Modulation of Macrophage Responses to Borrelia Burgdorferi in Acute Murine Lyme Carditis

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MODULATION OF MACROPHAGE RESPONSES TO BORRELIA BURGDORFERI IN ACUTE MURINE LYME CARDITIS

A Dissertation Presented

by

CHRIS M. OLSON JR.

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2009

Department of Animal Biotechnology and Biomedical Sciences
MODULATION OF MACROPHAGE RESPONSES TO BORRELIA
BURGDORFERI IN ACUTE MURINE LYME CARDITIS

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I give my most sincere love, appreciation and respect to my mother and father for
the sacrifices that they have made in an effort to ensure a better future for those they love.

Let this dissertation be a symbol of your combined efforts; may it represent the beginning
to that future you have graciously afforded.
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A special gratitude belongs to Carolina Village and the American Heart Association for their financial support during my graduate studies.
ABSTRACT

MODULATION OF MACROPHAGE RESPONSES TO BORRELIA BURGDORFERI IN ACUTE MURINE LYME CARDITIS

MAY 2009

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The Lyme disease spirochete *Borrelia burgdorferi* is the only known human pathogen that directly activates invariant natural killer T (iNKT) cells. The number and activation kinetics of iNKT cells vary greatly among different strains of mice. Here, we report the role of the iNKT cell response in the pathogenesis of Lyme disease using C57BL/6 (B6) mice, a strain with optimal iNKT cell activation that is resistant to the development of spirochetal-induced inflammation. During experimental infection of B6 mice with *B. burgdorferi*, iNKT cells localize to the inflamed heart where they are activated by CD1d-expressing macrophages. Activation of iNKT cells in vivo results in the production of IFNγ, which we demonstrate controls the severity of murine Lyme carditis by at least two mechanisms. First, IFNγ greatly enhances the recognition of *B. burgdorferi* by macrophages, leading to increased phagocytosis of the spirochete. Secondly, IFNγ activation of macrophages increases the surface expression of CD1d, thereby facilitating further iNKT activation. Collectively, our data demonstrate that in the resistant background, B6, iNKT cells modulate acute murine Lyme carditis through the
action of IFNγ, which appears to self-renew through a positive feedback loop during infection.

Inflammation during infection with *B. burgdorferi* is dependent on the ability of the spirochete to evade local mechanisms of clearance. Even though macrophages are the main infiltrating cell during Lyme carditis, the identification of a receptor capable of mediating phagocytosis of *B. burgdorferi* has been elusive. Here, we demonstrate that the integrin CR3 is able to mediate binding to the spirochete and facilitate phagocytosis in a complement-dependent and independent manner. Expression of CR3, but not CR4, in CHO cells markedly enhanced their capacity to interact with *B. burgdorferi*, in the absence and presence of complement opsonization. Furthermore, the interaction between CR3 and *B. burgdorferi* is dependent on the metal-ion-dependent adhesion site (MIDAS) and could be blocked with EDTA. Inhibition of CR3 with blocking antibody was able to completely abrogate phagocytosis of *B. burgdorferi* by the macrophage-like RAW264.7 cells and partially block uptake by bone marrow-derived macrophages (BMMs), a finding that was recapitulated with CD11b-deficient BMMs. We further show that activation with recombinant IFNγ increases the transcription of CD11b and CD18, which correlates with increased surface expression of CR3, and that the effect of IFNγ on the phagocytosis of *B. burgdorferi* is circumscribed to CR3 activity, because inhibition of CR3 is able to completely diminish the effect of IFNγ on the phagocytosis of the *B. burgdorferi*. Lastly, our results demonstrate that CR3 is a negative regulator of proinflammatory cytokine induction in macrophages responding to *B. burgdorferi*. Overall, our data demonstrate roles for CR3 in the binding, phagocytosis and proinflammatory cytokine elicited by *B.
*burgdorferi* and shed light on the role of IFNγ in mediating the clearance of the spirochete during Lyme disease.
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CHAPTER I

THE ROLE OF V\textsubscript{α}14-J\textsubscript{α}18 INVARIANT NKT CELLS DURING LYME BORRELIOSIS

Introduction

Lyme disease was first discovered in 1976 as a tick-borne spirochetosis affecting a cluster of children in Lyme, Connecticut. In 1982 Dr. Willy Burgdorfer and colleagues identified the agent of Lyme disease as a new Gram-negative, extracellular bacterium belonging to the eubacteria phylum of spirochetes (24). This spirochete was named *Borrelia burgdorferi*. Since then, it has been established that at least three species of the genus *Borrelia* are agents of Lyme disease. These include *B. burgdorferi* sensu stricto, *B. afzelii* and *B. garinii*, which are collectively referred to as *B. burgdorferi* sensu lato (5, 25). Eight minimally pathogenic or nonpathogenic genospecies of *B. burgdorferi* have been identified, which geographically span the United States and Eurasia (Table 1.1) (45, 67, 81, 90, 91, 110, 134). *B. burgdorferi* sensu stricto is the etiologic agent of Lyme disease in the United States and other parts of the world, while *B. afzelii* and *B. garinii* are agents of Lyme disease that are restricted to Europe and Asia (5, 25). Infection with Lyme disease-causing spirochetes has also been observed in Japan, Russia and China. In the United States, transmission of the spirochete is by hard-bodied ticks of the *Ixodes* complex, mainly *I. scapularis* and *I. pacificus*. Importantly, Lyme disease is the most commonly reported arthropod-borne disease in the United States and Europe.
Enzootic life cycle of *B. burgdorferi*

*B. burgdorferi* and its associated genospecies have complex enzootic life cycles involving ticks of the *Ixodes* complex and a wide range of mammals (139). Ixodid ticks have three feeding periods during their usual 2-year life cycle. During these periods, ticks feed to repletion on the blood of certain vertebrate hosts. In the case of *I. scapularis*, larval ticks feed during the late summer and then molt into a nymphal tick, which feeds the following year during late spring or early summer. Larval and nymphal *I. scapularis* ticks feed on certain rodents, including the white-footed mouse and chipmunk (Figure 1.1) (83, 88). The life cycle of *B. burgdorferi* depends on horizontal transmission, meaning that the larval and nymphal tick must share a host. As such, during late spring or early summer rodents are infected with *B. burgdorferi* by infected nymphs, and in the late summer, larvae acquire *B. burgdorferi* from the infected rodents. Adult *I. scapularis* feed on large mammals during the fall, in particular, the white-tailed deer, and then lay eggs, which persist through the winter and spring and hatch the following summer (Figure 1.1) (136). Although the white-tailed deer is not directly involved in the enzootic life cycle of *B. burgdorferi*, it is required for survival of *I. scapularis* (136). Humans are not involved in the maintenance of *B. burgdorferi* in the wild, and thus are not part of the enzootic life cycle of the spirochete. Instead, humans are an incidental host, which acquire the infection from infected ticks questing for their next blood meal. Most human infections occur during late spring or early summer, which is coincident with the nymphal feeding period.
The vector ecology of *B. burgdorferi* and genospecies is regional. For example, in the Western United States, two intersecting enzootic cycles are required for human infection with *B. burgdorferi*. One cycle, involving the dusky-footed wood rat and *I. spinipalpis* ticks, which do not bite humans, maintain the *B. burgdorferi* in nature. The other cycle, involves dusky-footed wood rats and *I. pacificus* ticks, which are less often infected, but which do bite humans (23). In the southeastern United States, nymphal *I. scapularis* ticks feed primarily on infection-resistant lizards, and as a consequence, the prevalence of *B. burgdorferi* in nature is low, along with the incidence of human infection (77). In Europe, *B. burgdorferi* sensu lato is maintained in nature by *I. ricinus*; however, the preferred host of these ticks is being debated because they feed on more than 300 animal species (50). In Asia, the enzootic life cycle of *Borrelia* species involve larval and nymphal *I. persulcatus* ticks, which feed on voles, shrews and birds and adult ticks, which feed on larger animals (72).

**The biology of *B. burgdorferi***

The *B. burgdorferi* (strain B31) genome has been completely sequenced. It has a single linear chromosome of around one megabase and 21 plasmids, many of which are indispensable (44). *B. burgdorferi* does not possess LPS-encoding genes (44) or any recognizable toxins, despite the ability of this microorganism to cause human disease. In addition, *B. burgdorferi* express very few proteins with recognizable biosynthetic activity, and therefore, the organism depends on the host for most of its nutritional requirements.
*Borrelia burgdorferi* is approximately 0.2 to 0.3 µm by 20 to 30 µm, and is a vigorously motile bacterium due to the presence of periplasmic flagella, which run lengthwise between the peptidoglycan layer and the outer membrane. Flagella originate at the ends of the spirochete where they are anchored to the cytoplasmic membrane and wind around the protoplasmic cylinder (Figure 1.2B) (49). The presence of flagella also gives *B. burgdorferi* its distinctive corkscrew shaped morphology and may be important in the invasiveness of the spirochete into target tissues and organs (Figure 1.2A).

One of the most striking features of the *Borrelia* genome is the large number of open reading frames (ORF's) encoding lipoproteins (44), including outer-surface lipoproteins (Osp's) A through F and VLsE, an important lipoprotein that undergoes antigenic variation and is involved in immune evasion (144). Five percent of the chromosomal ORF's encode putative lipoproteins, whereas 14.5% to 17% of the functionally complete ORFs contained in these *B. burgdorferi* plasmids encode lipoproteins (44). Lipoproteins are distributed throughout the outer leaflet of the inner-membrane (cytoplasmic membrane) and both the inner and the outer leaflets out the outer-membrane. The abundant lipoprotein coding potential of *B. burgdorferi* suggests that lipoproteins may be important for the survival of the spirochete. During tick feeding, as a result of increased temperature and nutrient availability, as well as reduced pH, expression of several *B. burgdorferi* lipoproteins is augmented (3).
Transmission of *B. burgdorferi* into the human host

The Ixodid tick, unlike any other hematophagous arthropod vector, engorges for 4 to 8 days on the vertebrate host in order to feed to repletion (123). Transmission of *B. burgdorferi* into the human host requires between 40 and 48 hours following *I. scapularis* attachment. During the acquisition of the bloodmeal, *B. burgdorferi* differentially regulates several genes that enable the passage from the midgut through the hemolymph and into the salivary glands (3). For instance, the lipoprotein OspA is expressed at high levels in the gut of the unfed tick, but is downregulated upon tick feeding, while the expression of OspC increases 90 fold (128). OspA is involved in the attachment of *B. burgdorferi* to the midgut of the tick through its interaction with the tick receptor TROSPA, which protects the spirochete from the digestive and defense pathways of the tick (105-107). Thus, downmodulation of OspA facilitates the transmission of the spirochete into the human host (106). Furthermore, upregulation of OspC is required for the survival of the spirochete in the human host during the initial phase of infection (54, 108), although its exact role both in the tick and the mammalian host remains unclear (54, 108).

The first barrier *B. burgdorferi* encounters during its colonization of the human host is serum complement, which occurs during its deposition into the dermis. Complement is a collection of serum proteins and cell surface receptors that are involved in the early response to pathogens, including *B. burgdorferi* (80). Destruction of microorganisms via complement involves the formation of a pore in the microbial cell membrane by the membrane attack complex (MAC), which results in the lysis of the
organism. Three different pathways elicit complement activation: the classical (antigen antibody-mediated), lectin and alternative (pathogen surface) pathway. These pathways converge at the level of C3 convertase, a protease that cleaves complement component C3 into C3a and C3b. As a result, C3b can 1) bind to the surface of the bacteria and facilitate internalization of the spirochete via opsonization or 2) bind C3 convertase and facilitate the deposition of downstream components onto the bacterial surface resulting in the formation of the MAC and lysis of the cell.

*B. burgdorferi* activates the classical and alternative pathways of the complement cascade (69). The activation of complement has been associated with decreases in spirochetal numbers in different tissues of infected mice (80), indicating the importance of the complement system early in *B. burgdorferi* infection. The members of the *B. burgdorferi* sensu lato group, which includes *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii*, have evolved a variety of mechanisms enabling them to escape complement-mediated lysis, including the expression of complement regulator-acquiring surface proteins (CRASPs) and OspE-related proteins (ERPs), which serve as binding sites for the complement inhibitor factor H and factor H-like protein 1 (FHL-1) (59, 73, 74) The interaction of factor H with these proteins recruits a protease (factor I) that cleaves and inactivates the complement serum proteins, C3b and C4b. Cleavage of these two complement proteins prevents the deposition of downstream components onto the surface of the spirochete; thereby, halting the formation of the membrane attack complex. *B. burgdorferi* also express a CD59-like molecule on the outer membrane, which can inactivate MAC and prevent complement-mediated lysis (109). It has been speculated that most borreliae are able to evade complement-mediated lysis, and recently a novel
protein expressed by *B. hermsii* (a relapsing fever spirochete) has been discovered which affords protection to spirochete by inactivating C3b (61). However, iC3b serves as a ligand for the complement receptor CR3, which mediates the internalization of iC3b-opsonized pathogens. Thus, although the expression of CRASP-1 could circumvent MAC-mediated killing, it could also generate iC3b in close proximity to the spirochete resulting in CR3-mediated uptake and subsequent phagocytic destruction. The relative contribution between these two modes of complement-mediated killing during Lyme pathogenesis is not clear. Because *B. burgdorferi* express CRASPs, we hypothesize that CR3-mediated uptake of iC3b opsonized spirochetes would represent an important mechanism of bacterial clearance, especially during the initial deposition of the bacterium in the skin, which occurs during the bloodmeal and also during the dissemination of the spirochete through the mammalian circulation. Moreover,

During the acquisition of the blood meal and transmission of the spirochete, the tick hypostome punctures the host dermis activating the host's hemostatic and innate immune defenses resulting the recruitment of innate immune cells including neutrophils and macrophages, among other cell types (135). One way *B. burgdorferi* overcomes this cellular challenge is by usurping tick salivary proteins that are potent suppressors of the host defense mechanisms, including neutrophil, macrophage, B cell and T cell activation (4, 38, 51, 57, 70, 71, 78, 111, 112, 131-133).

**Lyme disease pathogenesis**

Following deposition into the dermis and successful colonization, the highly motile *B. burgdorferi* establishes local infection (Stage I, Lyme disease). This is marked
by the appearance of a slowly expanding skin lesion called erythema migrans (EM), which forms at the site of the tick bite 70 to 80 percent of the time and may take between 3 and 32 days to appear (122, 126). In the United States, other symptoms that may appear at this stage include fever, headache, malaise, myalgia and/or arthralgia and clinical signs that suggest spirochete dissemination (125).

The spirochete then disseminates throughout the body by binding to blood components, including plasminogen and its activators, activated platelets and integrins lining the endothelium of blood vessels (Stage II, Lyme disease) (30-32). Symptoms of stage II can include conduction system abnormalities, meningitis, and acute arthritis, which appear in 60% of untreated individuals in the United States (124). Some untreated individuals develop stage III Lyme disease, which is generally characterized by prolonged infection with the spirochete. Late stage symptoms may include chronic arthritis, neuroborreliosis, or cutaneous lesions such as acrodermatitis chronica atrophicans.

Clinical presentations of Lyme disease are the result of local inflammatory reactions to the spirochete. Consequently, _B. burgdorferi_ is the subject of intensive investigation in order to elucidate the mechanisms that contribute to its ability to cause persistent infection and multi-system disease, despite the development of strong immune responses. The control of the number of bacteria in the mammalian host likely depends on several immune effector mechanisms, such as the development of antibody responses and the capacity of phagocytic cells to clear spirochetes.

**Murine models of Lyme disease**
Among the inflammatory conditions observed in infected humans, Lyme arthritis and carditis are reproduced to some extent in the murine model of infection with the spirochete (7). Mice become persistently infected upon experimental infection and develop joint and cardiac inflammation that resembles the human disease (6). The incidence and severity of murine Lyme borreliosis is dependent on the genetic background of the infected animals. Thus, C3H mice develop Lyme carditis and arthritis with a greater incidence and severity than strains such as DBA/2, B6 or 129 (6, 22, 141). As a result, we prefer to use the B6 model of *B. burgdorferi* infection, because the modulatory affects of specific cell types are not constrained by the high degree of inflammation observed in highly susceptible mouse strains, such as C3H. The basis for the genetic susceptibility to the development of Lyme inflammation is unknown. Among the differences among diverse inbred strains of mice, a notable factor that can influence the response to infection and the outcome of infectious inflammation is the capacity of iNKT cells to respond to specific ligands. Thus, recent reports have demonstrated that iNKT cells from B6 mice respond more vigorously and with faster kinetics than those from other strains, including BALB/c and C3H (116). These results make B6 mice an ideal model to test the response of iNKT cells in the context of murine infection.

**T cell activation during experimental Lyme borreliosis**

The T cell-derived cytokine, IFNγ, has been shown to enhance cellular responses to various pathogens, most notably intracellular pathogens, including viruses, bacteria and parasites. Reports have suggested that an intact IFNγ response is important for controlling the pathogenesis of Lyme carditis but not Lyme arthritis (16, 20, 22, 52). This is an interesting observation given that *B. burgdorferi* is an extracellular spirochete.
Although the presence of T cells in the absence of B cells results in the exacerbation of Lyme carditis (94), mice lacking T cells failed to resolve cardiac inflammation at 60 days of infection (93) and CD4⁺ T cells have been shown to mediate the regression of murine Lyme carditis 45 days after infection (16). The contribution of invariant NKT cells to these responses has until now not been determined.

**Invariant Natural Killer T cell activation**

Natural Killer T (NKT) cells are a unique and complex population of T lymphocytes that co-express αβ T cell (TCR) and NK receptors (53, 75). In humans, NKT cells nearly always have a Vα24-Jα18 TCR (75), whereas mouse NKT cells possess a Vα14-Jα18 rearrangement. In both humans and mice, this invariant TCR is capable of antigen recognition in the context of an MHC class I-like molecule, CD1d, which is adapted for the presentation of lipid antigens (130). Experimental infection of B6/129 mice deficient in CD1d with *B. burgdorferi* revealed that CD1d has an important role in controlling spirochete burdens as well as Lyme arthritis (76). Although CD1d-deficient mice lack Vα14-Jα18i NKT cells because of a developmental failure in the thymus, they also lack other CD1d-reactive T cells with more diverse TCRs (26, 27, 76) and further show deficient marginal zone B cell responses, which are involved in the response to *B. burgdorferi* (10, 11). This pleiotropy complicates the assignment of the observed phenotype to a single cell type, such as iNKT cells. In contrast, Jα18-deficient mice specifically lack Vα14-Jα18i NKT cells and thus represent a suitable model to determine the role of iNKT cells in the pathogenesis of Lyme disease. Upon activation, Vα14-Jα18i NKT cells rapidly produce large quantities of cytokines such as IFNγ, IL-4, IL-17, IL-5 and IL-13, which can modulate many immunological processes, including
tumor immunity, maintenance of self-tolerance, prevention of autoimmune disorders and protection from a variety of pathogens during experimental infections (75, 96).

Pathogenic bacteria can also activate Vα14-Jα18i NKT cells indirectly through Toll-like receptor (TLR) 4, 7 or 9-driven maturation of dendritic cells, which then present self-glycolipids in the context of CD1d and soluble factors such as IL-12 or type 1 interferon (19, 92). Until recently, the identification of an iNKT cell antigen expressed by a human pathogen capable of directly activating iNKT cells eluded investigators. Kinjo and co-workers reported that the Lyme disease spirochete *Borrelia burgdorferi* expresses a diacylglycerol glycolipid, BbGL-IIc, which acts as a natural antigen for murine and human iNKT cells (68). To date *B. burgdorferi* is the only known human pathogen containing an antigen capable of activating iNKT cells.

**Antibody responses during Lyme borreliosis**

Prior to the development of an antibody-specific immune response, the degree of clearance by circulating phagocytes is very limited, indicating that the humoral response to *B. burgdorferi* might be necessary for resolution of spirochetal infection. Plasma cells are the principal effector cells involved in the humoral response, and antibodies are specific and powerful effector molecules of the adaptive immune response. Once antibodies bind to their specific foreign antigen, they confer protection to the host using a variety of effector mechanisms. In general, antibodies contribute to adaptive immunity by neutralizing microbes or their products, activation of complement, and opsonization, which leads to phagocytosis of microorganisms. The activation of B cells and their differentiation into antibody secreting plasma cells often requires an interaction with helper T cells, which controls isotype switching as well as somatic hypermutation.
However, in response to *B. burgdorferi*, T cell independent humoral responses also confer protection to the host. T cell independent humoral immune responses are critical for resolving symptoms associated with *B. burgdorferi* infection (93). T cell deficient mice infected with *B. burgdorferi* mount a protective antibody response, which upon passive serum transfer affords protection to severe combined immune deficient (SCID) mice from homologous challenge (93, 118). However, mice that lack both B and T cells developed severe arthritis and carditis in response to infection with *B. burgdorferi* (94).

Stimulation of CD40L, which interacts with CD40 on T cells, is crucial for T cell dependent activation of B cells and subsequent antibody production. Mice deficient for CD40L are also able to generate protective antibody responses against *B. burgdorferi* and therefore resolve infection (43). Additionally, Class II MHC deficient mice are able to resolve symptoms associated with *B. burgdorferi* infection (42). Thus, it is implied that *B. burgdorferi* antigens have the ability to stimulate B cells, to expand clonally, and to differentiate into antibody producing plasma cells, and that T cell independent humoral responses are extremely important for the protective immunity against *B. burgdorferi*.

The importance of antibodies in controlling *B. burgdorferi* infection was underscored by the discovery of complement-independent bactericidal antibodies, which have the capacity to control an infection. An IgG1k monoclonal antibody termed CB2 binds to *B. burgdorferi*-OspB via a single lysine residue causing an alteration in the structure of OspB; thereby, increasing its susceptibility to protease degradation, and ultimately leading to the lysis of the spirochete (41). Another bactericidal antibody, H6831, with identical specificity to OspB was reported. Both CB2 and H6831 are so
effective at killing *B. burgdorferi* that their selective pressure on the growth of *B. burgdorferi* was used to generate escape mutants of *B. burgdorferi* that lacked the lysine necessary for antibody binding to OspB, which made the escape mutants less infectious (34).
<table>
<thead>
<tr>
<th>Pathogenic species (B. burgdorferi sensu lato)</th>
<th>Principal tick vector</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. burgdorferi</em> sensu stricto</td>
<td><em>I. scapularis, pacificus &amp; ricinus</em></td>
<td>NE, NC &amp; W USA, Europe</td>
</tr>
<tr>
<td><em>B. garinii</em></td>
<td><em>I. ricinus, I. persulcatus</em></td>
<td>Europe &amp; Asia</td>
</tr>
<tr>
<td><em>B. afzelii</em></td>
<td><em>I. ricinus, I. persulcatus</em></td>
<td>Europe &amp; Asia</td>
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<tr>
<th>Non-pathogenic species</th>
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<tbody>
<tr>
<td><em>B. andersonii</em></td>
<td><em>I. dentatus</em></td>
<td>Eastern US</td>
</tr>
<tr>
<td><em>B. bissettii</em></td>
<td><em>I. spinipalpis &amp; I. pacificus</em></td>
<td>Western US</td>
</tr>
<tr>
<td><em>B. valaisiana</em></td>
<td><em>I. ricinus</em></td>
<td>Europe &amp; Asia</td>
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<tr>
<td><em>B. lusitaniae</em></td>
<td><em>I. ricinus</em></td>
<td>Europe</td>
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<tr>
<td><em>B. japonica</em></td>
<td><em>I. ovatus</em></td>
<td>Japan</td>
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<td><em>B. tanukii</em></td>
<td><em>I. tanukii</em></td>
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<tr>
<td><em>B. turdae</em></td>
<td><em>I. turdus</em></td>
<td>Japan</td>
</tr>
<tr>
<td><em>B. sinica</em></td>
<td><em>I. persulcatus</em></td>
<td>China</td>
</tr>
</tbody>
</table>

**Table 1.1. Pathogenic versus non-pathogenic *B. burgdorferi* and their respective tick vectors and geographic distribution.**

*B. burgdorferi* sensu lato is comprised of the three genospecies of *B. burgdorferi* that cause human Lyme disease. In addition to these, eight species of *Borrelia* have been identified which are non- or minimally pathogenic. *B. burgdorferi* sensu stricto is the causative agent of Lyme disease in North America.
Larval *Ixodes scapularis* ticks feed during the late summer and then molt into a nymphal tick, which feeds the following year during late spring or early summer. Larval and nymphal *I. scapularis* ticks feed on certain rodents, including the white-footed mouse and chipmunk. During late spring or early summer rodents are infected with *B. burgdorferi* by infected nymphs, and in the late summer, larvae acquire *B. burgdorferi* from the infected rodents. Adult *I. scapularis* feed on large mammals during the fall, and in particular, the white-tailed deer, and then lay eggs which persist through the winter and spring and hatch the following summer. Although the white-tailed deer is not directly involved in the enzootic life cycle of *B. burgdorferi*, it is required for survival of *I. scapularis*.
Figure 1.2. The morphological characteristics and structure of *B. burgdorferi*.

(A) *Borrelia burgdorferi* is approximately 0.2 to 0.3 µm by 20 to 30 µm, and (B) is a vigorously motile bacterium due to the presence of periplasmic flagella, which run lengthwise between the peptidoglycan layer and the outer membrane and originate from either end of the spirochete where they are anchored to the cytoplasmic membrane and wind around the protoplasmic cylinder. The presence of flagella also gives *B. burgdorferi* its distinctive corkscrew shaped morphology and may be important in the invasiveness of the spirochete into target tissues and organs.
Materials and Methods

Mice and infection with *B. burgdorferi*

Jα18-deficient mice (36) were bred at UMass Amherst. The mice have been backcrossed to the C57Bl/6 (B6) background more than 10 generations. Age-matched B6 mice were purchased from Jackson laboratories. IFNγRα-deficient mice in a 129 Sv/J background were purchased from Jackson laboratories and bred at UMass Amherst. Six to 8 week old mice were infected by subcutaneous injection with $10^5$ *B. burgdorferi* strain 297 in the midline of the back, as previously described (58). At 2 weeks of infection, which represents the acute phase of disease, the mice were sacrificed and analyzed for inflammation and bacterial burdens, as previously described (100). Arthritis and carditis were evaluated histologically in formalin-fixed sections processed for H&E staining. The joints were also decalcified. The hearts were cut in half through bisections across the atria and ventricles prior to sectioning. Signs of arthritis were evaluated as described (100), based on a combined assessment of histological parameters of *B. burgdorferi*-induced inflammation, such as exudation of fibrin and inflammatory cells into the joints, alteration in the thickness of tendons or ligament sheaths, as well as hypertrophy and hyperplasia of the synovium. Signs of carditis were evaluated based on the cardiac inflammatory infiltrate, including the infiltration of connective tissue with macrophages at the base of the heart, including surrounding the aortic valve and the atria. Carditis was scored on a scale of 0 (no inflammation), 1 (mild inflammation with less than two small foci of infiltration), 2 (moderate inflammation with 2 or more foci of infiltration), or 3 (severe inflammation with focal and diffuse infiltration covering a large area). Both joint and heart tissue sections were blindly examined. The Institutional
Animal Care and Use Committee at UMass Amherst approved all procedures involving animals.

**Cells and stimulations**

Mouse bone marrow cells were collected from the femoral shafts by flushing 3 times with 1 ml of cold RPMI (10% FCS and 1% penicillin/streptomycin) supplemented with 20% L929-conditioned RPMI. The cell suspensions were cultured in 100mm x 15mm petri dishes (Fisher Scientific) in 20% L929-conditioned RPMI for 8 days at 37°C with 5% CO₂. Following incubation, non-adherent cells were eliminated and adherent macrophages scraped, counted and resuspended in serum-free RPMI medium without antibiotics prior to use. RAW264.7 macrophages were cultured in RPMI but washed and resuspended in serum- and antibiotic-free RPMI before use. 10⁶ BMMs or RAW264.7 cells per ml were incubated with recombinant murine IFNγ (rmIFNγ; carrier- and endotoxin-free; eBioscience, Inc., San Diego, CA) (0, 0.5, 5, 50 ng ml⁻¹) for 12h and analyzed for expression of CD1d (PE-anti-mouse CD1d, eBioscience, Inc.) or stimulated with live *B. burgdorferi* (strain 297) at a 25:1 multiplicity of infection (m.o.i) for 12h. Following stimulation with *B. burgdorferi*, culture supernatants were analyzed by ELISA for TNFα and IL-6, as previously described (103).

**B. burgdorferi-specific antibodies**

Analysis of total IgG and IgM were determined by coating 96 well plates with 0.5 µg/ml (IgG) or 1.0 µg/ml (IgM) of a *B. burgdorferi* strain 297 lysate and incubated with serial two-fold dilutions starting at 1:100 followed by incubation with HRP-conjugated anti mouse IgM or IgG (BD Pharmingen) (1:10000 dilution). The reactions were
developed using 1-component TMB substrate and stopped with TMB stop solution (KPL, Inc., Gaithersburg, MD).

**Quantitative analysis of IFNγ expression**

The relative expression of IFNγ in cardiac tissue was determined using total RNA extracted from the base of the heart using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA was treated with DNase I (Promega, Madison, WI), and reverse transcribed using random primers (Invitrogen) and SuperScript II reverse transcriptase (RT) (Invitrogen). The cDNA was amplified using primers specific for glyceraldehyde 3-phosphate dehydrogenase (gadph; 5’-CCA TCA CCA TCT TCC AGG AGC GAG-3’ and 5’-CAC AGT CCT CTG GG TGC AGT GAT-3’) and *ifnγ* (5’-GCG TCA TTG AAT CAC ACC-3’ and 5’-GGA CCT GTG GGT TGT TGA CC-3’) in an Mx3005P® QPCR System (Stratagene, La Jolla, CA) and SYBR green-containing reaction buffer (Roche, Nutley, NJ). Relative expression of the gene is referred to control-infected mouse heart tissue, as described (65).

**Detection of invariant NKT cells**

DNA from the hearts of control and infected mice were utilized to perform a nested-PCR reaction using primers specific for the Vα14-Jα18i TCR. For the first reaction, non-specific TCR (5’-GTC CTC AGT CCC TGG TTG TC-3’ and 5’-CTG CCT CCG AGG TAG TGA C-3’) was amplified using 5 µl of DNA in a total volume of 50 µl with the following conditions: 94°C 2 min, (94°C 1 min, 55°C 1.5 min, 72°C 1.5 min) x 30 and 72°C 2 min. A second reaction using primers specific for the Vα14-Jα18i TCR (5’-GAC AGT CCT GGT TGA CC-3’ and 5’-AAT GCA GCC TCC CTA AG-3’) was
performed using 5 µl of template from reaction 1 with the same conditions, except that 35 cycles were used.

**Confocal Microscopy**

At sacrifice, hearts were collected and snap-frozen in O.C.T. freezing medium (Sigma) and stored at -80ºC. Hearts were grossly cut through the ventricles parallel to the atria, sectioned using a Triangle Biomedical Sciences Cryostat (Durham, NC) and every sixth 10 micron section through the aortic sinus was placed on Fisherbrand Superfrost microscope slides (Fisher Scientific, Pittsburgh, PA). Sections were fixed in -20 ºC acetone for 5 min, rehydrated in distilled water for 3 min, blocked with Fc Block (anti-mouse CD16/32, clone 2.4G2; BD Pharmingen, Franklin Lakes, NJ) and stained with the following monoclonal antibodies: Alexa Fluor\textsuperscript{488} anti-mouse CD3 (clone 145-2C11), Alexa Fluor\textsuperscript{647} anti-mouse CD11b (clone M1/70), PE-anti-mouse CD1d (clone 1B1; BD Pharmingen). Nuclear staining was done with DAPI (Roche). The sections were imaged using a Zeiss LSM 510 META confocal laser scanning microscope (Zeiss Microimaging, Thornwood NY) operating in the multitrack mode, as described before (62).

**Analysis of phagocytosis**

BMM or RAW264.7 cells were activated with different doses of rmIFN\textgamma for 12 hour in serum- and antibiotic-free RPMI prior to the addition of GFP-expressing *B. burgdorferi* strain 297 at an m.o.i. of 10 for 4h. To distinguish between binding and internalization of spirochetes, the mixtures were incubated at 4 ºC and 37 ºC, in parallel. The incubation at 4 ºC results in the binding of particles, but not their internalization (15). After the incubation, the cells were washed and analyzed by flow cytometry. For Apotome epifluorescence analysis of phagocytosis, RAW264.7 cells were activated with
50 ng ml\(^{-1}\) of rmIFN\(\gamma\) and challenged with GFP-\textit{B. burgdorferi} at 37\(^\circ\) C, as described. The cells were then washed, cytospun and stained with the macrophage cell membrane marker F4/80 (clone BM8; rat anti-mouse F4/80, eBioscience, Inc.) followed by a secondary stain with Alexa Fluor\(^{594}\) goat anti-rat IgG (Invitrogen).

**Statistical analysis**

The results are presented as means \(\pm\) SE. Significant differences between means were calculated with the Student’s \(t\) test. P values of 0.05 or less were considered statistically significant.
Results and Discussion

\( V_{\alpha 14-J_{\alpha 18}} \) NKT cells modulate the pathogenesis of Lyme carditis

The identification of \( B. burgdorferi \)-derived diacylglycerol glycolipids as natural antigens to \( V_{\alpha 14} \) and \( V_{\alpha 24i} \)-NKT cells (68), prompted us to examine the role of \( V_{\alpha 14-J_{\alpha 18}} \) NKT cells in the pathogenesis of Lyme disease. To determine whether \( V_{\alpha 14-J_{\alpha 18}} \) NKT cells contribute to the host response to \( B. burgdorferi \), we syringe-infected wild type and \( J_{\alpha 18} \)-deficient C57Bl/6 mice with \( 10^5 \) spirochetes in the midline of the back and allowed infection to progress for 2-3 weeks, which represents the acute phase of infection (100). Histological evaluation of the joints and hearts of the infected mice revealed that \( J_{\alpha 18} \)-deficient animals are more susceptible to both arthritis (Figure 1.3A) and carditis (Figures 1.3B and C). Although there was a trend towards worse arthritis in mice that lacked \( J_{\alpha 18} \), the difference was not significant (Student t test; \( p = 0.07; n = 15 \)). In contrast, carditis severity in \( J_{\alpha 18} \)-deficient mice was significantly increased (\( p < 0.01 \)).
Figure 1.3. Jα18-deficiency results in increased carditis during infection with *B. burgdorferi*.

Joint (A) and cardiac (B) inflammation in wild type and Jα18-deficient mice 2 weeks following syringe infection with *B. burgdorferi*. Data are representative of 2 to 3 experiments with 5 mice per group. (C) Representative H&E-stained cardiac sections from uninfected, and infected wild type and Jα18-deficient mice.
Infection of Jα18-deficient mice results in increased spirochetal burdens in the heart

Inflammation during experimental infection with *B. burgdorferi* is dependent on the ability of the spirochete to persist in cardiac and joint tissue (141). Thus, we evaluated the ability of the mice to clear *B. burgdorferi* in the absence of Vα14-Jα18i NKT cells by assessing bacterial loads in the heart tissue of the infected animals, where the differences in inflammation are more evident as a result of the absence of iNKT cells. Compared to wild type mice, Jα18-deficient mice harbored more *B. burgdorferi* in their heart, while the bacterial burden in the ear was unaffected by the loss of Vα14-Jα18i NKT cells (Figure 1.4). Vα14-Jα18i NKT cells have been shown to potentiate the antibody response to various pathogens (46, 79). Since the antibody response to *B. burgdorferi* is critical to clear spirochetal infection (34), we assessed the *B. burgdorferi*-specific IgM and IgG responses 2 weeks following infection. The sera levels of antigen-specific IgM and IgG in wild type and Jα18-deficient mice were similar (Figures 1.5A and B) as well as IgG antigen specificity as determined by Western blot (Figure 1.5C).

Moreover, studies have demonstrated that iNKT cells can promote naïve CD4⁺ T cell (Tₕ0) polarization towards a Tₕ1 or Tₕ2 effector phenotype, depending on the infectious agent (130). For example, iNKT cells enhance *Chlamydia trachomatis* infection through augmentation of the Tₕ2 response (14). The mechanism behind iNKT-directed T helper cell polarization has not been defined; however, it is intriguing to speculate that the early production of IFNγ by iNKT cells during infection with *B. burgdorferi* can promote differentiation of Tₕ0 cells toward the Tₕ1 phenotype by inducing IL-12 production. We therefore determined the contribution of iNKT cells to
Th1 differentiation during infection with *B. burgdorferi*. Restimulation of Jα18-deficient splenocytes, obtained from infected mice, with *B. burgdorferi* did not result in diminished levels of IFNγ, compared to wildtype controls (Figure 1.6).

These results demonstrate an important role for Vα14-Jα18i NKT cells in the control of *B. burgdorferi* at the tissue - rather than at the systemic - level where acute inflammation occur, and that the differences in *B. burgdorferi* control and pathology between the Jα18-deficient and wildtype mice are not due to distinct antibody responses or altered T cell polarization.
Figure 1.4. Jα18-deficient mice harbor increased bacterial burdens in the hearts during *B. burgdorferi* infection.

Quantitative real-time PCR determination of spirochete burden in the ears and heart of infected wild type (n=13 and n=8, respectively) and Jα18-deficient mice (n=14 and n=5, respectively) 2 weeks following infection in 2 separate experiments.
Figure 1.5. Jα18-deficient mice have a normal IgM and IgG response following B. burgdorferi infection.

Serum ELISA titers for total IgM (A) or IgG (B) from 2-week infected wild type and Jα18-deficient mice. Results depicted were derived from 2 uninfected mice and 7-15 mice per infected group and are representative of at least 2 independent experiments. (C) Immunoblot for total IgG from wildtype and Ja18-deficient mice 2-weeks post infection with B. burgdorferi. Numbers correspond to individual mice.
Figure 1.6. Vα14-Jα18i NKT cell-derived IFNγ does not impair normal Th1 polarization during infection with *B. burgdorferi*.

ELISA analysis of IFNγ following *B. burgdorferi*-specific restimulation of whole splenocytes obtained from wildtype or Jα18-deficient mice. Results depicted were derived from 3 wildtype mice and 7 Jα18-deficient mice per group.
Vα14-Jα18i NKT cells and CD1d-positive macrophages migrate to the inflamed heart of B. burgdorferi-infected mice

Vα14-Jα18i NKT cells have been shown to migrate to sites of inflammation in the lung, liver and spleen (1, 86, 95, 101). We therefore analyzed the presence of Vα14-Jα18i NKT cells in the hearts of uninfected and B. burgdorferi-infected mice using a nested PCR approach (37, 117). While control mice did not harbor Vα14-Jα18i NKT cells in the heart, the presence of the invariant TCR alpha chain was readily detected in the inflamed cardiac tissue of wild type, but not Jα18-deficient mice (Figure 1.7A). We also analyzed by confocal microscopy the hearts of the infected mice for the presence of CD1d, as a potential contributor to the activation of iNKT cells. The infected hearts contained, as expected, CD11b- and CD3-expressing lymphocytes, (Figure 1.7B), and confirmed (115) a predominant macrophage infiltration during Lyme carditis. The inflamed hearts also showed increased expression of CD1d compared to uninfected controls (Figure 1.7B). Because CD1d expression is a requirement of APC-mediated activation of iNKT cells, we determined the identity of the iNKT cell activator in the inflamed heart. Confocal analysis revealed that CD1d expression colocalized with CD11b expression (Figure 1.7C). The presence of CD1d in the inflamed hearts indicates the potential for the direct activation of Vα14-Jα18i NKT cells through presentation of B. burgdorferi glycolipids. Taken together, the appearance of iNKT cells in a B. burgdorferi colonized organ competent in iNKT antigen presentation, which presents with exacerbated disease and increased bacterial burden in the absence of iNKT cells, is indicative of an important role for this cell type in protection against murine Lyme carditis.
Figure 1.7. Vα14-Jα18i NKT cells and CD1d positive macrophages migrate to the inflamed heart during infection with *B. burgdorferi*.

(A) Nested PCR was used to detect the presence of the Vα14-Jα18i TCR in the inflamed heart of wild-type infected mice. *gapdh* amplification was used to verify the presence of DNA. The data are derived from 2 control mice and 3 mice per infected group. (B) Confocal microscopy analysis of heart sections of control or infected B6 mice to determine the presence of CD1d⁺ (red), CD11b⁺ (purple) and CD3⁺ cells (green). Nuclei were stained with DAPI. Results depicted here are representative of 2 experiments. (C) Confocal microscopy of a *B. burgdorferi*-infected heart showing the presence of CD11b⁺ (green) cells and their co-localization with CD1d (red) staining. The panel on the right shows co-localization of both signals.
**Vα14-Jα18 iNKT cells are responsible for the production of IFNγ in the infected heart**

*B. burgdorferi* glycolipid-pulsed dendritic cells induce IFNγ production by Vα14-Jα18i NKT cells in vivo (68). In addition, IFNγ-producing CD4+ T cells facilitate the regression of murine Lyme carditis (16), while Stat1-deficient mice infected with *B. burgdorferi* develop exacerbated Lyme carditis, compared to control mice (20). We therefore addressed the contribution of Vα14-Jα18i NKT cells to the pool of cardiac IFNγ in *B. burgdorferi*-infected mice. *B. burgdorferi* infection of wild type mice resulted in increased expression of the *ifnγ* gene in the cardiac tissue compared to uninfected controls, as determined by quantitative RT-PCR (Figure 1.8). However, the level of *ifnγ* gene expression in the hearts of *B. burgdorferi*-infected mice was reduced in Jα18-deficient mice at the peak of disease (Student’s t test, p < .05; Figure 1.8). These results indicate that Vα14-Jα18i NKT cells are critical for generating an IFNγ response in the heart, which might play a role in the pathogenesis of Lyme carditis.

We next tested the direct contribution of IFNγ to the pathogenesis of Lyme carditis by infecting IFNγ receptor alpha (IFNγRα)-deficient 129S mice. Like Jα18-deficient mice, IFNγRα-deficient mice developed increased cardiac inflammation following syringe infection with *B. burgdorferi* (Student t test; p < .05; Figure 1.9A), supporting a role for IFNγ in the protection against Lyme carditis. The quantification of *B. burgdorferi* burdens by real-time PCR in the heart of the infected mice showed a significant (p < .006) increase in the levels of bacteria in the absence of IFNγ-mediated signals (Figure 1.9B), indicating that IFNγ contributes to the control of bacterial burdens.
during infection. Overall, these data indicate that Vα14-Jα18i NKT-derived IFNγ mediates protection against cardiac inflammation during infection with B. burgdorferi.
Figure 1.8. Vα14-Jα18i NKT cells regulate the expression of IFNγ in *B. burgdorferi*-infected hearts.

Relative IFNγ mRNA expression in the heart by real-time RT-PCR. Hearts were removed from 2-week *B. burgdorferi*-infected wildtype or Jα18-deficient C57Bl/6 mice. Results are the average of 3 mice per group.
Figure 1.9. IFNγ mediates protection against Lyme carditis and facilitates clearance of *B. burgdorferi* from the heart.

(A) Increased cardiac inflammation in IFNγRα-deficient mice at 2 weeks of infection with *B. burgdorferi*, compared to wild type mice. Results are derived from 2 independent experiments. (B) Bacterial burdens in the heart of wild type (n=5) and IFNγRα-deficient, infected mice at 2 weeks of infection. * Student’s T test, p<0.006.
IFN\(\gamma\) signaling enhances macrophage responses to \(B.\) burgdorferi

The mechanism underlying the control of cardiac inflammation during murine Lyme borreliosis by IFN\(\gamma\) has not been elucidated. IFN\(\gamma\) enhances cellular responses, including macrophages, to various pathogens (17). Indeed, activation of bone marrow-derived macrophages (BMMs) with IFN\(\gamma\) enhances spirochete recognition, as evidenced by augmented TNF\(\alpha\) and IL-6 production elicited against live \(B.\) burgdorferi (Figures 1.10A and B). Increased \(B.\) burgdorferi burdens in the heart of IFN\(\gamma\)R\(\alpha\)-deficient and J\(\alpha\)18-deficient mice during the presence of a strong antibody response suggested a defect in phagocytic clearance of the spirochete by infiltrating macrophages. We therefore defined the contribution of IFN\(\gamma\) to macrophage-mediated clearance of \(B.\) burgdorferi by phagocytosis. First, we determined by flow cytometric analysis that BMMs readily phagocytose GFP-expressing infectious \(B.\) burgdorferi (Figure 1.10C). Activation with recombinant murine IFN\(\gamma\) increased the already high phagocytic capacity of BMMs for \(B.\) burgdorferi (Figure 1.10C). We also performed this assay using the macrophage cell line RAW264.7. Incubation of RAW264.7 cells with GFP-\(B.\) burgdorferi resulted in a modest amount of phagocytosis, compared to BMMs, confirming previous reports indicating their relatively low phagocytic capacity (39). The activation of these cells with IFN\(\gamma\) markedly enhanced the percentage of cells that had internalized GFP-expressing \(B.\) burgdorferi, as determined by flow cytometric analysis and fluorescence microscopy (Figures 1.10D and E). These results suggested that iNKT cells control the levels of spirochetes in the infected heart through the production of IFN\(\gamma\) and increased phagocytosis.
Figure 1.10. IFNγ enhances the effector function of macrophages responding to B. burgdorferi.

(A) rmIFNγ activation (0, 0.5, 5, 50 ng/ml) of BMMs for 16 h leads to enhanced production of TNFα (A) and IL-6 (B) in response to live B. burgdorferi. (C) BMMs were stimulated with (green histogram) or without (black histogram) 50 ng/ml of rmIFNγ for 16 h. The cells were washed and assessed their phagocytic capacity for GFP-B. burgdorferi at an m.o.i of 25:1. (D) RAW264.7 cells were stimulated with 50 ng/ml of rmIFNγ for 16 h and assessed their phagocytic capacity, as before. (E) The cells were also cytopspun in positively charged slides, stained for the macrophage surface marker F4/80 (red) and the nucleus (blue) and analyzed by fluorescence microscopy (ApoTome) for B. burgdorferi phagocytosis. Results are representative of at least 3 experiments.
**IFNγ enhances the surface expression of CD1d in macrophages**

Previous reports have suggested that IFNγ can increase CD1d surface expression by macrophages (75). We thus assessed the effect of IFNγ on the expression of CD1d in primary macrophages and RAW264.7 cells. We stimulated BMMs (Figure 2.9A) and RAW264.7 cells (Figure 1.11B) with 50 ng ml\(^{-1}\) of recombinant IFNγ for 16 h. The cells were subsequently assessed for surface CD1d expression by flow cytometry. IFNγ treatment increased the expression of CD1d in both BMMs and RAW264.7 cells (Figures 1.11A and B). Since *B. burgdorferi* induces the upregulation of CD1 molecules on human monocytes (33), we also assessed the effect of live spirochetes on CD1d expression by BMMs and RAW264.7 cells. In contrast to human monocytes, the stimulation of both BMMs and RAW264.7 cells with live *B. burgdorferi* resulted in a minimal increase of CD1d expression (data not shown). These results suggest that during infection with *B. burgdorferi*, Vα14-Jα18i NKT-derived IFNγ is self-renewing through its capacity to induce CD1d expression and presentation to Vα14-Jα18i NKT cells (Figure 1.12).
Figure 1.11. IFNγ increases the surface expression of CD1d in macrophages.

BMMs (A) and RAW264.7 cells (B) were stimulated with 50 ng/ml of rmIFNγ for 16 h (red histogram) or left unstimulated (black histogram). The cells were washed and stained with an anti-CD1d antibody labeled with phycoerythrin (PE). The grey histogram represents unlabeled cells. Results are representative of at least 2 experiments for each cell type.
Macrophages phagocytose invading *B. burgdorferi* and present antigen in the context of CD1d to local Vα14-Jα18 iNKT cells resulting in the production of IFNγ. IFNγ activation of macrophages results in enhanced phagocytosis of *B. burgdorferi* and upregulation of CD1d. These events further perpetuate iNKT cell activation and thus results in self-renewal of IFNγ.
CHAPTER II

COMPLEMENT RECEPTOR 3 MEDIATES THE PHAGOCYTOSIS OF B. BURGDORFERI AND NEGATIVELY REGULATES PROINFLAMMATORY CYTOKINE INDUCTION

Introduction

The recognition of pathogenic bacteria or their constituents by pattern recognition receptors (PRRs) on the surface of innate immune cells is critical for the induction of an effective immune response. In addition to regulating cytokine secretion, nitric oxide production and cell maturation, engagement of these receptors can trigger phagocytosis of microbes and also orchestrate adaptive immunity. The Toll-like receptors (TLRs) 1 and 2 function as PRRs for B. burgdorferi lipoproteins (2, 85), including the prototypical borrelial lipoprotein, OspA. Studies using the murine model of Lyme disease have shown that TLRs 1 and 2, as well as the TLR adaptor protein, MyD88, are important for controlling the expansion of B. burgdorferi in target organs and tissues (8, 18, 87, 137, 138). Thus, it is well established that TLRs, their adaptor proteins or their co-receptors, such as CD14, are important in the recognition of B. burgdorferi (120, 138), but in regards to the identification of other PRRs involved in B. burgdorferi recognition, not much progress has been made. Moreover, although TLR signaling might control phagocytosis of B. burgdorferi to some extent (121), the identification of a bonafide receptor involved in spirochete phagocytosis has eluded investigators.

Of particular interest are the β2 integrins, which include LFA-1 (CD11a/CD18), CR3 (CD11b/CD18) and CR4 (CD11c/CD18), because it was reported that reduced
expression of the common beta chain, CD18, results in exacerbated Lyme carditis during experimental infection with *B. burgdorferi* (55). Furthermore, CR3 and CR4 have been shown to participate in the recognition and phagocytosis of a number of pathogens, including *Bacillus anthracis* spores (102), *Neisseria meningitidis* (66), *Bordetella pertussis* (98) *Francisella tularensis* (12) and serum opsonized *B. burgdorferi* (29). The importance of β2 integrin-mediated immunoprotection is highlighted in patients suffering from leukocyte adhesion deficiency-1 (LAD-1) syndrome who suffer from severe symptoms, including defects in phagocytosis and recurrent infections. In these patients, mutations in CD18 result in little or no expression of LFA-1, CR3 or CR4 (60).

In this study, we determine the role of CR3 in mediating phagocytosis of the Lyme disease spirochete *B. burgdorferi* and the production of proinflammatory cytokines.

**Innate immune responses to *B. burgdorferi***

The recognition of pathogens or their constituents by cells of the innate immune system constitutes the ‘first line’ of host defense. The detection of conserved bacterial motifs, known as pathogen associated molecular patterns (PAMPs) initiates a cascade of responses that lead to the upregulation of chemokines and cytokines, adhesion molecules and a vast array of other effector molecules. These events are aimed at the recruitment of phagocytic cells, their activation and the eventual development of the adaptive immune response and depend on the presence of germline-encoded PRRs. These pattern recognition molecules are being extensively studied because they not only mediate the
initial response to microorganisms, but they also modulate the extent and quality of acquired immune response. Toll-like receptors (TLRs), identified as the mammalian counterparts of the Toll protein in *Drosophila* that mediates the immune response to fungi and Gram-positive bacteria are the most studied, but do not constitute the only mechanism by which innate immune cells recognize infecting microorganisms. C-type lectins, Scavenger receptors, integrins and Nod-like receptors are also PRR that are uniquely suited to recognize specific structures that are present in a group or groups of microorganisms and that distinguishes them from the more specific recognition of antigens by the T and B cell receptors. The main sentinel cells of innate immunity (epithelial, phagocytic and dendritic cells) express unique combinations of PRRs, which likely result in tissue-specific responses to pathogen (64). It is the stimulation of complex sets of pattern recognition receptors that induce an effective immune response. For example, cooperative signaling between the C-type lectin, Dectin-1 and TLR2 enhance the innate response to *Candida albicans* (47) and *Mycobacterium tuberculosis* (140), while cooperation between Mannose Binding Lectin (MBL) and TLR2/6 signaling from within the phagosome enhance the response to *Staphylococcus aureus* (63). Thus, multiple PRRs contribute to defining pathogen-specific innate immune responses.

TLRs collectively recognize conserved lipid, carbohydrate, peptide and nucleic-acid structures expressed by different groups of microorganisms. *B. burgdorferi* does not contain LPS, in contrast with other members of the spirochete group and therefore, does not engage TLR4. The most important pattern recognized by innate immune receptors is constituted by the triacylated outer-surface lipoproteins of the spirochete and this seems to be true for whole spirochetes and lysates. It is not clear what the specific interaction
between whole spirochetes and antigens exposed by bacterial lysis with immune cells is \textit{in vivo}. However, it is conceivable that both elicit immune responses since \textit{B. burgdorferi} is likely to die \textit{in vivo}, for example in the presence of antibody in a process that does not required complement or phagocytosis (34, 41, 89).

The interaction of \textit{B. burgdorferi} lipoproteins with complexes formed by TLRs 1 and 2 on the surface of innate immune and other cell types initiates a series of incompletely defined signaling cascades that results in the production of proinflammatory cytokines (IL-1\(\beta\), TNF\(\alpha\), IL-12, and IL-18, among others), chemokines (IL-8, MCP-1, KC), metalloproteinases and adhesion molecules (E-selectin, VCAM-1 and ICAM-1) (56). These proteins mediate the inflammatory response to the spirochete through the production of proinflammatory cytokines and chemotactic factors that further induce the recruitment of inflammatory cells. In addition TLR1/2 complexes also modulate adaptive immune responses, through increased antigen presentation, induction of costimulatory signals on antigen presenting cells and the production of cytokines that influence CD4\(^+\) T cell differentiation and effector function. Detection of \textit{B. burgdorferi} lipoprotein is important for defining the in vivo response to \textit{B. burgdorferi}, because TLR1-(2), TLR2-(137), or MyD88-deficient (18, 87) mice have significant increases in bacterial burden, compared to wildtype mice. Moreover, the development of arthritis in the absence of TLRs, CD14 (13) or MyD88 suggests that TLR-independent PRRs exist that trigger the inflammatory response to the spirochete. So, although innate sensing of the spirochete has solely been attributed to its recognition by TLR2/1 heterodimer complexes, the TLR response is likely amplified and further defined by cooperation with additional PRRs, which may or may not occur within the phagosome. Indeed, the induction of a potentiated
inflammatory response following phagocytosis of *B. burgdorferi*, compared to cell-surface stimulation, indicates the existence of additional PRRs involved in the recognition of *B. burgdorferi* (35, 99). Whether this augmented response is the result of cooperative signaling with the phagocytic receptor or other PRRs recruited to the phagosome is unclear.

**Mechanisms of phagocytosis**

Phagocytosis is an evolutionary conserved phenomenon whereby large particles are ingested and destroyed via an extraordinarily complex process involving cognate receptors that distinguish foreign particles from self. While lower, single-celled organisms use phagocytosis primarily for the acquisition of nutrients, phagocytosis in multi-cellular Metazoans is carried out by specialized cells and is critical for the uptake and degradation of apoptotic cells and infectious agents. The contribution of phagocytosis to Lyme disease pathogenesis has not been studied, primarily because the mechanisms involved in this process have yet to be elucidated. Presumably, this cardinal mechanism of bacterial clearance is necessary for control of *B. burgdorferi* infection prior to and following the development of the adaptive immune response.

Phagocytic cells possess several pathways of phagocytic uptake, which vary depending on the origin of the phagocytic signal. Opsonin-dependent pathways are mediated by FcR and complement receptors, including complement receptor 3 (CR3, CD11b/CD18 or Mac-1), which was shown to mediate phagocytosis of non-specifically opsonized *B. burgdorferi* by human polymorphonucleocytes. Opsonin-independent phagocytosis can be mediated by multiple pattern recognition receptors, including C-type
lectins, scavenger receptors and integrins. Following adherence of the pathogen to the phagocytic cell through receptor interaction, phagocytic uptake is initiated by F-actin remodeling. Actin remodeling at the site of attachment is required for all forms of phagocytosis. The signaling cascade involved in the reorganization of actin is highly dynamic and can vary depending on which receptor(s) the phagocytic signal emanates from. Following attachment and actin remodeling, particles are internalized into phagosomes. It is here that microbial destruction and antigen presentation are initiated, through subsequent fusions with acid rich vesicles called lysosomes.

**Recruitment of phagocytic cells**

The recruitment of phagocytic cells into sites of infection precedes the phagocytosis and destruction of pathogenic organisms. Recruitment is mediated by the production of chemokines, increased vascular permeability and upregulated expression of cell adhesion molecules in endothelial cells. *B. burgdorferi* induces the upregulation of these factors in different cell types. Chemokine production at sites of pathology in disease susceptible C3H/HeJ mice and –resistant C57BL/6J mice show that inflammation is related to increased production of neutrophil and monocyte-macrophage chemokines, KC and MCP-1, respectively (21). In Lyme disease patients, the production of chemokines, especially IL-8, during the initial response to *B. burgdorferi* correlates well with the onset of symptoms known to occur during the early stages of infection, suggesting that their production is increased during the beginning of the infection to recruit phagocytic cells, which are involved in the initial clearance of the spirochete. Overall, these results also suggest that the chemokine response is modulated once the infection progresses, and that
progression of the disease may be dependent on the regulation of these chemoattractants whose production might be altered by the presence of the infiltrating cells.

**Complement opsonization**

In addition to initiating the alternative pathway of complement activation and MAC-mediated lysis of microbes, C3b acts as a serum opsonin that enhances the phagocytic ingestion and destruction of microbes through recognition by Complement Receptor 1 (CD35) (113). The members of the pathogenic *B. burgdorferi* sensu lato group have evolved a variety of mechanisms enabling them to escape complement-mediated lysis, including the expression of complement regulator-acquiring surface proteins (CRASPs), which serve as binding sites for the complement inhibitor factor H and factor H-like protein 1 (FHL-1) (59, 73, 74). Expression of CRASPs ultimately results in the cleavage of bound serum complement protein C3b into the serum opsonin inactivated C3b (iC3b), which is recognized by Complement Receptor 3 (CR3, CD11b/CD18, Mac-1). Thus, although the spirochete has evolved mechanisms to evade MAC-mediated lysis through the cleavage of C3b, this very mechanism could also generate iC3b fragments in close proximity to the *B. burgdorferi* organism making complement-mediated phagocytosis through CR3 a particularly relevant form of host defense against the invading and disseminated spirochete.

**Complement Receptor 3**

CR3 (CD11b/CD18) is a transmembrane glycoprotein of the β2 integrin family with multiple substrate specificity that is primarily found on phagocytic cells. The
multifarious nature of CR3 is borne from its molecular structure, which consists of two distinct functional domains in the extracellular portion of the CD11b subunit. One such domain, the inserted domain (I-domain), of CR3 binds iC3b and intercellular adhesion molecule-1 (ICAM-1), among others, and thus participates in opsonin-mediated phagocytosis and diapedesis, respectively (114). The other domain, called the lectin-like domain (LLD), of CR3 mediates binding to several polysaccharide-containing pathogen associated molecules, such as filamentous hemagglutinin (FHA) of Bordetella pertussis and zymosan of Saccharomyces cerevisiae, where it participates in the formation of transmembrane signaling complexes with GPI-anchored glycoproteins resulting in adhesion, degranulation or phagocytosis (40). In addition, CR3 has been shown to mediate the phagocytosis of Neisseria meningitidis (66) and Bacillus anthracis spores (102) by macrophages. Overall, the extensive binding repertoire of CR3 facilitates intercellular interactions that are important for immunological maintenance of the host.
Materials and Methods

Mice

C57Bl/6 mice were bred at UMass Amherst. TLR2-deficient and CD11b-deficient C57Bl/6 mice were purchased from Jackson laboratories. The Institutional Animal Care and Use Committee at UMass Amherst approved all procedures involving animals.

Cell culture and stimulations

Bone marrow-derived macrophages were generated as described previously (104). Briefly, bone marrow cells were collected from the femoral shafts of wildtype or CD11b-deficient C57Bl/6 mice and cultured in 20% L929-conditioned RPMI 1640 supplemented with 10% FCS (Hyclone), 2.4 mM L-glutamine and 10% penicillin-streptomycin (Invitrogen) in 100mm x 15mm petri dishes (Fisher Scientific) for 8 days at 37º C with 5% CO₂. Following incubation, non-adherent cells were eliminated and adherent macrophages scraped, counted and resuspended in serum-free RPMI medium without antibiotics overnight prior to use. Where indicated, 10⁶ wildtype, TLR2- or CD11b-deficient BMMs per mL were used for stimulation with different MOIs of live or sonicated B. burgdorferi in the absence or presence of wildtype or C3-deficient normal mouse serum (NMS) for 12h. Following stimulation, TNF secretion was determined by ELISA.

The CHO cells transfected with human CR3 or CR4 were maintained in Ham's F-12 medium supplemented with 10% FCS and 10% penicillin-streptomycin. 24 hours prior to use, the cells were washed and resuspended in FCS- and penicillin-streptomycin-free Ham's F-12.
The macrophage-like RAW264.7 cells were maintained in RPMI 1640 supplemented with 10% FCS, 2.4 mM L-glutamine and 10% penicillin-streptomycin, as described. RAW264.7 cells were washed and resuspended in FCS- and penicillin-streptomycin-free Ham's F-12, 24 hours before use.

**Bacteria**

GFP-expressing *B. burgdorferi* was generated by stably inserting a GFP-pflaB construct into the bbb20-bbb21 intergenic region of the virulent parental strain 297 (clone CE162). The GFP-expressing *B. burgdorferi* clone is completely stable even without antibiotic selection. Furthermore, the strain is comparable in virulence to the parental strain by needle inoculation and goes through the tick-mouse cycle as efficiently as the parental strain. A complete characterization of the clone, BB914, has been made and is the subject of a forthcoming manuscript (Ems et al. in preparation). The bacteria were cultured in BSK-II (Sigma) medium at 33°C and used at log phase of growth.

**CR3 receptor blocking**

Before co-culture of *B. burgdorferi* with CHO cells or macrophages, cells were incubated for 15 minutes with 1, 10 or 20 μg ml\(^{-1}\) of rat anti-mouse CD11b clone M1/70 antibodies (BD Pharmingen). For control experiments, cells were preincubated with 10 μg ml\(^{-1}\) of rat anti-mouse CD11a clone M1/74 (BD Pharmingen) or Armenian Hamster anti-mouse CD11c clone HL3 (BD Pharmingen). Alternatively, cells were cultured in the presence of .1 or 1mM EDTA (Sigma) during incubation with *B. burgdorferi* to chelate divalent cations, including Mg\(^{2+}\), and to block ligand binding to the inserted domain of CR3.
Phagocytosis assay

Internalization of *B. burgdorferi* was determined by a combination of flow cytometry and fluorescence microscopy. CHO-cells or macrophages (10⁶ ml⁻¹) were cultured in fetal calf serum-free and antibiotic-free medium with *B. burgdorferi* in the absence or presence of 5% normal mouse serum at different MOIs for 6h, except where indicated. Following incubation of the cells with *B. burgdorferi*, the cells were washed extensively and resuspended in PBS supplemented with 10% FCS and analyzed by flow cytometry or were further prepared for microscopic analysis.

To confirm phagocytosis, cells were examined by fluorescence microscopy. Following incubation of cells with bacteria, the cells were washed extensively and fixed in 3.7% para-formaldehyde for 7 minutes and then washed with PBS. The cells were then permeabilized with 0.1% Triton-X for 5 minutes and washed in PBS. Following blocking of non-specific binding with 5% BSA for 60 minutes, the permeabilized cells were stained with rhodamine phalloidin to visualize the actin skeleton for 30 minutes at 37°C and DAPI to stain the nuclei (Molecular Probes) for 5 minutes at 37°C. After extensive washing in PBS, the cells were mounted with Prolong Gold Anti-fade mounting reagent (Molecular Probes). Photographs were taken using a Zeiss Axiovert 200M inverted microscope (Thornwood, NY) equipped with a Hamamatsu Orca camera (Bridgewater, NJ).

Analysis of CR3 expression

The relative expression of CD18 and CD11b in RAW264.7 cells following activation of 10⁶ cells with 100 ng/ml rmIFNγ was determined using total RNA extracted
from harvested cells using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA was treated with DNase I (Promega, Madison, WI), and reverse transcribed using random primers (Invitrogen) and SuperScript II reverse transcriptase (RT) (Invitrogen). The cDNA was amplified using primers specific for glyceraldehyde 3-phosphate dehydrogenase (gadph; 5’-CCA TCA CCA TCT TCC AGG AGC GAG-3’ and 5’-CAC AGT CTT CTG GGT GGC AGT GAT-3’), cd18 (cd18; 5’-CGG TCT TCG ACT TGA AGT GAC CTG-3’ and 5’-GGG TCC ATG ATA TCA TCG GC-3’), and cd11b (cd11b; 5’-GGG TCC AGA AAC CTA ACT ACG TC-3’ and 5’-CCA GTG TAT AAT TGA GGC GC-3’) in an Mx3005P® QPCR System (Stratagene, La Jolla, CA) and SYBR green-containing reaction buffer (Roche, Nutley, NJ). Relative expression of the gene is referred to non-activated RAW264.7 cells.

We confirmed elevated levels of CR3 expression by RAW264.7 cells following activation with IFNγ by flow cytometry and fluorescence microscopy. Following activation with rmIFNg, RAW264.7 cells were harvested and washed with PBS before incubation with FITC-conjugated rat anti-mouse CD11b (BD Pharmingen) for 30 minutes at 4°C. The cells were next washed extensively and resuspended in PBS supplemented with 10% FCS and analyzed by flow cytometry for the expression of CR3.

For the microscopic analysis of CR3 expression, the IFNγ-activated or non-activated cells were washed extensively and fixed in 3.7% para-formaldehyde for 7 minutes and then washed with PBS, as described. Following blocking of non-specific binding with 5% BSA for 60 minutes, the cells were stained with FITC-conjugated rat anti-mouse CD11b (Molecular Probes) for 60 minutes at 37°C and DAPI, as described before. After extensive washing in PBS, the cells were mounted and analyzed.
**Statistical analysis**

The results are presented as means ± SE. Significant differences between means were calculated with the Student’s t test. P values of 0.05 or less were considered statistically significant.
Results and Discussion

Complement opsonization enhances phagocytosis of *B. burgdorferi* in a CR3-dependent fashion

The binding of complement-opsonized *B. burgdorferi* to human neutrophils has been shown to occur via a CR3-dependent mechanism (29). CR3-mediated phagocytosis of *B. burgdorferi* might be a particularly relevant mode of bacterial clearance during initial deposition of the spirochete into the skin and also during disseminated disease. This is because *B. burgdorferi* express surface exposed proteins that halt the terminal complement cascade by cleaving and inactivating C3b. This process generates iC3b, a serum opsonin and ligand for CR3, in close proximity to the pathogen. We therefore tested the role of complement opsonization in the phagocytosis of *B. burgdorferi* by murine BMMs. Incubation of GFP-expressing *B. burgdorferi* with BMMs in the presence of 10% normal mouse serum (NMS) greatly increases the amount of phagocytosis, as determined by immunofluorescence microscopy and flow cytometric analysis (Figures 2.1A and B). To determine the contribution of CR3 and CR4 in mediating the interaction with complement opsonized *B. burgdorferi*, we utilized CHO cells expressing either CR3 or CR4. Our results demonstrate that CR3 but not CR4 binds complement-opsonized *B. burgdorferi*, as previously demonstrated (29) (Figure 2.1C). Using a CR3 blocking antibody directed to the CD11b beta chain, we determined the contribution of CR3 to the phagocytosis of serum opsonized *B. burgdorferi* by RAW264.7 macrophage-like cells. The enhanced level of phagocytosis of *B. burgdorferi* in the presence of complement was abrogated by CR3 blocking, indicating that CR3 is the primary complement receptor
involved in the phagocytosis of complement opsonized *B. burgdorferi* (Figure 2.1D). Our results also show residual binding of *B. burgdorferi* to CHO-CR3 cells in the presence of heat-inactivated serum (Figure 2.1C), suggesting that CR3 may represent a means of interacting with *B. burgdorferi* at the host-cell/spirochete interface in the absence of complement. Furthermore, CR3 blocking reduced the level of *B. burgdorferi* phagocytosis in the presence of heat-inactivated NMS (Figure 2.1D), suggesting that this receptor may mediate the phagocytosis of the spirochete through a direct interaction.
Figure 2.1. Complement opsonization enhances phagocytosis of *B. burgdorferi* in a CR3-dependent fashion.

(A) Microscopic and (B) flow cytometric analysis of *B. burgdorferi* phagocytosis by BMMs in the absence or presence of 10% NMS. F4/80 (red) was used as a cell-surface marker and Dapi (blue) was used to stain the nucleus. (C) Increased *B. burgdorferi* interaction with CR3-expressing CHO cells in the presence of active or heat-inactivated 10% NMS. (D) Anti-CD11b antibodies (10 µg/ml) inhibit the phagocytosis of *B. burgdorferi* by RAW264.7 cells in the presence of 10% NMS. Heat-inactivated serum was used to control for phagocytosis of *B. burgdorferi* proceeding in the absence of complement opsonization. A 4º binding control (solid gray histogram) was included and consists of *B. burgdorferi* incubated with RAW264.7 cells in the presence of 10% NMS.
CR3 is a receptor for *B. burgdorferi*

Our assessment of the interaction between CR3 and *B. burgdorferi* in the absence of serum consisted of a combination of apotome microscopy and flow cytometry, as previously described (104). Our results show that CHO cells expressing CR3 acquire the ability to interact directly with *B. burgdorferi*, because CHO-CR3 but not CHO-CR4 cells are positive for GFP fluorescence, above background binding, as determined by flow cytometric analysis (Figures 2.2A and B). In order to further elucidate the nature of the interaction between CR3 and *B. burgdorferi*, we analyzed the interaction of *B. burgdorferi* with CR3- or CR4-expressing CHO cells by fluorescence microscopy. These data confirmed our flow cytometric finding that CR3 facilitates the interaction of *B. burgdorferi* to CHO cells, but further suggested that CR3 expression alone is unable to confer phagocytic capacity to these cells (Figure 2.2C), because the spirochetes sustained their elongated morphology consistent with extracellular localization. We therefore utilized an immunoglobulin (Ig) protection assay to determine whether CHO cells expressing CR3 are able to internalize *B. burgdorferi*. In this Ig protection assay, internalized spirochetes are protected from *B. burgdorferi*-specific Ig and therefore remain only GFP-positive while extracellular spirochetes will be stained with mouse *B. burgdorferi*-specific Ig and anti-mouse alexafluor 594 and therefore will be double-positive. Using this approach, we were unable identify CHO-CR3 cells that had internalized *B. burgdorferi*, indicating that CR3 is able to mediate cellular binding to *B. burgdorferi* but is not sufficient for phagocytosis of spirochete (Figure 2.2D).

The most important integrin domain in ligand binding is the inserted (I) domain (84), which contains at their apex a metal-binding site known as the metal-ion-dependent
adhesion site or MIDAS. The active conformation of CR3 requires the presence of a divalent cation in the inserted domain coordinated by the MIDAS. Mg\(^{2+}\) is the physiological metal that comprises integrin MIDAS, but Mn\(^{2+}\) is also able to orchestrate ligand binding in CR3 (82, 84, 97). Because CR3 binding to *B. burgdorferi* is disrupted by anti-CD11b antibody directed to the inserted domain (data not shown) and was previously shown not to depend on the lectin domain of CR3 (29), we tested whether this binding was dependent on free divalent cation. Using EDTA, a divalent cation chelator, we were able to completely disrupt the binding between CHO-CR3 cells and *B. burgdorferi*, indicating that CR3 MIDAS is required for binding to *B. burgdorferi* (Figure 2.2E).
Figure 2.2. CR3 is a receptor for *B. burgdorferi*.

(A) Flow cytometric analysis of *B. burgdorferi* interaction with CR3- or CR4-expressing CHO cells after 6h at an MOI of 10 or 100. Result is a single representative of three independent experiments. (B) Average percentage of *B. burgdorferi* associated CHO-CR3 and CHO-CR4 cells obtained from three separate experiments. (C) Microscopic analysis of CHO-CR3 or CHO-CR4 cellular interaction with *B. burgdorferi*. Cells were incubated with GFP-expressing *B. burgdorferi* at an MOI of 100. Rhodamine phalloidin was used to actin filaments (red) and the nucleus (blue) was stained with Dapi. (D) CHO-CR3 cells were incubated with GFP-expressing *B. burgdorferi* at and MOI of 100 and extracellular spirochetes were stained with *B. burgdorferi*-infected mouse serum followed by anti-mouse IgG 594. The nucleus was stained with Dapi. (E) CHO-CR3 cells were incubated with *B. burgdorferi* at an MOI of 100 in the absence or presence of 1mM EDTA.
Phagocytosis of *B. burgdorferi* by macrophages is mediated by CR3

Since CR3 is able to facilitate the attachment of *B. burgdorferi* to CHO cells (Figure 2.2), we hypothesized that CR3-mediated attachment could be important for phagocytosis of the spirochete by professional phagocytes, such as macrophages, in the absence of serum opsonization. We therefore determined the contribution of CR3 to the phagocytosis of *B. burgdorferi* by RAW264.7 macrophage-like cells by using the CR3 blocking antibody that we previously demonstrated was able to disrupt the attachment of *B. burgdorferi* to CHO-CR3 cells and serum opsonized *B. burgdorferi* to RAW264.7 cells. Anti-CD11a or -CD11c control antibodies do not affect phagocytosis of *B. burgdorferi*, while the presence of 5 µg/ml of anti-CD11b antibody results in a reduction in phagocytosis by approximately 50%, compared to the control group, as determined by flow cytometric analysis (Figure 2.3A) and microscopic analysis (Figure 3.3B). These data indicate that binding of *B. burgdorferi* by CR3 is important in mediating the phagocytosis of the spirochete by RAW264.7 cells.

We next determined the contribution of CR3 in the phagocytosis of *B. burgdorferi* by bone marrow-derived macrophages, using RAW264.7 cells for comparison. The effect of the blocking antibody on the phagocytosis of *B. burgdorferi* by RAW264.7 cells is dose-dependent with complete inhibition occurring between 10-20 µg/ml of anti-CD11b (Figure 2.4A), underscoring the importance of this receptor in the phagocytosis of *B. burgdorferi* in this cell type. Importantly, the chelation of divalent cation with EDTA is also able to fully prevent phagocytosis of *B. burgdorferi* (Figure 2.4B), as expected. Although the effect of the blocking antibody on the phagocytosis of the spirochete by BMMs is also dose-dependent, the amount of inhibition is not as pronounced (Figure
2.4C). Even at a moderately high concentration, the effect of the blocking antibody on the phagocytosis of *B. burgdorferi* does not appear to reach a saturating level. Arguing that higher expression of the receptor requires a higher concentration of blocking antibody to achieve a similar level of inhibition between the two cell types, we determined the level of surface CR3 expression between RAW264.7 cells and BMMs. Indeed, expression of CR3 is more than 1 order of magnitude higher in BMMs, compared to RAW264.7 cells and EDTA does not affect CR3 expression in either cell type (Figure 2.4E). Moreover, the presence of EDTA is able to fully inhibit the phagocytosis of *B. burgdorferi* by BMMs when used at the same concentration with RAW264.7 cells (Figure 2.4D). We therefore utilized CD11b-deficient BMMs in our study to eliminate the effect of variable CR3 expression. Using CD11b-deficient macrophages, we are able to demonstrate that phagocytosis of *B. burgdorferi* is at least partially dependent on the presence of CR3 (Figure 2.4F).
Figure 2.3. CR3 mediates phagocytosis of *B. burgdorferi* by RAW264.7 cells.

(A) Flow cytometric analysis or (B) microscopic analysis of RAW264.7 cell phagocytosis of *B. burgdorferi* in the presence of 5 µg/ml anti-CD11a, anti-CD11b or anti-CD11c at an MOI of 50 for 4h. A 4º control was used to determine background-binding levels. For microscopic analysis, rhodamine phalloidin was used to stain actin filaments (red) and the nucleus (blue) was stained with Dapi.
Figure 2.4. CR3 partially mediates the phagocytosis of *B. burgdorferi* by BMMs.

RAW264.7 macrophages were incubated with GFP-expressing *B. burgdorferi* at an MOI of 50 for 4h in the absence (black histogram) or presence of increasing concentrations of (A) anti-CD11b antibody (green histogram) or (B) in the absence or presence of 1 mM EDTA. The shaded histogram indicates the 4° control. (C) Analysis of BMM phagocytosis of *B. burgdorferi* in (C) the presence of increasing concentrations anti-CD11b blocking antibody or (D) EDTA, as before, with the exception that spirochetes were used at an MOI of 10. (E) Analysis of CR3 expression in RAW264.7 cells (green) and BMMs (blue) in the absence (solid histogram) or presence (dotted histogram) of 1 mM EDTA. (F) Wildtype (black) and CR3-deficient BMMs (red) were incubated with *B. burgdorferi* at MOIs of 1 and 10, as before. For A-E, results depicted are representative of at least 3 experiments. For F, results are representative of 2 experiments.
**IFNγ upregulates CD11b and CD18 transcription resulting in increased CR3 cell surface expression and phagocytosis of *B. burgdorferi***

We previously reported that IFNγ enhances phagocytosis of *B. burgdorferi* by RAW264.7 cells and BMMs (104). Although this phenomenon has important biological consequences in terms of infection, the mechanism underlying the IFNγ-mediated increase in spirochete phagocytosis is unknown. Now with a potential target, we determined the effect that IFNγ has on the expression of CD11b and CD18 by RAW264.7 cells. Quantitative RT-PCR analysis of CD11b and CD18 in macrophages revealed that both of these transcripts were upregulated in response to IFNγ activation (Figure 2.5A). Since IFNγ increased CD11b and CD18 transcription, we next assessed the level of CR3 protein on the surface of RAW264.7 cells. Microscopic analysis revealed that CR3 expression is increased following activation with IFNγ, compared to non-activated control cells (Figure 2.5B). This finding was confirmed by flow cytometric analysis, which also demonstrated increased CR3 surface staining following IFNγ activation (Figure 2.5C).

Because increased cell surface expression of CR3 could account for the IFNγ-mediated increase in the phagocytosis of *B. burgdorferi*, we next determined the contribution of CR3 to the phagocytosis of *B. burgdorferi* by macrophages activated with IFNγ. As previously shown, IFNγ activation enhances the phagocytosis of *B. burgdorferi* by RAW264.7 cells (Figures 2.5D and E). However, the presence of anti-CD11b blocking antibodies significantly decreased the phagocytosis of *B. burgdorferi* by both...
IFNγ-activated and non-activated RAW264.7 cells (Figures 2.5D and E). Importantly, our data demonstrate that the effect of IFNγ on the phagocytosis of *B. burgdorferi* is circumscribed to CR3 activity because disruption of CR3-mediated phagocytosis reduces phagocytosis to the same level in both IFNγ-activated and non-activated cells (Figures 2.5D and E).
Figure 2.5. IFNγ increases phagocytosis of *B. burgdorferi* through transcriptional upregulation of CR3

(A) Quantitative real-time PCR analysis of CD11b and CD18 expression following activation RAW264.7 macrophages with (empty bars) or without (black bars) rmIFNγ for 12h. Determination of CR3 expression by fluorescence microscopy (B) and flow cytometry (C) following activation with or without rmIFNγ. For (C), the dashed line represents IFNγ-activated cells, and the gray line represents the non-activated cells. (D) Flow cytometric analysis of the role of CR3 in IFNγ-mediated enhancement of *B. burgdorferi* phagocytosis. Spirochetes were incubated with RAW264.7 cells at an MOI of 50 for 4h in the presence or absence of 50ng/ml rmIFNγ, as before. Anti-CD11b blocking antibody (red bar) or control anti-CD11c antibody (black bar) was used at 10 µg/ml. The gray histogram is the 4°C control and represents background binding. The results depicted here are representative of three experiments. (E) Graphical analysis of the average percentage of cells positive for *B. burgdorferi* phagocytosis. Results depicted are the average of 3 experiments. * Student’s T test, p<0.05
CR3 is a negative regulator of proinflammatory cytokine induction in response to *B. burgdorferi*

Innate recognition of *B. burgdorferi* is mainly controlled by TLR1/2 complexes and results in the activation of an inflammatory gene profile. Having identified CR3 as a bonafide receptor for the spirochete, we next determined the contribution of the CR3-*B. burgdorferi* interaction to the TLR1/2-mediated response against *B. burgdorferi* by measuring the secretion of TNF in wildtype, TLR2- and CD11b-deficient BMMs. The secretion of TNF by wildtype BMMs challenged with live *B. burgdorferi* is almost completely abrogated in TLR2-deficient macrophages, confirming the predominant role for TLR1/2 complexes in the inflammatory response against the spirochete (Figure 2.6A). However, CD11b-deficient macrophages respond more vigorously to the spirochete compared to wildtype macrophages (Figure 2.6A), suggesting that CR3 is a negative regulator of TLR-induced TNF secretion. We next analyzed the contribution of CR3-mediated signals derived from iC3b-opsonized *B. burgdorferi* to the secretion of TNF. Although phagocytosis of live *B. burgdorferi* potentiates TNF secretion, as compared to sonicated *B. burgdorferi*, and complement-opsonization increases phagocytosis of *B. burgdorferi* in a CR3-dependent fashion (Figure 2.1A-C), complement-opsonization significantly reduces the secretion of TNF by BMMs responding to live and sonicated *B. burgdorferi* (Figure 2.6B; Student t test; \(p < .05; n = 5\)). Furthermore, the effect of complement-opsonization on TNF secretion in response to live and sonicated *B. burgdorferi* was reversed by complement component C3-deficiency (Figure 2.6B), supporting a role for CR3 as a negative regulator of proinflammatory cytokine secretion in response to *B. burgdorferi*. Therefore, although phagocytosis of *B. burgdorferi*
potentiates the secretion of inflammatory cytokines (35), our data indicate that a *B. burgdorferi* phagocytic receptor, CR3, serves to modulate the TLR-mediated response against the spirochete by dampening the secretion of TNF.
Figure 2.6. CR3 is a negative regulator of cytokine induction in macrophages responding to *B. burgdorferi*.

(A) BMMs obtained from wildtype, TLR2-deficient or CD11b-deficient mice were incubated with *B. burgdorferi* at an MOI of 10 or 50 for 6h. TNF secretion was analyzed by ELISA. Results depicted are from a single experiment. (B) BMMs were incubated with live or sonicated *B. burgdorferi* at an MOI of 10 in the absence or presence of 10% NMS obtained from wildtype or C3-deficient mice. TNF secretion was determined by ELISA, as before. Results are representative of 5 (live *B. burgdorferi*) and 3 (sonicated *B. burgdorferi*) experiments.
CHAPTER IV

CONCLUSION

Invariant natural killer T cell responses are important for host protection during a variety of microbial infections (75). The discovery that \textit{B. burgdorferi}-derived diacylglycerol glycolipids act as natural antigens to V\(\alpha14\)- and V\(\alpha24i\) NKT cells is the sole demonstration of an V\(\alpha14\)-J\(\alpha18i\) NKT cell antigen expressed by a human pathogen (68). The experimental infection of CD1d-deficient mice, which lack V\(\alpha14\)-J\(\alpha18i\) NKT cells, with \textit{B. burgdorferi} also suggested their potential involvement in the genesis of inflammation during Lyme borreliosis. The absence of CD1d results in augmented \textit{B. burgdorferi} levels in the urinary bladder of infected mice, in addition to increased Lyme arthritis incidence and severity (76), while the effect of CD1d-deficiency on Lyme carditis was not reported. However, these findings could not be assigned exclusively to V\(\alpha14\)-J\(\alpha18i\) NKT cell function, since CD1d-deficiency also leads to the lack of CD1d-reactive cells other than V\(\alpha14\)-J\(\alpha18i\) NKTs (26, 27, 76). Furthermore, CD1d-deficient mice have impaired marginal zone B cell function (10), which may also affect the systemic response to the spirochete. These findings prompted us to examine the specific role of V\(\alpha14\)-J\(\alpha18i\) NKT cells in the pathogenesis of Lyme disease.

Our studies, using a mouse line deficient for a component of the invariant alpha TCR, which specifically precludes the development of V\(\alpha14\)-J\(\alpha18i\) NKT cells in the thymus (75), show that infection of J\(\alpha18\)-deficient mice with \textit{B. burgdorferi} results in higher pathogen burden in the heart and, as a consequence, more severe Lyme carditis. Although V\(\alpha14\)-J\(\alpha18i\) NKT cells are able to potentiate B cell responses to various
pathogens (127), our data indicate that this does not occur during infection with \textit{B. burgdorferi}. Furthermore, J\(\alpha\)18-deficient mice infected with \textit{B. burgdorferi} did not develop an altered Th1 response to \textit{B. burgdorferi} antigens, as determined by antigen-specific restimulation experiments. Thus, the effect of V\(\alpha\)14-J\(\alpha\)18i NKT-deficiency on the pathogenesis of murine Lyme disease is likely the result of the modulation of cell responses at sites of acute inflammation and not in secondary lymphoid organs. Indeed, we show that spirochete colonization and expansion in the heart leads to cardiac inflammation, which is marked by the migration to the heart of CD3\(^+\) T cells as well as CD1d- and CD11b- antigen presenting cells, while no infiltration of CD11c\(^+\) cells was detected (data not shown). Our data show that CD3\(^+\) T cells include V\(\alpha\)14-J\(\alpha\)18i NKT and other T cells (CD4\(^+\) and/or \(\gamma\delta\) T cells), because J\(\alpha\)18-deficient infected hearts still contained CD3\(^+\) cells (data not shown).

Since \textit{B. burgdorferi} glycolipid-pulsed dendritic cells induce IFN\(\gamma\) production by V\(\alpha\)14-J\(\alpha\)18i NKT cells \textit{in vivo} (68), we tested the contribution of these cells to the pool of IFN\(\gamma\) mRNA found in the hearts of \textit{B. burgdorferi}-infected mice. Our results show that induction of \textit{ifn}\(\gamma\) gene expression in the hearts of \textit{B. burgdorferi}-infected mice was almost completely abrogated in J\(\alpha\)18-deficient mice 2 weeks post infection, indicating that V\(\alpha\)14-J\(\alpha\)18i NKT cells are critical for generating an IFN\(\gamma\) response \textit{in vivo} during the peak of disease. Reports have suggested that an intact IFN\(\gamma\) response is important for controlling pathogenesis of Lyme carditis and not Lyme arthritis (16, 20, 22, 52). Although the presence of T cells is not sufficient to prevent the onset of Lyme carditis (93), CD4\(^+\) T cells mediate the regression of murine Lyme carditis (16). Consistent with this, our results demonstrate that V\(\alpha\)14-J\(\alpha\)18i NKT cells are important for controlling the
severity of inflammation in the heart; a phenomenon circumscribed to their role in the
production of IFNγ and modulation of the macrophage response to *B. burgdorferi*. Thus,
we propose that the similar disease phenotype between IFNγRα- and Jα18-deficient mice
is explained by the critical contribution of Vα14-Jα18i NKT cells to the pool of IFNγ
found in the hearts of *B. burgdorferi*-infected mice.

Studies have reported great variation among different inbred strains of mice in
terms of the presence of Vα14-Jα18i NKT cells in different organs and their capacity to
respond to antigen (116). Among a large panel of strains tested, Vα14-Jα18i NKT cells
from B6 mice showed the highest production of cytokines and the fastest response
kinetics to the prototypical ligand αGalCer (116). Since B6 mice are less susceptible to
inflammation than C3H mice (141), and due to the difference in the number of Vα14-
Jα18i NKT cells and their response to specific antigen among these strains, it is tempting
to speculate that the differential susceptibility to cardiac inflammation is associated with
the presence and capacity of Vα14-Jα18i NKT cells to respond to *B. burgdorferi*
antigens. Indeed, Tupin and coworkers recently demonstrated that iNKT cells do not
migrate to the inflamed hearts of *B. burgdorferi*-infected BALB/c mice, and that Jα18-
deficient mice on the BALB/c background are not more susceptible to the development
of Lyme carditis upon infection with *B. burgdorferi* (129). Whether these differences in
quantity and/or their capacity to respond to antigen are also present in human subjects
remains to be elucidated.

A mechanism underlying IFNγ-mediated control of *B. burgdorferi* pathogenesis
has not been determined. Since macrophages are the predominant leukocyte of the
inflamed heart (115) and IFNγ activation of macrophages has been shown to enhance
their response to various pathogens (119), we determined the effect that IFNγ had on the macrophage response to *B. burgdorferi*. Our results indicate that IFNγ greatly enhances proinflammatory cytokine production by macrophages challenged with live *B. burgdorferi*. Most notably, IFNγ enhanced the phagocytic capacity of macrophages, suggesting their role in spirochete clearance at sites of active inflammation, such as the heart. This is an interesting observation given that IFNγ has historically been regarded as a cytokine with a biological activity confined to the response to intracellular pathogens, including viruses, bacteria and parasites. Here, we show that IFNγ is important for protection against the extracellular spirochete, *B. burgdorferi*. The lack of involvement of IFNγ in the pathogenesis of Lyme arthritis might be explained by the cellular profile of the inflammatory infiltrate. Indeed, lesions in the heart of *B. burgdorferi* infected mice are predominantly comprised of macrophages, while neutrophils are primarily found in the joint. The contribution of cell types such as synoviocytes, chondrocytes and other cells (9, 142) to joint but not heart inflammation further distinguish both inflammatory phenomena. As a consequence of the more complex cellular interplay, iNKT cell responses to *B. burgdorferi* in the joint might not be as effective at controlling Lyme arthritis.

These studies define the role of Vα14-Jα18i NKT cells in the host response elicited against the Lyme disease spirochete, *B. burgdorferi*. Our results show that Vα14-Jα18i NKT cells migrate to the inflamed heart of *B. burgdorferi*-infected mice and are exposed to an environment conducive for their activation by *B. burgdorferi* glycolipids. Activation of Vα14-Jα18i NKT cells is critical for the induction of IFNγ, which enhances
macrophage effector functions required for efficient clearance of *B. burgdorferi* from the inflamed heart and the sustained activation of Vα14-Jα18i NKT cells.

The etiology of Lyme disease is not well understood. This is due, in part, to the complex interplay between different cell types, which varies depending on the affected site. Because macrophages are the principal immune cells found in the heart during experimental infection with *B. burgdorferi* (104, 115), the interaction between the macrophage and the spirochete has important consequences in terms of pathogenesis of Lyme carditis. However, the identification of a receptor that lies at the interface between the spirochete and macrophage and is able to mediate phagocytosis has remained elusive. Here we have identified the β2 integrin, CR3, as a receptor able to mediate the phagocytosis of *B. burgdorferi* in a complement-dependent and-independent manner and that CR3-dependent phagocytosis of *B. burgdorferi* is enhanced by activation with IFNγ.

One of the main endogenous ligands of CR3 is the complement component iC3b (inactivated C3b), which is a serum opsonin with broad-range specificity for pathogens. Previous work demonstrated a role for CR3 in the phagocytosis of complement opsonized *B. burgdorferi* (29) by human neutrophils. Initial experiments here were in agreement with those findings because the phagocytosis of *B. burgdorferi* by RAW264.7 macrophage-like cells and BMMs was dramatically enhanced by complement opsonization. Complement opsonization is not required for the phagocytosis of *B. burgdorferi*, however, and the addition of anti-CD11b blocking antibodies reduced the phagocytosis of complement-opsonized *B. burgdorferi* to levels below that achieved with
non-opsonized *B. burgdorferi*, suggesting that CR3 mediates the phagocytosis of *B. burgdorferi* in a complement-dependent and independent manner.

When CHO cells were transfected with CR3 they acquired the ability to interact with *B. burgdorferi*, in the absence of complement opsonization. Studies using different pathogens, including *N. meningitidis* (66) and *B. anthracis* spores (102), showed that CR3 expression alone conferred phagocytic capacity for these pathogens to CHO cells. This is in contrast to our findings using *B. burgdorferi*, which show that the interaction between CR3-expressing CHO cells and *B. burgdorferi* is not productive in terms of phagocytosis, but instead is critical for attachment of the spirochete to the cell. These results indicated that CR3 is a *B. burgdorferi* receptor and suggested that the interaction between *B. burgdorferi* and CR3 might result in internalization of *B. burgdorferi* by a phagocytic cell.

Our studies using RAW264.7 cells demonstrated that inhibition of CR3 with blocking antibody was able to completely abrogate phagocytosis of *B. burgdorferi*. Thus, CR3-mediated binding is a requirement for phagocytic uptake in this cell type. Interestingly, however, BMM phagocytosis of *B. burgdorferi* was only partially affected by inhibition of CR3-mediated binding, but completely blocked by the non-specific divalent cation chelator EDTA, suggesting the presence of alternative receptors that mediate binding through divalent cations. C-type lectins are a family of PRRs, including phagocytic receptors, whose binding to ligands usually proceeds in a Ca$^{2+}$ dependent fashion. The mannose receptor (MR) is a C-type lectin that has been shown to mediate the binding of *B. burgdorferi* to human monocyte-derived macrophages (28). We speculate the involvement of MR in BMM phagocytosis of *B. burgdorferi*, based on the
complete abrogation of phagocytosis in the presence of EDTA. In contrast, RAW264.7 cell phagocytosis of *B. burgdorferi* is completely dependent on CR3. Importantly, preliminary studies in our lab have demonstrated that BMMs but not RAW264.7 cells express MR.

CR3 is a promiscuous receptor that recognizes an array of host and microbial ligands. Outer surface lipoproteins A and B (OspA and OspB) are two major molecular constituents of *B. burgdorferi*, which contribute to the maintenance of the spirochete in nature but whose expression is downregulated during transmission into a mammalian host. These proteins were shown to bind to CR3 in an iC3b-independent fashion, suggesting that they may drive CR3-mediated phagocytosis (48). Moreover, experimental infection with an OspA/B-deficient mutant led to higher bacterial loads in the joint and more pronounced Lyme arthritis (143). An evaluation of Lyme carditis was not made in this study, but proliferation of the OspA/B mutant in the heart was statistically similar to that of the wildtype *B. burgdorferi* (143). Current studies are determining the contribution of Osps A and B to the phagocytosis of *B. burgdorferi* and whether this is dependent on an interaction with CR3. Preliminary results suggest that in BMMs the expression of Osps A and B are dispensible for the phagocytosis of the spirochete.

We and others have shown that IFNγ-signaling is important for controlling the severity of Lyme carditis (16, 20, 22, 52, 104) via the modulation of macrophage-mediated phagocytosis of *B. burgdorferi* (104). We show here that activation of macrophages with IFNγ resulted in upregulation of both components of CR3, CD11b and CD18, at the transcriptional level and that this correlated with increased cell surface expression of CR3. Importantly, the effect of IFNγ on the phagocytosis of *B. burgdorferi*
was completely diminished in the presence of CR3 blocking antibodies, indicating that the role of IFN\(\gamma\) in the phagocytosis of \textit{B. burgdorferi} is circumscribed to the activity of CR3.

Macrophages express unique combinations of PRRs, which likely result in tissue-specific responses to pathogen (64). Because we defined CR3 as a receptor for \textit{B. burgdorferi}, we were interested in the role of CR3 in defining the proinflammatory response to the spirochete. Our results indicate that CR3 is a negative regulator of the TLR1/2-induced signals elicited by \textit{B. burgdorferi}, even in the context of complement. This is in contrast to most pathogens, in which complement opsonization increases the proinflammatory response and therefore our results warrant further investigation.

In summary, we have defined the contribution of the complement receptor CR3 to the phagocytosis of the Lyme disease spirochete, \textit{B. burgdorferi}. Our results place CR3 as a central PRR involved in the recognition of \textit{B. burgdorferi} by macrophages, because CR3 is able to mediate the phagocytosis of the spirochete. Our results also demonstrate that IFN\(\gamma\) regulates phagocytosis of \textit{B. burgdorferi} by controlling the transcriptional activation of the components of CR3, CD11b and CD18. Furthermore, our results indicate CR3 is a negative regulator of TLR-induced proinflammatory signals elicited in response to \textit{B. burgdorferi} both in the presence and the absence of complement opsonization. Taken together, these data shed light on the role of IFN\(\gamma\) and CR3 in macrophage-mediated control of spirochete expansion during Lyme borreliosis.


