[Frontiers in Bioscience S4, 699-712, January 1, 2012]

Deciphering the role of Toll-like receptors in humoral responses to *Borreliae*

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1. ABSTRACT

The bacteria of the genus *Borrelia* are arthropod-borne spirochetes that cause relapsing fever and Lyme disease in humans. Like most arthropod-borne pathogens, *Borreliae* must survive in the periphery of their vertebrate hosts to allow for transmission to another arthropod vector. These spatial and temporal restrictions require that *Borreliae* evade the adaptive immune response. *Borreliae* have evolved genetic mechanisms that alter their surface protein expression, thereby altering the antigenic target presented to the host. To control *Borreliae* infection, the host relies on a rapid humoral response. While it is clear that B cell antigen receptor signaling is a critical requirement for the specific antibody responses, growing evidence suggests that additional signaling by innate immune receptors such as Toll-like receptors is necessary for optimal T cell-dependent and T cell-independent antibody responses. This review is focused on the role of Toll-like receptors in B cell responses to relapsing fever and Lyme disease *Borreliae*.

2. INTRODUCTION

The genus *Borrelia* encompasses a large group of arthropod-borne spirochetes that cause either relapsing fever or Lyme disease in humans (1). Whether bacterial, protozoan, or viral in nature, pathogens requiring an arthropod vector face unique challenges regarding transmission to new hosts. The intermittent feedings and seasonal restrictions of the vector species require that the pathogens remain in the periphery of the infected host for extended periods of time. In order to evade the adaptive immune response of the vertebrate host, most arthropod-borne pathogens, including all *Borrelia* species, utilize complex genetic expression systems that allow the pathogen to alter surface protein expression, thereby changing the primary antigenic target presented to the host immune system (2, 3). It has been shown that relapsing fever and Lyme disease *Borreliae* have evolved distinct antigenic variation systems. Relapsing fever *Borreliae*, such as *B. hermsii*, achieve extremely high levels in the blood of infected host organisms and the antigenic variation

system in this organism switches the surface expression of antigenically distinct variable major proteins (Vmp) in 10^{-4} to 10^{-3} bacteria per generation (4). This system results in waves of bacteremia, with each wave associated with an antigenically distinct and largely antigenically uniform bacterial population. In contrast, Lyme disease *Borreliae* tend to colonize soft tissue and maintain relatively low levels in the blood compared to relapsing fever *Borreliae* (2, 3). Additionally, Lyme disease *Borreliae* such as *B. burgdorferi* possess a very different system of antigenic variation, which relies on segmental recombination, resulting in the development of a large repertoire of antigenically distinct bacteria possessing a unique surface protein named variable major protein-like sequence, expressed, or VlsE (3, 5). The consequence of this difference is that unlike relapsing fever *Borreliae*, infection by Lyme disease *Borreliae* produces an infection consisting of a mosaic of bacterial clones. In fact, while the vast majority of *B. hermsii* sampled during a bacteremic episode express the same Vmp, a skin biopsy of a *B. burgdorferi*-infected mouse found that each isolated clone possessed a unique VlsE variant (6). This mosaic infection seems to account for the fact that while relapsing fever resolves over several weeks, Lyme disease *Borreliae* can persist months or years if not treated with antibiotics (7-9).

3. HUMORAL IMMUNE RESPONSES

3.1. T cell-dependent and T cell-independent humoral responses

To combat pathogens that rapidly alter their surface antigens, the host relies heavily on robust and rapid antibody responses. Some antibody responses require B cell interactions with cognate T cells within germinal centers and these humoral responses are categorized as being T cell-dependent (TD) (10-12). In this context CD40-CD40L interaction between B and T cells plays a critical role, as it promotes several events including affinity maturation of B cell antigen receptor (BCR), and the development of long-lived plasma cells capable of secreting high-affinity antibodies of different isotypes. Additionally, germinal center reactions result in the development of conventional memory B cells, which are capable of responding quickly upon antigen re-exposure (10-12). Though responses to TD antigens following secondary exposure are rapid, the development of primary responses takes at least a week following initial antigen exposure. Therefore TD responses have limited utility during the earlier stages of an infection.

Antigens that are not efficiently presented to T cells in the context of MHC-II molecules are typically resistant to proteolysis (e.g., polysaccharides) and therefore have a restricted immunogenicity for TD responses. Nevertheless these type of antigens can efficiently activate B cells in the absence of T cell-help and are referred to as T cell-independent (TI) responses (13, 14). TI type 1 (TI-1) responses from B cells are induced following stimulation of mitogenic receptors, such as Toll-like receptors (TLRs). The prototypical example of a TI-1 antigen is bacterial lipopolysaccharide (LPS), which elicits a predominantly polyclonal antibody response following stimulation of TLR4 on the B cell surface. TI type 2 (TI-2) responses are B cell responses generated in the absence of T cells through the process of BCR cross-linking and utilization of Bruton's tyrosine kinase (Btk), a protein necessary for optimal BCR signaling. Since TI-2 antigens must induce BCR cross-linking, they tend to either have a highly repetitive structure, such as bacterial polysaccharides (PS), or are arranged in a highly repetitive manner, such as capsid proteins on the surface of a viral particle. X-linked immunodeficient (*xid*) mice have a point mutation in *Btk* and are severely deficient in TI-2 responses (15-18). The fact that TI-2 responses occur independent of germinal center reactions has several important effects on both the temporal and qualitative characteristics of the resulting antibody response. Since CD40-CD40L interactions during TD responses are largely responsible for the induction of class-switch recombination and TI-2 responses do not involve such interactions, IgM tends to be the dominant Ig isotype in TI responses. Additionally, without germinal center reactions, there is significantly less somatic hypermutation of the BCR. Therefore the antibodies developed against TI-2 antigens will be lower affinity and of the IgM isotype. Although these TI responses may appear to be an inferior response, it is important to note that these responses occur several days before any TD responses are possible, providing a key advantage in defending against a bacterial or viral infection, particularly during the early phase of infection (19). Additionally, it was previously thought that germinal center reactions were required for the development of B cell memory, but recent research has revealed that TI responses can induce long-term, protective memory against a number of important human pathogens (20).

3.2. B cell subsets

Mature B cells can be divided into four subsets: follicular (FO) or B2, marginal zone (MZ), B1a, and B1b subsets (19, 21). While each of these subsets has distinct properties and functions within the broader immune response, it should be noted that several, or even all subsets could play a complimentary role in response to a single infection. FO B cells make up the majority of B cells in the body. These cells recirculate through secondary lymphoid organs and take part in germinal center reactions following TD antigen exposure. MZ B cells express high levels of the complement receptor CD21 and populate the marginal zone of the spleen. Their surface receptor expression and anatomical localization make MZ B cells ideally suited to respond to particulate TI antigens in the systemic circulation and MZ B cells are involved in both the secretion of natural antibody and also specific responses to TI antigens (22). B1a cells are abundant in the peritoneal cavity, but can be found in the spleen and systemic circulation at lower frequencies. B1a cells, like MZ B cells respond to TI antigens (23). B1a cells along with MZ B cells are responsible for spontaneously produced natural antibodies, which recognize evolutionarily conserved antigens such as phosphorylcholine found on a wide variety of bacterial species (21). B1b cells share a number of features with B1a cells, including the expression of cell-surface markers and a relative abundance in the peritoneal cavity (24). In fact, the only known surface marker that is able to distinguish these two subsets is the expression of

CD5 on B1a cells, but not B1b cells (24). While these cells appear phenotypically similar, these subsets play very different roles within the immune system. While B1b cells can also respond independent of T cell help, the antigens that drive the B1b cell responses are not evolutionarily conserved (20). They include specific bacterial proteins and polysaccharides as well as synthetic haptens (20, 25-29). Additionally, unlike either MZ B cells or B1a cells, the antigen-specific B1b cell population expands significantly following antigen encounter and remains elevated for several months after exposure (29, 30). These B1b cells do not secrete antibody constitutively, like memory B cells they respond only in response to specific antigen encounter. It has also been demonstrated that compared to responses following primary antigen exposure, secondary antigen exposure results in B1b cell responses that occur more rapidly in terms of both antigen-specific B cell expansion and antibody production, even in T cell-deficient mice. Thus, B1b cells are uniquely capable of developing T cell-independent memory (20).

3.3. The role of costimulation by TLRs in B cell responses

TLRs are expressed on a wide range of cell types and play a critical role in immune responses to a wide range of pathogens (31). TLR signaling results in the activation of NF-kappaB, which in turn can induce both proinflammatory and proliferative responses (31). Considering both the widespread expression of TLRs on immune cells, including B cells, and the ability of TLRs to effect dramatic changes in host immune responses, it is not surprising that TLR stimulation or signaling would play a significant role in antibody responses (32). Bernasconi *et al.* demonstrated that TLR9 and TLR10 expression can be dramatically upregulated following BCR stimulation and that several TLRs are expressed at constitutively high levels in memory B cells (33). Furthermore, this group demonstrated that memory, but not naïve B cells, were able to differentiate into antigen secreting plasma cells following exposure to CpG ODN, a potent TLR9 agonist, even in the absence of specific antigen (33). This was some of the first evidence suggesting that TLR signaling could be playing a significant role in memory B cell responsiveness. Additionally, it has been demonstrated that the administration of TLR ligands can enhance antibody responses to TD antigens (32). Early work seeking to improve the efficacy of protein conjugate vaccines against *Streptococcus pneumoniae* found that the administration of CpG ODN is able to enhance antibody responses to protein-conjugated pneumococcal polysaccharides (PPS). In addition to enhancing the overall anti-PPS antibody titers, CpG ODN administration also drove class-switch recombination, resulting in enhanced PPS-specific IgG2a and IgG3 production (34). Furthermore, *Latz et al.* demonstrated that TLR2 stimulation by a vaccine component was partially responsible for the robust protective responses brought about by immunization with the Hib-OMPC vaccine against *Haemophilus influenzae* (35).

It has also been demonstrated that TLR ligands can enhance TI antibody responses. It was shown that the *in vitro* responsiveness of *xid* splenocytes to TI-2 antigens could be restored with the administration of TLR agonists (36). Furthermore, responses to a plain PPS vaccine have been shown to be enhanced when the polysaccharide was co-administered with monophosphoryl lipid A, a TLR4 agonist (37). Similarly to the studies with protein conjugate vaccines, this study found that TLR stimulation not only enhanced overall antibody responses, but also increased the diversity of the response by stimulating class-switch recombination. More recently, a study involving another non-conjugated PPS vaccine, revealed TLR ligand contamination in the commercial preparations (38). Furthermore, it was shown that this contamination with TLR2 and TLR4 ligands significantly enhanced both the primary and secondary anti-PPS antibody responses. In addition, Taillardet *et al.* demonstrated that ligands for TLR2, 3, 4, 7/8, or 9 could enhance IgM responses to PPS (39). Collectively, these findings suggest that many TLRs are capable of significantly enhancing antibody responses to both TD and TI antigens.

4. RELAPSING FEVER *BORRELIAE*

4.1. Relapsing fever

The bacterial species within the genus *Borrelia* can be divided into two groups: those that cause relapsing fever and those that cause Lyme disease (40). There are at least seventeen known species of relapsing fever *Borreliae* and the number continues to grow due largely to the widespread use of genetic sequencing (41). Nearly all species in this group cause tick-borne, or endemic relapsing fever, which is transmitted by soft-bodied ticks of the genus *Ornithodoros* (40). In areas where these vectors are endemic, these bacterial infections are responsible for significant morbidity and though these infections are only rarely fatal in adults, there is evidence that *Borreliae* infection of pregnant women is a significant cause of fetal loss and neonatal death in some disease-endemic regions (41, 42). In contrast, *Borrelia recurrentis* is transmitted exclusively by the human body louse, *Pediculus humanus humanus*, and has been responsible for relapsing fever epidemics throughout history. Today this infection is primarily found in parts of Africa and unlike tick-borne relapsing fever, louse-borne relapsing fever results in mortality as high as 40% in untreated patients and 2-4% following appropriate antibiotic intervention (43). Additionally, a significant proportion of treated patients suffer from mild to severe Jarish-Herxheimer reactions (43, 44). The precise cause for the significantly increased mortality following *B. recurrentis* infection is not known and studies seeking to understand the pathophysiology of this infection are severely hampered by the extremely narrow host range (humans and some non-human primates) and the subsequent lack of any easily manipulated immunocompetent animal models. Despite the utilization of different arthropod vectors and the differences in mortality rates, the general characteristics of relapsing fever caused by these species of *Borrelia* are largely conserved (45).

Relapsing fever *Borreliae* infection results in recurrent episodes of high-level bacteremia (~10^8 bacteria/mL blood), with each wave of bacteremia accompanied by a febrile episode. There is some variation in

the number of bacteremic episodes observed following infection by specific *Borrelia* species, but in most cases the bacteremia and subsequent febrile episodes become less severe over time until the disease is resolved. Aside from febrile episodes accompanied by headaches and malaise, relapsing fever is also capable of causing neurological manifestations in a condition termed neuroborreliosis (46). It has been shown that between 10 and 30% of patients infected with relapsing fever *Borreliae* demonstrate neurological involvement at some point during infection. The most common development is cranial neuritis resulting in facial palsy, though meningismus, and encephalitis are occasionally observed. Like relapsing fever borreliosis itself, the neurological manifestations observed during the infection typically resolve without antibiotic intervention (46, 47). It should also be noted that the incidence of neuroborreliosis varies significantly depending on which species of bacteria is involved. For example, it has been reported that *B. duttonii* and *B. turicatae* infection can induce neurological manifestations in up to 80% of infected patients while *B. hermsii* and *B. persica* infections causes neuroborreliosis in less than 5% of patients (46).

4.2. Humoral Responses to Relapsing Fever *Borreliae*

Borrelia hermsii is one of the best studied of the relapsing fever *Borreliae. B. hermsii* is endemic to the western United States where it is naturally transmitted to both humans and mice. Infections in mice have been shown to recapitulate the key pathophysiological characteristics of the human disease (48). Although immunocompetent mice suffer multiple episodes of bacteremia, the infection is typically resolved by four-weeks post-infection. In contrast, mice lacking B and T cells are unable to clear the infection, highlighting the importance of the adaptive immune system in the resolution of *B. hermsii* bacteremia. Furthermore, *B. hermsii*-infected B cell-deficient mice develop persistently elevated bacteremia and become moribund by two to three weeks post-infection demonstrating the critical role for B cell responses in controlling relapsing fever *Borreliae* (30, 49-54). It has been demonstrated that IgM is the primary antibody isotype generated in response to *B. hermsii* infection (50-52, 55, 56). Furthermore, mice deficient in the secretion of IgM, have been found to suffer persistently elevated bacteremia and eventually become moribund following *B. hermsii* infection (51). Conversely, mice that generate IgM but no other immunoglobulin isotypes showed no impairment in resolving *B. hermsii* bacteremia (30). Additionally, it has been shown that the administration of IgM from convalescent mice confers protection against infection (30). The rapid and nearly complete clearance of bacteria from the systemic circulation and the importance of a robust, specific, and protective IgM response suggest a humoral immune response that develops independent of T cell help (49-51). In fact, it has been shown that *B. hermsii* infection of TCRbeta$^{-/-}$ x delta$^{-/-}$ mice is indistinguishable from infection of wildtype mice in terms of bacteremia, antibody responses, or kinetics of the resolution of infection (30, 50, 51).

The control of *B. hermsii* bacteremia in IL-7$^{-/-}$ mice, which are deficient in FO B cells, but not B1a, B1b, or MZ B cells revealed that FO B cells are not required for efficient responses to *B. hermsii* (51). Furthermore, bone marrow chimeric mice deficient in B1a cell compartment (<5% normal levels) are also fully capable of mounting a protective immune response to *B. hermsii* indicating that this B cell subset is dispensable for clearing *B. hermsii* (30). A role for MZ B cells in protective antibody responses against *B. hermsii*, was revealed using splenectomized mice. The *B. hermsii*-specific IgM during the initial wave of bacteremia was lower in splenectomized mice compared to sham operated mice and this impaired antibody response correlated to a significantly higher initial peak bacteremia (51, 57). These data were supported further by other groups, which used anti-LFA1 and anti-integrin alpha4-beta1 antibodies to selectively deplete MZ B cells without altering *B. hermsii* trafficking through the spleen (58). Collectively, these data suggest that MZ B cells are important for the production of optimal IgM responses to the initial wave of bacteremia, but that this cell subset is not necessary for clearance of the infection, leaving the B1b cell subset as the likely B cell subset responsible for protective responses to *B. hermsii*. It has been shown that mutations that impair BCR signaling result in a dramatic reduction in the B1 cell compartment (both B1a and B1b cells). For example, *xid* mice are impaired in BCR signaling and are also severely deficient in B1a and B1b cells. *B. hermsii*-infected *xid* mice develop more severe bacteremia compared to wildtype mice, but the mice survive and resolve the infection (51). Interestingly, while naïve *xid* mice have a reduced number of B1b cells, *B. hermsii* infection induces a dramatic expansion in the B1b cell compartment such that convalescent mice have roughly as many B1b cells as naïve wildtype mice (51). These data suggest that B1b cells are important in protection from *B. hermsii*. To determine the exact role of B1b cells in protective responses to *B. hermsii*, B1b cells from convalescent mice were transferred into naïve Rag1$^{-/-}$ recipients and mice were infected with fully virulent *B. hermsii*. Mice without any B1b cell transfer developed chronically elevated bacteremia, whereas B1b cell reconstituted Rag1$^{-/-}$ mice were able to clear the infection, clearly demonstrating the role of B1b cells in protective responses to *B. hermsii* (30). The resolution of bacteremia in the absence of T cell help brought up an important question with regard to the resistance of convalescent mice to re-infection, as T cells were thought to be required for the development of memory B cells. To test whether or not B1b cells can generate B cell memory independent of T cell help, B1b cells from convalescent TCRbeta$^{-/-}$ x delta$^{-/-}$ mice were transferred into a naïve Rag1$^{-/-}$ recipient mice and infected with *B. hermsii* 1 day post-transfer. The reconstituted mice were able to clear the infection and were resistant to re-infection out to at least 60 days post-infection, suggesting not only that B1b cells were sufficient for the resolution of bacteremia following *B. hermsii* infection, but that the B1b-mediated bacterial clearance and long-lasting immunity were T cell-independent (30).

4.3. The Role of TLRs in the Resolution of Relapsing Fever

TLRs are expressed on a wide variety of immune cells, including B cells (59) and the ability of TLRs to influence host immune responses has been well described

(60, 61). Furthermore, the fact that TLR ligands have been shown to enhance antibody responses to TD and TI antigens would seem to indicate that TLR signaling could play an important role in antibody-mediated clearance of relapsing fever *Borreliae*.

TLRs recognize a wide range of structurally conserved components of bacterial cells, including lipoproteins, lipopolysaccharides, flagellin, and unmethylated DNA (31). Myeloid differentiation primary-response gene 88 (MyD88) is an adaptor molecule required for signaling for all TLRs except TLR3 and because it was thought that *Borrelia hermsii* could possess the ability to stimulate several different TLRs, early work seeking to determine the role of TLR signaling in relapsing fever utilized systems lacking MyD88 (62, 63). Infection of MyD88-deficient mice with fully virulent *B. hermsii* was shown to result in bacteremia more than 10-fold higher than that seen in infections of wildtype mice. Furthermore, ELISA and immunoblot analysis revealed that the elevated bacteremia correlates to a significant delay in the onset of *B. hermsii*-specific IgM responses in MyD88$^{-/-}$ mice, though these mice generate specific antibody responses similar to wildtype mice by 6 days post-infection (62, 63). Interestingly, significant mortality was observed among infected MyD88-deficient mice, starting around 8 days post-infection with most mice dying by 16 days post-infection (62). Together, these results suggest a role for TLRs in the development of a protective TI antibody response. While the data generated with MyD88$^{-/-}$ mice suggest a role for TLR signaling in antibody responses and subsequent protection from *B. hermsii* infection, it must be noted that MyD88 signaling is not specific to TLRs, but is also required for efficient signaling by other surface receptors such as the IL-1R and IL-18R (64, 65). This means that MyD88$^{-/-}$ mice may have additional signaling defects involving receptors other than TLRs, which may complicate the interpretation of the previously described data.

More recently, an effort was made to uncover precisely which TLRs are involved in the detection of *B. hermsii* and the subsequent antibody response (66). Lipoproteins make up the majority of the outer membrane of *B. hermsii*, suggesting that TLR2 likely detects those bacterial components. In fact, it was shown using a transfection system with luciferase reporter plasmids that TLR2 is able to induce robust NF-kappaB activation in response to sonicated *B. hermsii* (66). This supports previously published data demonstrating that loss of TLR2 delays the generation of specific IgM following *B. hermsii* infection (63). Additionally, it was shown that the loss of TLR1 and CD14 also results in impaired antibody responses. CD14 is a pattern recognition receptor capable of binding to a wide range of ligands, though it relies on other surface proteins, such as TLR2 and TLR4 for signaling. Considering its ability to enhance TLR2 signaling, a role for CD14 in this system is not surprising. TLR1 has been shown to heterodimerize with TLR2 for recognition of triacylated lipoproteins, explaining the phenotype observed in *B. hermsii*-infected TLR1$^{-/-}$ mice (63).

TLR4 is predominantly thought of as a sensor of bacterial lipopolysaccharide, but it has been shown to detect a wide range of pathogen components from bacterial lipoproteins to viral glycoproteins and parasitic glycosylphosphatidylinositols (31). Using the same transfection system described above, it was demonstrated that *B. hermsii* sonicate was not able to induce TLR4-mediated NF-kappaB activation (66). Mice deficient in TLR4 showed no impairment in antibody responses and no difference in the initial peak bacteremia (63). While *B. hermsii* possesses flagellin, a known agonist of TLR5, *B. hermsii* flagellin lacks the consensus sequences necessary for recognition by TLR5. Taken together, these data suggest that TLR2 is the only extracellular TLR involved in the detection of *B. hermsii*. Indeed, it has been shown that bone marrow-derived macrophages and peritoneal exudate cells from mice lacking TLR2 are able to induce a robust inflammatory response to whole heat-killed *B. hermsii*, but that these macrophages are unable to respond when internalization is blocked by the administration of cytochalasin D, an actin polymerization inhibitor (66). These data confirm the role of TLR2 as the primary extracellular sensor of *B. hermsii* and also support the conclusion that TLR4 and TLR5 are not involved in the detection of *B. hermsii*. Additionally, the fact that untreated TLR2-deficient macrophages can respond to *B. hermsii* suggests the involvement of intracellular sensors of bacteria (66).

Although we have been able to demonstrate that *B. hermsii* induces TLR9-mediated NF-kappaB activation (66), we found no delay in the onset of *B. hermsii*-specific IgM or heightened bacteremia in TLR9-deficient mice (63). While it is possible that TLR2 and TLR9 could work in a cooperative or redundant manner, subsequent work has shown that TLR2$^{-/-}$ x TLR9$^{-/-}$ mice display an impaired antibody response and course of infection that is indistinguishable from infections of TLR2-deficient mice, suggesting that while TLR9 can respond to *B. hermsii* components, it does not seem to play a role in bacterial clearance *in vivo* (66). Additionally, this suggests that TLR2 is the only MyD88-dependent TLR involved in the detection of *B. hermsii* and the induction of specific antibody responses (66).

Significant differences have been described between *B. hermsii* infection of TLR2$^{-/-}$ mice and MyD88$^{-/-}$ mice (62, 63). For example, while MyD88$^{-/-}$ and TLR2$^{-/-}$ mice have roughly the same overall mortality, the time course is significantly different, as TLR2$^{-/-}$ mice tend to die within the first week post-infection (63, 66). Additionally, infected TLR2$^{-/-}$ mice appear to clear the initial waves of bacteremia better than MyD88$^{-/-}$ mice, as the average duration of the first episode of bacteremia was nearly three times longer in MyD88$^{-/-}$ mice compared to TLR2$^{-/-}$ mice (63, 66). Significant lung injury, including intra-alveolar hemorrhaging, cellular infiltration and thickened septae has been reported following *B. hermsii* infection of MyD88$^{-/-}$ mice, while infected TLR2$^{-/-}$ mice develop no discernable lung injury at any time point following infection (62, 66). Considering that TLR2 is likely the only TLR involved in the detection of *B. hermsii in vivo*, it seems likely that the

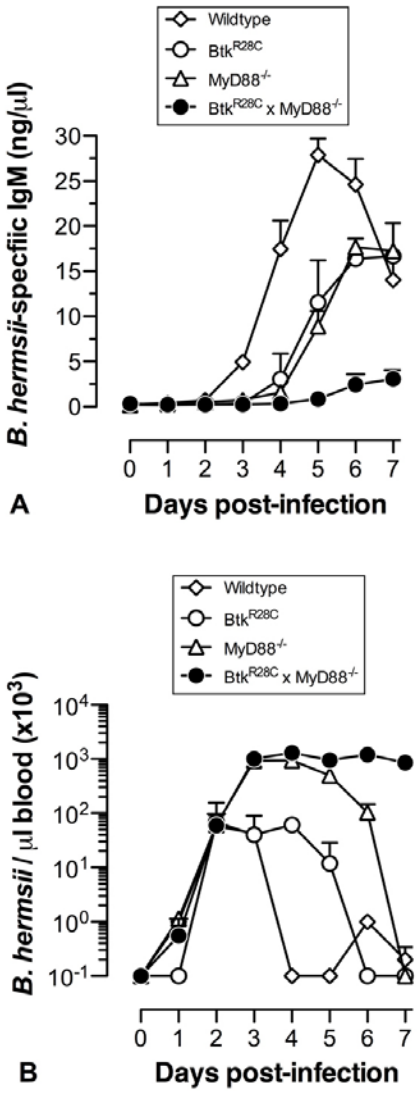

Figure 1. BCR and TLR mediated signaling are required for anti-*B. hermsii*-specific IgM response as well as for the resolution of bacteremia. Wildtype (C57BL6; n=3), Btk^{R28C} (C57BL6.*xid*; n=3), $MyD88^{-/-}$ (C57BL6 MyD88-/-; n=3) or Btk^{R28C} x $MyD88^{-/-}$(n=4) were infected intravenously with 5 x 10^4 *B.hermsii* strain DAH-p1. (A). *B. hermsii*-specific IgM responses in the blood on the indicated days post-infection were measured by ELISA and (B). Bacteremia was determined by microscopic counting. Mean and standard deviation values are shown.

differences observed in *B. hermsii* infection of TLR2-deficient mice and MyD88-deficient mice are due to the pleiotropic effects of MyD88 deletion, possibly through interference with IL-1R signaling, or some additional non-specific defect in the innate immune compartment.

While humoral responses to *B. hermsii* are necessary for the resolution of bacteremia, other non-humoral immune responses may also be important and TLRs could also be playing a role in these processes. For example, it has been demonstrated that *B. hermsii*-infected $TLR2^{-/-}$ x *scid* mice suffer significantly higher bacteremia than $TLR2^{+/+}$ x *scid* mice, suggesting another role for TLR2 in the clearance of bacteremia that is independent of its effects on the humoral response (62). In line with this, it has been shown that bone marrow-derived macrophages from mice lacking TLR2 are significantly impaired in their ability to secrete pro-inflammatory cytokines such as IL-6 in response to whole heat-killed *B. hermsii* compared to macrophages from wildtype mice (62, 66).

As discussed above, TI-2 antigens are defined by their ability to induce antibody response in the absence of T cell help through antigen-specific BCR cross-linking and the activation of Btk. Therefore, classical TI-2 antigens, such as purified PPS or NP-Ficoll are unable to induce an antibody response in *xid* mice (15-18). *Xid* mice express a substitution mutation (R28C) in the pleckstrin homology domain of Btk, a region critical for docking to the inner leaflet of the B cell plasma membrane during BCR signaling. This mutant Btk protein is expressed at 70% of the normal level and contains an intact kinase domain (15, 67). Remarkably, we found that unlike NP-Ficoll and purified PPS immunization, *B. hermsii* infection induces a protective, albeit delayed antibody response in *xid* mice and induces an expansion of B1b cells (51, 63). To test if Btk is necessary for protective antibody responses, we have examined *B. hermsii* infection in mice that have a targeted deletion in the kinase domain of Btk. While the mutant *Btk* gene is transcribed as detected by Northern blots, no detectable Btk protein is produced in these mice, effectively making them $Btk^{-/-}$ mice (17). These $Btk^{-/-}$ mice, like *xid* mice exhibited a similar delay in the anti-*B. hermsii* antibody response and eventually resolved all episodes of *B. hermsii* bacteremia (63).

Since *B. hermsii* activates at least two TLRs, both of which signal through MyD88, the role of TLR signaling in $Btk^{-/-}$ mice was examined in $Btk^{-/-}$ x $MyD88^{-/-}$ mice. Compared to mice deficient either in Btk or MyD88, mice deficient in both MyD88 and Btk mice exhibit a more severe impairment in anti-*B. hermsii* specific IgM responses (63). Consistent with this, mice deficient in both MyD88 and Btk suffer persistently high-level bacteremia (63). Furthermore these mice continue to exhibit a paucity of B1b cells even after *B. hermsii* infection (63), unlike *xid* mice (30, 51). Since *xid* mouse B cells express a mutated protein that contains a functional kinase domain, we explored the extent to which this mutation plays a role in the context of a TLR signaling deficiency by generating $MyD88^{-/-}$ mice that contained an *xid* mutation (i.e., R28C). Like $Btk^{-/-}$ x $MyD88^{-/-}$ mice, these mice are more impaired in anti *B. hermsii* responses than $MyD88^{-/-}$ mice and suffer persistent bacteremia (Figure 1). These data demonstrate that antibody responses to *B. hermsii* involve both BCR signaling through Btk and TLR signaling through MyD88 (63).

In summary, it has been shown that signaling through TLRs is critically important for the resolution of relapsing fever through the efficient generation of protective *B. hermsii*-specific antibody responses.

Furthermore, TLR2 is the primary TLR responsible for enhancing the efficiency of the specific antibody response. Additionally, it has been shown that both BCR signaling through Btk and TLR signaling through MyD88 are required for optimal responses to *B. hermsii*, indicating that the type of TI responses that occur during *B. hermsii* infection cannot be simply defined as TI-1 and TI-2 responses. It seems likely that this is characteristic of pathogens that induce T cell-independent humoral responses, since during a normal infection the immune system would likely be exposed to antigens that induce BCR cross-linking as well as mitogens, such as pathogen associated molecular patterns, that trigger innate receptors such as TLRs.

5. LYME DISEASE *BORRELIAE*

5.1. Lyme disease

Lyme disease is the most common vector-borne disease in North America and Western Europe, accounting for roughly 30,000 cases/year in the United States alone (68). Lyme disease can be caused by a variety of bacterial species within the genus *Borrelia*. All Lyme disease *Borrelia* species fall within the *Borrelia burgdorferi* sensu lato species complex, which can be further divided based on genomic analysis into individual species (69). The exact species responsible for Lyme disease varies based on geographical location, with *B. burgdorferi* sensu stricto responsible for most US infections. European cases stem from infections by a wider range of bacterial species including, but not limited to *B. afzelii*, *B. garinii*, as well as *B. burgdorferi* sensu stricto and new species continue to be isolated and identified (69-71). Unlike relapsing fever *Borreliae*, which are transmitted by soft-bodied ticks or the human body louse, Lyme disease *Borreliae* are transmitted by hard-bodied ticks of the genus *Ixodes*. Furthermore, the global distribution of Lyme disease within the northern hemisphere is limited to those areas with populations of Ixodid tick vectors (69). Infected ticks transmit the bacteria during feeding, depositing the spirochetes in the skin. It has been demonstrated in murine infection models that the spirochetes remain localized in the skin near the bite site for at least two days after feeding (72). Studies in both humans and animal models have suggested that from the initial site of infection, the bacteria spreads through soft tissue into adjacent areas. For example, it was shown that when dogs were exposed to infected ticks, joint inflammation and lameness always arose first in the leg closest to the site of tick exposure before eventually spreading to joints around the body (73). While earlier studies showed that fewer that 5% of Lyme disease patients yielded positive blood cultures, more recent techniques have shown a much higher prevalence of bacteremia, as several groups of researchers have been able to recover *B. burgdorferi* from roughly 40% of adult patients with erythema migrans indicating that in addition to spreading through soft-tissues, hematogenous dissemination may also significantly contribute to the distribution of Lyme disease *Borreliae* throughout the body (74). Erythema migrans is one of the first clinical manifestations of Lyme borreliosis and the most important from a diagnostic standpoint (69, 75). Erythema migrans begins as a small red macule or papule at the site of the tick bite that grows larger over several days while clearing in the middle, giving a classic "bull's-eye" pattern. Erythema migrans typically evolves within a few days or weeks of infection, but it can develop at sites on the body distal to the bite site following hematogenous spread later in the infection (69).

Roughly 70% of patients with untreated erythema migrans go on to develop arthritis, making it one of the more common sequelae of Lyme disease *Borrelia* infection (69). Lyme arthritis is marked by the rapid onset of asymmetrical joint pain and inflammation, primarily in the large joints of the leg, most commonly the knee and ankle (69). The duration of joint inflammation and pain is variable, ranging from days to weeks or even months and patients may suffer from several intermittent bouts of arthritis before ultimate resolution (69, 76). It should be noted that arthritis may persist well after antibiotic-mediated resolution of active infection, and though many groups have speculated that the persistence of bacterial components or the induction of autoimmunity through molecular mimicry may be playing a role, the precise cause of prolonged arthritis following the resolution of infection is not currently known (69). Carditis is also observed in human patients, with roughly 5% of untreated patients developing acute cardiac involvement (77). Carditis in Lyme disease typically develops within a few weeks of infection and manifests most commonly as fluctuating atrioventricular blocks, though acute perimyocarditis and mild left ventricular dysfunction also occur in some patients and rarely patients may develop cardiomegaly or lethal pancarditis (77). Lyme disease carditis typically resolves by 3-6 weeks after the onset of symptoms even without antibiotic intervention (78). Neuroborreliosis, with a wide range of clinical symptoms, develops in roughly 15% of untreated patients during the course of infection by Lyme disease *Borreliae* (77). Most commonly, neurological manifestations of neuroborreliosis include radiculoneuritis, meningitis, and cranial neuritis, typically involving facial nerves and resulting in either unilateral or bilateral facial palsy (69, 79). Similar to other symptoms of Lyme disease, the severity and duration of the symptoms of neuroborreliosis can vary significantly over the course of the infection and resolve spontaneously (77). In addition to specific disease manifestations, a large percentage of patients with Lyme disease suffer from non-specific complaints such as myalgia, fatigue, malaise, or headaches of varying severity. The high prevalence of these non-specific symptoms has led to significant confusion, as many patients without any documented evidence of primary Lyme disease are being diagnosed with "chronic Lyme disease" and prescribed long-term antibiotic therapy. This subject has been thoroughly reviewed elsewhere (80).

5.2. Humoral responses to Lyme disease *Borreliae*

The humoral immune response to *B. burgdorferi* is critical to both protective and disease-resolving immunity. *Scid* mice or mice deficient in B but not T cells suffer more severe *B. burgdorferi* infection compared to wildtype mice. Transfer of serum from infected mice into naive recipient mice either prior to or at the time of infection prevents *B. burgdorferi* infection (81). Furthermore, studies have demonstrated that the passive transfer of immune serum is able to induce arthritis remission in infected *scid* mice and maintain this remission for up to 60 days (82). Passive

immunization of mice with sera from individuals with late-stage Lyme disease that contained strong antibody reactivity to several proteins including outer surface proteins (Osps) A and B, can provide a partial protection against *B. burgdorferi* challenge (83). These findings reveal that humoral immune responses generated in Lyme disease patients and experimentally infected mice play an important role not only in clearing the infection, but also in the resolution of some of the most commonly reported clinical manifestations of Lyme disease, namely arthritis and carditis.

CD40-CD40L interactions as well as MHC-II-mediated presentation of antigens are critical for mounting TD responses. No difference has been observed in the rate of infection, incidence or severity of arthritis, or the incidence of carditis in $CD40L^{-/-}$ (84) or class II transactivator-deficient ($CIITA^{-/-}$) mice, which are deficient in MHC-II (85), compared to wildtype mice. Furthermore, both $CIITA^{-/-}$ and $CD40L^{-/-}$ mice resolve arthritis and carditis following *B. burgdorferi* infection, though $CIITA^{-/-}$ mice appear to exhibit a slight delay in carditis resolution compared to wildtype mice. Collectively, these data suggest that neither CD40-CD40L nor MHC-II interactions are critical for the control of *B. burgdorferi* infection. Furthermore, it was shown that the passive transfer of immune serum from $CD40L^{-/-}$ or $CIITA^{-/-}$ mice into *scid* recipient mice was just as effective as wildtype immune serum at preventing infection, arthritis and carditis when administered 1 day post-infection (84). Interestingly, there were marked shifts observed in the antigens targeted in $CD40L^{-/-}$ or $CIITA^{-/-}$mice compared to wildtype mice. For example, IgG responses to flagellin, but not P39 and OspC appear to require CD40L and MHC-II (84). Taken together, these studies clearly demonstrate that even in mice that are unable to undergo cognate T cell interactions with APCs, there exists a capacity to produce robust, antigen-specific antibody responses capable of protecting from infection and inducing disease remission.

The role of T cells in *B. burgdorferi* infection has been evaluated in TCRbeta x $TCRdelta^{-/-}$ mice, which lack all mature T cell subsets (86). The prevalence and severity of arthritis in these mice is similar to wildtype mice and the incidence of carditis is comparable (86). The specific Ig isotype profile in T cell-deficient mice appears to be distinct from that of wildtype mice. For example, T cell-deficient mice generated mainly IgM and IgG3 responses, whereas wildtype mice generated IgG2b and IgG3. The serum of the infected T cell-deficient mice recognized a number of *B. burgdorferi* antigens, though the serum from these mice did not react to as many antigenic targets as the serum of infected wildtype mice. For example, the immune serum from T cell-deficient mice reacts to antigens such as OspA, DbpA, P12, and P61, but not proteins such as Arp, indicating that responses to certain antigens require T cell help (87). Despite these differences, the passive transfer of serum from immune T cell-deficient mice prevents *B. burgdorferi* infection as efficiently as that of the immune wildtype serum. These data reveal that protective antibody responses can develop even in the absence of T cells.

In addition to its protective capability, sera from immune wildtype mice has been shown to induce arthritis and carditis remission when passively transferred into infected *scid* mice indicating that the antibodies generated during a natural infection have the capability to induce disease remission (88). Interestingly, immune serum from T cell-deficient mice, which has reactivity to several *B. burgdorferi* proteins including DbpA but not Arp, also exhibited such capability (88). When DbpA or Arp antiserum was passively transferred to infected C3H/scid mice, both induced significant decreases in the prevalence and severity of carditis at 28 days post-infection. Additionally, both Arp and DbpA antiserum caused a decrease in arthritis prevalence and severity. Though the level of *B. burgdorferi* in the inflamed tissues was the same in the treated mice, immunohistochemical studies revealed that the antibodies were altering the distribution of bacteria within the tissues (88). For example, spirochetes were eliminated from the loose connective tissue of the heart base and in the inflamed synovium, but were then abundant within the highly collagenous aortic wall of the heart and adjacent ligaments and tendons of the leg. These data suggest that antibody responses to both TD and TI antigens can induce disease remission.

Another important question with regard to humoral responses to Lyme disease *Borreliae* is which B cell subsets are responsible for protective antibody responses. While *B. burgdorferi* has been shown to spread via soft tissue, it also spreads through the bloodstream. Early control of the hematogenous distribution of the bacteria can help prevent additional pathological sequelae, as Lyme disease patients with demonstrable bacteremia are significantly more likely to be symptomatic and are more likely to develop multiple erythema migrans distal to the bite site (74). Considering their anatomical localization, their surface receptor repertoire, and their capacity for producing TI antibody responses, the MZ B cell compartment seems an appropriate subset for the production of protective antibody responses to systemic *B. burgdorferi*. In fact, it has been shown that following MZ B cell depletion by the administration of anti-LFA-1 and anti-alpha4 beta1 integrin antibodies, mice exhibited decreased *B. burgdorferi*-specific IgM and IgG and increased bacteremia compared to untreated mice at 7 days post-infection (58, 89). MZ B cell-depleted mice also show an increased prevalence and severity of arthritis early in the infection (10 and 14 days post-infection). Increased bacterial burden was observed in the urinary bladder by 14 days post-infection indicating increased systemic spreading compared to untreated mice, though this is corrected if the mice are administered 10 day post-infection immune serum (89). Collectively this study demonstrates that at least early in *B. burgdorferi* infection, MZ B cells are significant contributors to the protective immune response. Additional work is needed to assess the role of other B cell subsets, such as the FO and B1b subsets in the humoral responses throughout the course of *B. burgdorferi* infection.

5.3. The role of TLRs in humoral responses to Lyme disease *Borreliae*

The outer membrane of *B. burgdorferi* is largely composed of lipoproteins that have long been shown to have the ability to induce NF-kappaB activation and inflammation (90-92). In fact, *B. burgdorferi* outer membrane protein OspA was one of the first bacterial

ligands that exhibited an ability to activate TLR2-mediated signaling (93). The induction of the TLR2-mediated inflammatory response was abrogated if the OspA lacks the normal tripalmitoyl-S-glycerylcysteine (Pam_3Cys)[3] lipid modification (94). Furthermore, it was demonstrated that TLR2 is able to induce NF-kappaB activation in response to a wide range of *B. burgdorferi* surface proteins (94). Subsequently, extensive research has been focused on the role of TLRs in Lyme disease, some of which focused exclusively on TLR2, while other work examined the role of MyD88, which is involved in signaling for nearly all TLRs.

Since *Borreliae* are likely to stimulate many TLRs, the MyD88$^{-/-}$ mouse system provides a unique opportunity to follow the progression of Lyme disease in the absence of nearly all TLR signaling. Mice deficient in MyD88 harbored significantly more bacteria in a variety of tissues such as the ear, joint and bladder compared to wildtype mice (95-97). When antibody responses in these mice were examined, it was found that at 4 weeks post-infection MyD88$^{-/-}$ mice had significantly lower levels of circulating *B. burgdorferi*-specific IgG than wildtype mice. Conversely, the *B. burgdorferi*-specific IgM was significantly higher in MyD88$^{-/-}$ mice compared to wildtype mice (95). Additionally, it was found that MyD88-deficient mice exhibited significantly lower levels of IgG2b and significantly higher levels of IgG1 compared to wildtype mice (97). Moreover, CD4^{+} T cells from MyD88$^{-/-}$ mice produced more IL-4 and less IFN-gamma following stimulation *ex vivo* with *B. burgdorferi* lysates, suggesting, along with the antibody data, that a Th2 skewing is occurring in infected MyD88$^{-/-}$ mice (95). Assays seeking to determine the antigen specificity of immune serum collected from infected MyD88$^{-/-}$ mice revealed antibodies with a specificity to a wide range of *B. burgdorferi* antigenic targets, though immune serum from MyD88$^{-/-}$ mice appeared to recognize fewer antigens than immune serum of wildtype mice. Despite this potential decrease in the number of antigens recognized, immune serum from MyD88$^{-/-}$ mice (28 days post-infection) was just as effective as wildtype immune serum in conferring protective immunity (97). Collectively, these data suggest that when TLR signaling is lost through the deletion of MyD88, there is a significant impairment in the immune response to *B. burgdorferi*, resulting in a significantly elevated bacterial burden in tissues throughout the body.

TLR2 has long been implicated in immune responses to *B. burgdorferi* and significant work has been done to determine the role of this particular TLR in the development of immune responses to *B. burgdorferi*. TLR2$^{-/-}$ mice have significantly higher numbers of bacteria in the ankle, heart, and ear following infection (97-99). Additionally, it has been shown that mice deficient in CD14, which enhances responses to TLR2 and TLR4 ligands, also develop an elevated bacterial burden in multiple tissues following *B. burgdorferi* infection. Together, these data suggest that a loss of TLR2 signaling alone significantly impairs the ability of the immune system to efficiently limit bacterial growth and dissemination during the initial infection. Interestingly, MyD88$^{-/-}$ mice have higher levels of bacteria in the aforementioned tissues at 4 weeks post-infection compared to TLR2$^{-/-}$ mice, suggesting that either other TLRs are contributing to host defense in the absence of TLR2, or that the loss of MyD88 is having additional TLR-independent effects (e.g., cytokine signaling), which is affecting the course of the infection (97). Unlike in MyD88-deficient mice, TLR2$^{-/-}$ mice did not show a significant shift in the IgG isotype distribution generated in response to infection (98, 99). Considering that TLR2-deficient mice are not impaired in the generation of anti-*B. burgdorferi* antibodies and do not show any significant alteration in the antibody isotypes generated in response to infection, it is possible that the impaired control of *B. burgdorferi* infection in TLR2$^{-/-}$ mice could be attributed to defects in the innate immune cell-mediated responses to the bacteria (97-99).

A vaccine involving recombinant OspA was developed to prevent Lyme disease in the United States and protection in immunized humans and mice correlates to the development of antibodies specific to OspA. It was found that a small cohort within the vaccination group failed to generate a strong antibody response to OspA even after repeated immunizations, though these same individuals showed serological evidence of effective antibody elicitation following previous vaccination efforts; these participants were labeled “low responders” (100). While normal responders showed local reactions at the site of injection, low responders also had less apparent soreness, redness, and swelling following injection, consistent with a lack of responsiveness to the bacterial protein. When peripheral blood mononuclear cells (PBMCs) from normal and low responders were analyzed by flow cytometry, it was found that while they had similar levels of TLR2 expressed on their surface, TLR1 expression was lower on PBMCs of low responders compared to normal responders (100). Since TLR1 heterodimerizes with TLR2 to induce efficient recognition of triacylated lipoproteins, decreased expression of TLR1 could result in significantly lower antibody responses following OspA immunization. In fact, it was demonstrated that co-expression of TLR1 significantly enhances TLR2-mediated NF-kappaB activation in response to OspA. Additionally, mice deficient in either TLR2 or TLR1 produce significantly lower levels of OspA-specific antibodies following immunization compared to wildtype mice (100). Taken together, this work suggested that TLR signaling, specifically TLR1-assisted TLR2 signaling is important for protective anti-OspA responses in vaccinated individuals. Though the anti-OspA antibody response is significantly impaired in TLR2-deficient mice, the diminished response may still be protective. In fact, Yoder *et al.* immunized wildtype and TLR2$^{-/-}$ mice with two doses of recombinant OspA three weeks apart and then challenged the mice with a low dose of *B. burgdorferi* one week later (101). There was no significant difference observed in protection from challenge in TLR2$^{-/-}$ mice compared to wildtype mice, suggesting that the reduced antibody responses to OspA following immunization of TLR2- or TLR1-deficient mice or low-expressing humans could be protective (101).

Collectively, the extensive work done by many groups has revealed a complicated role for TLRs in Lyme borreliosis. It does appear that TLRs contribute to the

development of humoral repsonses to *B. burgdorferi*, as evidenced by the altered antibody isotype profiles. Additionally, there is evidence suggesting that efficient antibody responses to certain antigenic targets may be compromised in mice lacking TLR signaling and optimal immune responses to isolated *B. burgdorferi* OspA require TLR2 and TLR1. Furthermore, though the work is beyond the scope of this review, there is significant evidence that TLRs contribute significantly to innate immune responses to *B. burgdorferi*, suggesting that innate immune defects may account for the elevated bacterial loads observed in mice lacking TLR signaling, though additional work is needed to parse out precisely how TLR signaling aids in controlling this infection.

6. CONCLUDING REMARKS

Bacteria of the genus *Borrelia* have developed several adaptations necessary for persistence in the periphery of mammalian host organisms, most notably an ability to alter the proteins expressed on their surface. These antigenic variation systems provide these bacteria with the ability to evade the humoral immune response. The relatively uniform antigenic nature of each wave of bacteremia in relapsing fever means that the rapid generation of specific antibody can clear the vast majority of bacteria, resulting in a transient period of well being. TI antibody responses are critical for the rapid resolution of these bacteremic waves and TLRs have been shown to provide important co-stimulatory signals for the efficient generation of TI antibody responses. In fact, the loss of TLR2 alone results in overwhelming bacteremia and septic shock in infected mice (66), demonstrating the importance of TLRs in the resolution of infection by relapsing fever *Borreliae*. In the case of Lyme disease, TLRs seem to play a more complex role by assisting in the development of specific antibodies to certain bacterial targets, driving efficient innate immune responses, and also potentially contributing to the pathological inflammation. While the work done to date has contributed significantly to our understanding of these important pathogens, additional work is necessary to fully decipher the role of TLRs in *Borreliae* infections. A more complete understanding could lead to significant advancements in vaccination strategies or the development of novel therapeutic interventions designed to alleviate pathological inflammation in those infected.

7. ACKNOWLEDGEMENTS

We acknowledge the support from NIH/NIAID RO1 AI065750.

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Abbreviations: Vmp: Variable major protein; VlsE: Variable major protein-like sequence, Expressed; TD: T cell-dependent; TI: T cell-independent; BCR: B cell antigen receptor; LPS: Lipopolysaccharide; TLR: Toll-like receptor; *xid*: X-linked immunodeficient; Btk: Bruton's tyrosine kinase; FO: Follicular; MZ: Marginal Zone; PPS: Pneumococcal polysaccharide; *scid*: severe-combined immune deficiency; MyD88: Myeloid differentiation primary-response gene 88; CIITA: Class II-transactivator; TCR: T cell antigen receptor; Osp: Outer surface protein.

Key Words: *Borrelia hermsii*, *Borrelia burgdorferi*, Toll-like receptors, Antibody, Relapsing fever, Lyme disease, Review

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