocompetent humans and other vertebrates for extensive periods of time, even for the animal's lifetime (Moody et al., 1990; de Souza et al., 1993; Steere, 2001; Miller et al., 2003; Stanek et al., 2012). As an extracellular pathogen, *B. burgdorferi* interacts with cells, tissues, and components of the ECM. *In vivo*, *B. burgdorferi* is frequently found associated with connective tissues (Kornblatt et al., 1984; De Koning et al., 1987; Barthold et al., 1991, 1992a,b, 1993; Defosse et al., 1992; Häupl et al., 1993; Pachner et al., 1995; Franz et al., 2001; Coburn et al., 2002; Cadavid et al., 2003; Cabello et al., 2007) and detected in infected cartilaginous tissues, such as skin and joints (Kornblatt et al., 1984; Schwan et al., 1988; Sinsky and Piesman, 1989; Barthold et al., 1991, 1992b; Melchers et al., 1991; Berger et al., 1992; Defosse et al., 1992; Schwartz et al., 1992; Shih et al., 1992; Pachner et al., 1995; Coburn et al., 2002; Liveris et al., 2002; Miller et al., 2006; Bykowski et al., 2007). Indeed, the ability of *B. burgdorferi* to invade collagenous tissue has been suggested as a possible mechanism of immune evasion (Cadavid et al., 2003; Barthold et al., 2006; Cabello et al., 2007). *B. burgdorferi* interactions with the host ECM are therefore likely critical in both the spirochete's pathogenesis as well as its persistence in mammals.

B. burgdorferi binds various components of the ECM, including glycosaminoglycans (GAGS), fibronectin, decorin, collagen, laminin, and integrins. In addition, *B. burgdorferi* adheres to numerous host cell types and binds components of host serum and extracellular fluids, such as plasminogen and complement regulators (**Table 1**). A number of *B. burgdorferi* adhesins were recently reviewed by Antonara et al. (2011). However, considerable research into the functions and biomolecular interactions of these adhesins has taken place since then, including the identification of novel Lyme spirochete adhesins. In this review, we provide an update on *B. burgdorferi* adhesins, focusing specifically on the bacterial factors that interact with components of the ECM, plasminogen, and complement regulators. For readers interested in adhesins involved in spirochete/tick interactions, please refer to the recent review by Kung et al. (2013). We would also refer the reader to the excellent discussion of novel *in vivo* imaging techniques and their use in delineating the roles of *B. burgdorferi* adhesins in a infectious mouse model in the work of Coburn et al. (2013).

Table 1 | *B. burgdorferi* adhesins.

Function	Adhesin (genetic locus)	Comments	References
FIBRONECTIN BINDING			
	BBK32 (*bbk32*)	Also binds GAGs; mutants attenuated in mice; role in vascular interactions in mice	Probert and Johnson, 1998; Probert et al., 2001; Kim et al., 2004; Raibaud et al., 2005; Fischer et al., 2006; Li et al., 2006; Seshu et al., 2006; Norman et al., 2008; Hyde et al., 2011; Chan et al., 2012; Lin et al., 2012; Moriarty et al., 2012
	RevA (*bbM27, bbP27*)	*bbM27* attenuated in mice; early antigen of Lyme disease; not involved in vascular interactions in mice	Gilmore and Mbow, 1998; Carroll et al., 2001; Mbow et al., 2002; Brissette et al., 2009a, 2010; Lin et al., 2012; Moriarty et al., 2012; Floden et al., 2013
	RevB (*bbC10*)	Attenuated in mice; not involved in vascular interactions in mice	Brissette et al., 2009a, 2010; Lin et al., 2012; Moriarty et al., 2012
	BB0347 (*bb0347*)	Not involved in vascular interactions in mice	Moriarty et al., 2012; Gaultney et al., 2013
	CspA (*bbA68*)	Also binds complement regulator proteins, plasminogen, laminin, others	Hallstrom et al., 2010
	CspZ (*bbH06*)	Also binds complement regulator proteins, laminin	Hallstrom et al., 2010
GAG BINDING			
	DbpA/B (*bbA24, bbA25*)	Bind decorin and dermatan sulfate; attenuation of infection in mice	Fischer et al., 2003; Shi et al., 2006; Blevins et al., 2008; Shi et al., 2008a,b; Benoit et al., 2011; Hyde et al., 2011; Chan et al., 2012; Lin et al., 2012; Wang, 2012; Imai et al., 2013; Morgan and Wang, 2013
	Bgp (*bb0588*)	Attenuated in mice	Parveen et al., 2006; Lin et al., 2012
	BbhtrA (*bb104*)	Binds and cleaves aggregan; first cell-associated protease described in *B. burgdorferi*	Coleman et al., 2013; Kariu et al., 2013; Russell and Johnson, 2013; Russell et al., 2013
LAMININ BINDING			
	BmpA (*bb0382*)	Potential role in arthritis	Pal et al., 2008; Yang et al., 2008; Verma et al., 2009
	ErpX (on lp56 of B31)		Brissette et al., 2009c
	CspA (*bbA68*)	Also binds complement regulator proteins, plasminogen, fibronectin, others	Hallstrom et al., 2010
	CspZ (*bbH06*)	Also binds complement regulator proteins, plasminogen, fibronectin, others	Hallstrom et al., 2010
COLLAGEN BINDING			
			Zambrano et al., 2004
	CspA (*bbA68*)	Also binds complement regulator proteins, plasminogen, fibronectin, others	Hallstrom et al., 2010
	CspZ (*bbH06*)	Also binds complement regulator proteins, laminin, fibronectin	Hallstrom et al., 2010
INTEGRIN BINDING			
	P66 (*bb0603*)	Binds α1β3; also porin; essential for infection of mice	Antonara et al., 2007; Behera et al., 2008; Lafrance et al., 2011; Ristow et al., 2012
	BB0172 (*bb0172*)	Binds α3β1; MIDAS motif	Wood et al., 2013
PLASMINOGEN BINDING			
	BB0337(*bb0337*)	Enolase; moonlighting protein	Floden et al., 2011; Nogueira et al., 2012; Toledo et al., 2012
	OspC (*bbB19*)	Required for establishment of infection	Grimm et al., 2004; Tilly et al., 2006; Antonara et al., 2007, 2010; Tilly et al., 2007; Xu et al., 2008; Lin et al., 2012; Onder et al., 2012
	CspA (*bbA68*)	Also binds complement regulator proteins, laminin, others	Hallstrom et al., 2010; Hammerschmidt et al., 2014
	BBA70 (*bbA70*)		Koenigs et al., 2013
	ErpA/C/P (*bbP38, bbI39, bbN38*)	ErpA mutants have slight attenuation in mice	Alitalo et al., 2002; Brissette et al., 2009b; Lin et al., 2012
COMPLEMENT REGULATOR BINDING			
			Kraiczy and Stevenson, 2013
	CspA (*bbA68*)	Binds FH, FHL-1	Hellwage et al., 2001; Alitalo et al., 2002; Metts et al., 2003; Hallstrom et al., 2010; Caesar et al., 2013b; Hammerschmidt et al., 2014
	CspZ (*bbH06*)	Binds FH, FHL-1	Hartmann et al., 2006; Rogers and Marconi, 2007; Kraiczy et al., 2008; Rogers et al., 2009
	ErpA/C/P (*bbP38, bbI39, bbN38*)	Bind FH, different binding affinities for FHR1, 2, 5	Kenedy et al., 2009; Hammerschmidt et al., 2012; Bhattacharjee et al., 2013; Caesar et al., 2013a; Meri et al., 2013

Abbreviations: Bgp, Borrelia glycosaminoglycan-binding protein; Bmp, Borrelia membrane protein; Dbp, decorin binding protein; Erp, OspEF-related protein; FHL, factor H-like; FHR-1, factor H related-1; GAG, glycosaminoglycan.

FIBRONECTIN BINDING PROTEINS

Fibronectin is a large plasma glycoprotein and a prominent component of the ECM and host serum (Romberger, 1997). In the uninfected host, fibronectin has many important roles including involvement in cell adhesion, cell migration, cell-cell signaling, ECM remodeling, and cell proliferation, survival and differentiation (Hynes, 1999; Pankov and Yamada, 2002; Midwood et al., 2006). Many bacterial pathogens interact with fibronectin, including several pathogenic spirochetes (Fitzgerald et al., 1984; Umemoto et al., 1993; Kopp et al., 1995; Grab et al., 1998; Probert and Johnson, 1998; Merien et al., 2000; Probert et al., 2001; Cameron et al., 2004; Schwarz-Linek et al., 2004, 2006; Choy et al., 2007; Lee and Choi, 2007; Stevenson et al., 2007; Atzingen et al., 2008; Brinkman et al., 2008; Hauk et al., 2008; Brissette et al., 2009a; Bamford et al., 2010; Henderson et al., 2011). *B. burgdorferi* binds to fibronectin *in vitro* (Kopp et al., 1995; Grab et al., 1998; Probert and Johnson, 1998). Additionally, soluble fibronectin or anti-fibronectin antibodies inhibit interactions between *B. burgdorferi* and cultured endothelial cells and their secreted matrices (Szczepanski et al., 1990; Kopp et al., 1995). *In vivo*, fibronectin interactions are critical in the initiation of borrelial interactions with the microvasculature (Norman et al., 2008). To date, there have been five fibronectin binding proteins identified in *B. burgdorferi*: BBK32, RevA/B, BB0347, and CspA (CRASP-1).

BBK32

BBK32, a 47 kDa protein encoded on linear plasmid 36 (lp36), binds the glycosaminoglycans heparin sulfate and dermatan sulfate, in addition to fibronectin. BBK32 was the first fibronectin-binding protein identified in *B. burgdorferi* (Probert and Johnson, 1998; Probert et al., 2001). BBK32 deletion mutants demonstrate a slight, but significant, defect in infectivity in the mouse (Li et al., 2006; Seshu et al., 2006). BBK32 has sequence similarities with fibronectin-binding adhesins of Gram-positive pathogens such as *Staphylococcus aureus* and the streptococci. Indeed, BBK32 interacts with fibronectin via the "tandem beta zipper" mechanism that is common for fibronectin-binding adhesins of streptococci (Kim et al., 2004; Raibaud et al., 2005). Several recent studies have investigated the functions of BBK32 in *B. burgdorferi* pathogenesis (Hyde et al., 2011; Wu et al., 2011; Chan et al., 2012; Moriarty et al., 2012; Ranka et al., 2013).

Recently, a whole body imaging system was utilized to examine the role of BBK32 in dissemination throughout the murine host (Hyde et al., 2011). Luciferase-expressing *B. burgdorferi* lacking BBK32 were injected into mice. The authors demonstrated a decrease in the infectious load of *bbk32* mutants at days 4 and 7 compared to the parental strain. Dissemination defects were more pronounced at lower inocula, and disappeared with higher doses of bacteria and with later time points (14 days post-inoculation). These data are consistent with the infectivity phenotypes observed in previous studies, which implied that BBK32 is important in the earlier stages of infection (Li et al., 2006; Seshu et al., 2006). The imaging results correlated well with quantitative PCR data, suggesting that whole body imaging may be a useful tool to discern subtle differences in colonization and dissemination with other borrelial mutants. These studies are also significant as the first use of bioluminescent imaging in mice with *B. burgdorferi* mutants (Hyde et al., 2011).

Elegant studies recently have demonstrated the involvement of BBK32 in adhesion to the vasculature through the use of intravital microscopy (Moriarty et al., 2012). BBK32-fibronectin interactions were shown to be important for the initial tethering of the bacterium to the endothelium, a process termed "molecular braking." This transient slow- down allowed for more stable interactions between BBK32 and glycosaminoglycans, leading to dragging of the bacterium along the endothelium, stationary adhesion of the bacterium to the endothelium, transmigration, and, finally, escape from the vasculature in a manner akin to the diapedesis of leukocytes (Muller, 2009). BBK32-deficient bacteria had significantly reduced interactions per minute, but still adhered to the endothelium, signifying that other adhesins are involved in the initial tethering step. The authors looked at the potential role of other fibronectin-binding proteins; gain of function studies were performed with a high passage nonadherent strain of *B. burgdorferi* overexpressing RevA, RevB, or BB0347. None of these proteins acted as vascular adhesins in fluid shear forces under the conditions of this experiment (Moriarty et al., 2012), suggesting additional borrelial adhesins (fibronectin binding or otherwise) are involved.

Another study evaluated a *B. burgdorferi* strain, N40D10/E9, which naturally lacks *bbk32* and has decreased binding to Vero (epithelial) cells. This strain disseminated at lower doses than the *B. burgdorferi* type strain, which does possess BBK32. The authors postulate this difference may be due to the lack of binding of the bacteria to epithelial cells at the site of inoculation (Chan et al., 2012). An important point gleaned from the above two studies is that while adhesion is a critical virulence factor for *B. burgdorferi*, that adhesion must be modulated to allow the bacterium to disseminate, either hematogenously or through the tissues. Adhering too strongly to tissues or cells is likely just as detrimental as not adhering at all.

B. burgdorferi is generally considered an extracellular pathogen, although there are numerous studies demonstrating at least transient intracellularity (Ma et al., 1991; Klempner et al., 1993; Dorward et al., 1997; Livengood and Gilmore, 2006). Recently, specific integrins were shown to be involved in *B. burgdorferi* invasion of mouse fibroblasts and human umbilical vein endothelial cells (HUVEC) (Wu et al., 2011). Since these integrins interact with fibronectin, Wu et al. examined the role of BBK32 as a bridge in the uptake of *B. burgdorferi*. BBK32 was not involved in the invasion of *B. burgdorferi* into murine fibroblasts, human fibroblasts or HUVECs, suggesting other components (such as additional fibronectin binding proteins) of *B. burgdorferi* interact with integrins to trigger uptake (Wu et al., 2011).

However, BBK32 protein fragments were used for the successful intracellular targeting of nanoparticles into epithelial cells, based on a Hepatitis B core protein system. Hepatitis B virus core is a 21 kDa protein which self-assembles into 35 nm particles (Wynne et al., 1999). Using region 130–166 of BBK32 (fibronectin binding region) fused into the major immunodominant region of the Hepatitis B core antigen, the authors demonstrated both fibronectin binding and uptake of the engineered nanoparticles into baby hamster kidney cells (Ranka et al., 2013). Targeting

nanoparticles to cells or tissues of interest using a bacterial adhesin might be an efficient approach for drug delivery.

RevA/B

RevA is a 19 kDa surface protein encoded on the circular plasmid 32 (cp32) family of plasmids. RevB is encoded on cp9, and shares 28% overall amino acid sequence identity with RevA. RevA expression is upregulated in the mammal compared to the tick vector, and its expression pattern and surface exposure suggest a potential role in *B. burgdorferi* pathogenesis (Gilmore and Mbow, 1998; Carroll et al., 2001; Mbow et al., 2002; Brissette et al., 2009a). Both RevA and RevB were shown to bind fibronectin *in vitro*, and anti-RevA antibodies block binding of whole *B. burgdorferi* to fibronectin (Brissette et al., 2009a).

Recently, the potential for RevA to serve as a diagnostic tool or vaccine was evaluated. RevA is expressed early upon mammalian infection, and patients in various stages of Lyme disease, including patients with erythema migrans, exhibited antibodies to this protein (Brissette et al., 2010). In contrast, RevB lacked utility as a potential diagnostic marker; patients rarely possessed antibodies to RevB, probably because cp9 is a plasmid often lost even in infectious wild-type *Borrelia* (Brissette et al., 2010). In mice, there was a rapid IgM response to RevA, which remained steady over months, and was followed by a variable IgG response. Polyclonal rabbit anti-RevA antibodies were bactericidal *in vitro*, but serum from vaccinated mice was not. Mice vaccinated with RevA were not protected when exposed to *B. burgdorferi* by needle or tick bite (Floden et al., 2013); however, passive immunization with the bactericidal rabbit anti-RevA antibodies did prevent infection. The reason for this disparity is not known; a bolus of anti-RevA antibody concentrated near the site of infection may be necessary for the observed bactericidal effect.

In addition to their bactericidal effects, antibodies against adhesins can block critical interactions between bacteria and their host. Schmit and colleagues investigated the ability of various monoclonal antibodies against *B. burgdorferi* outer surface proteins to block binding of the spirochete to human cells. Monoclonal antibodies against RevA did not prevent binding of *B. burgdorferi* to HUVECS or H4 neuroglial cells (Schmit et al., 2011). Since monoclonal antibodies were used, it is possible that polyclonal antibodies recognizing multiple epitopes of RevA could block binding of the bacterium to cells. Indeed, a polyclonal antibody against RevA prevents binding of *B. burgdorferi* to immobilized fibronectin (Brissette et al., 2009a). However, nonadherent *B. burgdorferi* overexpressing RevA did not exhibit increased binding to the vascular endothelium *in vivo,* which suggests this adhesin is not involved in endothelial cell interactions (Moriarty et al., 2012). RevA's role in pathogenesis remains to be elucidated; however, a *revA* mutant uncovered in a global signature-tagged mutagenesis study did demonstrate an infectivity defect (Lin et al., 2012). It is likely that many borrelial adhesins have distinct tissue and cell type tropisms, as well as overlapping and redundant functions.

BB0347

The chromosomally-encoded protein BB0347 was recently confirmed to bind fibronectin by two independent studies (Moriarty et al., 2012; Gaultney et al., 2013). Previously, BB0347 had been annotated as a putative fibronectin binding protein due to sequence similarity with other such proteins (Fraser et al., 1997). Gaultney et al. demonstrated that BB0347 was expressed and surface exposed, and that mice infected with live *B. burgdorferi* produced anti-BB0347 antibodies (Gaultney et al., 2013). The importance of BB0347 in murine infection has not been thoroughly tested; however, overexpression of this protein does not restore the ability of noninfectious, nonadherent *B. burgdorferi* to bind host vasculature (Moriarty et al., 2012). Additionally, results suggest that BB0347 has a higher dissociation constant (K_D) than BBK32, suggesting this protein does not bind fibronectin as strongly (Moriarty et al., 2012; Gaultney et al., 2013). Ultimately, the role of BB0347 in *B. burgdorferi* pathogenesis is still largely unknown, and further research is needed to determine the functionality of this protein in the Lyme spirochete.

CspA (CRASP-1)

CspA, also known as Complement Regulator Acquiring Surface Protein-1 (CRASP-1), is a factor H binding protein important in *Borrelia* resistance to complement (Kraiczy et al., 2001b; Brooks et al., 2005). Like many *B. burgdorferi* adhesins, CspA has multiple functions and binding partners (Kraiczy et al., 2001a; Hallstrom et al., 2010). Recent work by Hallstrom et al. demonstrated that CspA binds to fibronectin *in vitro,* as well as to human bone morphogenic protein 2 (BMP2), plasminogen, collagens I, III, and IV, and laminin. CspZ (CRASP-2), another factor H binding protein, also bound fibronectin and laminin *in vitro* (Hallstrom et al., 2010).

PROTEOGLYCAN AND GAG BINDING PROTEINS

Proteoglycans are a diverse group of highly viscous macromolecules with a core protein and attached polysaccharide chains called glycosaminoglycans, or GAGs (Lodish and Darnell, 2000). Glycosaminoglycans, including chondroitin sulfate, dermatan sulfate, keratin sulfate, heparin, heparin sulfate, and hyaluronan, are long unbranched polysaccharides comprised of a repeating disaccharide unit (Varki, 2009). *B. burgdorferi* binds to both proteoglycans and their GAG chains through a number of proteins, including decorin-binding proteins A and B (DbpA/B), *Borrelia* glycosaminoglycan- binding protein (Bgp), and BBK32 (Parveen and Leong, 2000; Fischer et al., 2003, 2006).

Decorin-binding protein A/B

Decorin is a small leucine rich proteoglycan that associates with collagen; it possesses a collagen binding core protein and single GAG chain, either dermatan sulfate or chondroitin-6 sulfate (Danielson et al., 1997; Keene et al., 2000; Zhang et al., 2006). Decorin is widely expressed, and has important functions not only as a structural molecule of the ECM, but also as a cell signaling molecule influencing growth, differentiation, and inflammation (Dugan et al., 2006). Decorin also interacts with fibrinogen, allowing interaction with the hemostasis and thrombosis cascades (Dugan et al., 2006; Seidler, 2012). *B. burgdorferi* has two decorin-binding proteins that recognize decorin and other proteoglycans with GAG chains (Guo et al., 1995, 1998; Leong et al., 1998; Parveen et al., 1999). The genes encoding decorin-binding

proteins A and B (*dbpA/B*) are encoded in a bicistronic operon on lp54. Mutants deficient in decorin- binding proteins are still infectious; however, *dbpA/B* deficient bacteria are impaired in both colonization of various tissues and in persistent infection (Shi et al., 2006, 2008a,b; Weening et al., 2008). Decorin deficient mice are resistant to *B. burgdorferi*, exhibiting fewer bacteria in joints upon infection as well as less severe arthritis than that induced in wild type mice (Brown et al., 2001). These data highlight the importance of decorin interactions to the establishment of disseminated infections by *B. burgdorferi*.

The recently solved structure of DbpA lends insight into the biophysical interaction of this protein with GAGs (Wang, 2012). DbpA was shown to be a helical bundle protein with 5 helices and a strong hydrophobic core. The authors demonstrated that a patch composed of basic amino acids is formed by portions of two helices and two flexible linkers. Residues K163, K170, and R166 formed a basic stretch on helix 5, reminiscent of motifs present in other GAG-binding helices. Heparin titration of DbpA showed that perturbed residues were in the linker domain between helices 1 and 2, demonstrating an important role for this linker in binding GAGs. Dermatan sulfate binds with a lower affinity than heparin, but still binds DbpA (Wang, 2012). In a separate study, the heparin binding epitope was identified as a BXBB motif ($B =$ basic amino acid) in the linker between helices 1 and 2 (Morgan and Wang, 2013).

Based on the structural data, it seems likely that DbpA allelic variation, particularly in the GAG binding region, would affect the ability of *B. burgdorferi* to bind to decorin, GAGs, and mammalian cells. In gain of function studies, B314, a noninfectious and nonadherent *B. burgdorferi* strain, was used to examine the binding characteristics of various DpbA alleles. Expression of various alleles of DbpA in the nonadherent background allowed adhesion to epithelial but not endothelial or glial cells (Benoit et al., 2011). The authors also demonstrated that decorin and dermatan sulfate binding were separable, suggesting different binding sites on the DbpA protein for decorin and dermatan sulfate. The DbpA protein from strain N40 showed reduced adhesion to GAGs *in vitro*; however, the N40 strain caused disseminated infection *in vivo* (Chan et al., 2012), suggesting that other adhesins are crucial for adhesion and dissemination in this strain. An alternative explanation is that the recombinant protein used for *in vitro* studies may have different binding activities than the native borrelial protein.

Another group examined decorin binding of various genospecies including *B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto (Salo et al., 2011). There is only 40–60% similarity among the decorin binding proteins of these genospecies. Interestingly, *B. afzelii*, despite its preference for skin and its association with the Lyme disease skin manifestation known as acrodermatitis chronica atrophicans, doesn't bind decorin. The authors evaluated the binding abilities of both DbpA and DbpB independently. DbpA of *B. garinii*, as well as DbpB of *B. garinii* and *B. burgdorferi*, promoted strong binding to decorin. In contrast, DbpA of *B. burgdorferi* and DbpA and DbpB of *B. afzelii* exhibited lower binding to decorin. Despite differences in their binding to decorin *in vitro*, both DbpA and DbpB of *B. garinii*, as well as *B. burgdorferi*, promoted adhesion of whole spirochetes to decorin or decorin-expressing human foreskin fibroblasts (Salo et al., 2011). The authors suggest that the Dbps of *B. afzelii* have different biological functions and ligands than the Dbps of *B. garinii* or *B. burgdorferi*.

In a previously mentioned study with BBK32, luciferase-expressing *B. burgdorferi* lacking *dbpAB* were also injected into mice, and mutants were only visible at 1 h post infection (Hyde et al., 2011), suggesting efficient clearance of the spirochetes. These data again demonstrate a role for decorin-binding proteins in the initial colonization by *B. burgdorferi*.

Another group demonstrated that DbpA/B mutants have decreased infectivity and an early dissemination defect in immunocompetent but not immunodeficient mice. Development of arthritis and carditis decreased only in early stages of infection with DbpA/B mutants in either strain of mice. Up to 14 days post infection, the bacterial load in the lymph nodes of mice infected with DbpA/B mutants was decreased compared to lymph nodes of mice infected with wild type bacteria. The authors postulate that the immune response restricts early dissemination through the lymphatic system, which suggests a role for nonantibody mediated clearance, perhaps by iNKT cells. A particular adhesin may not be essential for infection, but can still influence pathogenicity by altering the course of infection through changing the ability to disseminate, colonize, or persist (Imai et al., 2013).

Finally, anti-DbpA antibodies were shown to significantly reduce the ability of whole *B. burgdorferi* to bind to H4 (neuroglial) and HUVEC cells. Indeed, *dbpA* expression was increased when *B. burgdorferi* was incubated with either cell type (Schmit et al., 2011). Overall, the data amassed in the last 3 years demonstrate that decorin binding proteins of *B. burgdorferi* are important in both the initial establishment of infection and persistence of the Lyme spirochete.

BbhtrA

The proteoglycan aggrecan is found in the ECM and cartilage, particularly articular cartilage of joints. Aggrecan is comprised of three globular domains, with 2 sites for GAG attachment. The N terminus of aggrecan binds hyaluronan, which is important for linking of aggrecan molecules to form aggregates (Watanabe et al., 1998). Aggrecan thus adds to the resiliency of functional cartilage. *B. burgdorferi* binds aggrecan, and both BbhtrA and Bgp were recently identified as aggrecan-binding proteins (Russell and Johnson, 2013). BbhtrA is significant as the first identified outer surface protease of *B. burgdorferi*. BbhtrA is expressed during human disease, is immunogenic, and is conserved among *B. burgdorferi* strains. BbhtrA both binds and cleaves aggrecan at a site known to eradicate its function, suggesting that this protease may contribute to the damage seen in Lyme arthritis (Russell and Johnson, 2013). Proteolytic activity for BbhtrA against *B. burgdorferi* proteins BB0323, BmpD, and CheX was also demonstrated, in addition to cleavage of host ECM proteins (Coleman et al., 2013; Kariu et al., 2013; Russell et al., 2013).

COLLAGEN-BINDING PROTEINS

Collagens are triple helical proteins and the most abundant ECM proteins. Collagens are important structural components of many tissues, allowing tissues to withstand stretching

(Lodish and Darnell, 2000). There are numerous collagens and related proteins. Collagens I, II, and III are the major fibrous collagens, while collagen IV is a major constituent of basement membranes (Labat-Robert et al., 1990). *B. burgdorferi* has long been known to bind collagen *in vitro*, but until recently no collagen binding adhesin had been identified (Zambrano et al., 2004). Hallstrom and colleagues recently demonstrated that CspA (CRASP-1), a factor H binding protein, also binds to collagen I, III, and IV (Hallstrom et al., 2010). More studies are necessary to elucidate the multiple roles of CspA in *B. burgdorferi* pathogenesis. Given the spirochete's redundant interactions with other ECM proteins, it is likely that other collagen binding adhesins remain to be discovered.

LAMININ-BINDING PROTEINS

Laminin is a glycoprotein component of the ECM consisting of α, β, and γ subunits that self-assemble into a heterotrimer. Laminin serves a scaffolding function and interacts with integrins as well as other matrix components (Colognato and Yurchenco, 2000). *B. burgdorferi* binds laminin through BmpA and ErpX (Brissette et al., 2009c; Verma et al., 2009). Recently, the promiscuous CspA and CspZ proteins were also shown to bind laminin *in vitro* (Hallstrom et al., 2010).

INTEGRIN-BINDING PROTEINS

Integrins are transmembrane bidirectional signaling molecules involved in cell-cell signaling and cell-matrix interactions. Intergins serve as ECM ligands and partner with many multiadhesive proteins including fibronectin, laminin, and collagen (Takada et al., 2007). These heterodimeric proteins consist of both α and β subunits. *B. burgdorferi* binds αIIβ3 (Coburn et al., 1993), αvβ3, α5β1 (Coburn et al., 1998), as well as α3β1 integrins (Behera et al., 2006).

B. burgdorferi binds α1β3 integrins through the P66 outer membrane protein and porin (Lafrance et al., 2011). Because of the ability of integrins to signal "outside-in," the authors examined the effects of P66 on gene expression by human cells in culture. *B. burgdorferi* strains with and without P66 were incubated with EA-hy926 (endothelial) cells or human embryonic kidney cells (HEK 293) and subjected to microarray analysis. For both cells, but especially the endothelial cells, there were dramatic changes in the expression of ECM interacting proteins, components of the immune response, and genes encoding proteins involved in actin dynamics. The authors postulate that P66 may decrease the endothelial cell response, which in turn affects the ability of the endothelium to respond to the bacterial threat (Lafrance et al., 2011).

ΔP66 strains were not infectious in wild type, TLR2-, or MyD88- mice, suggesting that even in the absence of innate immune signaling, P66 is required to establish infection (Ristow et al., 2012). Interestingly, restoration of P66 on a shuttle vector did not restore infectious ability, but complementation on the chromosome did, supporting the hypothesis that transient adhesion is important (i.e., the overexpressing complemented strain was not infectious). ΔP66 mutants were cleared from site of inoculation, but survived in rat dialysis chambers. These data demonstrated that the inability to infect mice was not due to a nutritional defect, an important point as P66 is a porin as well as an adhesin. P66 was not required for survival in ticks, as ΔP66 mutants survived in the tick through the molt from larvae to nymph. Increased mast cells were present at the site of inoculation of ΔP66 strains, and the mutants did not show increased susceptibility to serum. The authors suggest that P66 binding to integrins is necessary to move from the site of inoculation to distant tissues (Ristow et al., 2012). The exact connection between the lack of P66 and bacterial clearance remains to be established.

Integrins were also shown to be involved in invasion of human endothelial cells and mouse fibroblasts by *B. burgdorferi*. This process required β1 integrins but not α5β1 or the fibronectin binding protein BBK32. Internalization did require actin reorganization, as well as Src kinase activity (Wu et al., 2011).

Wood et al. recently described a novel integrin binding factor (Wood et al., 2013). The protein encoded by *bb0172* was shown to be associated with the outer membrane by proteinase K digestion and Triton X-114 extraction. This protein bound α3β1 integrin, and has a von Willebrand factor (vWF) domain, which is oriented to the extracellular environment. vWF domains are important in both cell adhesion and protein-protein interactions (Schneppenheim and Budde, 2011). Changes in temperature affected *bb0172* expression, which was increased during the change from unfed to fed tick conditions *in vitro*. BB0172 was shown to be a metal ion-dependent integrin binding protein, and this paper marks the first report of a metal ion-dependent adhesion site (MIDAS) motif in *Borrelia* species. Of particular note from this study is the use of an *in vitro* system to determine the orientation of transmembrane helices in microsomal membranes; this may be a helpful tool for studying other helical membrane proteins of *B. burgdorferi* and other spirochetes (Wood et al., 2013).

PLASMINOGEN-BINDING PROTEINS

Plasmin is a serine protease synthesized as the inactive zymogen plasminogen. Plasminogen is activated by physiological activators uPA (urokinase plasminogen activator) or tPA (tissue plasminogen activator) to the active serine protease plasmin. Plasmin's main role *in vivo* is to degrade fibrin-containing thrombi, but it can also degrade ECM components (Castellino and Ploplis, 2005). Plasminogen is abundant in serum and extracellular fluids, and is a favorite target of many pathogenic bacteria. Binding plasminogen to the surface of a bacterium can provide a potent protease that can assist in dissemination and invasion of the pathogen (Bhattacharya et al., 2012; Sanderson-Smith et al., 2012). Several plasminogen-binding proteins have been identified in *B. burgdorferi*, including OspA, OspC, ErpA/C/P, CspA, CspZ, BBA70, enolase, and a 70 kDa protein (Fuchs et al., 1994; Hu et al., 1997; Lagal et al., 2006; Brissette et al., 2009b; Hallstrom et al., 2010; Koenigs et al., 2013; Hammerschmidt et al., 2014). Plasminogen deficient mice have decreased spirochetemia upon *B. burgdorferi* infection (Coleman et al., 1997). Plasmin on the surface of *B. burgdorferi* facilitates movement through endothelial cell monolayers and degrades components of the ECM (Coleman et al., 1995, 1999). Clearly, acquisition of plasminogen is vital for efficient dissemination of *B. burgdorferi*.

Enolase

Enolase is a highly conserved glycolytic enzyme that should be cytosolic, as it lacks classical cell sorting sequences and cell anchoring moieties. Yet, enolase is a prime example of a "moonlighting" protein (Jeffery, 1999) that is found on the surface of a variety of eukaryotic cells where it can function as a plasminogen receptor (Pancholi, 2001). A number of bacteria express enolases, which likewise can be found on the surface of bacterial cells and function as plasminogen receptors (Pancholi and Fischetti, 1998; Lahteenmaki et al., 2001; Pancholi and Chhatwal, 2003).

Three research groups published that *B. burgdorferi* enolase, BB0337, is a plasminogen-binding protein (Floden et al., 2011; Nogueira et al., 2012; Toledo et al., 2012). Floden et al. demonstrated that this binding was dependent on lysine residues but not influenced by ionic charge. Enolase was localized to the outer surface as shown by proteinase K digestion and immunogold electron microscopy. Plasminogen bound to *B. burgdorferi* enolase was converted to active plasmin by the plasminogen activator uPA (Floden et al., 2011). Noguiera et al. also showed that BB0337 was associated with the outer membrane and surface exposed. BB0337 retained its enzymatic activity intrinsic to the glycolytic pathway, and interacted with plasminogen via lysine residues. The *B. burgdorferi* enolase showed variable temporal and spatial expression in ticks, with expression relatively high in fed and unfed nymphs. In a mouse model of infection, enolase demonstrated its highest expression in joints and heart at 28 days post-infection, while expression in skin disappeared by day 28. Immunization with BB0337 did not evoke protective immunity in mice, but did prevent acquisition by ticks (Nogueira et al., 2012). In contrast, a third group found BB0337 in outer membrane vesicles, but did not detect enolase on the outer surface of *B. burgdorferi* by proteinase K digestion or by electron microscopy (Toledo et al., 2012). In addition, Toledo et al. demonstrated that enolase was recognized by host antibodies from tick-infected mice, rabbits, and human Lyme disease patients (Toledo et al., 2012).

OspC

In a recent study, Önder et al. make the case for OspC as the principal plasminogen-binding protein of *B. burgdorferi* (Lagal et al., 2006; Onder et al., 2012). The authors demonstrated that OspC bound plasminogen *in vitro*, and also showed co-localization of plasminogen and OspC on the surface of intact bacteria. Finally, OspC-deficient bacteria did not appreciably bind plasminogen, and plasminogen binding was effectively blocked by anti-OspC antibodies (Onder et al., 2012). The authors note that these results do not preclude a role for the other identified plasminogen-binding proteins under different conditions.

CspA (CRASP-1)

CspA binds plasminogen *in vitro*, and this interaction was inhibited by 6-ACA, a lysine analog. In the presence of uPA, plasminogen bound to CspA was converted to active plasmin (Hallstrom et al., 2010).

BBA70

Recently, Koenigs et al. described another plasminogen binding protein of *B. burgdorferi*, BBA70 (Koenigs et al., 2013). As with other plasminogen binding proteins, the interaction between BBA70 and plasminogen was dose-dependent and affected by ionic strength. This interaction was mediated by lysine residues in the C-terminus of BBA70. The authors demonstrated that BBA70 is located on the borrelial outer surface. Plasminogen bound to BBA70 was converted to active plasmin by urokinase-type plasminogen activator, and was able to degrade fibrinogen. Interestingly, BBA70-bound plasmin was able to degrade the central complement proteins C3b and C5 (Koenigs et al., 2013).

COMPLEMENT REGULATOR ACQUIRING SURFACE PROTEINS

Factor H is a 155 kDa protein present in mammalian serum that is important for homeostasis of the complement system. Factor H binds C3b, and accelerates the decay of the alternative pathway C3 convertase C3bBb, as well as serving as a cofactor for Factor I-mediated proteolytic inactivation of C3b (Rodriguez de Cordoba et al., 2004). Factor H consists of 20 short consensus repeats (SCRs); SCRs 19–20 interact with GAGs, such as heparin, on the surface of host cells.

There are additional Factor H-like proteins; Factor H-like protein-1 (FHL-1) is encoded by the same gene, but is a product of alternative splicing of the Factor H pre-mRNA. Factor H related proteins (FHRs) are encoded by separate genes but have sequence similarities with SCRs 18–20 of Factor H. FHR-1 blocks the action of the C5 convertase, as well as the membrane attack complex (MAC) formation, while FHR-5 has cofactor activity for Factor I-mediated inactivation of C3b (McRae et al., 2005). The function of FHR2 is unknown.

B. burgdorferi has several proteins which bind Factor H and its related proteins, initially identified as CRASPs (Complement Regulator-Acquiring Surface Proteins), including CRASP-1 (CspA), CRASP-2 (CspZ), and CRASP-3 (ErpP), CRASP-4 (ErpC), and CRASP-5 (ErpA) (reviewed in Kraiczy and Stevenson, 2013). Many of these proteins have multiple designations in the literature; to avoid confusion, the designations CspA, CspZ, ErpP, ErpC, and ErpA will be used here.

A number of exciting biochemical and biophysical studies recently have provided important insights into the interaction of borrelial Factor H-binding proteins with their ligands. One study examined the structural basis for complement evasion. The authors solved the solution structure of ErpP interacting with Factor H by NMR and X-ray crystallography. Factor H SCR 19–20 interacts with ErpP, reminiscent of how Factor H binds to GAGs (Bhattacharjee et al., 2013). Another study resolved the atomic resolution structure of ErpC. This protein consists of 10 antiparallel β strands capped by two α helices. The outer surface is charged, while the inner surface is hydrophobic. ErpC bound Factor H through residues within the loops between the β strands (Caesar et al., 2013a). In contrast, CspA, which forms homodimers, was shown to consist of 7 α helices joined by short loops. The flexibility between the subunits may allow for increased access of Factor H to the binding site. The C terminus of CspA is required for assembly of a stable dimer (Caesar et al., 2013b). Finally, Meri et al. showed that ErpA of *B. burgdorferi*, as well as Factor H binding proteins from the relapsing fever spirochete *B. hermsii* and other pathogens, binds Factor H through a common site in SCR 20. The authors showed that binding of Factor H by these

pathogens was inhibited by heparin binding, and that a tripartite complex between the microbial protein, Factor H and C3b is formed, facilitating complement evasion (Meri et al., 2013).

The contribution of the Factor H-binding proteins to complement evasion is being heavily investigated not just for *B. burgdorferi*, but also *B. afzelii* and *B. spielmannii*. Kenedy and Akins overexpressed ErpP and ErpA in a *cspA*-deficient strain. The overexpression of these proteins enhanced serum resistance and the amount of Factor H bound to the bacterial surface. Deposition of complement components C3 and C5b-9 (the MAC complex) were reduced on the surface when ErpP or ErpA were overexpressed (Kenedy and Akins, 2011).

ErpC immobilized on magnetic beads captured Factor H, as well as FHR-1, -2, and -5 from human serum. However, *B. garinii*, which is sensitive to complement killing, expressing ErpC were still killed by complement (Hammerschmidt et al., 2012), suggesting ErpC alone is insufficient to protect the Lyme bacterium from complement.

Borrelia spielmanii binds both Factor H and plasminogen, and is serum resistant. *B. spielmanii* has a 15 kDa CRASP protein (akin to the 17–20 kDa ErpA, C, and P proteins of *B. burgdorferi*) that was shown to bind Factor H, FHR-1 but not FHL-1. Factor H bound to BsCRASP maintained its cofactor activity for Factor I-mediated C3b inactivation. Mutating H79 to alanine abrogated Factor H binding but not plasminogen binding, demonstrating independent binding sites on BsCRASP for plasminogen and Factor H. *B. spielmanii* also expresses a CspA-like protein (Seling et al., 2010).

Finally, *B. burgdorferi* ErpP and ErpA bind FHR-2 and FHR-5. In contrast, *B. garinii* CRASPs bind FHR-1, -2, and -5 but not Factor H. ErpA, ErpC, and ErpP bind Factor H and FHR-1, but not FHL-1. The binding properties of ErpA, ErpP, and ErpC are different for the recombinant proteins as compared to the native proteins. *B. burgdorferi* lacking CspA and CspZ, which bind both Factor H and FHL-1, were killed by complement. The balance of the data suggest that the Lyme *Borrelia* require acquisition of Factor H, but not FHRs, to evade complement-mediated killing (Siegel et al., 2010). A recent review by Kraiczy and Stevenson neatly summarizes the acquisition of Factor H, FHL-1, and FHRs by the various *B. burgdorferi* proteins (Kraiczy and Stevenson, 2013).

CONCLUDING REMARKS

Although much progress has been made in the field, further work is needed to elucidate the roles of the many identified borrelial ECM-binding proteins and other adhesins (**Table 1**). Obstructing progress are several characteristics inherent to these types of proteins. For instance, redundancy for specific host substrates can prevent phenotypic characterization of adhesin mutants, as the lack of one protein may be compensated for by the presence of several others. Another obstacle for research is the ability of multiple borrelial proteins to each interact with several substrates. Defining a role for a given protein during an infection may be difficult when that protein may fulfill different functions during the course of an infection. Compounding these issues are the different potential roles for every protein in the infection process of the bacteria. To be infectious, *B. burgdorferi* must colonize, disseminate, and persist in the host, all of which are facilitated by proteins discussed herein. To this end, these studies highlight the need to examine adhesins *in vitro*, *in vivo*, and with both loss and gain of function mutants. Determining which stage or stages of infection each protein participates in will help us to further understand the biology of the bacteria and potentially allow for novel treatment strategies for Lyme disease. For example, anti-adhesive therapies that target the initial interactions between bacteria and their host are garnering considerable interest in this era of antibiotic resistance (Krachler and Orth, 2013). Once the precise roles of these sticky *B. burgdorferi* proteins are well understood, these adhesins themselves may prove to be responsive targets for Lyme disease therapies.

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METHODS

This review covers new research published on *B. burgdorferi* adhesins from January 2010–October 2013. Search terms on PubMed: Borrelia + adhesin, Borrelia + fibronectin, Borrelia + laminin, Borrelia + decorin, Borrelia + integrin, Borrelia + collagen, Borrelia + proteoglycan, Borrelia + GAG, Borrelia + Factor H, Borrelia + plasminogen.

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