RESEARCH ARTICLE

The role of host immune cells and *Borrelia burgdorferi* antigens in the etiology of Lyme disease

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ABSTRACT. Lyme disease is a zoonosis caused by infection with bacteria belonging to the *Borrelia burgdorferi* species after the bite of an infected tick. Even though an infection by this bacterium can be effectively treated with antibiotics, when the infection stays unnoticed *B. burgdorferi* can persist and chronic post-treatment Lyme disease syndrome is able to develop. Although a cellular and humoral response is observed after an infection with the *Borrelia* bacteria, these pathogens are still capable to stay alive. Several immune evasive mechanisms have been revealed and explained and much work has been put into the understanding of the contribution of the innate and adaptive immune response. This review provides an overview with the latest findings regarding the cells of the innate and adaptive immune systems, how they recognize contribute and mediate in the killing of the *B. burgdorferi* spirochete. Moreover, this review also elaborates on the antigens that are expressed by on the spirochete. Since antigens drive the adaptive and, indirectly, the innate response, this review will discuss briefly the most important antigens that are described to date. Finally, there will be a brief elaboration on the escape mechanisms of *B. burgdorferi* with a focus on tick salivary proteins and spirochete antigens.

Key words: Lyme disease, antigen presentation, adaptive immune cells, innate immune cells

Each year, 20,000 to 30,000 new Lyme disease cases are diagnosed in the United States of America according to the Centers of Disease Control and Prevention [1]. Lyme disease, also known as Lyme's borreliosis, is one of the most frequently occurring zoonosis. It is caused by the bacteria belonging to the *Borrelia burgdorferi sensu lato* strain. Through a tick bite (mostly *Ixodes ricinus* species), these bacteria are transferred to the host. Even though antibiotic therapy is often sufficient to cure most patients, some patients still develop persistent Lyme disease, which then leads to a multisystemic disease affecting either the joints (Lyme's arthritis), the heart (Lyme's carditis), or the central peripheral nervous system (neuroborreliosis) and causing atrophic chronica acrodermatitis (inflammatory patches or lesions) [2, 3].

It was already known in 1945 that *Ornithodoros moubata* tick bites could lead to relapsing fever caused by a spirochete of the *Borrelia* species [4]. Lyme disease has been clinically described as an infectious illness by Steere *et al.* in 1977 [5]. Ever since the discovery of the disease, the understanding and involvement of the immune system has developed excessively. This review provides an overview of the cells of the innate and adaptive immune systems with respect to their potential roles during *Borrelia* infection or in persistent Lyme disease. Additionally, antigens of *B. burgdorferi* are described with special mention of the recent advances that discuss the function or role of these antigens in the life cycle of *B. burgdorferi*.

THE INNATE IMMUNE SYSTEM AND ITS ROLE IN BORRELIA BURGDORFERI INFECTIONS

Innate immune responses are facilitated by the recognition of pathogen-associated molecular patterns (PAMPs). These PAMPs are molecules associated with pathogens and can be recognized by Toll-like receptors (TLRs) or a variety of other pattern recognition receptors (PRRs) expressed on the cell surface or intracellularly in cells of the innate immune system such as macrophages, dendritic cells (DCs), and neutrophils [6]. This chapter provides an overview of the cells of the innate immune system and how they are involved in the recognition of *Borrelia*. In addition, the role of nonimmune cells such as keratinocytes, mast cells, and fibroblasts will also be discussed.

Macrophages and monocytes

Human skin-residing macrophages are one of the first innate immune cells that *B. burgdorferi* encounters when

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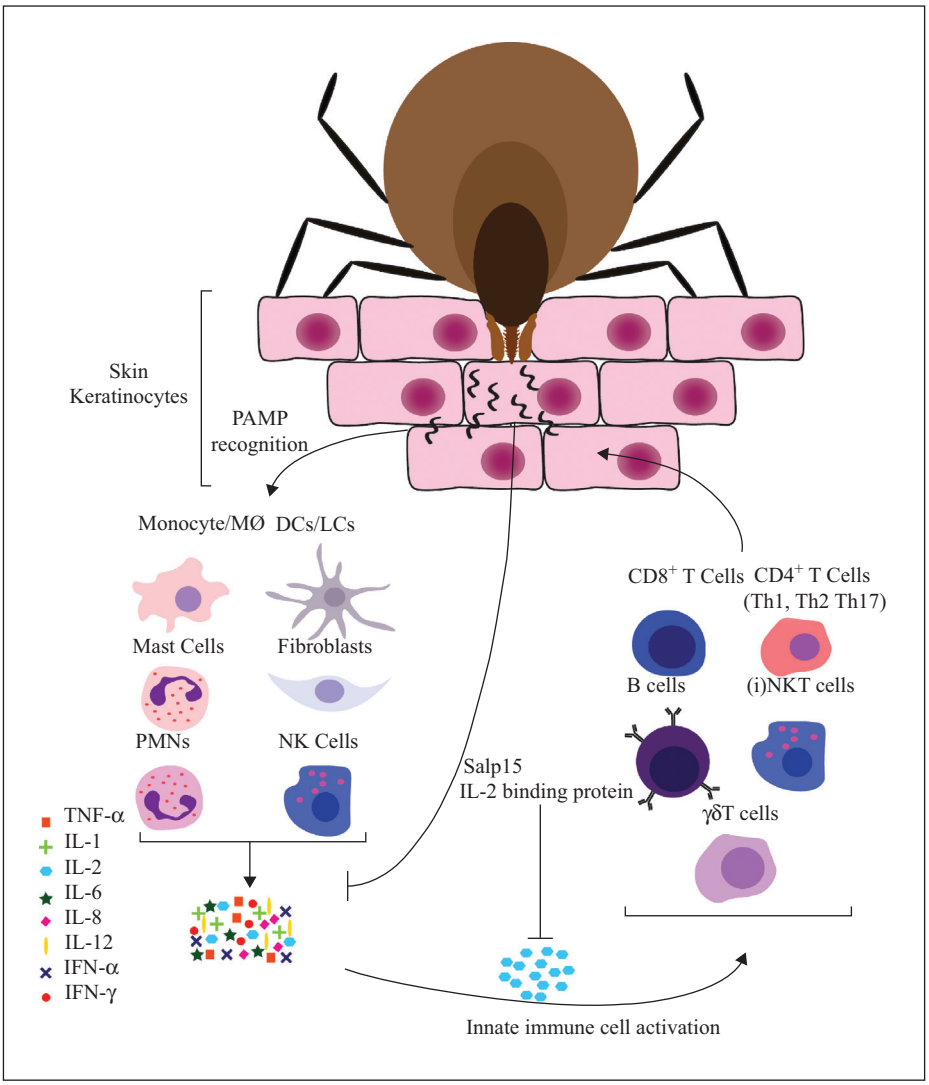

Figure 1

Overview of the immune system in response to *Borrelia burgdorferi* infection. A tick crawls its way into the skin, and while sucking up blood, the spirochete is transmitted to the host. Upon recognition of PAMPs, a variety of proinflammatory mediators are being released, which subsequently activate the adaptive immune cells. The adaptive immune system is then able to effectively respond to the infection by *Borrelia burgdorferi* bacteria.

entering the host (*figure 1*). In addition, macrophages are also found in the tissues of the heart in Lyme's carditis, a generally early and nonrecurring disease manifestation of Lyme disease [7]. Recognition of *B. burgdorferi* by macrophages is mediated by several TLRs, including TLR2/1 heterodimers [8], the receptor of TLR4; CD14 [9], and intracellularly located TLR7 and TLR8 [10, 11]. Other TLRs such as TLR3, TLR5, and TLR9 also have a role or an involvement in *B. burgdorferi* recognition [2]. TLR3 is known to recognize double-stranded RNA and, as a result, induces the production of type I interferons (IFN). *B. burgdorferi* is thereby known to induce transcription and production of type I IFNs. However, it is not known if this is mediated by recognition through TLR3 or by other TLRs such as TLR7 and TLR8, which are known to be able to induce the production of *Borrelia*-dependent type I IFNs [11]. The role of TLR5 in the recognition of *B. burgdorferi* remains a topic of debate. TLR5 recognizes bacterial flagellin and contradictory papers have been published, which will be discussed later in this review, on whether there is a role for TLR5 in PAMP recognition on *B. burgdorferi*. The same contradiction seen in TLR3 and TLR5 applies for TLR9 as well. Petzke *et al*. suggested a role for TLR9 since blocking the receptor resulted in an abrogated production of type I IFNs by monocytes in response to live *B. burgdorferi* [10], whereas Shin *et al*. show no inflammatory response in TLR9 knockout macrophages incubated with live *B. burgdorferi* [12]. In 2001, a novel TLR was identified and described as TLR10 [13]. The phylogeny of TLR10 resembles that of TLR1 and TLR6 and its mRNA has been found to be expressed in the spleen, lymph node, thymus, and tonsil [13]. Additionally, TLR10 was found to be expressed on plasmacytoid DCs and B cells [14]. Research on TLR10 is hindered by the lack of a rodent homologue. However, two studies have found expression of *TLR10* homologues in rat but not in mouse [14, 15]. The role of TLR10 is not yet fully understood and its ligand has to be identified yet. The function of TLR10 seems to be of a suppressive nature and blocking the receptor elicits a higher cytokine response compared to control in PBMCs stimulated with viable *B. burgdorferi* [16]. As a result of pattern recognition of *B. burgdorferi*, an upregulation of the macrophage attractant chemokines C-C motif ligand (CCL) 2, CCL3, and CCL4 has been observed [17].

In addition, upregulation of inflammatory and regulatory cytokines such as IL-1, IL-6, IL-8, IL-10, IL-12, IFN-α, IFN-γ, and TNF-α in macrophages/monocytes could be observed after exposure to *Borrelia* [10]. Activation of the TLR pathways not only results in the expression of chemokines, cytokines, and IFNs but also helps in the maturation and survival of cells [18, 19]. Interestingly, live *B. burgdorferi* elicited a higher expression of chemokines and cytokines compared to heat-killed bacteria in monocytes found by Cruz *et al*. in an *ex vivo* model [20]. It was hypothesized that this indicated the presence of one or more nonlipoproteins as PAMPs, which signal intracellularly. Thereby, monocytes phagocytosing live *B. burgdorferi* showed pathogen-induced apoptosis [20]. Even though this is a common feature, also seen in macrophages [21], whether this is beneficial for the host or *B. burgdorferi* remains a topic of debate. It could be that either self-induced apoptosis of macrophages or monocytes prevents collateral damage by limiting the inflammatory response, or that pathogen-induced apoptosis promotes the survival of opsonized *B. burgdorferi via* a parasitic suicide strategy [20].

Polymorphonuclear leukocytes

In addition to first-line defense mechanisms by the innate immune system is the presence or invasion of polymorphonuclear leukocytes (PMNs) at the site of *Borrelia* infection. As seen for macrophages, pathogen recognition of PMNs is not only mediated by PAMPs interacting with TLRs but also complement receptors (CR). Neutrophils express mRNA for TLRs 1 through 10 with exception of TLR3, and upon recognition of PAMPs, they secrete inflammatory chemokines [22]. Clearance of *B. burgdorferi* by PMNs has been shown to be both dependent and independent in the presence of anti-*B. burgdorferi* antibody [23, 24]. Upon recognition, *B. burgdorferi* is cleared through conventional phagocytosis or by two other specialized techniques, that is, coiling phagocytosis, by which the PMNs form pseudopods that wrap around the pathogen in a spiral pattern [25, 26], and tube phagocytosis, by which thin cellular protrusions surround and engulf the spirochete [27, 28]. In addition to the phagocytic killing mechanisms by PMNs, *B. burgdorferi* has also been shown to be susceptible for either oxidative or nonoxidative mechanisms, which contribute to killing or inhibiting growth of the pathogen, respectively [7, 28]. Nevertheless, *B. burgdorferi* is able to stagnate neutrophil chemotaxis by inhibiting IL-8 and N-formyl-met-leu-phe (fMLP), a potent chemotactic factor for PMNs released by tissue bacteria. Phagocytosis is inhibited by OspB expression, and tick saliva helps *B. burgdorferi* by inhibiting neutrophil function [29-32]. Besides phagocytosis, another clearance mechanism, exclusive to neutrophils, is neutrophil extracellular traps (NETs) formation. It was found in 2004 by Brinkmann *et al*. that neutrophils were able to release these NETs that can trap and kill pathogens [33]. This specific mechanism, better known as NETosis, kills bacteria by high concentrations of elastase and myeloperoxidase and has also been observed in the entrapment of *B. burgdorferi* [29, 34]. Menten-Dedoyart *et al*. suggested a possible evasion mechanism from NETosis by *B. burgdorferi* by keeping in mind their formation of round bodies. These round bodies are formed under unfavorable conditions enabling the spirochetes to survive longer, and these round bodies are also observed in NET entrapment. In addition, *B. burgdorferi* secretes anti-elastases, which serves as an alternative survival strategy [17].

Mast cells, fibroblasts, and keratinocytes

Mast cells are effector cells found throughout the body, but higher numbers are observed in the epithelial surface of the skin, adding up to the cells that could directly encounter *B. burgdorferi* in early stages of infection. The granules present in the mast cells are mostly known to contain heparin and histamine, diluting the blood and increasing the permeability of capillaries [35]. However, mast cell granules also contain cytokines, lysosomal enzymes, and proteases. In response to external stimuli, such as recognition of the Fc-region of IgE, mast cells are capable of phagocytosis and antigen presentation on either MHC class I or II, to CTLs or $CD4^+$ cells, respectively. In addition, they are also capable of producing IL-4, IL-5, IL-6, IL-8, and TNF-α [36-38]. Though mRNA expression levels of IL-3, IL-4, IL-5, IL-9, IL-10, IL-13, and IL-15 did not increase upon incubation with *B. burgdorferi*, increased production of proinflammatory cytokine TNF-α was detected [39].

Fibroblasts reside in the dermis, which is tightly connected to the epidermis. Due to their contribution in modeling the extracellular matrix and signaling to DCs, macrophages, mast cells, and nonimmune cells, such as keratinocytes, are seen as a part of the immune system [40]. Microarray analysis of fibroblasts treated *in vitro* with different strains of *B. burgdorferi* showed upregulation of 47 genes, which including extracellular matrix remodeling genes, proinflammatory genes, and chemokines. Many of these genes are under control of the signaling/regulating pathway mediated by NFκB, such as IFN-related genes [40, 41]. Further validation of the upregulated proinflammatory genes by qRT-PCR showed IL-6, IL-8, and CXCL1 to be upregulated when fibroblasts were coincubated with *B. burgdorferi*, which is in line with other studies, suggesting an important role for fibroblast in inflammation regulation and recruitment of immune cells such as leukocytes, DCs, and macrophages [40, 42].

Stratified keratinocytes, differentiating from the stratum basale up to the protective physical barrier of the stratum corneum, in the epidermis form the first line of defense against pathogens. Thereby, keratinocytes maintain an important immunological function by continuously producing antimicrobial peptides [43]. TLR1, TLR2, TLR4, and TLR5 have been found to be expressed on keratinocytes, enabling to respond to PAMPs [44-46]. As a result, an upregulation of the antimicrobial peptides is observed, which assist in the killing of pathogens [43, 44]. IFN-γ even shows upregulation of MHC class II on keratinocytes, indicating an immunocompetent role for keratinocytes and antigen presentation to $CD4^+$ T cells [47]. Keratinocytes are also affected by tick saliva proteins, which inhibit activation and subsequent antimicrobial peptide or cytokine secretion [32].

Dendritic cells

The key in bridging the innate immune system with the adaptive immune system lies within DCs. DCs are

professional antigen presenting cells (APCs) able to phagocytose and process antigens. They can be distinguished into two classes: the myeloid dendritic cells (mDCs) and the plasmacytoid dendritic cells (pDCs). Both classes of DCs have a distinct pattern in expression of TLRs; mDCs express TLR1 – through TLR8, and TLR10, whereas pDCs express TLR1, TLR6, TLR7, TLR9, and TLR10, which express high levels of type I IFNs in response to viruses [48]. Langerhans cells (LCs), a subclass of mDCs, reside in the skin and are possibly the first DCs to encounter *B. burgdorferi* lacking the expression of TLR4 and TLR5 [48, 49]. Recognition of *B. burgdorferi* and phagocytosis leads to the degradation of the bacteria in phagosomes. Activation of TLRs in DCs results in the same MyD88-mediated pathway, increasing expression of MHC class II, costimulatory molecules CD80/CD86, and a large subset of cytokines [49, 50]. Expression of these surface molecules indicates the maturation of the DCs allowing them to migrate toward the lymphoid tissues for antigen presentation to T lymphocytes, initiating an adaptive immune response. The antigenic peptides are presented to the T lymphocytes on the major histocompatibility complex (MHC) class I or II with additional stimulation by CD80/CD86 and cytokines [20, 22, 48]. Since *B. burgdorferi* can also be found alive in the cytoplasm, antigens of these microbes can be presented on MHC class I [49]. In *in vitro* studies, a CTL response has been observed and their presence is also shown in biopsies of erythema migrans lesions. However, their function or role in the defense against *B. burgdorferi* infection is not yet understood [49]. Infectivity of *B. burgdorferi* in the host is increased through saliva proteins Sialo L, Sialo L2, and prostaglandin E2. Sialo L inhibits IL-12 production; this is important for T lymphocyte differentiation. Sialo L2 inhibits chemokine production, and prostaglandin E2 affects DCs maturation [32, 51, 52].

THE ADAPTIVE IMMUNE SYSTEM AND ITS ROLE IN BORRELIA BURGDORFERI INFECTIONS

The adaptive immune system comprises of $CD4^+$ cells (T helper 1, T helper 2 cells, and T helper 17 cells), $CD8^+$ cells (cytotoxic T cells), gamma delta T cells ($\gamma\delta$ T cells), natural killer T cells (NKT cells), and B cells that are able to effectively kill pathogens or prevent pathogen growth (*figure 1*). In addition to the cell-mediated immunity, the adaptive immune response also consists of a humoral part in the form of antibodies.

$CD4^+$ T cells

When naive $CD4^+$ T cells interact with an antigen-presenting cell, they are activated and start to differentiate into T helper 1 or T helper 2 cell, depending on the cytokine environment. Other subsets to differentiate from CD4 naive T cells, discussed later, are T helper 17 cells, regulatory T cells, NKT cells, $\gamma\delta$ T cells, and memory T cells, which are also possibly depending on cytokine stimulation [53].

Both Th1 and Th2 responses have been observed in Lyme disease but the ratio between the two seems to determine the severity of the disease. Patients with a predominant Th1 response are associated with the development of a more severe Lyme's arthritis phenotype [54]. This has also been shown in the synovial fluid of Lyme's arthritis patients [55]. Patients with a predominant Th2 response seem to be more susceptible to a chronic Lyme disease manifestation [54, 56]. The reason for the different outcomes as a consequence of Lyme's borreliosis is probably multifactorial, but remain largely unknown to date [57].

For differentiation into T helper 17 cells (Th17), naive $CD4^+$ T cells have to be stimulated by IL-6, IL-21, IL-23, and transforming growth factor β [53]. As stated before, Lyme disease is able to develop into Lyme's arthritis, in which T cells are shown to play an important role. Autoimmune diseases with comparisons between clinical signs, such as rheumatoid arthritis, show that Th17 cells play an important role in the autoimmune disease [58, 59]. The Th17 response in *B. burgdorferi* infections seems to arise from the recognition of the neutrophil-activating protein A (NapA), encoded by *B. burgdorferi*, by TLR2, which stimulates the expression of cytokines involved in Th17 differentiation [60]. Taken together, the Th17 response might be crucial for Lyme disease to become a chronic condition in patients.

$CD8^+$ T cells

$CD8^+$ T cells differ from the $CD4^+$ positive cells in the recognition of MHC class I compared to MHC class II for $CD4^+$ T cells. For the development of immature $CD8^+$ T cells into effectors cytotoxic T cells (CTLs), an antigenic signal is required, delivered by the MHC class I, which is recognized by the TCR on the $CD8^+$ T cell. In addition, costimulatory molecules expressed by APC signals such as CD80 or CD86 are recognized by CD28 on CTLs, which are thought to help develop an optimal CTL response. IL-12 and type I IFNs are thought to be the third type of signal. The role of CTLs in Lyme disease is not well understood. In a mouse model susceptible to Lyme disease, depletion of CTLs led to a decrease of the Lyme's arthritis phenotype and to an increase in disease exacerbation [61, 62]. This suggests that CTLs might have a negative role in *B. burgdorferi* infections in the host by contributing to Lyme's arthritis. In neuroborreliosis, it could be demonstrated that there is high infiltration and clonal expansion of CTLs in the cerebrospinal fluid (CSF). Cytokine profiling using CD3/CD28-containing beads to *in vitro* activate CTL clones, derived from patient's CSF after infection of *B. burgdorferi*, shows excretion of high amounts of IFN-γ, little IL-2, and lesser IL-4, IL-5, and TNF-α [63]. However, DCs that were preincubated with *B. burgdorferi* lysate only showed an increase in TNF-α [63]. The animal model indicates a MHC class I interaction *via* phagocytosis of *B. burgdorferi* and an IFN-γ response rather than with antigens of extracellular proteins [61, 62, 64]. Infiltration of CTLs in the CSF might be explained by the fact that DCs are also capable of MHC class I antigen presentation and are present in the CSF [63]. Differences in cytokine profiles could be explained by the fact that DCs were treated with *B. burgdorferi* lysates instead of live bacteria, which might induce a different MHC class antigen presentation and subsequent induction of the T cell response. Another reason could be the use of a different model system that might affect cytokine profiles.

Gamma delta T cells

In contrast to the more conventional αβ T cells that reside in the peripheral lymphoid organs, the gamma delta (γδ) T cells predominate the epithelial tissues. The function of γδ T cells in *Borrelia* infection is not yet fully understood. In the autoimmune disease psoriasis, γδ T cells are found to produce IL-17 and contribute to disease pathology [65]. Another, and maybe more important, difference compared to αβ T cells is the recognition of antigens. Instead of activation through MHC class I or II antigen presentation, γδ T cells recognize a more diverse collection of intact, unprocessed ligands including nonclassical MHC molecules (*e.g.*, heat shock proteins, lipids, and stress-induced molecules) expressed on CD1a/b/c on DCs [65, 66]. In Lyme disease, γδ T cells have been shown to be activated through DCs upon *B. burgdorferi* infection. The interaction between DCs and γδ T cells seems to be mediated by Fas:FasL interactions, physical contact, and γδ TCR ligand recognition by DCs [66, 67]. Additionally, expression of cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP) by DCs inhibits the apoptotic pathway and induces the NFκB pathway, leading to an increased proinflammatory cytokine expression and cell survival for γδ T cells [66]. As a result of Fas:FasL interaction, caspase 8 is activated, which only recently has been appreciated to be required for T cell growth [68]. Subsequent to γδ T cell growth and activation, IL-17 levels are increased, and in contrast to their beneficial effects, they have also been found to contribute to the pathology caused by psoriasis [69].

(Invariant) natural killer T cells

A subgroup of lymphocytes, 0.2% of the T cells, express surface antigens associated with both adaptive and innate immunity and are described as natural killer T cells (NKT cells), that is, CD56 and CD16 with CD3 to discriminate between NK and NKT cells [70]. NKT cells are not only potent producers of cytokines but also provide protective immunity to microbes and tumor cells, and maintain tolerance. Recognition of lipids occurs through the CD1 family, which are structurally related to the MHC complex. Based upon which member of the CD1 family they recognize and the expression of specific α and β chains of the TCR, NKT cells are being classified. The invariant NKT cell (iNKT cell) is the most studied subtype of NKT cell and is able to recognize lipid antigens, including one from *B. burgdorferi*, presented on CD1d by the specific Vα24-Jα18 α chain and the Vβ11 β chain [71-73]. As a result of antigen recognition, iNKT cells produce and secrete IFN-γ and IL-4, similar to the innate NK cells [72]. Like mice lacking γδ T cells, iNKT-deficient mice also show a reduced ability to respond and clear *B. burgdorferi* infection [74]. Due to the overlapping characteristics of both the innate and adaptive immune responses of iNKT cells and γδ T cells, they may link both systems as an immune response to *B. burgdorferi* and other pathogens. Thereby, they help in the clearance of *B. burgdorferi* and limit the severity of Lyme's arthritis. Interestingly, numbers of iNKT cells in peripheral blood of humans vary greatly, from almost undetectable to several percentages, and might be a risk factor for the development of Lyme's arthritis [75].

B cells

Antigen recognition by B cells is mediated by the B cell receptor (BCR) and occurs in the secondary lymphoid organs. Upon antigen recognition, B cells can be activated either with or without the presence of T cells. T cell-dependent activation occurs through MHC class II antigen presentation by the B cell [76]. Plasma cells are the result of T cell-dependent B cell activation and secrete large amounts of IgG antibodies. T cell-independent activation is mediated by the costimulation of TLRs and results in short-lived plasma cells secreting antibodies mostly of the IgM class [76, 77]. The T cell-independent response occurs after 3 to 4 days of infection and is the first humoral response against pathogens, and thus *B. burgdorferi*. B cells rapidly differentiate into IgM secreting cells in response to antigens. Marginal zone B cells are the first cells to produce pathogen-specific IgM, and defects in this system make the host more susceptible to the development of Lyme's arthritis in a murine model [78]. Due to the development of Lyme disease, patients start to develop autoantibodies in response to *B. burgdorferi* proteins and glycolipids. Nonresponding patients, treated with antibiotics, undergo excessive inflammation and an uncontrolled immune response, causing autoantibody formation to annexin A2 (autoantibodies found in multiple autoimmune diseases), endothelial cell growth factor, and apolipoprotein B-100, ultimately leading to Lyme's arthritis development [79]. In addition, they also found that these patients had a T and B cell response and include higher levels of IFN-γ production.

ANTIGENS OF THE BORRELIA BURGDORFERI BACTERIUM

Bridging the innate immune system to the adaptive immune system occurs through APCs. Phagocytosis and subsequent breakdown of *B. burgdorferi* initiates antigen presentation by APCs to T and B cells (*figure 2*). This process not only initiates an adaptive immune response to effectively respond to an invading pathogen but also creates immunological memory. The combination of immunological memory and antigens is particularly interesting since it would accelerate the development of vaccines to prevent *B. burgdorferi* causing Lyme disease. In addition, gaining specific knowledge on *B. burgdorferi* antigens could also help in developing a more sensitive and specific diagnostic tool for Lyme disease.

Outer surface proteins A-F; OspA-OspB

The first protein identified on the surface of *B. burgdorferi* is outer surface protein (Osp) A, which was described as a surface antigenic determinant [80]. A similar protein was described later by the same group and it became clear that OspA and OspB are cotranscribed and their amino acid sequence are identical for at least 53% [81-83]. Even though protein identification and sequence were described, it took a while before its function was determined. In 2000, OspA was identified as a mediator for *B. burgdorferi* attachment to the gut of the tick [84]. Later, it was found that OspA binds specifically to the tick receptor for OspA (TROSPA) [85]. The importance of these proteins led to the development of a vaccine with recombinant

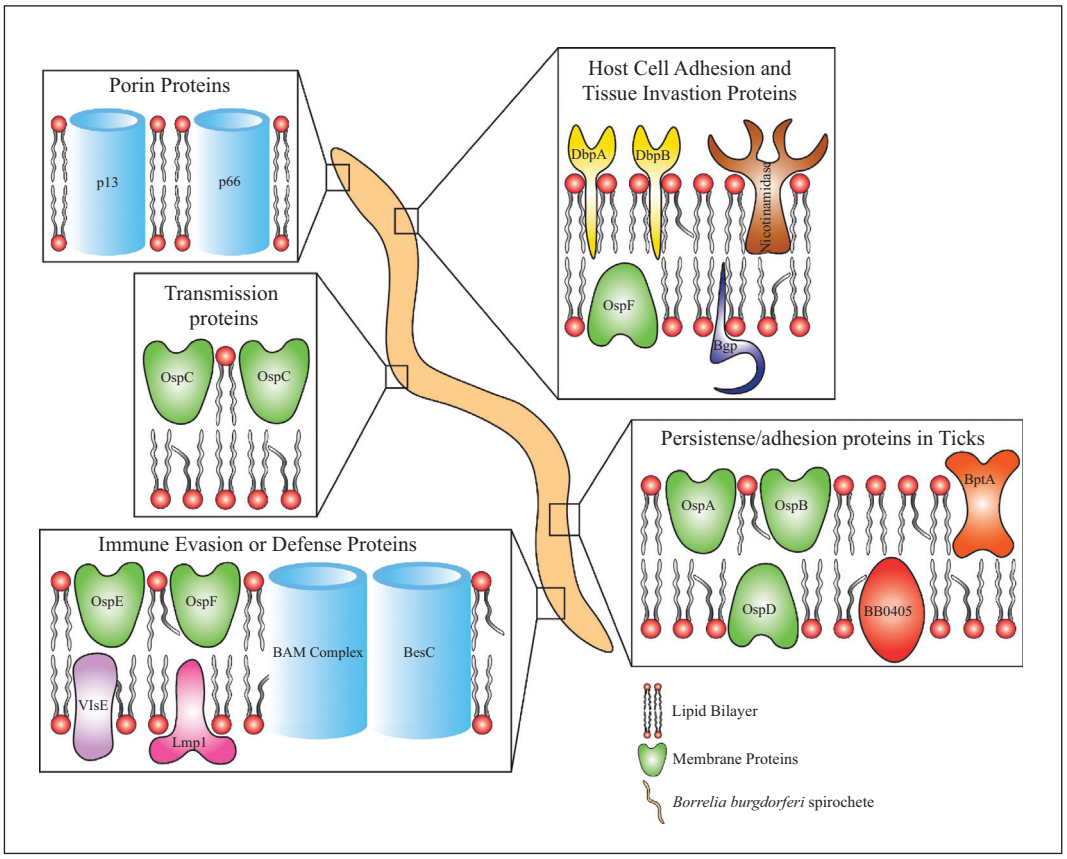

Figure 2
Overview of the *Borrelia burgdorferi* spirochete and its outer surface proteins and antigens, which are ordered according to their (predicted) function.

OspA. Despite high protection levels against Lyme disease (80-90%), OspA antibody responses in vaccine recipients are generally low and result in vaccine failures [86, 87].

OspC

Through clonal expansion and expression of an unknown *pc* gene, encoding a major and prevalent protein on *Borrelia*, a pC protein was purified and later identified as OspC [88]. The expression pattern of OspC is mutually exclusive to OspA and OspB and is observed during tick feeding; and the expression pattern suggests a role for OspC in dissemination from the tick and infestation in the host [89]. How this expression is regulated is still unknown. Nevertheless, it seems that changes in temperature and tick feeding might be the factors responsible for gene expression patterns [90, 91]. That OspC may serve as virulence factor was further validated using a *B. burgdorferi* OspC mutant that was unable to infect the host [92]. Moreover, OspC has shown to bind to tick salivary protein 15, which seems to help the survival of *B. burgdorferi* in early infection stages by complement- or phagocytosis-mediated killing mechanisms [19, 93]. When OspC is not regulated and constitutively expressed on *B. burgdorferi*, it was shown that the ability of spirochetes to evade humoral immunity is reduced [94, 95]. The observed humoral response to OspC was a reason to investigate the potency of a vaccine directed against OspC. Unfortunately, the OspC sequence displays high variation between *Borrelia* species and the humoral response tends to be directed to the variable domains of the protein complicating the vaccine design [96]. Therefore, better understanding of the variable antigenic domains could improve further studies on vaccine development.

OspD

Norris *et al*. discovered OspD expressed by a low-passage strain of *B. burgdorferi*. Molecular cloning and sequence analysis revealed a lipoprotein of about 28 kDa, representing OspD [97]. The function of OspD was later determined when recombinant OspD was found to bind to tick gut extracts. Furthermore, its expression decreased when subjected to host-specific factors in a dialysis membrane system mimicking the host environment, and when temperature rose from 23 °C to 35 °C, providing more evidence of it as a adhesion protein for *B. burgdorferi* in the tick gut [91, 98]. Unless there was tight regulation pattern, OspD mutant strains did not alter spirochete transmission during tick feeding, which indicates that *B. burgdorferi* is able to compensate for the lack of OspD and therefore OspD seems not required for the spirochete to survive [99].

OspE

OspE is the first lipoprotein that has been found to bind to factor H and/or factor H-like protein-1 (FH/FHL-1) [100]. All proteins interacting with FH/FHL-1-factors are referred to as complement regulator-acquiring surface proteins (CRASPs) [101]. OspE plays a role in the evasion of the immune system of the host. The expression of OspE is increased when the temperature rises to 35 °C, providing

more evidence for its immunoevasive role [102]. OspE function was demonstrated by adding soluble OspE to human serum. The addition of soluble OspE inhibited factor H binding to the spirochete, and complement-mediated killing was initiated [100]. It is therefore involved in immune evasion, which is in line with a recent paper that shows that *B. burgdorferi* spirochetes protect themselves from a complement attack by mimicking host protection mechanisms [103].

OspF

OspF is found downstream of OspE in the same operon and they are cotranscribed genes. This seems to be a unique feature of the *B. burgdorferi* N40 strain [104]. As is seen with OspE, gene expression of OspF is also upregulated when there is a shift in temperature to 35 °C. While the function of OspF is yet unknown, this behavior in gene expression implies a role in the suppression of the complement system during spirochete infection in the host [91]. Additionally, OspF was found to be as a candidate adhesion molecule in Lyme's carditis [105].

Osp17

Osp17, first described in 1999, was found to be abundantly expressed on the surface of the *Borrelia afzelii* strain pKo. Unexpectedly, its expression was found in patients with Lyme's borreliosis in a late disease stage as well [106]. However, the sequence of the *osp17* gene showed 88-100% sequence homology with Decorin-binding protein A (DbpA) within the same *B. afzelii* strain [107]. DbpA sequence homology in *B. burgdorferi sensu stricto* and *Borrelia garinii* was shown to be around 45%. Altogether, these data imply that the protein is species-specific and conserved over time [107]. This finding could be confirmed by another group, which proposed Osp17 as a new component for serodiagnosis of Lyme's borreliosis [108].

Borrelia p83/100 antigen protein

The p83/100 antigen protein is known to elicit a strong IgM and IgG response in all stages of Lyme disease. The p83/100 antigen can be found in literature under different annotations, for example, p83, p93, p94, or p100. Nevertheless, p83, p93, and p100 are homologs [109]. The function of this protein is yet unknown, though it is currently being used in IgG Western blotting analysis as one of the 10 specific bands for Lyme disease diagnosis, as described by the CDC [110] after recommendation by Engstrom *et al*. and Dressler *et al*. [111, 112].

Flagellin

Briefly discussed earlier in this review for its potential role in PAMP recognition by TLR5 is flagellin. Even though the flagella of *B. burgdorferi* are contained within the outer envelope, early immune responses still show an increase in antibody response against the 41 kDa flagellin protein. It seems that processed spirochetes result in the production of antiflagellin antibodies [113]. Besides flagellin, also described as p41 or flagellin B [114], flagellin A is an outer sheath protein of the flagella of *B. burgdorferi* and has a molecular weight of 37 kDa [114]. In contrast, only the 41 kDa flagellin is described by the CDC as a positive signal in the test for diagnosis of Lyme disease [110]. In addition to diagnostic purposes, flagellin could also be a factor for the innate immune system to recognize the spirochete. Flagellin is recognized by TLR5 and its recognition has been described to induce a response by monocytes [12]. However, it has been recently reported that *B. burgdorferi* recognition is independent of TLR5, which is likely to assume since the flagella are encased within the outer surface membrane [12, 115]. It might be that processing the dead bacteria results in the unwrapping of the flagella and thereby exposing the protein to TLR5, or recognition through transient gaps within the membrane of the spirochete. While both have yet to be demonstrated, it was shown that TLR5 is able to recognize the flagellin protein of the spirochete *Treponema pallidum* [116]. It remains an open question whether TLR5 and its ligand, flagellin, are involved in the recognition of *B. burgdorferi* during infection or disease.

Borrelia persistence protein A

The *Borrelia* persistence protein A (BptA) is a protein that is conserved in all *sensu lato* strains and was shown to be an Osp as well [117]. The sequence for BptA is located on the linear plasmid 25 (lp25). Although its function is not yet known, it was shown to be involved in infectivity of the bacteria since a BptA-deficient strain was not only less virulent but also had drastically reduced survival within the tick [117].

P13 and P66

P13 and P66 proteins were found as surface antigens with a size of 66 kDa and 13 kDa, respectively [118, 119]. Both proteins were found to be pore-forming proteins, which were expressed on the outer surface of *B. burgdorferi*. In addition, the porin protein P66 was found to have adhesive properties and bind to human β1- and β3-chain integrins. A P66-mutant strain was severely impaired during binding to β3-chain integrins and was shown to be a virulence factor for *Borrelia* to establish infection in the host [120, 121]. The pore formed by P66 consists of 7-8 subunits, in total accounting for a membrane complex of about 460 kDa, and build up by oligomeric P66 proteins [122]. It is of note that results from a recent study led the authors to conclude that the integrin-binding characteristic of P66 does not seem to play a role in infectivity of *B. burgdorferi*, but is important for transmigration of the bacteria through the endothelial cell layer and further dissemination [123]. In contrast, the P13 pore formed is approximately 300 kDa and is composed of monomeric P13 subunits [124]. Even though the function of P66 is partly described, the function of both P66 and P13 as porins is not well understood to date. Their function as pore proteins was investigated, but when the spirochete was faced with osmotic pressure, P13 and P66 showed no active participation [125].

Decorin-binding proteins A and B

In addition to P66 that binds to host ligands, decorin-binding proteins A (DbpA) and B (DbpB) have been shown to bind host decorin and glycosaminoglycans [126]. Both are important elements of proteoglycans found in the connective tissues. The ability of *B. burgdorferi* being able to bind to proteoglycans facilitates tissue invasion

[127]. The role of DbpA and DbpB was further validated by Shi *et al*., who demonstrated that with mutant DbpA or DbpB *B. burgdorferi* strains, the infectivity was not decreased whereas the colonization in the tissues was decreased [126]. This hypothesis was substantiated based on the observation the expression of DbpA and DbpB was induded in a temperature-dependent manner when the spirochete was transferred from an environment of 23 to 37°C [91].

Nicotinamidase

In a study with a *B. burgdorferi* strain lacking lp25, another gene, *pncA* (BBE22), was found to be important for the infectivity of the spirochete. Loss of the lp25 plasmid made the strain deficient for infectivity. However, infectivity was restored to almost wild-type levels when the spirochete was transfected with a shuttle vector containing the *pncA* gene [128]. The *pncA* gene was found to be responsible for the transcription of a protein with nicotinamidase activity, thus catalyzing the reaction of nicotinamide to nicotinic acid [128]. The latter is being used for the production of NAD^+. Interestingly, the genome of *B. burgdorferi* does not seem to contain genes encoding for enzymes that are required for NAD^+ synthesis. This indicates that *B. burgdorferi* relies on the salvage of nicotinamide in the host for the production and homeostasis of NAD^+ [129]. In a recent study by Gilmore *et al*., it was investigated whether *pncA* alone or *pncA* and *bptA* would restore the infectivity to *Ixodus scapularis* in a lp25-deficient strain. Even though some of the infectivity was restored, it was still far from wild type (45% in *pncA*- and *bptA*-restored strains vs. 100% wild type) [130]. They rationalize this finding by the fact that a complete loss of lp25 not only results in a loss of *pncA* and *bptA* but also other proteins that may be involved in *B. burgdorferi* infectivity and persistence [130].

Barrel assembly machinery

B. burgdorferi is known as a diderm, meaning that their cell envelope consists of outer and inner membranes. All diderms can be characterized by the presence of a β-barrel assembly machinery (BAM complex) that is dependent on the presence of BamA [131]. The BAM complex is involved in the localization and assembly of other β-barrel-containing integral outer membrane proteins into the bacterial outer membrane [132]. These barrel-containing membrane proteins may be involved in the defense against environmental factors including antibiotics. Besides BamA, the BAM complex in *B. burgdorferi* is composed of other lipoproteins, namely, BamB and BamD [133, 134]. These lipoproteins may act as interaction modules. This has been shown by Iqbal *et al*. in which BamA was shown to interact with translocation and assembly module B (TamB) [135]. A TamB mutant strain showed deformed morphology and was essential for *B. burgdorferi* viability. These data give new insights regarding outer membrane biogenesis and suggest an important role for TamB orthologues in this field [135]. Furthermore, since the BAM complex is located in the outer membrane, it might be a potential antigen for novel vaccination strategies.

Surface-located membrane protein 1

The 128 kDa surface-located membrane protein 1 (Lmp1) was originally discovered by Antonara *et al*. [105, 136]. This led to the identification of several new and already verified adhesins in *B. burgdorferi*; Lmp1 was found to be a new protein with an unknown function and was found in heart, joint, and bladder tissues [105], suggesting a role in adhesion, tissue invasion or persistence in the host. In addition, it was found to be a chromosomally encoded protein, and serum antibodies of *B. burgdorferi* infected mice did recognize a certain fragment of Lmp1 (BB0210) [105]. Additional investigation of the protein's function and antibody responses showed that the antibody response of the host was directed against Lmp1 protein [137]. When mice were immunized with Lmp1, antibody responses significantly interfered with the persistence of *B. burgdorferi* in an organ-specific manner [137]. The most significant results were found in Lmp1N-immunized mice which showed a significant decrease of the spirochete in the heart and joint tissues but not bladder tissue [137]. Furthermore, the Lmp1-N-terminal region shows more beneficial effects for *B. burgdorferi* since *lmp1* mutant strains were highly susceptible to immune sera whereas *lmp1N*-complemented strains showed viability levels compared to wildtype, suggesting a role in bacterial resistance to the humoral immune response [136, 137]. The middle region of the Lmp1 protein is involved in cell adhesion and mutant strain of Lmp1-expressing Lmp1M was sufficient to support *B. burgdorferi* infectivity in a murine host [138].

Borrelia efflux system protein C

Multidrug resistance in bacteria is mainly provided through membrane transporter proteins such as *Borrelia* efflux system protein (BesC). These efflux systems are able to pump out toxins that otherwise would kill the pathogen, hence the interest in these proteins to circumvent drug resistance. Efflux pumps with these properties are categorized in the resistance-nodulation-division (RND) superfamily [139]. A well-known member of this family is the TolC membrane channel found in *Escherichia coli*, providing a role in antibiotic resistance [139]. Borrelia efflux system protein C was previously known as BB0142 but Bunikis *et al*. found that this gene is likely a homologue of TolC [140]. They also identified two other genes, BesA and BesB, located within the same putative operon and cotranscribed with BesC [140]. Besides the efflux properties, *Borrelia* strains with mutated *besC* were unable to establish infection in the host heart, bladder, knee, and ear tissues. Additionally, *besC* mutants were up to 64-fold more sensitive to inhibitory concentrations for certain antibiotics [140].

BB0405

An addition to the outer membrane proteins is BB0405. The gene is cotranscribed with its paralog BB0406. However, the interest in BB0405 is due to the fact that a mutant strain failed in transmission to the host, whereas loss of BB0406 had no influence on infectivity [141]. That BB0405 would be important in the infectivity of the spirochete was hypothesized before since the *bb0405* gene is upregulated when temperature rises, similar to when *B. burgdorferi* is in the environment of the host [142].

Nevertheless, its expression is ubiquitous through the life-cycle of *B. burgdorferi*, and the function of BB0405 is still not known [143]. Recent reports have not found an antibody response against this protein despite its location on the surface of the bacterium. Nevertheless, mice that were immunized with a recombinant protein were found to develop a longer lasting and higher antibody titer response, which protected the mice against infection through tick bite, indicating a potential interesting target for vaccine studies [143].

Borrelia glycosaminoglycan-binding protein

Glycosaminoglycans (GAG) are polysaccharides that are ubiquitously expressed throughout the body. Distinct classes of GAGs are known such as heparin sulphate and dermatan sulphate, which differ with respect to disaccharide repeat and the degree of modification. These GAGs are covalently bound to proteins to form proteoglycans of which decorin is a well-known example by binding to fibrils in connective tissue [144]. Borrelia glycosaminoglycan-binding protein (Bgp) is known to bind this protein in addition to DbpA; DbpB binds to decorin and BBK32 binds to fibronectin [144, 145]. In a study to determine the importance of Bgp for infection of *B. burgdorferi*, two *bgp*-mutant strains were used and compared to wild-type and a lp25-knockout strain, having the plasmid required for infectivity [146]. Results showed that mutations in the *bgp* gene mutation abrogated Bgp protein expression. However, the mutant strains were still able to maintain their infectivity in immunodeficient mice by collecting them from injection site, blood, skin, joints, and bladder [146]. This indicates that Bgp is not required in establishing infection and suggests that other proteins such as DbpA and DbpB may be more important or that Bgp functions as a redundant protein.

Surface lipoprotein E

A widely studied protein on the surface of the *B. burgdorferi* spirochete is surface lipoprotein E (VlsE). This protein is of interest since it contributes to immune evasion and persistence in the host, which is mediated through antigenic variation. It consists of an invariable C- and N-terminus, 6 other invariable regions, as well as 6 variable regions. The Vmp-like sequence (*vls*) is located on plasmid lp28-1 and consists of a *vlsE* protein coding region and an array of 15 unexpressed *vls* cassettes [147]. These cassettes replace the variable regions within the *vlsE* cassette and thereby contribute to antigenic variation. Interestingly, the antigenic variation activity only occurs in the host environment. Loss of the lp28-1 plasmid results in a decreased infectivity in *B. burgdorferi* in immunocompetent and immunodeficient mice [148]. This already suggested an important role for VlsE, which was verified by Bankhead and Chaconas who found that VlsE is an absolute requirement for persistence in immunocompetent mice [149]. In humans, a strong humoral response against VlsE is seen after infection, mainly against the invariable region 6; however, fortunately, this region is located in the membrane-distal region and not surface exposed, and thus it is likely that antibodies cannot bind to this region [150]. However, antibody reactivity against other domains of the VlsE regions are seen throughout the disease and may possibly be linked to the adaption state of *B. burgdorferi* and/or disease stage [151, 152]. Furthermore, experiments have been carried out using a nonvariable VlsE gene to determine if the spirochete would be able to colonize the *Ixodus scapularis* tick again. Results show that colonization of this mutant strain was impaired [152]. Moreover, it was found that reinfection in *Peromyscus* mice only occurred when a different strain of *Borrelia* was used. Intrastrain reinfection was not able to establish another infection in the same host. This would suggest that reinfection as it occurs in nature would go through strains that are heterologous to the strain that originally infected the host [152].

BORRELIA BURGDORFERI IMMUNE EVASION STRATEGIES

Despite the contribution of the aforementioned immune cells, the *B. burgdorferi* spirochete remains capable of establishing a chronic disease pathology. It is well known that several immune evasion strategies exist for the spirochete. Although some strategies are dependent on the interaction with tick-derived proteins or presented antigens, other strategies of *B. burgdorferi* are a direct result of the migratory behavior of the spirochete upon infection in the host. More knowledge with respect to these evasion strategies could potentially contribute to new vaccines. Herein, we will elaborate on the mechanisms to escape from the immune system utilized by *B. burgdorferi* with respect to tick-assisted immune evasion or specific strategies exhibited by the spirochete.

Tick-assisted immune evasion

The first step in the infection cascade is the tick that crawls into the skin of the host with the sole goal of sucking up blood. Injury in the vascular system of the host normally results in the coagulation of blood to prevent blood loss. Tick saliva however contains a panel of molecules that target and thereby inhibit the platelet aggregation pathway, for example, integrin antagonists and collagen receptor inhibitors [153]. In addition, ticks secrete vasodilatory molecules that allow more blood circulation at the site of infection, which contributes to the infectivity of *B. burgdorferi* [153]. Other proteins present in the saliva of the tick are more directed to inhibiting innate and adaptive immune pathways, as well as target various key elements of the host defense mechanisms. Tick salivary protein (Salp) 20 is a protein that is involved in inhibiting complement-mediated opsonisation by interfering with the active C3 convertase [154]. Besides the help of salivary proteins to escape the deleterious effects of complement system, OspE expression on the outer surface of *B. burgdorferi* interacts with complement regulating factor H in an inhibitory fashion [103]. Salp15 on the other hand is a protein derived from tick saliva that is involved in inhibiting $CD4^+$ T cell activation. The target of Salp15 is T cell receptor ligation and subsequent calcium fluxes. As a result, lower levels of IL-2 are observed and development of $CD4^+$ T cell responses are hampered [155, 156]. Moreover, tick saliva also contains IL-2 binding protein, which results in even more reduced levels of IL-2 [156]. Another salivary protein, Salp25D, that shares homology with glutathione peroxidases, reduces the production of reactive oxygen species,

which allows extended feeding time for the tick since wound healing is impaired [157].

Recently, Bernard *et al*. found that tick saliva suppresses TLR2/TLR3-induced cytokine responses in keratinocytes when exposed to live *Borrelia* [32]. TLR3 recognizes dsRNA, which is found at the site of skin injury, whereas TLR2/1 recognizes OspC present on the outer surface of the spirochete. These results were found *in vitro* and were verified later in an *in vivo* mouse model. They rationalize this finding as a two-step process of infection. The first step involves transmission of *B. burgdorferi* with TLR inhibition through tick saliva proteins. This allows multiplication of the spirochete. The second step would be the migration of *Borrelia* to other tissues. As a consequence, tissue injury would arise, and since the saliva is no longer active at the time, inflammation arises in the form of erythema migrans [32].

Not only are TLRs inhibited on keratinocytes, TLRs on DCs are also affected by the presence of tick saliva. Phagocytosis, lymph node migration, the expression of costimulatory molecules such as CD40, CD80, and CD86, and secretion of proinflammatory cytokines such as IL-12, IL-6, IFN-γ, TNF-α, and IL-1β have all been shown to be inhibited or diminished in DC subsets in various treatments in combination with tick saliva [51, 158-160]. In contrast, IL-10 was upregulated, which is a well-known suppressor of inflammation [160]. In addition, antigen presentation and a Th1/Th17 polarization was impaired but Th2 differentiation was promoted, which is supposed to be in favor of *B. burgdorferi* and the tick itself [158].

Escape mechanisms by Borrelia burgdorferi

The aforementioned escape mechanisms all rely on the assistance of tick salivary proteins. However, after some time of feeding by the tick, the saliva and its content are cleared and the *B. burgdorferi* spirochete has to survive without further help. To fully develop into a chronic disease, the spirochete relies on some other evasion strategies that are independent of the tick and its proteins.

One of the strategies is infiltration into the lymph nodes. Even though the lymph nodes contain high numbers of naive B and T cells, which are being activated upon recognition of antigens, whether or not presented by APCs, *B. burgdorferi* is able to suppress long-lived humoral immunity. Lymphadenopathy, an enlargement of the lymph node which is abnormal in size, number, or consistency, is a common manifestation following an infection by *B. burgdorferi* [161]. It often occurs adjacent to the site of infection with *B. burgdorferi*. The strategy of the spirochete by infiltrating the lymph node is by evoking an inadequate humoral immune response by infiltrating the germinal centers. This provokes a rapid expansion of B cells in the germinal centers, which ultimately secrete IgM, IgG2, and IgG3a antibodies that are T-independent B cell response antibody subtypes [161, 162]. It is thought that the T cell-independent nature of the immune response is inadequat to remove *B. burgdorferi* in the host.

Recently, it has been shown that due to the infection of the spirochete into the lymph nodes, the germinal centers develop abnormally and become nonfunctional. As a result, no memory B cells and long-lived antibody-producing plasma cells will develop [163]. Even an induction of a T-dependent antibody response was not sufficient to elicit a long-lasting antibody response [162, 163].

In addition to infiltrating the lymph nodes and thereby disrupting the adaptive humoral immune response, it has also been shown that *B. burgdorferi* is better protected in the skin and joints compared to the heart and the bladder. Due to the expression of decorin in the skin and joints, the spirochete is able to establish a protective niche by upregulating its decorin-binding protein A expression [164].

CONCLUSION

The pathogenesis of Lyme disease has been well investigated over the last few decades. Even though a cellular immune response can be observed at the site of an infection with effector cells of both the innate and adaptive immune systems, *B. burgdorferi* has a variety of evasion strategies that result in its survival in the host. These strategies include, but are not limited to, the formation of round bodies, secretion of IL-2 binding protein, and the assistance of tick (salivary) proteins to protect the spirochete from killing mechanisms or to interfere with immune cell effector functions (*figure 1*). To overcome chronic infection by *B. burgdorferi* and the development of Lyme disease, vaccination of the highest-risk population groups could be beneficial in preventing Lyme disease. In the United States of America, 93% of all reported cases were located within 10 states in the Northeast and upper Midwest according to the CDC in 2009 [1]. Vaccine development already took place in the 1990s by two companies whose vaccines were directed against OspA. Results were promising as the phase III trial prevented most definite cases of Lyme disease or asymptomatic *B. burgdorferi* infection [87]. However, due to adverse events and other factors, the vaccine was taken off the market [165]. Prevention of Lyme disease has since then been focused on a practical basis instead of prevention through vaccines. The understanding of the infection mechanisms of *B. burgdorferi* and the antigens present on the spirochete could assist in the development in new and more promising vaccines. However, one of the difficulties to overcome while designing a vaccine is strain-specific antigens. This has been shown with respect with the OspA antigen, which showed protection against *B. burgdorferi sensu lato* strains expressing serotype-1 or serotype-2 OspA molecules in mice [166]. It is therefore of importance that vaccine design should provide a broad range of protection or vaccines be designed for specific geographic regions where only certain strains of the spirochete are observed. Additional ideas on vaccine design could be sought by targeting proteins of the tick itself, tick salivary proteins, or proteins that interact with *Borrelia* [167]. Further understanding of these proteins and their potential as vaccine candidate could mediate the development of new vaccines to prevent cases of Lyme disease or other zoonoses, particularly those transmitted through insects.

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