A NOVEL MOUSE MODEL OF NEUROBORRELIOSIS

A Dissertation

by

ALI DIVAN

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	M. Karen Newell-Rogers
Committee Members,	David C. Zawieja
	Cynthia J. Meininger
	Xu Peng
Head of Department,	Warren E. Zimmer

August 2017

Major Subject: Medical Sciences

ABSTRACT

Lyme disease (LD) is a tick-borne infection caused by Borrelia burgdorferi (Bb) that has a global impact and a high incidence in the United States. If not treated with antibiotics during the early stages of the disease within the first few weeks of infection, patients usually develop musculoskeletal symptoms and neurological complications that can include neurocognitive and neuropsychiatric manifestations. For many, regardless of the stage of the disease or duration of the infection, antibiotic treatment with oral or intravenous antibiotics is curative. Nevertheless, up to 20% of individuals that receive antibiotic treatment during late-stage infection do not find relief from their symptoms, despite multiple rounds of antibiotic therapy. Collectively, the symptoms that persist after antibiotic treatment are called Refractory Lyme arthritis (RLA) when symptoms are predominantly rheumatologic, or post-treatment Lyme Disease syndrome (PTLDS) when symptoms are predominantly neurologic, neuropsychiatric and/or neurocognitive. Neither of the syndromes is mutually exclusive. Currently there is no FDA approved treatment for PTLDS, and although autoimmune etiologies have been proposed, a definitive causal mechanism has not been identified.

A major limitation in our understanding of neurologic LD and its associated syndrome PTLDS is the lack of a suitable mouse model for modeling neurologic Bb infection. In section 1, we provide background on LD and summarize the hypotheses and approaches that we took in our research. In section 2, we review T cell and dendritic cell contributions to inflammation in RLA, to underscore what is known about complications

of LD. Section 2 closes with a brief discussion of PTLDS and neuroborreliosis, to emphasize the questions that are currently understudied in LD. In section 3, we present a novel mouse model of neuroborreliosis that results from Bb infection of C3H/HeN mice, using the North American CSF-tropic strain of Bb, Bb 297, and an intradermal route of inoculation. We demonstrate that intradermal infection with Bb 297 results in persistent infection of the meninges and the brain, that CD4+ T cells accumulate in the brain during early and late-stage LD, and that both persistent infection and neuroimmune changes are temporally associated with significant behavioral deficits in nociception, mobility, and movement. In section 4 we provide a discussion of the implications of the research that we have performed.

DEDICATION

This dissertation is dedicated to my wife, Debra, who patiently and fully supported my dream to obtain my doctorate; to my hardworking immigrant parents, Divan Fard and Simin Pouya, who taught me that no dream is unattainable; to my two children, Faith Aria Divan and Caleb Cyrus Divan, who always gave me something to be happy about when I came home from long hours in the laboratory; to my pastor, Louis Best, who helped advise me when I had to make tough personal decisions; and to the thousands of individuals that suffer from complications of Lyme Disease, yet have not received the cure they have been praying and hoping to receive.

ACKNOWLEDGEMENTS

I would like to thank my outstanding mentor, Dr. Karen Newell-Rogers for her encouragement, optimism, and willingness to take big risks on my wild ideas. I will forever be grateful for the opportunity that she gave me to work with her and to learn so much from her. I would also like to thank my committee members, Dr. David Zawieja, Dr. Cynthia Meininger, and Dr. Xu Peng, for guiding me through the scientific method and providing me with the constructive criticism that I needed to learn how to think like a scientist. I would also like to thank Dr. Jon Skare for teaching me the methodology for culturing Borrelia burgdorferi, and for generously continuing to support my work with culture reagents from his laboratory. Thank you to Dr. Lee Shapiro and Dr. Damir Nizamutdinova for helping me design behavioral studies, and thank you to Dr. Sanjib Mukherjee and Dr. Richard Tobin for teaching me many of the laboratory techniques that I utilized to perform this dissertation project. Thank you to Mr. Anand Narayanand for working closely with me to design experiments for immunostaining.

Thank you to the charitable organizations that funded my research in Lyme Disease, specifically the Global Lyme Alliance and Mr. Rob Kobre.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Dr. Karen-Newell Rogers [advisor, Department of Surgery], Dr. David Zawieja [co-advisor, Department Head, Department of Medical Physiology], Dr. Cynthia Meininger [committee member, Department of Medical Physiology], and Dr. Xu Peng [committee member, Department of Medical Physiology].

Dr. Karen Newell-Rogers contributed to experimental design, data analysis and interpretation of data, and helped write/edit all manuscripts. Dr. Ralph Budd and Dr. Richard Tobin assisted with writing the manuscript presented in chapter 2. Dr. David Zawieja and Mr. Anand (Sunny) Narayanand contributed to experimental design for immunostaining and imaging in the manuscript presented in chapter 3. Dr. Lee Shapiro and Dr. Damir Nizamutdinova contributed to experimental design for behavioral tests in manuscript presented in chapter 3. Ms. Anna Webb provided instruction and training on confocal microscopes that were used for imaging in the manuscript presented in chapter 3. Dr. Sanjib Mukherjee contributed to experimental design for tissue harvesting in the manuscript presented in chapter 3. Dr. Jon Skare provided instruction on culture methodology of Borrelia burgdorferi and provided the medium for Borrelia burgdorferi culture. Ms. Jessica Kain provided technical support with mouse colony management.

Funding Sources

This work was supported by the Global Lyme Alliance, the Texas A&M University Health Science Center, and the Baylor Scott & White Foundation.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

1. INTRODUCTION

1.1 The Current State of Lyme Disease

Lyme disease (LD) is a zoonotic bacterial infection caused by a variety of borrelia-species spirochetes including *B.burgdorferi*, *B.hermsii*, *B.miyamotoi*, *B.afzelli*, *B.garinii*, and *B.bissettii* (1-4). Collectively, these pathogenic species of Borrelia are called *Borrelia burgdorferi sensu lato* (Bbsl), meaning the broad sense of Borrelia burgdorferi. Bbsl occurs globally, has been reported in many countries, and it is most prevalent in North America and Europe (5). Epidemiologic and ecologic data provides evidence that Bbsl can infect a variety of vertebrate hosts including multiple species of rodents, birds, rabbits, and deer (5). In North America the most commonly observed species of Borrelia is *Borrelia burgdorferi sensu stricto* (Hereon referred to as Bb), meaning the strict sense of *Borrelia burgdorferi*, and it is especially prevalent in white-tailed deer and white-footed mice (5). Although Bb is the most prevalent and well-studied species of borrelia in North America, it is not the only borrelia species that exists, as evidence demonstrates that *B.hermsii*, *B.miyamotoi*, and the newly-discovered species *B.mayonii* are found in both animals and humans in the United States (1, 6, 7).

In the United States, Bb infection in humans was first observed in the 1970s among children presenting with polyarthritis, within three distinct communities in eastern Connecticut (8-11). At the time it was not known whether the cause of infection was viral or bacterial, but investigative efforts among physicians and researchers

demonstrated that a spirochetal bacterium was the causative agent of the disease (9-11). Later it was determined that the transmission of Bb depended on a vector that could harbor the infection from feeding on an infected host, and transmit it to another host, without itself succumbing to the pathological effects of the infection. Thus far, evidence has only supported Bb and Bbsl disease transmission to occur through the bite of a tick that carries the organism (9, 12). Hard-bodied ticks belonging to the family Ixodidae transmit Bb in North America, and Bbsl in Europe (13-15), and although it is unknown whether soft-bodied ticks of the family Argisidae can transmit Bb, it is well-established that soft-bodied ticks can transmit some of the Borrelia infections belonging to the Bbsl group, including *B.hermsii* (1). In rodent studies, evidence suggests that an infected tick will transmit Bb to a host 93% of the time if the tick remains attached for 72 hours (16). In humans, it is not known what the minimum time requirement is for a tick to transmit Bb to a host, but infection has been shown to occur commonly in human patients ~24 hours after attachment (17), and as early as 4 hours after attachment (18). Multiple factors can affect the kinetics of disease transmission between the tick and the host, and these factors include 1) bacterial burden and physical distribution of infection in the tick, 2) feeding behaviors of the tick, 3) species of host, 4) species of Borrelia, 5) species of tick 6) life cycle of the tick, and 7) presence of other co-infections, as summarized in Table 1.1(19). Overall, there remains much to be investigated regarding the tick as a vector for LD and associated borrelioses.

1.2 Clinical Signs and Symptoms

The pathognomonic clinical sign of LD is an erythema migran (EM) skin lesion that usually manifests during the early stages of the disease at the site of the tick bite, within the first 7-14 days after infection (20). The lesion begins as a small rash that expands in diameter, leaving an alternating ring of clearance that makes the rash resemble a bullseye. Besides changes in the appearance of the skin, an EM is asymptomatic, does not cause pain or itching, and as a result it may be overlooked, especially if it occurs in a location that the patient may not see, such as the back (21). Importantly, not all patients will develop EM, and the absence of EM does not rule out LD. Typically the EM clears within a matter of days to weeks, and during this time an overlap of acute LD symptoms begin to manifest in the musculoskeletal system, the cardiovascular system, and if left untreated, the nervous system (20, 22).

Musculoskeletal symptoms are the most commonly reported symptoms, and they include myalgias and arthralgias that are migratory and are typically accompanied by fever, and fatigue. Carditis is a sign of early disseminated LD, and it occurs in 1-4% of infected individuals, presenting as electrophysiological conduction disturbances, most often in the atrioventricular AV node (23). Men in their 20's – 40's have the highest risk for developing Lyme carditis, with a preponderance of men outweighing women by a factor of at least 3:1 (24). In rare cases, spontaneous cardiac arrest due to Lyme carditis has been reported (25). During the early stages of infection (1-4weeks), antibiotic treatment

Table 1.1 Transmission kinetics. Effects of different variables on the transmission time of Borrelia infections between ticks and vertebrate hosts

Variable	Example	Transmission time*
Bacterial Burden/location	Bb infection in midgut, salivary gland, hemolymph, or systemic	Systemic infection and salivary gland infection < hemolymph < midgut
Feeding behavior	Questing duration, prior partial feeding	Long questing duration and/or prior partial feeding < short questing duration and no prior partial feeding
Species of Host	Rodent, Human	Human < Rodent
Species of Borrelia	B.burgdorferi (ss), B.afzelli,	B.afzelli < B.burgdorferi
Species of Tick	I.scapularis, I.persulcatus, I.ricinus	I.ricinus and/or I.persulcatus < I.scapularis
Life Cycle of tick	Adult or nymph	Effect on transmission rate not reported
Co-infection	Tick co-infected by A.phagocytophylum, E.chaffensis, B.hensalae, B.microti	Effect on transmission rate not reported

*Data are presented in descending order, showing the variable that corresponding to the fastest transmission time first, followed by variables that require longer tick attachment times to transmit borrelia infections

with doxycycline is generally well-tolerated and most patients recover from the infection without complication. If clinical diagnosis is delayed, neurological symptoms may develop/worsen. The earliest that neurological symptoms have been reported is 14 days after infection, but they can occur as late as months after infection (23, 24). Neurological symptoms can include seventh cranial nerve palsy, meningitis, sensory or motor radiculopathies, encephalomyelitis or axonal polyneuropathy (25). Cognitive abnormalities are often reported by patients, and neuropsychiatric disorders can also occur, ranging from depression, mania, psychosis, and dementia (26). Cognitive abnormalities usually include difficulty with verbal fluency, short-term memory, and processing speed (often referred to as "brain fog"), and evidence from functional imaging studies suggests that these abnormalities are associated with encephalopathy, impaired blood flow, and altered metabolism in the brain (23, 27, 28).

1.3 Post-infectious Complications

In addition to increasing the risk of neurological symptoms, delayed treatment can also increase the risk of long-term or permanent disability subsequent to antibiotic treatment. When antibiotic treatment has been given, but neurological and/or neurocognitive symptoms persist beyond 6 months after therapy, the resulting combination of symptoms is called post-treatment Lyme disease syndrome (PTLDS) (29). It is estimated that up to 20% of individuals that have neurological symptoms go on to develop PTLDS, despite antibiotic therapy. Importantly, there is a difference between untreated LD, and PTLDS. In untreated LD, neurocognitive symptoms are accompanied by objective physical

findings such as recurring EM rashes, cranial nerve palsy, meningitis, or radiculitis (29). In PTLDS, the clinical spectrum usually excludes objective physical findings, although significant neurocognitive abnormalities in processing, fatigue, and widespread musculoskeletal pain persist (29). At this time there is no FDA approved treatment for PTLDS, and although the topic remains controversial, prolonged antibiotic therapy has not been shown to be beneficial (29). Another distinct manifestation of LD is Antibiotic-Refractory Lyme arthritis (RLA), and it is characterized by arthritic symptoms that persist subsequent to antibiotic treatment, in the absence of neurological sequelae (29, 30). RLA has an association with the DRB4 and DRB2 alleles of the DRB human leukocyte antigen (HLA-DRB), suggesting that antigen presentation and/or autoimmunity may underscore the development of RLA (30). Gamma delta T cells (γδ T) are also evidenced to play an important role in the development of RLA(31).

1.4 Coinfections

Acute or untreated LD can often be complicated by other co-infections that are transmitted via the bite of a tick at the same time as B.b transmission. Examples of tick borne coinfections include *Ehrlichia*, *Anaplasma*, *Babesia*, and *Bartonella*, and the prevalence of each co-infection depends heavily on the geographic setting (32). In the United States, the overall prevalence of *B.burgdorferi* and at least one other co-infection in ticks ranges from 9.4%-26% (33-35). Given the similarities between co-infection rates and the prevalence of PTLDS, it is tempting to speculate that co-infection may play a role in the development of PTLDS, or other post-treatment complications of LD.

Consistent with the possibility that coinfection facilitates PTLDS, *Babesia microti*, a protozoan parasite, has been shown to increase disease severity and duration of LD when transmitted as a co-infection with Bb (34, 36). There is also evidence that *Anaplasma phagocytophilum* co-infection with Bb increases production of multiple matrix metalloproteases and the cytokines IL-6, TNF-α, IL-10, and IL-8, resulting in increased permeability of brain microvascular endothelial cells *in vitro* (37). *Ehrlichia chaffensis* is capable of establishing central nervous system (CNS) infection, and thus when transmitted as a co-infection, it may provide permissive conditions for Bb to enter the CNS (38). Although tick-borne transmission of *Bartonella* species may be possible, *Bartonella* has not yet been demonstrated to be a major contributor to tick-borne coinfection in the human population. Collectively, the relatively high prevalence of co-infections suggests that studying Bb infection in isolation may not represent the full spectrum of LD that occurs in patient populations. Thus, in both research and clinical settings, it may be especially important to consider co-infections in LD, particularly as they relate to host-mediated physiological responses and parallel immune responses that may determine disease outcomes, including the development of post-treatment sequelae such as PTLDS.

1.5 Diagnosis and Treatment

The diagnosis of LD is most accurately made based on clinical the presentation of symptoms, and history of a tick bite (39). For individuals that are in Lyme endemic areas, a timely diagnosis is more likely, due to public health efforts to educate both

patients and physicians on LD signs and symptoms. The presentation of an EM at the time of a clinical visit is sufficient objective evidence that justifies antibiotic therapy (40). In the acute phase of the disease, 21 days of treatment with doxycycline (100mg twice per day) is the preferred therapy, because it is reported to be effective in the treatment of LD, human granulocytic anaplasmosis (HGA), human granulocytic ehrlichiosis (HGE), and human monocytic ehrlichiosis (HME) three coinfections that are caused by *Anaplasma* and *Ehrlichia* bacterial species (40, 41). Rifampin therapy has also shown efficacy in the treatment of HGA and HME (42). In cases where EM cannot be distinguished from community-acquired cellulitis (CAC), the Infectious Disease Society of America (IDSA) guidelines recommend antibiotic treatment with cefuroxime axetil or amoxicillin-clavulanic acid, as both of these antibiotics are reported to have activity against LD and CAC (40). If at the time of clinical presentation, coinfection by *Babesia* is suspected, either due to signs/symptoms or prevalence of the parasite in the geographical area, babesial parasites can be detected by blood smear, or by polymerase chain reaction (PCR) assay. The recommended treatment for babesiosis and LD is 7-10 days of combination therapy with either ativaquone and azithromycin, or in severe cases, intravenous clindamycin, quinine, and blood transfusion. In individuals with persistent symptoms of babesiosis, a longer duration of therapy may be considered (40).

Up to 20% of patients may not present with EM during the acute phase of LD, and for these patients, diagnosis may not occur until after the onset of arthritis or neurologic involvement (39, 40). Because many of the secondary and tertiary symptoms of LD can

resemble other diseases such as multiple sclerosis, amyotropic lateral sclerosis, and systemic lupus erythematosus, serology is usually necessary in the differential diagnosis (40, 43). The current Centers for Disease Control (CDC) recommended serology is a two-tiered approach consisting first of an enzyme-linked immunosorbent assay (ELISA), and if positive, followed by a western blot (WB) test. If an ELISA is positive, and the patient has been infected for < 30 days, then 2/8 IgM bands or 5/10 IgG bands must be positive on the WB for a positive LD diagnosis. If ELISA is positive, and the patient has been infected for ≥30 days, then only positive IgG bands (5/10) should be used to interpret the test result. A negative ELISA is recommended as exclusion criteria for LD.

The ELISA assay works by detecting host-derived IgM or IgG antibodies that are reactive against whole Borrelia proteins, derived from sonicated Borrelia cells. In the acute phase of the disease, ELISA may not be useful because seroconversion does not occur this early for all individuals. Consequently, up to 50% of individuals that are infected may produce negative ELISA results during the acute phase of the disease, although convalescent serology may improve sensitivity up to 80% (44, 45). Interestingly, the duration of EM positively correlates with ELISA sensitivity. For example, in patients that have had EM for a duration of <7 days ELISA sensitivity is 13% (44). It is unknown how sensitive ELISA may be for infected individuals that do not present with EM. During the secondary and tertiary stages of the disease, IgG ELISA sensitivity is reported to be as high as 89%, however the values can vary substantially between different test manufacturers, because even FDA-approved ELISA tests for LD

have not been standardized against panels of well-characterized sera from patients (44, 46, 47). Because, antibodies against other antigens can occasionally cross-react with sonicated Bb proteins, the specificity of ELISA is relatively lower than its sensitivity, generally about 70% (47). Thus, for surveillance purposes a positive ELISA may not definitively indicate LD, and for this reason, WB is recommended as a secondary confirmation test.

The WB assay uses immunodominant Bb antigens of known molecular weights to detect reactive antibodies in sera. Much like ELISA, WB is not a useful tool for detecting LD during early infection, however, for surveillance purposes it is a useful test to confirm LD infection when ELISA is positive (44). Overall, WB testing has sensitivity of approximately 85% (44). Generally, most WB tests use antigens derived from the B31 strain of Bb, and although useful, the focus on this particular strain may exclude antigens that would result in a positive diagnosis of individuals that are infected by other strains of Bb, such as strain 297, N40, or other Bbsl species such as *B.miyamotoi*, or *B.hermsii* (44). In contrast to the ELISA, a positive WB is highly specific for LD infection, with specificity reported as high as 95% (44).

The two-tiered CDC criteria that requires a positive ELISA, followed by a positive WB, increases the specificity of the LD test to 99%, with a respective sensitivity of 87% (44, 48). High specificity provides excellent guidelines for surveillance cases. From the perspective of research, it is most favorable to err on the side of specificity, rather than

sensitivity, because specificity ensures that positive samples are truly positive. In contrast, these criteria may not be ideal for a clinical setting. In the clinical setting it is not always possible to determine the exact disease, and in some cases it is most equitable/cost efficient to determine the most probable disease(s). Based on consideration for patients, and the low risk associated with oral-antibiotics in the treatment of LD, it seems more logical to err on the side of caution for the patient, and to prescribe antibiotic treatment if LD is a possibility. Based on the CDC criteria, up to 13% of patients that have LD will return negative serology, and may receive inaccurate diagnoses. This unfavorable outcome underscores the need for higher sensitivity testing that keeps specificity in an acceptable range. In the near future, multiplex platforms may provide a method for ELISA-based assays to test for seroreactivity to multiple Bbsl strains and species, thus potentially improving sensitivity and/or specificity.

1.6 Animal Hosts and Experimental Animal Models

Bbsl and Bb are prevalent in the wild, especially among birds, rabbits, and rodents (5). In north America, the most common animal reservoir for Bb is *Peromyscus leucopus*, the white-footed mouse. The white-footed mouse is an efficient host for Bb infection because it is readily accessible to ticks (especially larval and nymphal ticks), acquires Bb infection rapidly, but does not experience any selective disadvantage from acquiring infection (49, 50). With respect to infection efficiency, it is estimated that greater than 90% of white-footed mice that are bitten by a Bb-infected tick, become permanently

infected with Bb. Thus, a single feeding session by an infected tick can result in Bb transmission to the white-footed mouse, and the white-footed mouse can then transmit Bb to multiple ticks for the duration of its life. Deer, including white-tailed and mule deer, are important hosts for the maintenance of Bb-infected adult ticks, but contrary to popular belief, deer are not competent host reservoirs for LD, as they appear to clear Bb infection spontaneously. Therefore, the prototypical lifecycle of LD is characterized by infection of larval/nymphal ticks by a Bb infected white-footed mouse, followed by maturation of the tick into an adult that will either remain persistently infected while feeding on deer, or will remain persistently infected and transmit the infection to a dead-end host, such as a human or dog (5, 51) (Figure 1.1).

LD is also observed in livestock, making LD a relevant concern in large animal veterinary medicine. Evidence suggests that cattle are susceptible to LD due to a variety of Bbsl species, but infection of cattle by Bb is not supported in the literature (52, 53). Horses are also susceptible to LD due to Bbsl, although in contrast to cattle, evidence suggests that Bb can also establish infection and produce pathology in horses (54-56). Among domestic animals, dogs are the most common species infected by Bb, and much of the clinical presentation revolves around polyarthritis (57, 58). Although there are sporadic veterinary reports of neurological symptoms in dogs infected by Bb, CNS infection or involvement is not supported in studies using experimental infection (59). This does not rule out the possibility that dogs may be susceptible to CNS pathology during or subsequent to Bb infection under different circumstances. Interestingly, cats do

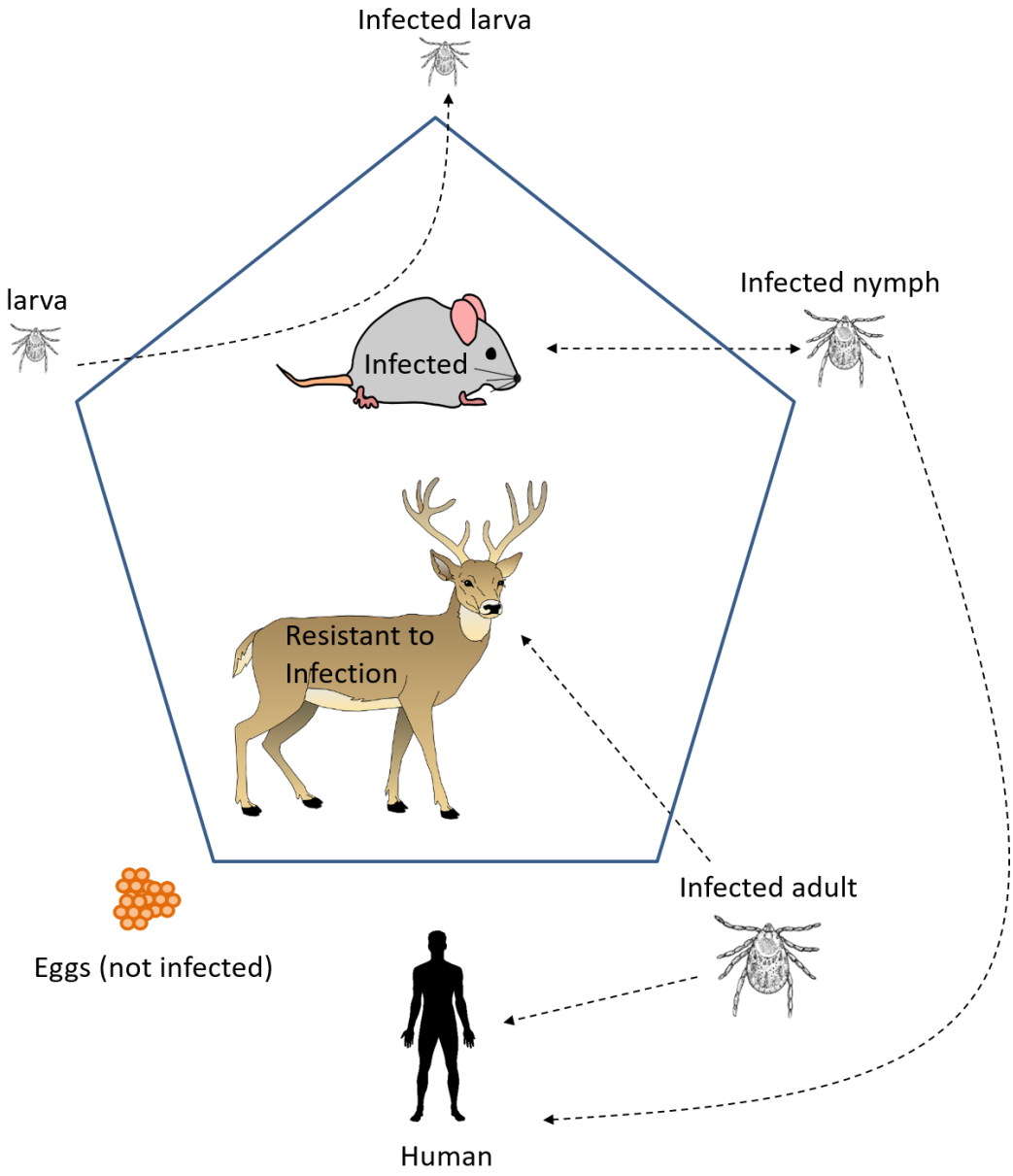

Figure 1. 1 Enzootic Life Cycle of Bb. Larval ticks feed on Bb-infected rodents, resulting in transmission of Bb to larva. Infected larva mature into nymphs and continue to feed on rodents resulting in new transmission of Bb to uninfected ticks and hosts. Infected adult ticks feed on Bb-resistant deer and lay uninfected eggs. Transmission of Bb to "dead end" targets, such as humans, can occur through the bites of infected nymph or adult ticks.

produce measurable serologic responses to Bb infection, but they do not typically succumb to any apparent pathologies. Some have hypothesized that in contrast to dogs, cats may produce a sufficient innate immune response that prevents the infection from disseminating (57, 58). As a zoonotic disease, LD is relevant in many different animal species, providing many potential options for experimental animal models.

Experimental infection of animals in the laboratory has produced important advances in our understanding of the mechanisms that Bb utilizes to establish infection. Thus far, the animal species have that have aided in understanding Bb pathophysiology are dogs, non-human primates (NHP), rats, and mice. Dogs were among the first species to provide evidence that Bb transmission by the tick vector may produce a unique microenvironment that facilitates a different course of disease than infection by needle inoculation of culture-grown bacteria (60). Subsequent studies have revealed that experimentally infected dogs remain culture positive and PCR positive for Bb after antibiotic treatment with doxycycline or amoxicillin, despite resolution of arthritis and negative serology after antibiotic treatment (61). Dogs have also been a good model for understanding histopathological changes in inflamed tissues due to Bb infection (62). Interestingly, even in sub-clinical cases, histopathological changes consistent with synovitis are evident. Finally, dogs have proven useful models for evaluating the efficacy of outer surface protein A (OspA) based vaccines, demonstrating that vaccination with OspA provides significant protection from Bb infection (63-65). Unlike humans, evidence does not support CNS involvement during Bb infection in dogs, and

dogs produce a measurable response to OspF that does not appear to occur in humans (59, 66). The last two observations indicate that although dogs are a useful laboratory model for understanding pathological changes in joints during Bb infection, the course of their immune responses might not faithfully replicate human immune responses to Bb, and mechanisms underlying CNS pathology in humans are unlikely to be elucidated by canine laboratory experiments.

The NHP model of LD replicates human Bb infection more closely than in any other species, presumably due to the overwhelming similarity (>95%) in the genetic composition between NHPs and humans (67). Notably, and in contrast to other species, Rhesus macaques (RM), a type of NHP, develop EM in the acute phase of Bb infection, and some investigators speculate that the immune response that contributes to EM formation conditions the Bb spirochete for disseminated infection (67). The brain structure of the RM closely resembles the relative size and morphology of a human brain, the exception being the number of folds in the brain. Comparatively, there is almost no morphological difference between the brain of a 6kg infant, and a 6kg RM (67). Like humans, RMs also develop chronic Bb infection if left untreated, and immunosuppression by corticosteroids exacerbates disease dissemination and tissue pathology, especially in the meninges of the spinal cord (68). Generally, when neuropathology occurs in RMs, it is associated with inflammation of the meninges, spinal nerve roots, and the dorsal root ganglion. In immunocompetent animals, inflammation is typically associated with intrathecal antibody production in the

cerebrospinal fluid (CSF), and PCR or culture positivity of tissues in the CNS (69). Consistent with increased intrathecal antibody production, the B cell attracting chemokine CXCL13 is also elevated in the CSF (70). Histopathological evidence reveals that Bb is typically observed attached to the meninges, nerve roots, or ganglia of the spinal cord, but not in the parenchyma of the spinal cord (68, 71). Overall, the NHP model is most appropriate for understanding neurological manifestations of human LD, due to the similarities in genetic composition between hosts, and in disease progression/pathology. The primary obstacles to using NHPs are the husbandry costs and the time associated with conducting such research. Unlike other laboratory animals, RMs take years to reach sexual maturity. The female reaches sexual maturity at 3 years of age, whereas the male is sexually mature at 4 years of age. When fertilization occurs, gestation time takes 164 days, and the interval between births ranges between 12 and 24 months. Thus, it can take as long as 8.5 years for two RM infants to produce a sexually mature F1 generation. Breeding strategies can be employed that take advantage of social dynamics within breeding groups to expedite the interval range between births, and after females reach sexual maturity, they remain sexually viable for up to 20 years. Despite the utility of the existing breeding strategies, the high cost of producing and maintaining NHPs makes them unsuitable for early investigations, or investigations that require high n values to model LD.

Rats are not typical reservoirs for LD in the wild, however, epidemiological studies in the United States and in Norway have revealed that rats are competent hosts for Bb and

Bbsl in urban settings (72, 73). Consistent with these observations, investigators have successfully used rats as experimental models for LD to study routes of infection transmission, and communicability of LD *in utero* and with respect to contact transmission between littermates (74). These early studies contributed to the current experimental models of LD by demonstrating that intradermal inoculation closely resembles a tick bite, and that LD is not communicable by physical or sexual contact, nor does the infection appear to be transmitted *in utero*. Sprague-Dawley rats have been used to reliably recapitulate polyarticular arthritis and to model neuroinflammation via microglial activation, although direct infection of the CNS is not supported in rats (75, 76). Rats were also among the first experimental models to reveal that Bb undergoes significant antigenic changes in the host's immune environment, as compared to when it is cultured *in vitro*, suggesting that a host-adapted spirochete may resemble tick-borne infection more accurately than culture grown Bb (77). Indeed, current studies in mice have corroborated the pathological similarities between tick-borne infection, and infection with host-adapted spirochetes (78). Overall, the rat has been demonstrated to be a useful experimental model for LD, however, as is discussed below, the laboratory mouse has even more utility than the rat, and is therefore the most widely used animal model for LD.

Although the rat model of LD preceded the mouse model, the development of transgenic mice provided incentive for investigators to identify suitable mouse models for LD, thus allowing them to use transgenic mice to study gene function (79-82). The decision to

switch to mouse models was further supported by observations that white-footed mice are natural reservoirs of LD in the wild (83, 84). Investigators identified three mouse strains that were useful for modeling different manifestations of LD, and many of those models currently remain in use (79). C3H/HeJ or C3H/HeN mice infected intradermally before 12 weeks of age develop severe polyarthritis, whereas Balb/c mice manifest cardiac symptoms of LD faithfully, characterized by conduction disturbances resulting in carditis (79, 85). Unlike the other two strains, C57/BL6 mice do not develop arthritis or carditis in response to Bb infection, but they do develop significant lymphoadenopathy, characterized by germinal center dysfunction and immature B cell proliferation in lymph nodes near the site of infection (78, 86, 87). Similar to human infection, untreated LD results in persistent infection in all three strains of laboratory mice (88). Remarkably, serum from human patients infected by Bb provides substantial protection against Bb infection in C3H/HeJ mice, suggesting that there is likely overlap in epitopes presented between humans and mice (89).

Another advantage of mice as experimental models for LD is that different inbred strains have different major histocompatibility complex (MHC) alleles that correspond to human HLA alleles, thus allowing investigators to determine whether there are factors related to antigen presentation that may contribute to disease severity or susceptibility. Although not yet studied in LD, the recent development of the human immune system mouse, provides an experimental mouse model that develops human innate and adaptive immune cells instead of murine immune cells (90-92). Human immune system mice

have been shown to reconstitute 40-60% of human CD45+ mononuclear cells in the peripheral blood and in the spleen, with relatively appreciable frequencies of B cells, T cells, NK cells, monocytes/macrophages, and DCs (93), suggesting that this model may provide a suitable method for investigating the effects of diseases on the human immune system.

Other practical considerations that make the mouse especially suitable for modeling LD (and many other diseases) include factors such as the availability/cost of reagents, and the cost of husbandry. By far, there are a greater variety of conjugated and unconjugated antibodies available for staining mouse tissues or cells, and the overall quantity of these reagents that are produced exceed that of any other laboratory animal species. Mice are also significantly smaller than rats, and as a result smaller quantities of reagent are needed to achieve a particular dosage of treatment. For example, a 1mg/kg dosage of lipopolysaccharide (LPS) for a 30g adult mouse would require 30µg of reagent. The same treatment administered to a 300g adult rat would require 300µg of LPS, a ten-fold increase in the quantity of reagent per treatment group. The smaller size of mice also requires relatively less space, and less food, thereby reducing overall costs for care and husbandry. Overall, an appropriate mouse model of neurological LD would greatly contribute to our understanding of pathophysiological mechanisms that may underlie neurological LD, and its associated complications that occur in patients. Below, we discuss recent discoveries in neuroimmunology and neuroanatomy that are potentially relevant to neurologic LD.

1.7 CSF, Brain Glymphatics and Meningeal Lymphatics

The primary region where CSF is produced is the choroid plexus (CP), anatomically located in the 3rd ventricle of the brain. Unlike the tight-junction connected capillaries of the blood brain barrier (BBB), the capillaries that vascularize the CP are semi-permeable, and interstitial fluids can exit the capillary beds with relatively little resistance (94). Despite the relative permeability of the CP capillary beds, interstitial fluids cannot easily escape the perivascular spaces surrounding the CP, because the epithelial layer of the CP is lined by tight-junction connected ependymal cells that regulate which molecules are transported into the ventricles of the brain. Thus, similar to the BBB, the blood-CSF barrier is tightly regulated(94).

Ependymal cells facilitate the production of CSF by selectively regulating Na+, HCO^{3-}, K^{+}, and Cl^{-}, ions across ion channels and shuttling water via aquaporin-1 or aquaporin-4 mediated transport(95). The overall contribution of water to the volume of CSF is substantial, as it comprises 99% of the volume of CSF, compared to only 92% in blood ((96). CSF continually bathes the brain, circulating through the 4 ventricles of the brain and through the perivascular Virchow-Robin spaces of the brain parenchyma. As CSF circulates through the Virchow-Robin spaces, it produces a convective effect that draws out interstitial fluids that are produced by blood circulation in the brain (94). Overall, CSF functions to continually remove interstitial fluid from the brain, ultimately draining it back into the lymph and peripheral blood. The collective term for CSF-mediated

transport of interstitial fluid inside the brain is called the glymphatic system, based on its similarity to the peripheral lymphatic system (97).

The glymphatic and lymphatic systems working together recycle and renew CNS 4-12 times per 24 hour period (94). There are three proposed models that explain CNS recycling. The first, and oldest model demonstrates that CSF exists the CNS through small fingerlike projections that protrude from the subarachnoid spaces of the spinal cord, called arachnoid villi. The arachnoid villi penetrate through the dura and into neighboring venous plexi, that drain the contents back into the blood and/or lymph (98). The second model shows that the contents of CSF can also drain through the subarachnoid space proximal to the olfactory bulb of the brain. Near the olfactory bulb, CSF flows behind the cribiform plate, and drains into the lymphatic vessels of the nasal mucosa that connect to the superficial and deep cervical lymph nodes (99, 100). The third, and most recent model, demonstrates that lymphatic vessels are present in the dura(101). Each meningeal lymphatic network appears to start from both eyes and runs above the olfactory bulb before connecting to the dural sinuses. The dural sinuses run parallel to the superior and transverse sagittal sinuses, and evidence suggests that these vessels transport T cells and some portion of CSF from the dura into the deep cervical lymph nodes (101). All three models of CNS fluid dynamics are supported by evidence, however, the mechanisms underlying immune surveillance and immune trafficking to and from the brain, especially in the context of infection, remained incompletely understood.

1.8 Major Gap in Knowledge

Overall, the incidence of LD that is caused by Bb in the United States is estimated to be approximately 300,000 per year (102). Recent investigations also reveal that other Borrelia species belonging to Bbsl are emerging in the United States, suggesting that the total incidence of LD caused by all Bbsl species would likely exceed the 300,000 estimate (1, 6, 7). Most individuals that are infected by Bb recover if diagnosed and treated promptly, but not all individuals develop the pathognomonic sign of an EM, while others do develop EM, but do not notice it due to its location on the body (i.e. scalp or back) (39, 40). Those that do not receive timely diagnosis may go on to develop secondary or tertiary symptoms that include polyarthritis, polyneuritis, encephalitis and carditis (40). Neurological symptoms are associated with a higher risk of developing PTLDS, a debilitating syndrome that up to 20% of individuals with neurological manifestations of Bb develop, despite antibiotic treatment (43). At this time there are no FDA approved treatments for PTLDS, and physicians are limited in the types of approaches that they can use for PTLDS patient management (29).

A key factor that limits our understanding of neuroborreliosis and its potential complications such at PTLDS is the lack of a high efficiency and cost-effective animal model that faithfully replicates human manifestations of neurological LD. The RM is an accurate model for human neuroborreliosis, but the high cost of care, long reproduction time, and small litter size make it unfeasible for early investigations or approaches that

require large sample sizes. Thus far, the laboratory mouse has been instrumental in understanding the pathological features of LD that relate to arthritis and carditis, as well as the mechanisms that Bb utilizes to subvert immune detection and to persist in an otherwise immunocompetent host (31, 78, 79, 85-88, 103-109). Although some investigators have attempted to develop a mouse model of neuroborreliosis, the inoculation route of Bb was intracerebroventricular, and thus it did not model the true course of infection, which is intradermal (110). The recent discovery of the meningeal lymphatics also highlights the importance that tissues within the CNS, especially the meninges, may have in regulating immunity, and understanding the role that these tissues play during LD may elucidate underlying pathophysiological mechanisms of LD. Thus, there is a major gap in our understanding of neuroborreliosis, and this is due in part to the lack of a suitable mouse model. Minimally, a mouse model that could replicate Bb infection, and/or Bb-induced inflammation in the CNS could provide an avenue to understand the course of the disease in humans, including the post-treatment complications such as PTLDS. Below, we propose a novel experimental approach, using the Lyme arthritis susceptible C3H/HeN mouse as a potential model for neuroborreliosis.

1.9 Research Overview

1.9.1 Central Hypothesis

Bb infection establishes persistent infection in brain-associated meninges, resulting in neuroimmunological changes and behavioral deficits

1.9.2 Background

LD is one of the fastest growing infectious diseases in the United States. Of the patients that acquire LD, up to 20% may develop chronic symptoms, in spite of antibiotic treatment, a condition known as PTLDS (29). Why some individuals do not recover from LD infection remains unknown, although the possibilities include 1) persistent infection in the CNS or associated tissues, 2) post-infectious autoimmunity that results in neuropathology, and/or 3) permanent damage to the CNS. The long-term effects of Bb infection on the brain and its associated tissues are not well understood, and relatively little is known about the neurological consequence of long-term Bb infection in mice. Typically, neurological Bb infection has been modelled in NHPs, and although the NHP model is useful for modelling the disease, its major limitation is the high cost of primates and their maintenance. In the work presented below, we sought to produce a mouse model of late-stage neurological Bb infection, using the LD susceptible C3H/HeN mice, a common American strain of Bb (Bb 297), and intradermal inoculation as the route of infection.

1.9.3 Approach

Although many strains of mice including C57BL6, Balb/c, and C3H are susceptible to infection with Bb, the C3H/HeN strain is an especially good model for cardiomyopathy and acute inflammation of the joints (88). In most mice, the highest susceptibility to Bb infection occurs at ~6-8 weeks of age (79, 88). Intradermal needle inoculation of Bb spirochetes has been established as a physiologically relevant method for modeling tick transmission of Bb (85), as it results in acute infection (within 3-5 days of inoculation), and disseminated infection that occurs within weeks. Similar to the course of LD in humans, in the absence of antibiotic treatment, Bb infection persists in most mice (88).

There are three strains of Bb that are commonly used to model experimental infection in the United States, strains B31, N40, and 297. Bb 297 is a particularly appropriate strain for modeling neurological infection because it is currently common in the human population in the United States, and this strain has been shown to result in infection of the CSF, suggesting that it has some tropism for tissues associated with the CNS (111). In patients, neurological infection can begin as early as 3 weeks after infection, and typically manifests within 6-12 weeks after tick-transmitted Bb infection (20). Accordingly, we infected mice with Bb 297, and we evaluated the presence of neurologic infection, changes in neuroimmune phenotypes, and behavior, early in disease and during late-stage disease, where neurologic infection was expected, corresponding to 5 days post infection (dpi), and 45 dpi, respectively.

In C3H/HeN mice, it has been demonstrated that Bb inoculum is typically most effective for transmitting chronic infection when the initial inoculum dose is transmitted intradermally and contains 10^4-10^6 spirochetes (88). Importantly, when Bb spirochetes are cultured *in vitro* for the purpose of experimental infection, they must be kept at low passage number (usually <20 passages), otherwise the spirochetes become adapted to *in vitro* conditions and they lose their virulence factors and the ability to produce experimental infection (112, 113). Furthermore, experimental infections require that the spirochetes are in the mid-log phase of growth, because this is the phase of growth where protein expression has the least variation between Bb spirochetes within the same culture (113).

1.9.4 Aim 1

Aim 1: Determine if intradermal infection with Bb 297 results in infection of brain or dura

Rationale: Bb 297 is a strain that was originally isolated from the CSF of a patient in New York (111). Because Bb 297 has tropism for the CSF, and the CSF is a fluid that encapsulates the brain, and circulates through perivascular spaces of the brain parenchyma (94), we reasoned that 1) spirochetes would be present in CSF of LD susceptible C3H/HeN mice, and 2) spirochetes would establish infection in the brain and its associated tissues, including the meninges. It has also been established that Bb has tropism for decorin, a collagen bound protein, as well as other components of the extracellular matrix (114, 115). Importantly, collagen and decorin are expressed in the

meninges, and the arterial walls of the blood vessels that supply the meninges (116, 117). Decorin is also expressed in the gray matter of the developing neocortex, hypothalamus, and thalamus (118).

Hypothesis: Intradermal infection of C3H/HeN mice with Bb 297 will result in infection of the brain and meninges within 45 dpi.

1.9.4.1 Aim 1.1

Aim 1.1: Determine if Bb spirochetes can be cultured from brain or meninges of Bb infected mice, early in infection (5 dpi), and late in infection (45 dpi).

Rationale: BSK-II medium is routinely used to culture Bb spirochetes *in vitro* until the concentration of spirochetes is sufficient for use in experimental infection. Many investigators have also used BSK-II medium to culture Bb out of the tissues of Bb-infected mice (88, 103).

Experimental design: For each experiment, we infected male, C3H/HeN mice that were 6-8 weeks old at the time of infection, with 10^6 low passage (<6), mid log-phase Bb 297 spirochetes in BSK-II medium. All injections were given via the intradermal route of inoculation in the dorsal thoracic midline. Mice were euthanized at time points of 5 dpi, or 45 dpi, to assess whether Bb could persist in the CNS at these time points. Sham mice, injected intradermally with BSK-II medium only, were used as negative controls. While under anesthesia, each mouse was perfused transcardially to remove all blood, followed by the removal and collection of brain and meningeal tissues. Tissues were evaluated for evidence of Bb infection by culturing the tissues in BSK II medium for 12

days. The number of spirochetes that grew out from the culture was quantified by dark field microscopy. Experiments were performed as described in Table 1.2.

Methods to assess Bb persistence:

Tissue culture: To determine if any spirochetes remained in the tissues of interest, we cultured the extracted tissues in BSK-II medium for up to 12 days. Culturing the extracted tissues provided us with insight about whether host-adapted Bb spirochetes in our tissues of interest were sufficiently viable or resilient to replicate in culture.

Table 1.2 Aim 1. Experimental treatment groups for completion of Aim 1.1 and Aim 1.2

Group Number	Treatment ID	Description	n per tx group	Number of groups	Tissues collected upon euthanasia	Methods of assessment	Rationale for each treatment
1	5dpi Bb	Mice infected with Bb 297. Euthanized after 5 days	5	1	Dura, Brain	tissue culture (n=5 for each)	Test borrelia persistence after 5 days of infection
2	5dpi Sham	Mice treated with BSK-II medium. Euthanized after 5 days	5	1			Negative control for vehicle after 5 days of inoculation
3	45dpi Bb	Mice infected with Bb 297. Euthanized after 45 days	5	2		immunostaining, tissue culture (n=5 for each)	Test borrelia persistence after 45 days of infection
4	45dpi Sham	Mice treated with BSK-II medium. Euthanized after 45 days	5	2			Negative control for vehicle after 45 days of inoculation
Total mice needed to address aim = 30							

n values were determined based on power analysis using GPower3 software and the following parameters: apriori analysis, difference between two independent means, Beta = 0.20, alpha = 0.5, and an effect size of 2.19 that was calculated based on preliminary data (not shown)

1.9.4.2 Aim 1.2

Aim 1.2: Determine if spirochetes or spirochete antigens persist in meninges of Bb infected mice late in infection (45 dpi).

Rationale: Although tissue culture is a suitable method for determining whether viable bacteria persist in tissues, it does not address whether any bacterial remnants, for example antigens, or "viable but nonculturable" Bb spirochetes may persist in tissues (103, 119). Furthermore, culture positivity does not provide information about the anatomical region in the tissue where persistent infection may occur. Therefore, we performed immunostaining followed by confocal imaging to determine whether any spirochetes or spirochete remnants remained in the meninges, and if so, where in the tissues they localized.

Experimental design: Mice were infected and euthanized as described in aim 1.1. All samples were immunostained for Bb, CD31, and LYVE-1, and they were imaged by confocal microscopy. Experimental groups are described in Table 1.2.

Methods to assess Bb persistence:

Immunostaining/confocal imaging: After euthanasia, the dura were removed and fixed in 2% paraformaldehyde overnight, followed by immunostaining. Immunostaining was performed for Bb, the blood vessel marker CD31, and the lymphatic vessel marker LYVE-1. All staining was performed using primary unconjugated polyclonal IgG antibodies, followed by secondary staining with fluorophore conjugated anti IgG. Tissues were mounted in DAPI containing mounting medium for the detection of

nucleated cells. Samples were imaged on Olympus and Leica confocal microscopes and images were processed using ImageJ software.

1.9.5 Aim 2

Aim 2: Determine if intradermal infection with Bb 297 results in changes in B or T cell populations in the brain or meninges

Rationale: Immune cells in the CNS have been shown to play a role in the etiology of neurodegenerative diseases such as Multiple Sclerosis, Alzheimer's disease, Parkinson's disease, and acute-onset neurological diseases such as Guillain-Barre syndrome (120, 121). Although, it has not been proven definitively, evidence suggests that post-infectious complications of LD, such as RLA and PTLDS, may have autoimmune etiologies that can involve subsets of B cells and T cells (23, 29, 31, 122). It is possible that immune related events that occur in the CNS during neurological LD may set the stage for the outcome of the disease, either in favor of disease resolution, or toward autoimmunity. Accordingly, we aimed to address whether any changes in B or T lymphocytes were detectable in the CNS during early infection (5 dpi), or late-stage infection (45 dpi).

Hypothesis: C3H/HeN mice infected by Bb have measurable differences in B cell and T cell immune subsets in the brain and meninges during early (5 dpi), and late-stage (45 dpi) infection.

Experimental design: Mice were infected and euthanized as described in Aim 1.1. After euthanasia, brains and meninges were removed and put in single cell suspension, stained

with fluorochrome conjugated antibodies, and evaluated for immunophenotypic changes by flow cytometry. Treatment groups are described in Table 1.3.

Methods to assess changes in leukocyte populations:

Isolation of leukocytes: While under anesthesia, each mouse was perfused with 60mL Ca^{++} / Mg^{++} free, phenol red-free Hank's balanced salt solution (HBSS). Each head was removed and the skullcaps were cut out for the isolation of the dura. Dura were incubated in collagenase to dissociate cells from tissue. The resulting suspensions of the brains and dura were triturated through a 100um strainer to produce single cell suspensions. To remove myelin, brain single cell suspensions were centrifuged over 37% Percoll and the myelin-containing top layer was removed by vacuum aspiration.

Staining and flow cytometry: All cells were stained with 1:100 dilution of fluorochrome-conjugated monoclonal antibody, as described, in serum block buffer for 20 minutes. Stain panels and gating strategies are described in Table 1.4. Samples were washed and run on Becton Dickinson (BD) FACS CANTO II flow cytometer, and data was analyzed using FlowJo software

1.9.6 Aim 3

Aim 3: Determine if long-term infection with Bb 297 results in behavioral deficits

Rationale: Neurological LD and its post-treatment complication, PTLDS, typically take several weeks to months to develop (20, 29, 40). In patients that have neurological LD or PTLDS, objective sensory/motor and cognitive deficits are often present (23, 29, 123). Typical deficits include difficulties with verbal fluency, impaired short-term memory,

impaired ability to process information, fatigue, sleep disturbances, peripheral neuropathy and depressed mood (23, 123, 124). To date, we have not identified any LD behavioral studies performed in any mouse models of LD. Thus, we aimed to determine if there were any underlying behavioral deficits in LD-susceptible C3H/HeN mice infected by the CSF-tropic strain of Bb, Bb 297.

Hypothesis: C3H/HeN mice infected intradermally with Bb 297 develop behavioral deficits manifesting as increased peripheral nociceptive response, decreased motor function, anxiety, and impaired short-term memory.

Experimental design: Mice were infected as described in Aim 1.1, for 45 dpi only, and euthanized. Nociceptive responses were be measured using the Von Frey pain test, as described (125). Changes in motor function, and anxiety behaviors were assessed by the open field test (OF) (126, 127). Short-term visual memory was assessed by the novel object recognition test (NORT) (126, 128). The OF and NORT tests were recorded digitally and performed on the same day as described in Table 1.5. OF and NORT video data were analyzed using Noldus Ethovision XT12 software.

Table 1.3 Aim 2. Experimental treatment groups for completion of Aim 2

Group Number	Treatment ID	Description	n per tx group	Number of groups	Tissues collected upon euthanasia	Methods of assessment	Rationale for each treatment
1	5dpi Bb	Mice infected with Bb 297. Euthanized after 5 days	5	1	Dura, Brain	Flow Cytometry	Test changes in leukocytes after 5 days of infection
2	5dpi Sham	Mice treated with BSK-II medium. Euthanized after 5 days	5	1			control for vehicle effects after 5 days of inoculation
3	45dpi Bb	Mice infected with Bb 297. Euthanized after 45 days	5	1			Test changes in leukocytes after 45 days of infection
4	45dpi Sham	Mice treated with BSK-II medium. Euthanized after 45 days	5	1			control for vehicle effects after 45 days of inoculation
Total mice needed to address aim = 20							

n values were determined based on power analysis using GPower3 software and the following parameters: apriori analysis, difference between two independent means, Beta = 0.20, alpha = 0.5, and an effect size of 2.19 that was calculated based on preliminary data (not shown)

Table 1.4 Flow cytometric approach. B cell and T cell antibody panels and flow cytometric gating strategy

Panel	Antibodies in Panel	Subset	Gating Strategy
T cell	Aqua	CD4	Singlet, Aqua-, CD45high, CD3+, CD19-, CD4+, CD8-
	CD45		
	CD3		
	CD19	CD8	Singlet, Aqua-, CD45high, CD3+, CD19-, CD4-, CD8+
	CD4		
	CD8		
B cell	Aqua	B1a	Singlet, Aqua-, CD45high, CD3-, CD19+, CD5+, CD1d-
	CD45		
	CD3	B1b	Singlet, Aqua-, CD45high, CD3-, CD19+, CD5-, IgDlow
	CD19		
	CD5	B2	Singlet, Aqua-, CD45high, CD3-, CD19+, CD5-, IgDhigh
	CD1d		
	IgD	B10	Singlet, Aqua-, CD45high, CD3-, CD19+, CD5+, CD1d+

Table 1.5 Proposed behavioral testing. Approach and timing for OF and NORT tests.

Test	Phase	Duration (min)	Subsequent resting interval (min)
Open field	Habituation	10	90
Novel object recognition - 1	Learning	5	90
Novel object recognition - 2	Testing	5	N/A

Methods to assess behavioral changes:

Von Frey pain test: The Von Frey pain test was performed on an elevated surface with small holes in the base. Each mouse was allowed to habituate to the environment for 3 minutes. The tip of the Von Frey filament was applied to the plantar surface of each hind paw. If a withdrawal response did not occur, the next largest filament was used. If a withdrawal response was elicited, the next lowest filament was used until no withdrawal response occured. The filaments were then increased in size again until a withdrawal response occured. This method was repeated until a single filament elicited 4 consecutive withdrawal responses, indicating the end of one trial. Two trials were conducted on each hind-paw of each mouse per treatment group.

OF test: Each mouse was placed in a 31 cm x 32 cm plexiglass OF box and behavior was recorded for 10 minutes. After a 10 minute recorded session in the OF box, each mouse was returned to its original cage, and the OF box was cleaned thoroughly with ethanol before the next mouse was tested.

NORT test: After the OF test, each mouse was given a 90 minute rest interval, and then each mouse was returned to the OF cage containing two identical, equally-spaced objects that met the criteria previously described (128). After a 5 minute learning interval, each mouse was returned to its original cage for 90 minutes. Next, each mouse was put back in the OF cage, this time containing one familiar object from the previous session, and a novel object. The mouse was recorded for 5 minutes, and behavior was analyzed using Noldus Ethovision XT 12 software.

1.9.7 Significance

The research proposal described above was designed to comprehensively evaluate whether the patient-derived CSF-tropic strain of Bb, Bb297, could produce persistent infection in the CNS of LD-susceptible C3H/HeN mice. Our approach also provided information about neuroimmune changes that were associated with Bb infection. Finally, we assessed whether any behavioral deficits were associated with Bb infection, an approach that to date has not been used in the context of experimental LD. Collectively, this research helped to determine whether infection with Bb 297 in C3H/HeN mice could serve as a novel experimental mouse model for neuroborreliosis, and it elucidated some possible mechanisms that Bb or other pathogens could utilize to establish infection in the CNS.

2. GAMMA DELTA T CELLS AND DENDRITIC CELLS IN REFRACTORY LYME ARTHRITIS*

2.1 Synopsis

LD is a multisystem infection transmitted by tick vectors, and it is the fastest growing vector-borne disease in the United States. The primary treatments are oral or intravenous antibiotics. Despite treatment, some individuals do not recover and have prolonged symptoms affecting multiple organs, including the nervous system and connective tissues. Inflammatory arthritis is a common symptom associated with Lyme pathology. In the past decades, γδ T cells have emerged as candidates that contribute to the transition from innate to adaptive responses. These cells are also differentially regulated within the synovia of patients affected by RLA. Here we review and discuss potential cellular mechanisms involving γδ T cells and DCs, in RLA. Toll-like receptor signaling (TLR) and antigen processing and presentation, will be the key concepts we review in aid of understanding the impact of γδ T cells in RLA.

2.2 Introduction

LD is a multisystem tick-borne infection primarily affecting the joints, heart, and nervous system (40, 129). As we have described, compared to other vector-borne diseases, there is a relatively high incidence of LD in the United States (102). The

* Reprinted with permission from "γδ T cells and Dendritic Cells in Refractory Lyme Arthritis" by Ali Divan, Ralph C Budd, Richard P Tobin and M.Karen Newell-Rogers, 2015. J. Leuk Biol, 97, 653-63, Copyright [2015] by Federation of American Societies for Experimental Biology.

infection is caused by the Bb spirochete, which is transmitted through the salivary glands of a tick during a blood meal (130). The minimum time requirement for tick-to-skin attachment and Bb dissemination is 24-48 hours, although this can vary depending on whether the tick has had previous blood meals in the same day (131). At present, only two species of ticks are known to transmit LD, the Blacklegged tick *Ixodes scapularis*, and the Western Blacklegged tick *Ixodes pacificus* (102). If LD is detected within the first few weeks of infection, treatment with oral or intravenous antibiotics is generally effective. If treatment is delayed, systemic dissemination and continued symptoms can present challenges to therapy. In some individuals, antibiotic treatment does not provide relief of symptoms. Instead, these patients manifest chronic states of inflammatory disease. Evidence suggests that the pathology of these chronic diseases can be attributed to either autoreactive immune cells and/or persistent infection (132-135). These chronic diseases are called PTLDS when there is multisystem involvement, or RLA when the involvement is predominantly in the joints. Lyme advocacy groups, researchers, and clinicians disagree as to whether the symptoms of these lingering diseases are from chronic infection or immune dysfunction or both (40, 134, 136-140). Recent reports in the literature describe persistent levels of flagellin B DNA and Bb cell bodies in multiple tissues up to 12 months after antibiotic treatment. Bb bodies that exist within these tissues are uncultivable, but surprisingly xenodiagnosis with ticks reveals new spirochetal forms within tick tissues (135). This suggests that non-cultivable spirochetes may either be dormant, or are sufficiently immunogenic to cause prolonged pathology and symptoms. Other work shows persistent increases in Bb antigens, DNA, and RNA in

the tissues of RMs, despite receiving aggressive antibiotic treatment in the late disseminated period of infection (133). This further supports the hypothesis that an initial tick bite can result in persistent infection. These studies underscore the importance of early treatment, and they bring into question whether treatment success may depend on a balance between effective adaptive immune responses, and long-lasting innate responses. Furthermore, the timing of clinical intervention, as it relates to host immunity and pathogen burden, may determine the outcome of LD on the basis of effective and regulated antigen processing and presentation. Thus, despite contention between paradigms of chronic infection versus immune dysfunction, a combination of Bb persistence and/or immune dysfunction could also result in a state of chronic disease.

Both PTLDS and RLA have associations with TLR1 polymorphisms, and HLA-DR haplotypes (141, 142). Symptoms of PTLDS include mild to severe musculoskeletal pain, fatigue, as well as difficulties in concentration, loss of cognitive abilities, and loss of memory. RLA is marked by persistent polyarthritis of the joints, especially in at least one knee (134, 143, 144). Both syndromes are debilitating and can decrease the quality of life.

An important T cell subset, the γδ T cell, has been at least partially characterized over the last two decades. Several laboratories and investigators have focused on these cells in both infectious and autoimmune diseases. Of particular interest, is our work that has focused on elucidating the signaling pathways and the molecular mechanisms used by

(DCs) and γδ T cells in RLA (105, 141, 143-146). Our group's work includes cloning γδ T cells from the synovial fluids of RLA patients, characterizing changes in Fas ligand (FasL) expression, and investigating caspase signaling events in response to FasL signaling (105, 145, 146). Here, we will focus on the immune responses in RLA, with specific focus on DCs and γδ T cells. Additionally, we will discuss an antagonist peptide that affects survival signals on polarized antigen presenting cells (APCs), and we will discuss its potential role in treating RLA.

2.3 γδ T Cells in Inflammatory and Infectious Diseases

γδ T cells were first observed in the peripheral blood of humans in the 1980's (147). These cells are characterized by the expression of the T cell receptor gamma and delta chain genes located on chromosomes 7 and 14, which rearrange in a manner similar to V(D)J recombination in αβ T cells (148). In contrast to αβ T cells, however, the γ and δ chains of the T cell receptors (TCRs) need not be disulfide linked. Whether disulfide linkage occurs depends on the expression of the constant regions Cγ1 vs Cγ2, both of which are contained within the TCR gamma locus (149, 150). Besides structural differences in their TCR, these cells are unique because they have the ability to respond to non-classical MHC antigens, such as CD1a-c (151, 152) or CD1d (153, 154). γδ T cells are also differentially regulated in a variety of infectious and inflammatory diseases. For example, in murine relapsing/remitting experimental autoimmune encephalitis (EAE), the γδ T cell subset expressing Vγ4 is elevated in the CNS both during disease onset and relapse, but not during remission. Interestingly, the γδ T cell

subset expressing the Vγ1 TCR is found at the highest frequency in the CNS during remission. Consistent with the above functional associations, treatment with activating antibodies against Vγ1 is associated with decreased disease severity and decreased IL-17 production. In contrast, during all phases of EAE, Vγ4 activating antibodies increase disease severity and IL-17 production (155). In rheumatoid arthritis (RA) Vγ9Vδ2 T cells are elevated in frequency in the synovial tissues of human patients, and in contrast to MS, this subset is the primary subset that produces IL-17 and IFN-γ (156-158). Importantly, γδ T cells can be regulated by different subsets of natural killer T cells (NKTs) (159). In murine Coxsackievirus-induced endocarditis, there are increases in Vγ4 T cells, while invariant NKT cells (iNKT), and T regulatory cell (Treg) cells and their responses are decreased. Protection against endocarditis is associated with increased iNKT activation, decreased activation of Vγ4 T cells, and increased Treg responses (159). γδ T cells have also been implicated in granulomatous mycobacterial infections, such as leprosy and tuberculosis (160, 161), as well as parasitic infections mediated by multiple Plasmodium species (162, 163). Collectively, these studies demonstrate that γδ T cells are important in inflammatory and autoimmune diseases. Below we summarize what is known about γδ T cells in LD and RLA.

2.4 Characterization of γδ T cells in Lyme Arthritis

Interest in γδ T cells expanded significantly during the 1990s. A few groups focused on γδ T cells in RLA. In these studies, synovial tissues from RLA patients showed distinct increases in the Vδ1+ subsets of γδ T cells (164). Although many studies have

documented the presence of γδ T cells in the synovial fluid of RLA, it remains unclear which APCs are most important γδ T cell activation. Some suggest that B-cells are the primary APCs, while others show that DCs may be more important (165-167). Sometimes γδ T cells themselves can serve as APCs. We will focus on DCs, as the APCs of interest, because some evidence suggests DCs are the most important antigen processing and presenting cells in RLA (146).

2.5 γδ T cells and the Dendritic Cell

In LD, γδ T cells require both an APC, and activating cytokine(s) (146). Flow cytometric analyses of RLA-derived γδ T cells demonstrate that IL-2, DCs, and Bb antigens are necessary to cause FasL and CD25 expression on γδ T cells. In the absence of DCs, or Bb sonicate, no increases in FasL or CD25 are observed. Consistent with these findings, others report that γδ T cells and DCs both contribute to the immune response against infectious pathogens, including malaria, West Nile virus, and sepsis (168-171). In RLA, Fas:FasL interactions are the initiating events for reciprocal maturation/activation of DCs and γδ T cells (105). These events are also associated with increased IL-12 production and increased cell surface expression of CD40 and CD86, well-described co-stimulatory molecules, on DCs. As a result of the increased co-stimulation, the DC serves to activate the γδ T cell as reflected by an increase in the activation marker CD25 (Figure 2.1 A) (105). The increase in FasL may be surprising because FasL expression is often assumed to be exclusively associated with cell death pathways, although recent work has demonstrated that this is not necessarily the case (172-176). In fact, Fas:FasL

signaling can have a variety of different outcomes (172-174, 176). Below we discuss the Fas:FasL signaling mechanisms that may be important in RLA.

2.6 Fas:FasL Signaling in Murine Lyme Polyarthritis Models

To understand the role of FasL expression on γδ T cells in RLA, Shi et al addressed the question using C3Hgld mice, a mouse strain with a non-functional mutation that results in defective FasL expression (145). Using this mouse model, the investigators show that mice deficient in FasL sustain reduced cytokine responses to Bb, and have decreased incidence and severity of polyarthritis (145). Interestingly, there is no difference in the Bb burden or antibody titers to Bb between C3Hgld mice and controls, suggesting that the Fas:FasL receptor:ligand pathway does not directly impact the number of spirochetes during the infection. Instead, Fas:FasL signaling between CD4 T cells, DCs, and γδ T cells has been proposed to be part of the proinflammatory response during Lyme polyarthritis and RLA (145). In parallel, in human synovial fluids collected from RLA patients, FasL selectively induces apoptosis of Fas^{high} CD4 T cells, whereas approximately 90% of Fas^{Low} CD4 T cells remain viable (164). It has not yet been reported whether the Fas^{Low} CD4 T cells become activated and/or produce inflammatory cytokines in response to FasL on γδ T cells. However, if they produce inflammatory cytokines, this finding would be consistent with the results obtained using the C3Hgld mouse model.

The consequence of Fas engagement by FasL appears to be determined by the activation of downstream accessory proteins, including Fas Associated Death Domain (FADD)-containing molecules, FADD-like IL-1β converting enzyme (FLICE), and/or cellular FLICE-inhibitory protein (c-FLIP). Cell death is associated with recruitment of FADD-containing molecules, whereas c-FLIP serves as an inhibitor of Fas-dependent apoptosis (177-179). DCs are known to produce high levels of c-FLIP, levels greater than those reported for other monocyte-derived cellular populations (180). Multiple studies in Lyme and non-Lyme models show that there is a direct correlation between c-FLIP expression and DC resistance to Fas:FasL mediated apoptosis (105, 180-182). In fact, downregulation of c-FLIP correlates with increased sensitivity to Fas:FasL-induced apoptosis, whereas overexpression of c-FLIP is associated with cell survival. Interestingly, c-FLIP expression in DCs increases in response to TLR4 activation with LPS (180). In addition, when T cells are activated via TCR/CD3 signaling, c-FLIP can serve to promote IL-2 production by the activated T cells through the ERK and NFκB pathways (182). Furthermore, c-FLIP confers protection from apoptosis for the TCR/CD3 activated cells (182).

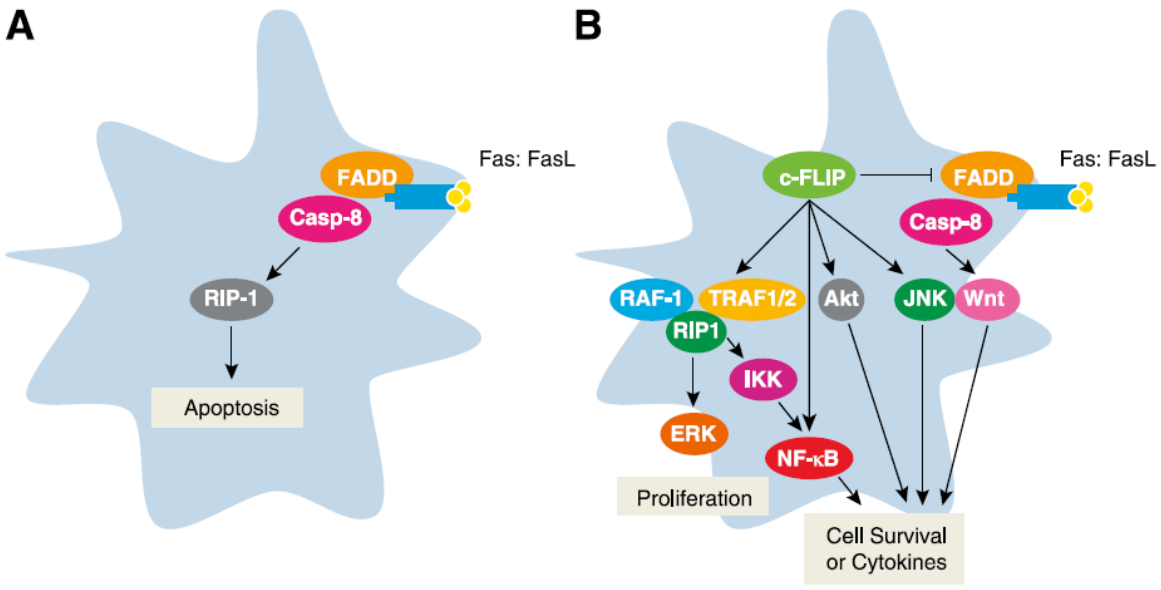

Figure 2.1 Fas:FasL interactions. A, Death signaling pathway initiated by Fas:FasL binding in a DC. Fas Associated Death Domain (FADD) binds the cytoplasmic domain of Fas and recruits caspase-8 to activate RIP-1 and initiate apoptosis. B, Alternative Fas:FasL signaling pathways are controlled by cellular FLICE-inhibitory protein (c-FLIP). c-FLIP inhibits signaling from the death-inducing FADD/caspase8 complex and diverts the signals toward the mitogen-activated protein kinase (MAPK) pathways (AKT, JNK, Wnt) NF-κB pathway, or ERK pathways resulting in survival, cytokine production, and/or proliferation. These pathways are not mutually exclusive

Based on these observations, Shi et al propose that c-FLIP is the Fas-associated element that is responsible for Fas-dependent proliferation, differentiation, and/or cytokine secretion in DCs. c-FLIP could act to prevent the activity of FADD in causing Fas-mediated cell death by diverting the Fas:FasL death signal from the FADD/caspase-8 death pathway, toward the MAPK/ERK pathway by way of the association of the serine/threonine kinase Raf1 with c-FLIP (183-186). Alternatively, TNF receptor associated factor 2 (TRAF-2) and receptor-interacting protein 1 kinase (RIP1) could bind c-FLIP and trigger the NF-κB pathway resulting in inflammation (187). In either case, both MAPK and NF-κB pathways regulate increases in TNF-α and the proinflammatory cytokine IL-12, as illustrated in Figure 2.1B (105, 188, 189). These mechanisms explain two possible ways in which Fas:FasL signaling between Bb-stimulated γδ T cells and DCs may promote production of TNF-α and IL-12, thereby contributing to RLA.

The above possibilities are consistent with the observation that C3Hgld mice deficient in Fas:FasL interactions have reduced joint inflammation in response to Bb infection (145).In fact, when C3Hgld mice are infected with Bb, their CD4 T cells produce significantly less IFN-γ and IL-4 than their wild type counterparts (145), suggesting that Fas:FasL signaling affects CD4 T cells as well as other lymphocyte populations. Currently, little is known about the outcome of Bb infection in Fas-deficient lpr mice. Because lpr mice do not express the Fas receptor, they will serve as an important model system to better elucidate the contribution of Fas:FasL interactions in murine Lyme polyarthritis and human RLA.

2.7 γδ T Cell Activation Depends on TLR2 in Lyme Disease

The lipidated outer surface proteins A and C (OspA, OspC), that are produced by Bb, have been demonstrated to cause inflammation via TLR2 signaling in monocytes and B cells (190). TLR2 signaling pathways are also important for γδ T cell activation (104). When DCs and γδ T cells are combined with IL-2 and either lipidated or non-lipidated OspA, the outcomes are quite different, respectively. In the case of lipidated OspA, γδ T cell proliferation and activation occurs with similar magnitude as exposure to whole Bb sonicates (104). In contrast, treatment with non-lipidated OspA results in no net proliferative effect. These contrasting outcomes reveal that the TLR2 signaling pathways, necessary for DC and γδ T cell signaling in response to Bb, depend heavily upon the lipid modifications of the TLR agonist. The contribution of TLR signaling in DCs and γδ T cells is further supported by studies using MyD88-/- mice. The downstream effects of TLR2 engagement require MyD88, a complex of signaling molecules that lead to cellular activation (191-193). Loss of MyD88 in DCs prevents activation of γδ T cells, regardless of the lipidation state (lipidated versus non-lipidated) of OspA. Antibodies blocking TLR2 binding also prevent γδ T cell activation (104), further supporting the hypothesis that TLR2 may be an important mediator of γδ T cells in RLA.

The requirements for physical contact between Bb-exposed γδ T cells and DCs has been investigated (104). In the absence of physical contact between γδ T cells and DCs, treatment with Bb sonicate failed to activate the γδ T cells, as measured by upregulation

of cell surface CD25. This suggests that contact between γδ T cells and DCs is necessary for γδ T cell activation. Furthermore, fixation of DCs prior to Bb antigen exposure prevents CD25 upregulation, underscoring the requirement for DC metabolic activity in γδ T cell activation (104). In contrast, γδ T cell activation is not affected by antibodies blocking MHCI or MHCII molecules, although blocking the γδ TCR does have an inhibitory effect (104). This indicates that signaling through the γδ TCR is necessary for cell activation in response to Bb sonicate, but that the γδ TCR is not necessarily restricted to classical MHC I or MHC II molecules. Interestingly, blocking caspase activity with the pan-caspase inhibitor zVAD also prevents CD25 upregulation and inhibits downstream cytokine responses as evidenced by decreases in IL-1β, IL-12, and IL-6 (190). Collectively, these data suggest that TLR2 signaling, and physical contact between metabolically active DCs and γδ T cells are required for γδ T cell activation in response to Bb. Furthermore, these interactions are not necessarily MHC restricted to classical MHCI or MHCII molecules, although both the γδ TCR and caspase activity are necessary in DCs.

Although some investigators have shown that γδ T cells from human blood can express TLR2 receptors, this does not appear to be the case in γδ T cells isolated from RLA synovial tissues (104). In fact, γδ T cells bearing Vδ2Vγ9 subsets of γδ T cells are the only known subsets that produce robust responses to TLR2 ligands without help from DCs (194, 195). The Vδ2Vγ9 subsets of γδ T cell subsets are the most common subset in human peripheral blood. However, this same subset represents a minority of the γδ T

cells in RLA synovia, where the most frequent subset expressed is Vδ1 (194, 195). Interestingly, both Vδ1 and Vδ2Vγ9 subsets can produce increased cytokine responses, including IFN-γ and IL-8, when their TCRs are stimulated with anti-γδ TCR antibody and TLR2 ligands (194, 195). This implies that TLR2 may costimulate γδ T cells, either directly or indirectly, resulting in cytokine production. Thus, TLR2 may serve a costimulatory role for γδ T cells analogous to the proposed costimulatory role for TLR2 in αβ T cell activation (196). Because of the abundance of Vδ1 subsets in RLA (164), and because these subsets are not directly activated in response to TLR2 stimulation, γδ T cells may depend on other cellular populations to become activated in RLA. Based on the evidence cited above, γδ T cells likely become indirectly activated by TLR2 agonists through physical cell:cell contact that triggers caspase activity in metabolically active DCs, as illustrated in Figure 2.2. A similar interpretation has been reported for other non-RLA models of disease (197).

2.8 Non-classical Antigen Presentation in Lyme Disease and RLA

Unlike the αβ TCR that has been widely characterized as recognizing antigens only when presented by classical MHC class I or II molecules, the specificities of γδ TCRs remain controversial, as they are thought to bind a variety of cognate ligands. The MHC-like non-classical molecules, the CD1 proteins, have become the focus of many studies involving γδ T cells, iNKTs, and NKT cells (154, 198, 199). CD1 molecules can present amphipathic moieties, including lipids, lipopeptides, and glycolipids, that appear to help γδ T cells differentiate self from non-self (200). The group two CD1 protein, CD1d, is

expressed constitutively on DCs (201). Evidence shows that Bb peptides can be presented via CD1d, and can activate NKT cells to respond to Bb infection (201-203).Unlike CD1d molecules, the group one CD1 proteins, CD1a/b/c, are not constitutively expressed in DCs (204), but instead upon TLR activation that results in IL-β secretion, CD1 group one molecules are synthesized *de novo* (109, 205, 206). The peak expression of CD1 group 1 proteins occurs approximately 72 hours after stimulation with microbial products, likely due to the time required for transcription, translation, and proper folding (205, 206).

In Bb infection TLR2 signaling contributes to the activation of DCs (109). The activated DCs produce IL-1β, that in turn, activates neighboring DCs to begin the *de novo* synthesis of CD1 group 1 proteins. In the absence of TLR2 activation, IL-1β is sufficient to induce CD1 group 1 surface expression, suggesting that other IL-1β inducing pathways could also produce similar results (109). In contrast, blockade of IL-1β completely prevents CD1 group one expression, highlighting the importance of IL-1β in CD1 group 1 expression. Interestingly, IL-1β has no apparent effect on CD1d, implying that the CD1 group one and group two proteins may be differentially regulated, and serve different functions (109). CD1a/b/c are especially relevant in LD (109). In humans, all three of these isoforms are inducible in dermal DCs exposed to Bb antigens (109). Of the three isoforms, CD1c levels are the most responsive and exhibit the highest levels of expression, upon encounter with Bb lipopeptides (109). Though it has yet to be studied in RLA, multiple investigators show that Vδ1 T cells can respond to lipopeptides

presented via CD1c on DCs (152, 207, 208). Because the Vδ1 subset is prevalent in the synovial tissues of RLA patients, it is possible that the Vδ1 subset of T cells can also respond to CD1c presenting Bb lipopeptides. In summary, Vδ1 γδ T cells are prevalent in RLA inflammatory sites, Bb antigens induce upregulation of CD1a/b/c on DCs, and Vδ1 γδ T cells respond to lipopeptides presented by CD1a/b/c on DCs. These observations provide evidence that CD1 group one proteins may be relevant in RLA.

One possibility is that CD1 group one proteins present self-lipopeptides, priming autoreactive γδ T cells that contribute to the pathology of RLA. Alternatively, CD1 group one proteins may be presenting Bb antigens that have been trapped in collagen, thereby causing γδ T cells to produce chronic inflammatory responses. Consistent with the latter scenario, Lyme antigens can persist from months to years in the joints and synovial fluids of Bb infected mice (103). Taken together, the data are consistent with the idea that non-classical antigen presentation may be relevant in the pathology of LD and RLA.

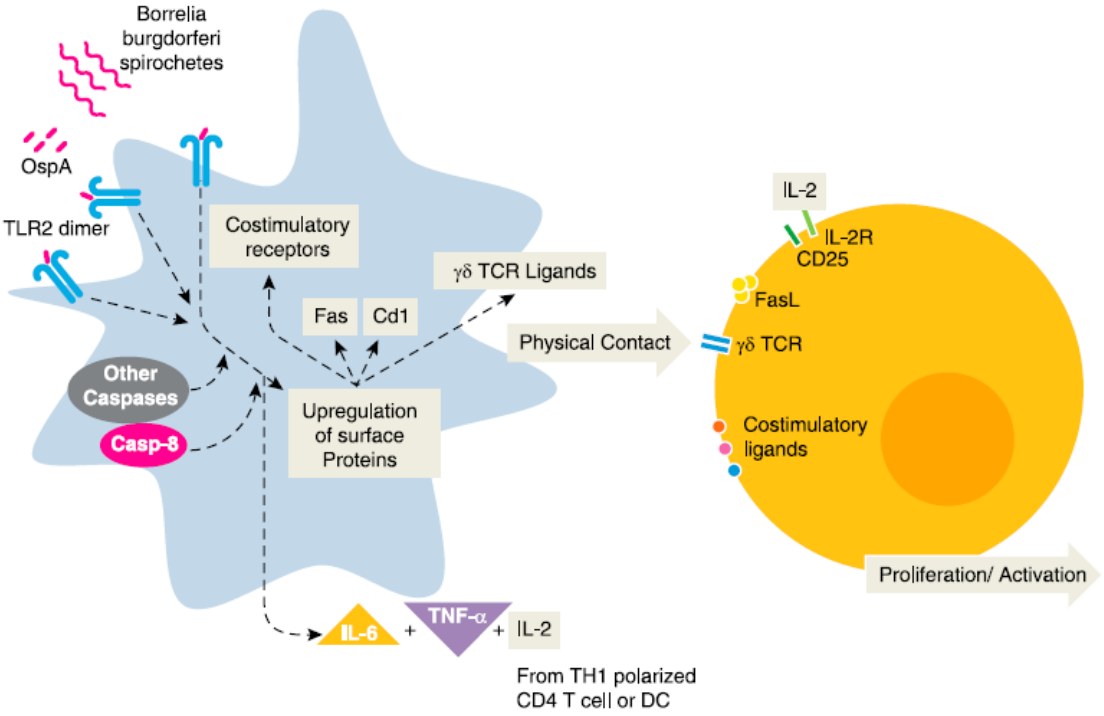

Figure 2. 2 OspA activates dendritic cells via TLR engagement. TLR signaling initiates a caspase dependent upregulation of surface proteins that are essential for binding and activating the γδ T cell. These signals may include Fas, Cd1, γδ TCR ligands, or a variety of co-stimulatory receptors that bind the TCR. In addition, IL-6 and TNF-α are secreted, while IL-2 is produced either by Th1 polarized CD4 T cells or DCs. Both γδ TCR and Fas:FasL binding are essential for CD25 upregulation and γδ T cell proliferation. Co-stimulatory ligands shown on γδ T cell are likely to play a role in the activation signal. These can include CD2, Inducible T cell costimulator (ICOS), CD28, CD27, CD30, and natural killer group 2, member D (NKG2D).

2.9 The Reciprocal Roles of Caspases

Caspases are cysteine-dependent proteases that cleave proteins at aspartic acid residues (209). The caspase family of proteins are involved in cell signaling pathways related to apoptosis, cell survival, and differentiation (210). Fas and FasL can have multiple, and sometimes opposing, roles in the Fas:FasL signaling process. For example, inhibition of caspase-8 by c-FLIP promotes DC survival and induces the proinflammatory cytokines IL-12 and TNF-α (105, 182). In contrast, caspase-8 activity is upregulated in Bb activated DCs, and it remains elevated above resting monocytes, suggesting that it is necessary for their activation. Furthermore, monocytes require some caspase-8 activity to differentiate into immature myeloid DCs (104). Thus, the regulation of caspase-8 appears to be a key determinant in cell fate decisions. Downstream accessory proteins that associate with caspase-8 can also determine cell behavior in monocytes and DCs. For instance, recent work shows c-FLIP can have at least two different ways of interacting with caspase-8 in DCs. Cleaved c-FLIP can compete with caspase-8 for the FADD death effector domain, which diverts the signal away from apoptosis. Interestingly, full length c-FLIP ligand (c-FLIPL) can heterodimerize with caspase-8 and produce a cleavage product called p43FLIP (211). In turn, p43FLIP induces proliferation and differentiation through the ERK and NF-κB pathways (211). Thus, levels of caspase-8, and changes in its associated downstream proteins can produce a variety of effects in DCs and monocytes (211).

Caspase-1 cleaves pro-IL-1β into IL-1β (212) and, as discussed, IL-1β is essential for non-classical antigen presentation. The inflammasome components that assemble caspase-1 have also been implicated in the pathology of RLA (213, 214). Although some investigators showed that the inflammasome component nod-like receptor family, pyrin domain containing 3 gene (NLRP3) is necessary for caspase-1 assembly (215), it does not appear to be relevant in Lyme arthritis (214). In Bb infected NLRP3 KO mice no differences were observed in either the quantity of caspase-1 production or joint inflammation when compared to infected WT controls. In contrast, the inflammasome component apoptosis-associated spec-like protein containing a carboxy-terminal CARD (ASC) contributes to RLA, as loss of this component significantly decreases the levels of caspase-1 and the magnitude of arthritis severity (214). Thus, caspase-1 is important in RLA, with significant contribution from ASC independent of NLRP3. Thus, both caspase-8 and caspase-1, and their respective neighboring proteins, are involved in pathologies associated with Bb infection. The effects of caspase-8 depend upon the accessory proteins, cFLIP and cFLIPL and the associated cleavage product, p43cFLIP, whereas the effects of caspase-1 and its contribution depend on the inflammasome component ASC that contributes to IL-1β production as an important step in non-classical antigen presentation. Interestingly, IL-1β can also potentiate Th17 type responses.

2.10 IL-17 in RLA

IL-17 is another important cytokine in inflammatory responses during infection (216-221). IL-17 has been implicated in multiple human diseases, including psoriasis, RA, multiple sclerosis, ankylosing spondylitis, inflammatory bowel disease, asthma, and LD (216-221). There are many cell types that can produce IL-17, the most well-known of which is a subset of retinoic acid receptor-related orphan receptor gamma/alpha (RORγ+/RORα+) T cells, called Th17 T cells (222, 223). Th17 cells are thought to develop and progress through three stages, including differentiation, amplification, and stabilization-all of which are dependent on continued signal transducer and activator of transcript 3 (STAT3) activation, through a variety of cytokine combinations, the most important of which are thought to be IL-6, TGFβ, IL-21 and IL-23 (223). For example, Th17 cells can differentiate from naïve CD4 T cells after exposure to IL-6 and TGFβ, resulting in the production of inflammatory cytokines, including IFN-γ, TNF-α, IL-17A and IL-17F , as a part of the key mechanism(s) in fighting infection (223). In the absence of IL-6, TGFβ can upregulate forkhead box P3 (FoxP3), that antagonizes RORγ, and RORα, thus inhibiting the Th17 program and causing differentiation of naïve CD4 T cells into inducible T regulatory cells (iTregs), that can function to suppress conventional antigen-specific T cell responses (223). Importantly, IL-21 can substitute for IL-6 in promoting Th17 cell differentiation, supporting the observation that multiple cytokine combinations can lead to Th17 differentiation (222, 223). Furthermore, IL-1β and TNFα prevent the Th1 and Th2 promoting cytokines, IFN-γ and IL-4, and in doing

so, IL-1β and TNFα provide an environment in which Th17 differentiation can occur (222).

Importantly, IL-17 can recruit and stimulate fibroblasts, endothelial cells, and epithelial cells to produce IL-6, TNFα, IL-1β, granulocyte macrophage colony stimulating factor (GMCSF), nitric oxide, and a number of matrix metalloproteases (MMP), all of which can contribute to IL-17 feedback loop amplification, germinal center formation and antibody production (222, 224). As an example of an inflammatory disease, IL-17 is relevant to the pathology of RA. In fact, clinical trials show that antibody-mediated blocking of the IL-17 receptor, or neutralizing antibodies to IL-17, delay RA progression and joint damage (225, 226). IL-17 is especially important in RLA. A study shows that 48% (13/27) of CDC confirmed Lyme+ RLA patients produce serum antibodies against the Bb virulence factor, neutrophil activating protein (NapA) (227). Importantly, the production of anti-NapA antibodies is unique to RLA, as patient control groups with RA, sepsis, or pneumonia do not produce any circulating NapA antibodies. Likewise, patients with acute diagnoses of Lyme do not produce antibodies to NapA, suggesting that this antibody is specific for RLA. Consistent with the interpretation that NapA is specific to RLA, in synovial T cells isolated from RLA patients that produce serum NapA antibodies, IL-17 production is abundant in response to NapA antigen challenge (227). Also, in accordance with other RLA models, synovial T cells can only produce IL-17 in response to NapA in the presence of DCs (227). Furthermore, NapA can induce DCs to produce Th17 promoting cytokines, which in turn prime the Th17 responses that

contribute to damaging inflammation and arthritis (227). These investigations demonstrate that IL-17 is a relevant pathological factor in RLA.

Multiple γδ T cell subsets can produce IL-17 in response to bacterial infections by *L.moncytogenes, E.coli, S.aureus,* and *M.tuberculosis* (MT) (228-231). In the case of MT, infected DCs also produce significantly increased levels of IL-23 that in turn, drive production of IL-17 by γδ T cells, but not CD4 T cells (232). These observations suggest that IL-17 is an important cytokine in recovery from infection, but other data suggest a pathological role for IL-17 in conditions of chronic inflammation, including arthritic autoimmune conditions, such as RA and RLA, as discussed above (225-227). While there is a clear association between elevated IL-17 production and RLA, it is not known whether the source of IL-17 is γδ T cells. If the immune responses in RLA are similar to those of MT infection, then DCs and γδ T cells could provide novel drug targets to treat RLA.

2.11 γδ T Cells in the Transition Between Innate and Adaptive, Specific Immunity

During infections with organisms such as *Listeria*, *Leishmania,* various mycobacterial, plasmodial, and *Salmonella* infections, γδ T cells are among the "first responders" as a part of the early response to an acute infection (233-237). However, an increased frequency of γδ T cells is also observed in the inflamed tissues in various inflammatory diseases, including celiac disease, RA, RLA, and sarcoidosis, suggesting that γδ T cells may be contributing to the pathology of these and other diseases, and implicating γδ T

cells in both recovery from infection as well as in chronic inflammatory syndromes (164, 175, 238, 239).Consistent with their roles in acute responses to infection and their roles in inflammatory diseases, some have proposed that γδ T cells may bridge the transition between innate and adaptive immune responses, as has been suggested in MT infection (240-242). Thus, although γδ T cells may be helpful in fighting an initial infection, they may also contribute to a variety of potentially post-infectious syndromes and inflammatory diseases in which some individuals develop chronic inflammation.

As an example of the dual contributions of γδ T cells, γδ T cells of the Vγ9Vδ2 subset produce cytokines in response to soluble MT antigens and lipopeptides during an acute MT infection. Consistent with their contribution to innate immunity during the infection, the Vγ9Vδ2 subset of γδ T cells can initially respond to MT infection in the absence of other APCs (243). However, the presence of professional APCs accelerates the production of pro-inflammatory cytokines by Vγ9Vδ2 T cells and suggests that γδ T cells can contribute to an immune response through mechanisms involving APCs (243, 244). Additionally, γδ T cells can exhibit memory phenotypes subsequent to their initial activation and expansion, and because memory is a key feature of adaptive immunity, the presence of memory phenotypes further supports the role of γδ T cells in adaptive, specific immunity (245). In support of this correlation, it has been reported that up to 90% of circulating Vγ9Vδ2 T cells exhibit memory phenotypes (246).

Shi et al, investigated γδ T cells in LD and RLA (108). They demonstrated that WT mice infected with Bb for 4 weeks show significant increases in the number of activated $CD44^+$ γδ T cells within their lymph nodes. In contrast, Bb infection of either TLR2-/- or γδ TCR-/- mice resulted in no net expansion of γδ T cells (108). Importantly, the γδ T cell deficient animals had significantly lower numbers of CD4 T cells, CD8 T cells, and B cells in the lymph nodes subsequent to Bb infection, supporting the notion that γδ T cells are required for an effective transition from an innate to an adaptive immune response during Bb infection. Both mutant strains of animals produce much lower levels of IL-6, IL-12, IFN-γ, TNF-α and IL-1β in response to Bb infection. Moreover, the DCs from TLR2-/- animals do not release IL-17 in response to Bb (108). These studies show that both TLR2 receptors on DCs and the γδ TCR on γδ T cells are necessary for pro-inflammatory cytokine responses, and expansion of B and T cells within the lymph nodes, during Bb infection. TLR2, and γδ T cells, may therefore be important contributors to the effective transition between innate and adaptive immunity to Bb, suggesting that γδ T cells may be important in the pathology of RLA.

2.12 Concluding Remarks on RLA

The discussion above provides an overview of γδ T cells and DCs in RLA. The TLR2 ligands (OspA, OspC, NapA) from Bb bind TLR2 receptors on DCs, which in the presence of IL-2 initiates caspase-8 dependent production of IL-1β (104). The sources of IL-2 are usually existing Th1 CD4 cells, though some have shown IL-2 can be produced by DCs as well (247-249). IL-2 binds its cognate receptor on γδ T cells and promotes

proliferation and activation, which increases surface CD25, and FasL (146). Meanwhile IL-1β binds neighboring DCs to initiate de novo production of non-classical antigen presenting proteins within the CD1 group one family (Figure 2.3 A). Within 72 hours, CD1a/b/c peak on DCs and they present amphipathic Bb lipids, lipopeptides and glycolipids to γδ T cells (109). Fas:FasL binding between DCs and γδ T cells induces γδ T cells to produce IFN-γ and IL-17 (108). At the same time γδ TCRs bind CD1 ligands presented by DCs, which in turn potentiates production of IL-6, IL-12, IFN-γ, and TNF-α (108) as illustrated in Figure 2.3 B. Together, the DC and γδ T signal back and forth to produce a cytokine environment intended to clear infection. If the cytokine environment is out of balance, or lasts too long, it may become pathological. Based on the evidence we have presented, we propose that RLA is due to chronically activated synovial γδ T cells exposed to either live Bb or trapped Bb antigens. Thus, strategies for eliminating chronically activated γδ T cells could provide novel therapeutic approaches for treating RLA.

2.13 Understudied Complications of LD

As we have described, RLA is a relatively well-studied disease that is characterized by inflammation of the joints, centered around interactions between DCs and γδ T cells. It is important to note, that although there is no single treatment for RLA, clinical interventions that include treatment with disease-modifying anti-rheumatic drugs, or surgical intervention by synovectomies, are usually effective in remediating RLA symptoms (250). Unlike RLA, PTLDS affects multiple organ systems, is not easily

diagnosed, and is relatively understudied. Furthermore, there are no therapies that have FDA approval for the treatment of PTLDS, and currently a general consensus of strategies for the treatment of PTLDS does not exist among clinicians. The paucity of research on PTLDS can be attributed in part to the lack of a suitable animal model for studying the disease. Moreover, the RM is the only animal that is routinely used to model neuroborreliosis, a condition that is thought to precede PTLDS (29). As we have described, use of the RM is a relatively inefficient and uneconomical approach. The lack of a mouse model that faithfully replicates neuroborreliosis underscores a major gap in our knowledge of LD and its associated complications, such as PTLDS. In the manuscript that follows, we will describe our approach and the results of our experiments that were focused on addressing this major gap in knowledge.

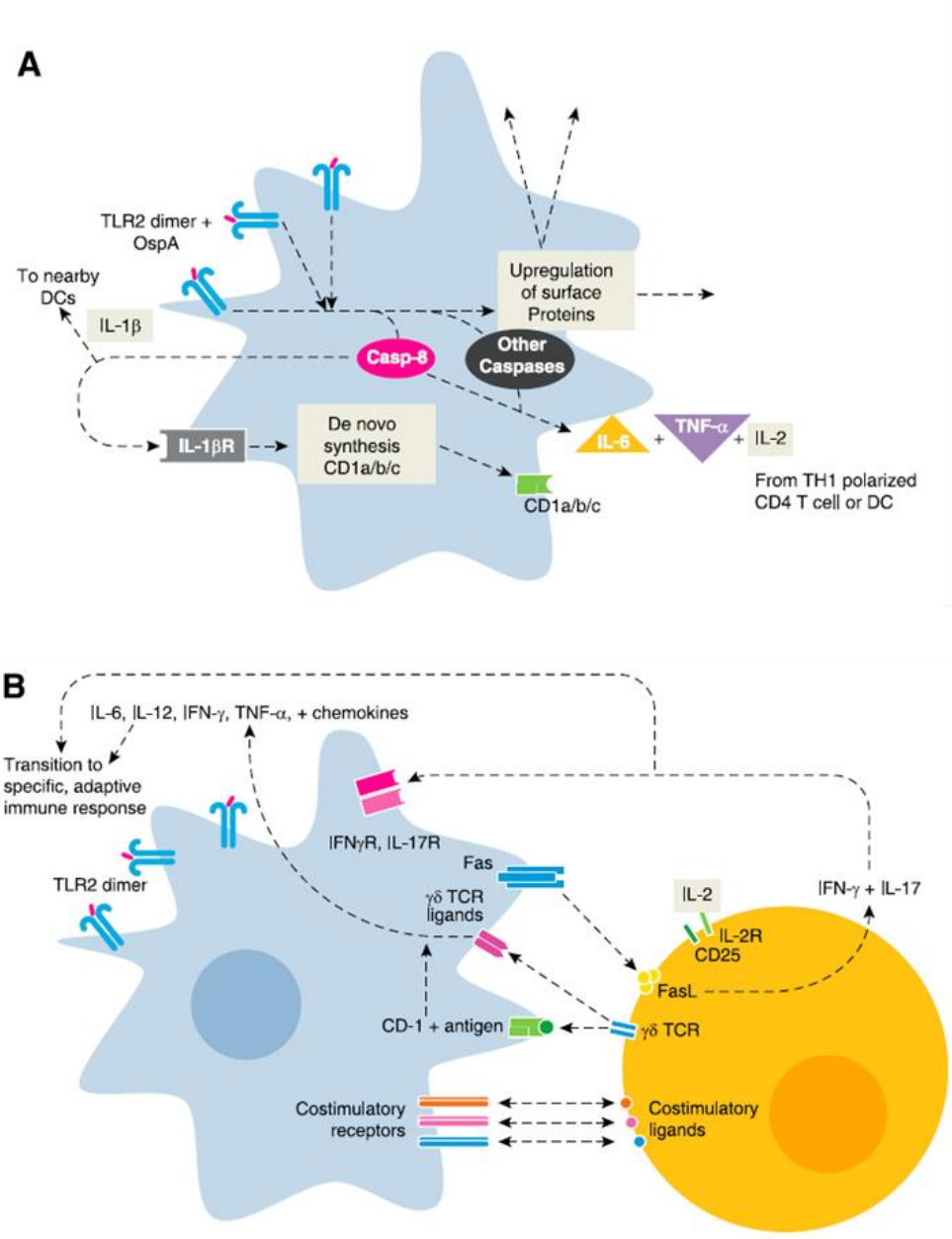

Figure 2.3 Cell signaling between γδ T cells and DCs during Bb infection. A, Activated TLR2 initiates caspase-dependent production of IL-1β, IL-6, and TNF-α. IL-1β acts in an autocrine and paracrine manner to initiate *de novo* synthesis of Cd1 group 1 proteins. Other cell surface proteins may become upregulated including Fas, γδ TCR ligands, and co-stimulatory molecules. B, Surface factors on DC and γδ T cells facilitate cognate interactions that initiate cytokine cascades involved in the transition to an adaptive immune response. Fas:FasL interactions lead to increased expression of CD25, production of IFN- γ, and of IL-17 by γδ T cells. Unknown γδ TCR ligands or CD1 group 1 proteins present antigen to the γδ TCR and induce the DC to secrete IL-6, IL-12, TNF-α, and other chemokines necessary for an effective transition to adaptive immunity.

3. EVIDENCE FOR LONG-TERM PERSISTENCE OF BORRELIA BURGDORFERI IN THE MENINGES

3.1 Synopsis

LD is a tick-borne infection caused by Bb, that has a global impact and a relatively high incidence in the United States (39). If not diagnosed and treated promptly, LD can result in musculoskeletal, neurological, and cardiac symptoms resulting in disability and sometimes death (40, 251). Some individuals that receive delayed antibiotic treatment experience persistent LD symptoms, a syndrome called PTLDS (29, 40). The etiology of PTLDS is unknown, however, persistent bacterial infection or neurologic auto-immune disease, or both, may underlie PTLDS (29, 252, 253). Few studies have focused on the effects of Bb on the CNS, and Bb has never been detected in the brain after experimental intradermal infection. We hypothesized that intradermal infection with a CSF-tropic train of Bb that was derived from the CSF of a patient, would result in dissemination of Bb into the CNS. Here we show that infection with Bb 297 results in bacterial persistence in the dura, in immunological changes in the brain, and in behavioral deficits. We found that intradermal infection by Bb 297 resulted in the presence of CD4 T cells and the B2 subset of B cells (B2 B cells) in the brain as early as 5 dpi, and CD4 T cells remained persistently elevated as long as 45 dpi. Remarkably, Bb spirochetes were detected in, and could be cultured from, the dura and the brain at 5 and up to 45 dpi. We observed significant behavioral deficits temporally associated with infection, including reduced pain threshold in the periphery, and impaired motor function. Overall, our data

provide insight into the neuropathophysiology of LD, and suggest that neuroimmune responses that occur early in the course of Bb infection are not sufficient to clear infection, or to resolve behavioral changes that are associated with Bb infection. Moreover, our findings reveal that the dura is a viable niche environment for long-term bacterial infection.

3.2 Introduction

As we described in Chapter 1, the central hypothesis of this dissertation project was that Bb would establish persistent infection in the meninges of the brain, and that Bb infection would be associated with neuroimmunological changes and behavioral deficits. As part of testing this overall hypothesis, we established three aims. Our first aim was to determine if intradermal infection with Bb 297 would result in infection of the CNS, namely the brain or the dura, with the working hypothesis that both the brain and dura would become infected as early as 45 dpi. We formulated this hypothesis based on the fact that Bb 297 has a particular tropism for the CSF (111), and because it is known that the dura expresses proteins that Bb has high affinity for, such as collagen and decorin (114-117). We demonstrate below, that Bb did establish infection in the dura and the brain as early as 5 dpi, and that the infection persisted as late as 45 dpi. Our second aim was to determine whether there were any immunophenotypic changes in B or T cells in the dura or the brain, in response to intradermal Bb infection. We hypothesized that we would observe measurable changes in B and T cells, in both the dura and the brain. Our rationale for making this prediction was based on published findings in the literature that

Bb can induce B and T cell mediated pathologies in the periphery, and that neuroborreliosis has a tendency to elicit cytokine responses in the brain (23, 26, 31). We observed that multiple B and T cell subsets were detectable in the brain and dura of sham treated and Bb infected mice, and of particular note, B2 and CD4 T cells frequencies and numbers increased in the brain in response to Bb infection. Our third and final aim was to determine if long-term infection by Bb would result in behavioral changes. Based on the prediction that mice would manifest behavioral changes that paralleled the signs and symptoms of infection in humans, we hypothesized that mice would also exhibit behavioral changes that corresponded to deficits in pain sensitivity, mobility, and neurocognition (29, 123). Our data demonstrate that Bb infection results in behavioral deficits pertaining to pain sensitivity and mobility, however, changes in neurocognition, specifically visual memory, were not evident. Overall, our research resulted in the production a novel mouse model of neuroborreliosis that may be useful for understanding the pathophysiology of neurological LD.

3.3 Methods

Animals. Male C3H/HeN mice were purchased from Charles River Laboratories and were housed in temperature and humidity controlled rooms, housed in 12h/12h light/dark cycles. All procedures were in compliance with and approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University Health Science Center and Baylor Scott & White Health. All animals were 7-8 weeks old at the time of initial needle inoculation.

Bb culture and infection. Low passage Bb strain 297 was purchased from the American Type Culture Collection (ATCC), and was cultured in BSK-II medium at 37°C, 5% CO2, until it reached an approximate concentration of $2x10^7$/mL. Bb spirochetes were quantified by dark field microscopy using a Petroff-Hausser chamber. Aliquots of Bb culture were diluted in BSK-II medium to achieve a concentration of $1x10^7$/mL, and 100uL of inoculum containing $1x10^6$ Bb was administered intradermally in the dorsal thoracic midline. Sham animals were needle inoculated intradermally with 100uL of BSK-II medium. Bb passages were recorded, and all Bb used for infection had passage number less than 6.

Tissue harvest and tissue culture. Prior to euthanasia, all mice were anesthetized by isoflurane, and each mouse was perfused transcardially with 60mL Ca^{++}/Mg^{++} free, phenol red free, HBSS (Gibco #14185-052) over the course of approximately 5 minutes. Brain and DCLN were dissected, and dura was isolated from the skullcap as described (101). Tissues were aseptically transferred to 10mL of BSK-II medium and cultured in an incubator at 37°C, 5% CO2 for 12 days. Samples were checked daily for Bb growth, and on day 12 of culture Bb concentration was quantified by darkfield microscopy. Acridine orange was added at a 1:1 ratio to the Bb culture, and samples were viewed by epifluorescence using a FITC filter set.

Dextran Assay. Mice were anesthetized with isoflurane and injected via the retro-orbital sinus with 100uL of 2.5mg/mL 10KD FITC-dextran. After 3 minutes of circulation time,

20uL of blood was collected by facial vein puncture, and plasma was collected by centrifugation. 2uL aliquots of plasma samples were placed in 300uL formamide. After blood collection, mice were transcardially perfused with PBS, as described in "tissue harvest and tissue culture". Mice were craniotomized, brains removed, weighed, and homogenized in 300uL formamide with a probe sonicator. All samples in formamide were incubated on a heating block at 55°C for 24 hours, followed by centrifugation at 14,000rpm for 20 minutes. Supernatant from brain homogenates was drawn by Pasteur pipette, and transferred to 50uL formamide, and centrifuged again. 50uL aliquots of final supernatant from brain homogenates, and 50uL aliquots of plasma samples in formamide were sampled in triplicate to detect fluorescent emission by plate reader, using wavelengths 420ex/520em. Concentrations of dextran were determined by linear regression using a standard curve.

Immunostaining. Skull caps and brains were removed and both were post-fixed in 2% paraformaldehyde for 24h at 4°C. Dura samples were permeablized in 0.1% Triton X-100, and serum blocked in 2.5% goat serum/PBS containing 1:100 dilution of Fc block (BD 553142). Each dura was incubated in 1:200 rabbit anti mouse LYVE-1 IgG , and 1:200 rat anti mouse CD31 IgG unconjugated polyclonal antibodies in 2.5% goat serum/PBS at 4°C overnight. Dura samples were stained with fluorochrome-conjugated secondary antibodies at dilutions of 1:200 of Alexa 546 goat anti rabbit IgG (Invitrogen A11071), and of 1:200 Alexa 633 goat anti rat IgG (Invitrogen A21094) for 1 hour at room temperature. Afterwards, dura samples were incubated with 1:50 biotinylated

rabbit anti Bb polyclonal IgG (Invitrogen PA1-73007) at 4C overnight. Next, dura samples were stained with secondary antibody 1:200 Alexa 488 streptavidin (Invitrogen S11223) for 1 hour at room temperature. All dura samples were washed 3x for 5 minutes at room temperature between each step, and after the final staining step. Brain samples were dehydrated in 30% sucrose overnight, followed by freezing in optimum cooling temperature (OCT) medium at -80°C. Each brain sample was cut into 50um sections using a cryostat, and every 3rd section was collected for immunofluorescent staining. Each brain sample was stained for 24 hours at room temperature in staining solution containing 0.1% Triton X-100, 2.5% goat serum, and 1:200 dilution of Alexa 555 Goat anti-mouse IgG. Each brain sample was washed 3x for 5 minutes at room temperature before mounting. All tissue samples were wet-mounted onto a positively charged glass slide and cover-slipped in Fluoromount-G with DAPI mounting medium (eBioscience 0100-20). Tissues were imaged on Olympus IX-71 and Leica DMIRE 2 confocal microscopes and images were analyzed using FIJI-Image J software.

Leukocyte isolation and flow cytometry. Dura were isolated as described, and leukocytes were freed from tissue by incubation for 30 minutes in 1mg/mL collagenase in HBSS supplemented with 5mM $CaCl_2$. Next, using a 10mL syringe plunger, dura and brain samples were pushed through 100um strainers. Myelin was separated from the brain leukocyte suspensions using a 37% Percoll gradient as described (254). All cells were counted using a hemocytometer. Cells were stained on ice for 20 minutes with appropriate dilutions of Aqua viability dye, 1:100 (Invitrogen L34966); PE-conjugated

anti CD45, 1:100 (eBioscience 12-0451-83); APC-Cy7 conjugated anti-CD19 1:400 (BD 557655); APC conjugated anti-CD5 1:100 (Biolegend 100625); FITC conjugated anti-CD1d 1:100 (Biolegend 123508); PacBlue conjugated anti-IgD 1:100 (Biolegend 405712); PacBlue conjugated anti-CD3 1:100 (Biolegend 100214); FITC conjugated anti-CD4 1:100 (BD 553651); or APC conjugated anti-CD8 1:100 (Biolegend 100712), as described in table 3.5. Samples were washed in 3% newborn calf serum (NBCS), and analyzed using a BD FACS CANTO II flow cytometer. Antibody compensations were performed using BD CompBeads (BD 552845), Aqua compensation was performed using ArC Amine Reactive Compensation Bead Kit (Life Technologies A10346) and all gating was done based on fluorescence minus one (FMO) controls (not shown). Flow cytometric data was analyzed using FlowJo software.

Von Frey pain test. The Von Frey pain test was performed on an elevated surface with small holes in the base. Each mouse was allowed to habituate to the environment for 3 minutes. The tip of the Von Frey filament was applied to the plantar surface of each hind paw. If a withdrawal response did not occur, the next largest filament was used. If a withdrawal response was elicited, the next lowest filament was used until no withdrawal response occurred. The filaments were then increased in size again until a withdrawal response occurred. This method was repeated until a single filament elicited 4 consecutive withdrawal responses, indicating the end of one trial. Two trials were conducted on each hind-paw of each mouse per treatment group.

OF test. Each mouse was placed in a 31 cm x 32 cm plexiglass OF box and behavior was recorded for 10 minutes. This test also constituted the habituation phase for the NORT tests that followed. After a 10 minute recorded session in the OF box, each mouse was returned to its original cage, and the OF box was cleaned thoroughly with ethanol before the next mouse was tested. Behavior was analyzed using Noldus Ethovision XT 12 software, and the following variables were analyzed: total distance moved, velocity, movement (averaging interval 15 samples, start velocity 2cm/s, stop velocity 0.5cm/s), time spent on floor, time spent on wall, time spent in each quadrant of floor, meandering, mobility state (Immobile threshold ≤ 5%, mobile threshold between 5% and 25%, highly mobile threshold ≥25%), and zone alteration between floor quadrants.

NORT Test. After the OF test, each mouse was given a 90-minute rest interval, and then each mouse was returned to the OF cage containing two identical, equally spaced objects that met the criteria described (128). After a 5-minute learning interval, each mouse was returned to its original cage for 90 minutes. Next, each mouse was put back in the OF cage, this time containing one familiar object from the previous session, and a novel object. The mouse was recorded for 5 minutes, and behavior was analyzed using Noldus Ethovision XT 12 software. The following variables were analyzed: visits to novel object zone (nose point within 4cm of novel object), visits to familiar object zone (nose point within 4cm of familiar object), nose touching familiar object, nose touching novel object, nose point distance from each object, novel object exploration (nose point facing novel object while within 4cm of novel object), familiar object exploration (nose point

facing familiar object while within 4cm of familiar object). Testing schematic is illustrated in Figure 3.10.

Statistical analysis. All data were analyzed and graphed using GraphPad Prism 7.0 software. For tests involving multiple comparisons, two-way ANOVA was used with Holmes-Sidak correction for multiple comparisons, alpha = 0.05. For tests that did not involve multiple comparisons, unpaired t-test was used, alpha = 0.05. Power analysis was completed using GPower3 software and the following parameters: a priori analysis, difference between two independent means, Beta = 0.20, alpha = 0.05, and an effect size of 2.2 (based on preliminary data). Outliers were excluded by outlier test, alpha = 0.05. Animal randomization was not used in our approach, and investigators were not blinded to treatment groups.

Data availability. Publicly available databases were not used to generate any of the data presented in this manuscript.

3.4 Results

3.4.1 Dura Supports Bb Growth During Late-stage Infection

Two species of ticks, *Ixodes scapularis* and *Ixodes pacificus*, are the accepted natural vectors for the transmission of Bb, the causative agent of LD (102). We aimed to establish a mouse model of neurological LD, by using an infection route that was relevant to the natural vector. Accordingly, we utilized needle inoculation via the intradermal route to inject ~$1x10^6$ Bb spirochetes belonging to the strain Bb 297, into the dorsal thoracic midline of LD susceptible C3H/HeN mice (88). Because Bb 297 is a strain that was originally isolated from the CSF of a LD patient in the United States (111), we expected that it could have a tropism for the CNS. Mice were evaluated for LD infection during the early stage of the disease (255), 5 dpi, or during late-stage disease, 45 dpi, when neurological involvement may be expected (20). After performing transcardial perfusions, the brain, dura, and DCLNs of each mouse were removed, and each tissue was placed in BSK-II medium and cultured for 12 days. We assessed the tissues daily by dark field microscopy and we observed spirochete growth in all tissue types at 5 dpi, 45 dpi, and as early as 4 days after culture (Table 3.1-3.4). Aliquots of the culture were stained with acridine orange, and fluorescent spirochetes were visualized in all three tissues (Figure 3.1a, Table 3.1-3.4). Paraformaldehyde-fixed culture samples also demonstrated fluorescence when treated with Bb-specific antibody, providing confirmation that the observed spirochetes were Bb (Figure 3.2a-b).

To determine whether Bb persistence in the brain was specific to Bb strain 297, we used a green fluorescent protein (GFP) expressing variant of another common Bb strain, known as B31. Although we did not attempt to culture the brains of mice that were infected with B31, immunofluorescent staining with fluorochrome conjugated anti-GFP antibody did not reveal any spirochetes in any sections of the brain after 45 days of infection (data not shown). To rule out the possibility that GFP expression was preventing B31 from entering the brain, we infected mice for 45 days with wild-type B31, and using fluorochrome-conjugated anti-Bb polyclonal antibody, we did not detect any B31 in the brain (data not shown). Thus, it seems that CNS tropism may be a unique characteristic of Bb 297.

A possible mechanism that could explain the presence of viable Bb in the brain is that Bb infection may compromise the integrity of the BBB, resulting in diffusion of Bb from the blood into the parenchyma or perivascular spaces of blood vessels in the brain. To test this possibility, we infected mice with Bb, and at the earliest time point that Bb was cultured from the brain (5dpi), we injected a 10 kilodalton fluorochrome-conjugated dextran (FITC-dextran) into the peripheral blood. We quantified the tracer signal spectrophotometrically, and we determined that the tracer signal was detectable in the plasma and brain homogenates of naïve mice (Figure 3.3a-b). No significant differences were apparent between sham and 5 dpi Bb-infected mice (Figure 3.3c). Our results suggest that the persistence of Bb in the brain or its associated tissues is unlikely to be due to a general increase in the permeability of the BBB. Importantly, our data does not

rule out the possibility that Bb could pass through the BBB via paracellular or transcellular routes using other mechanisms, for example ATP-dependent processes such as receptor mediated transport.

3.4.2 Bb Localizes to Blood Vessels in the Dura

Recently, it has been shown that the dura has functional primary lymphatic vessels that run parallel to the sagittal and transverse sinuses, and show the capacity to traffic lymphocytes from the meningeal compartment into the draining DCLNs (101). Recent investigations have revealed that Bb can adhere to endothelial cells on the vessel walls by adhesion molecules using mechanisms similar to selectin-mediated rolling of leukocytes (256). Seeking to identify where in the dura Bb may localize, we performed immunostaining on dura, gathered from perfused mice 45 dpi. We stained the samples with LYVE-1 and CD31, markers for lymphatic vessels and blood vessels, respectively. We observed Bb spirochetes in association with blood vessels, but not lymphatic vessels (Figure 3.1b-e, Figure 3.2c-f). We also observed Bb spirochetes in the lumen of a blood vessel, apparently adhering to the luminal wall (Figure 3.1c,d). These observations suggest that arterial or venous pathways may play a role the dissemination of Bb to and from the CNS.

Table 3. 1 Tissue culture. Summary results of Bb growth from dura, brain, and DCLN of Bb infected mice, after 12 days of culture

Infection duration (days)	5	45	5	45	45	5	45	5
Tissue	Dura	Dura	Brain	Brain	R-DCLN	R-DCLN	L-DCLN	L-DCLN
Culture Positive Samples	**(3/7)**	**(2/3)**	**(2/5)**	**(2/4)**	**(2/3)**	**(5/5)**	**(2/3)**	**(1/4)**

Table 3.2 Brain culture. Summary results of brain cultures from Bb-infected mice, checked daily for 12 days.

Infection Group	Sample	Days of culture 0	1	2	3	4	5	6	7	8	9	10	11	12	Final concentration on day 12 (/mL)
45dpi	Sample 1	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	NA
	Sample 2	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	NA
	Sample 3	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	1.5x10^5
	Sample 4	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	6.9x10^5
5dpi	Sample 1	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(+)	(+)	(+)	(+)(+)	4.38x10^5
	Sample 2	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	NA
	Sample 3	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)(+)	(+)(+)	(+)(+)(+)	6.36x10^7
	Sample 4	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	NA
	Sample 5	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	NA

Table 3.3 Dura culture. Summary results of dura cultures from Bb-infected mice, checked daily for 12 days.

Infection Group	Sample	Days of culture 0	1	2	3	4	5	6	7	8	9	10	11	12	Final concentration on day 12 (/mL)
45dpi	Sample 1	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)(+)	(+)(+)	1.16 x 10^6
	Sample 2	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	NA
	Sample 3	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)(+)	(+)(+)	(+)(+)	(+)(+)(+)	2.03 x 10^7
5dpi	Sample 1	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	NA
	Sample 2	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)(+)	(+)(+)	(+)(+)	5.8 x 10^5
	Sample 3	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	NA
	Sample 4	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	NA
	Sample 5	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(-)	(+)(+)	(+)(+)	(+)(+)(+)	1.85x10^7
	Sample 6	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	NA
	Sample 7	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)(+)	(+)(+)	(+)(+)(+)	1.25x10^7

Table 3.4 DCLN culture. Summary results of DCLN cultures from Bb-infected mice, checked daily for 12 days.

Infection Group	Sample	Days of culture 0	1	2	3	4	5	6	7	8	9	10	11	12	Final concentration on day 12 (/mL)
45dpi	R-DCLN 1	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)(+)	(+)(+)	(+)(+)(+)	8.6x10^6
	R-DCLN 2	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)(+)	(+)(+)	(+)(+)	(+)(+)(+)	8.1x10^6
	R-DCLN 3	(-)	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(-)	(-)	NA
5dpi	R-DCLN 1	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)(+)	(+)(+)	(+)(+)(+)	(+)(+)(+)	1.38x10^7
	R-DCLN 2	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)(+)	(+)(+)	(+)(+)(+)	3.36x10^7
	R-DCLN 3	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)(+)	(+)(+)(+)	(+)(+)(+)	(+)(+)(+)	7.92x10^7
	R-DCLN 4	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)(+)	(+)(+)(+)	(+)(+)(+)	(+)(+)(+)	7.62x10^7
	R-DCLN 5	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)(+)	(+)(+)(+)	(+)(+)(+)	(+)(+)(+)	8.4x10^7
45dpi	L-DCLN 1	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)(+)	(+)(+)	(+)(+)(+)	(+)(+)(+)	2.7x10^7
	L-DCLN 2	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)(+)	(+)(+)	(+)(+)(+)	(+)(+)(+)	2.6x10^7
	L-DCLN 3	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(-)	(-)	NA
5dpi	L-DCLN 1	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	NA
	L-DCLN 2	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)(+)	(+)(+)	(+)(+)(+)	(+)(+)(+)	(+)(+)(+)	(+)(+)(+)	6.15x10^7
	L-DCLN 3	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	NA
	L-DCLN 4	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	NA

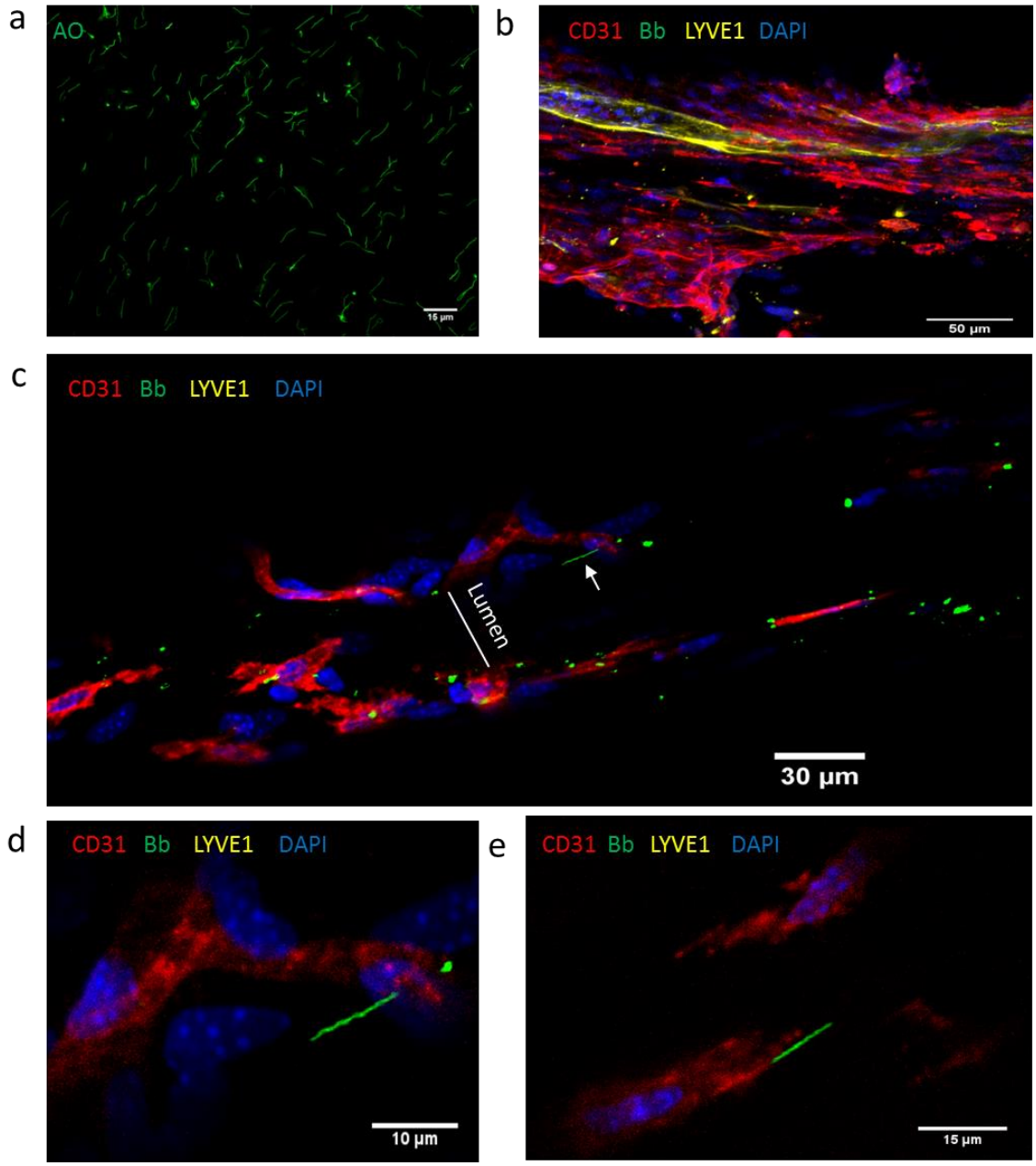

Figure 3.1 Borrelia persistence in dura 45 dpi. (**a)** Spirochetes cultured from 45 dpi dura on day 12 of culture in BSK-II medium. Spirochetes stained with acridine orange for fluorescent visualization of Borrelia; n=3. (**b**) Representative image of superior sagittal sinus in dura whole-mount showing blood vessels (CD31, red), lymphatic endothelium (LYVE-1, yellow), and nucleated cells (DAPI, blue). Bb (Bb, green) were not detected in dural lymphatic endothelium; n=5 (**c**) Representative image of blood vessel in dura containing a Bb spirochete (Bb, green) adhering to a nucleated cell (DAPI, blue) on the endothelial layer (CD31, red); n=5; (**d**) Higher magnification image of (**c**) showing a Bb spirochete (Bb, green) adhering to nucleated (DAPI, blue) endothelial cell (CD31, red). (**e**) Spirochete (Bb, green) in association with blood vessel (CD31, red); images (**c-e)** correspond to n=5.

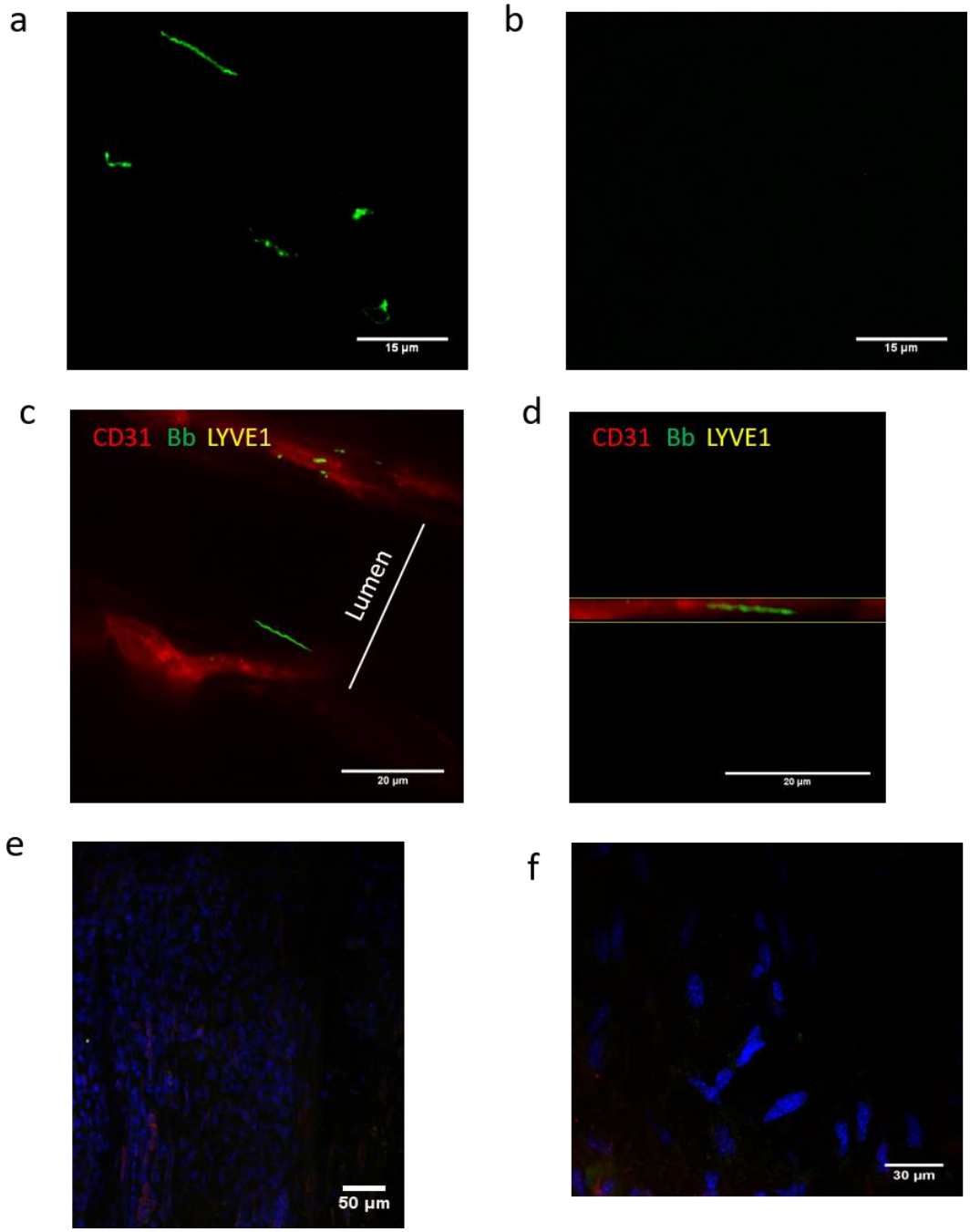

Figure 3. 2 Bb is culturable from 45 dpi dura and persists in association with blood vascular endothelium. (a) Bb spirochetes from 45 dpi dura on day 12 of culture in BSK-II medium; culture samples were fixed in paraformaldehyde and stained with Bb-specific primary antibody, followed by fluorochrome-conjugated secondary antibody; n=3; (**b**) Secondary antibody control of samples stained in (**a**); n=3; (**c**)Representative confocal image of Bb spirochete (Bb, green), in association with blood vascular endothelium (CD31, green); n=5; **d**, Y-Z projection of (**c**) demonstrating that Bb is located within blood vessel boundaries. (**e/f)** Secondary antibody controls for images presented in Fig 3.1b (**e**); n=1; and Fig 3.1c (**f**); n=1.

3.4.3 Elevated CD4+ T cells in the Brain During Early and Late-stage Infection

In approximately 20% of neurological LD cases, post-infectious complications can arise in spite of antibiotic treatment, as can be the case in PTLDS. Manifestations of PTLDS can include fatigue, musculoskeletal pain, and cognitive abnormalities that vary in characteristics between individuals (29). Other post-infectious complications of LD, such as RLA, have been linked to specific T cell subsets (31,34). HLA subsets belonging to the HLA-DR isoform also appear to play a role in RLA, suggesting that in the context of LD, an immune response can influence the probability of developing post-infectious diseases, and that immunogenetics may play a role in LD autoimmunity (31, 257-259). Although it has not been demonstrated definitively, it has been suggested that PTLDS may also have autoimmune etiologies (29). To determine whether any immunophenotypic changes were apparent in our mouse model of neurological LD, we performed flow cytometry on cells isolated from the meningeal compartments or the brain, specifically probing for differences in the B cell and T cell subsets that may reside in these tissues pre- and post-infection (gating strategy is provided in Table 3.5, Fig 3.4, and Fig 3.5).

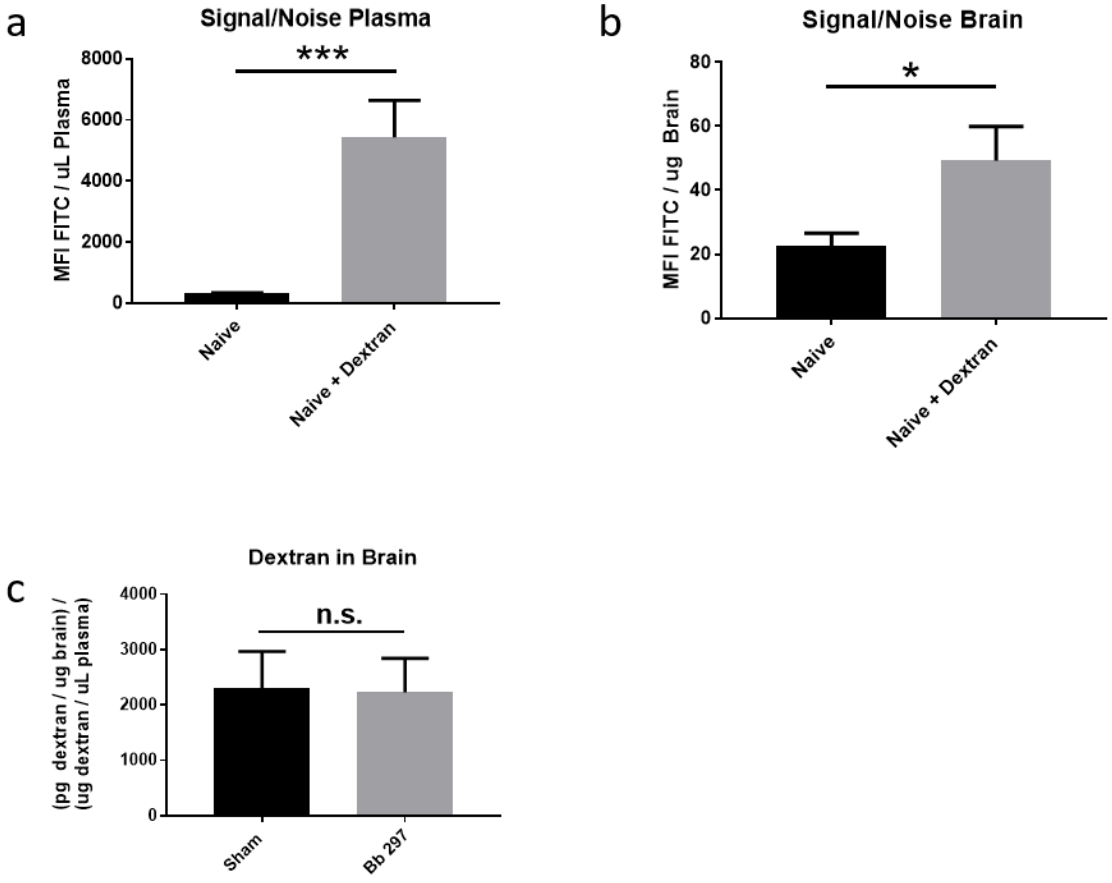

Figure 3. 3 Infection by Bb does not alter BBB integrity. (a) Mean fluorescent intensity of FITC-dextran signal per uL of plasma in naive mice that were either given no tracer (black bar), or IV injection of FITC-dextran (gray bar); n=3 naïve, n= 4 naïve + dextran; ***p <0.001; (b) Mean fluorescent intensity of FITC-dextran signal per ug of brain homogenate in naïve mice that were either given no tracer (black bar), or IV injection of FITC-dextran (gray bar); n=3 naïve, n=4 naïve + dextran; *p <0.05; (c) ratio of dextran mass in brain per dextran mass in plasma in mice that were injected with FITC-dextran and given sham infection for 5 days (black bar), or were infected by Bb 297 for 5 days (gray bar); n=7 sham, n=4 Bb 297; n.s. is not significant.

Our data show that within 5 dpi there is a transient increase in conventional T cell-dependent B2 type B cells (260) ($CD45^{high}$, $CD3^{-}$, $CD19^{+}$, $CD5^{+}$, $CD1d^{-}$, IgD^{high}), in the brain, and that the increase in these B cells coincides with an increase in CD4+ T cells in the brain (Figure 3.6 a-h). CD4+ T cells remained persistently elevated in the brains of Bb-infected mice as late as 45 dpi (Figure 3.6 e,f). These data suggest that a long-lasting subset of CD4+ T cells may respond to Bb infection in the CNS. Notably, we detected B1a ($CD45^{high}$, $CD3^{-}$, $CD19^{+}$, $CD5^{+}$, $CD1d^{-}$), B1b ($CD45^{high}$, $CD3^{-}$, $CD19^{+}$, $CD5^{-}$, $CD1d^{-}$, IgD^{low}), $CD4^{+}$ ($CD45^{high}$,$CD3^{+}$,$CD19^{-}$,$CD4^{+}$), and $CD8^{+}$ ($CD45^{high}$, $CD3^{+}$,$CD19^{-}$,$CD8^{+}$) cells in both the brain and dura, in sham and Bb infected mice (Figure 3.7 a-h, Figure 3.8 a-f). We also detected B10 B cells ($CD45^{high}$, $CD3^{-}$, $CD19^{+}$, $CD5^{+}$, $CD1d^{+}$), a unique B cell subset that has been shown to have anti-inflammatory function (261, 262). B10 B cells were only observed in the dura of Bb infected mice, at the 5 dpi time point (Figure 3.8 a,b). Collectively, these data support the emerging paradigm that the brain and meningeal compartments have a diverse repertoire of lymphocytes, and that this repertoire can change in response to Bb infection.

3.4.4 IgG Detected in Brain During Late-stage Infection

Immunoglobulins are not typically found in the brain or the CSF, due to the highly regulated tight-junction connections between endothelial cells that form the blood vessels of the BBB, and the ependymal cells that line the CP to form the blood-CSF-barrier (94). When immunoglobulins are detected in the CSF, or the brain, they are

usually associated with neuropathologies, as is the case in multiple sclerosis, and alzheimer's disease (263-266). Evidence suggests that antibodies directed against OspA, a common Bb antigen, can cross-react with neural peptides, leading to autoimmunity in PTLDS patients (122, 252). Because we detected B2 B cells and CD4+ T cells in the brains of Bb-infected mice (Fig 3.6 a,b,e,f), we expected that IgG could be present in the brain as well. Indeed, we observed deposition of IgG near the hippocampus and in the 3rd ventricle at 45 dpi, but not at 3 dpi, or in naïve mice (Fig 3.9 a-i). With the exception of the hippocampus (Fig 3.9 f) and 3rd ventricle (Fig 3.9 c), IgG was not detected in any other regions of the brain (Figure 3.9 i). We reason that IgG was not detected at 3 dpi (Fig 3.9 b,e,h), because sufficient time had not passed for seroconversion in response to Bb, as this can take as long as 4 weeks (267). Our observation that IgG accumulates in the brains of Bb-infected mice supports the possibility that B cells may contribute to the overall immune response during neuroborreliosis.

Table 3.5 Gating strategy. Flow cytometric gating strategy for T cell and B cell antibody panels that were used to stain brain and dural leukocytes.

Panel	Antibodies in Panel	Subset	Gating Strategy
T cell	Aqua	CD4	Singlet, Aqua-, CD45high, CD3+, CD19-, CD4+, CD8-
	CD45		
	CD3		
	CD19	CD8	Singlet, Aqua-, CD45high, CD3+, CD19-, CD4-, CD8+
	CD4		
	CD8		
B cell	Aqua	B1a	Singlet, Aqua-, CD45high, CD3-, CD19+, CD5+, CD1d-
	CD45		
	CD3	B1b	Singlet, Aqua-, CD45high, CD3-, CD19+, CD5-, IgDlow
	CD19		
	CD5	B2	Singlet, Aqua-, CD45high, CD3-, CD19+, CD5-, IgDhigh
	CD1d		
	IgD	B10	Singlet, Aqua-, CD45high, CD3-, CD19+, CD5+, CD1d+

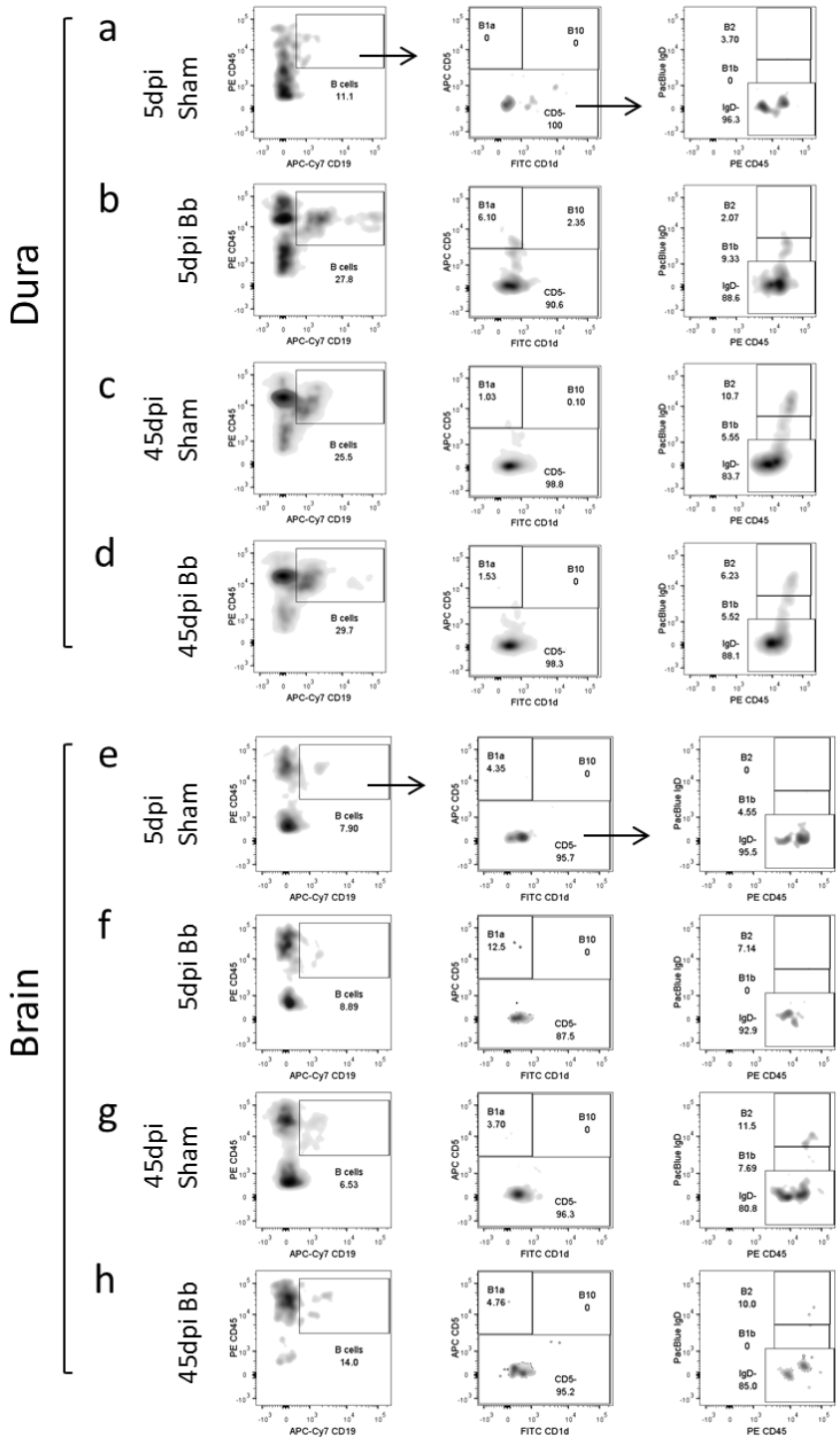

Figure 3.4 Flow cytometric gating strategy for B cells in the dura and brain. (a-d) Flow cytometric gating strategy for B cells in the dura from 5 dpi sham (**a**), 5 dpi Bb (**b**), 45 dpi sham (**c**), and 45 dpi Bb (**d**); All samples were first gated on Singlets, then live cells were gated based on Aqua exclusion viability dye (data not shown). e-f, Flow cytometric gating strategy for B cells in the brain from 5 dpi sham (**e**), 5 dpi Bb (**f**), 45 dpi sham (**g**), and 45 dpi Bb (**h**); All samples were gated as described in table 3.5. All gating was done based on fluorescence minus one (FMO) controls (data not shown).

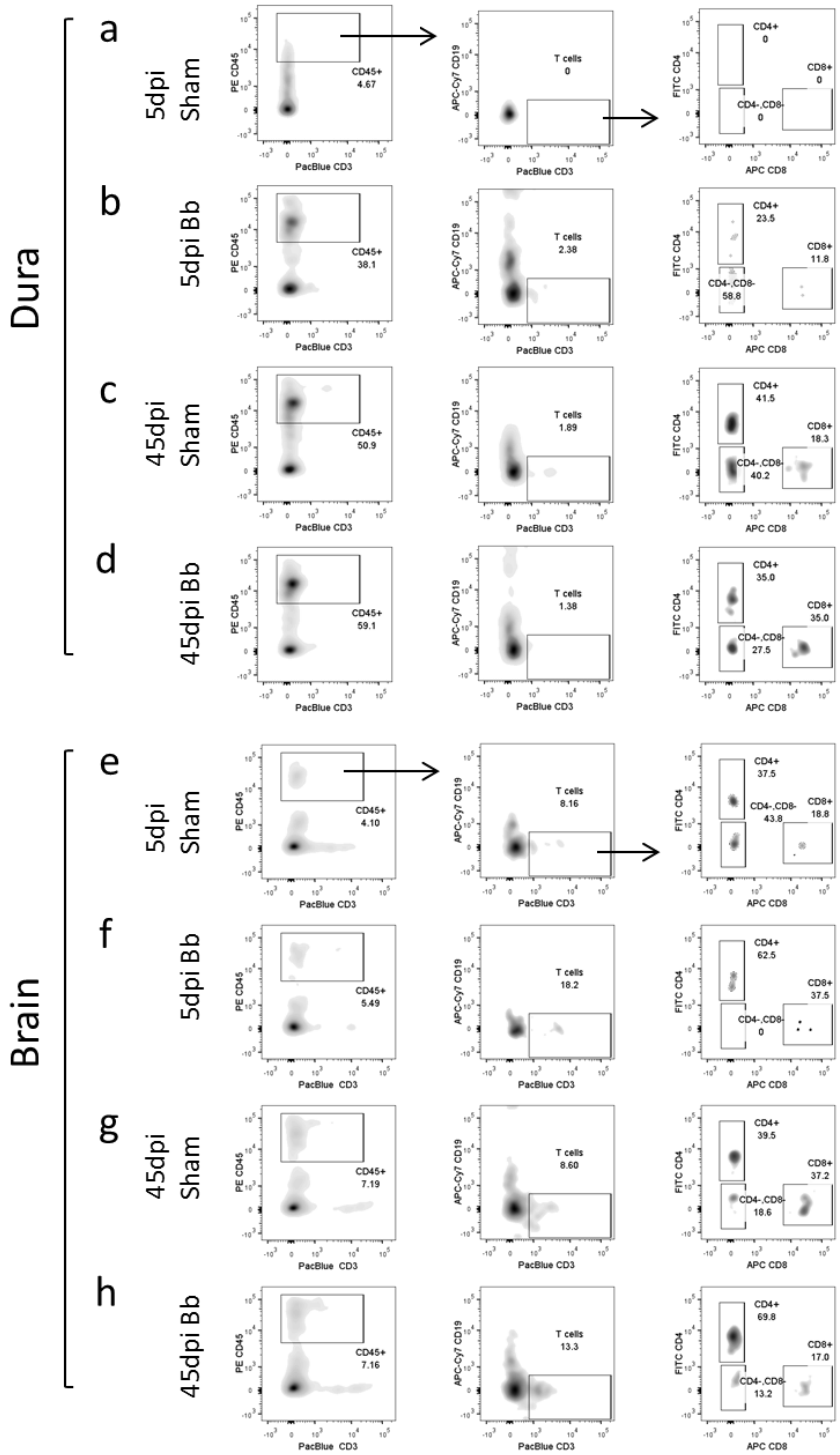

Figure 3.5 Flow cytometric gating strategy for T cells in the dura and brain. (a-d) Flow cytometric gating strategy for T cells in the dura from 5 dpi sham (**a**), 5 dpi Bb (**b**), 45 dpi sham (**c**), and 45 dpi Bb (**d**); All samples were first gated on Singlets, then live cells were gated based on Aqua exclusion viability dye (data not shown). (**e-f**) Flow cytometric gating strategy for T cells in the brain from 5 dpi sham (**e**), 5 dpi Bb (**f**), 45 dpi sham (**g**), and 45 dpi Bb (**h**); All samples were gated as described in table 3.5. All gating was done based on fluorescence minus one (FMO) controls (data not shown).

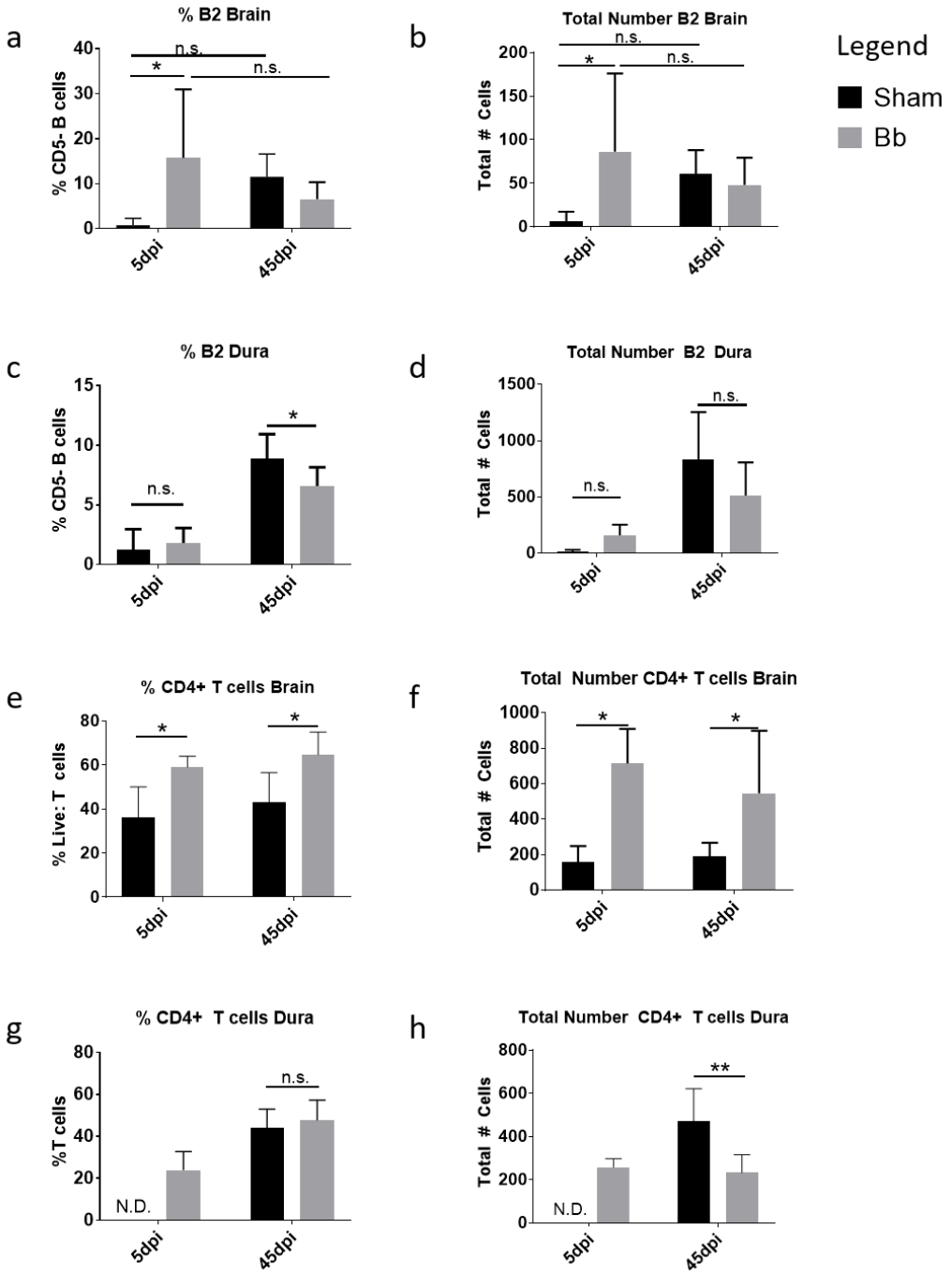

Figure 3.6 B2 B cells and CD4+ T cells in brain and dura. (**a/b**) Percent (**a**) and total number (**b**) of B2 B cells in the brain; Treatment groups had the following n values for brain samples: 5 dpi Sham n=4, 5 dpi Bb n=3, 45 dpi Sham n=5, 45 dpi Bb n=5; (**c/d**) Percent (**c**) and total number (**d**) of B2 B cells in the dura; Dura samples had the following n values: 5 dpi Sham n=5, 5 dpi Bb n=4, 45 dpi Sham n=5, 45 dpi Bb n=5; (**e/f**), Percent (**e**) and total number (**f**) of CD4+ T cells in brain; Treatment groups had n-values as described for (**a/b**); (**g/h**) Percent (**g**), and total number (**h**) of CD4+ T cells in dura; Treatment groups had n-values as described for (**c/d**). All data are representative of two independent experiments. Sham infection is represented by black bar, Bb infection is represented by gray bar; N.D. is not detected; * P <0.05; ** P <0.01, n.s is not significant; 2-way ANOVA w/ Holmes-Sidak correction for multiple comparisons; Error bars represent standard deviation

3.4.5 Behavioral Deficits During Late-stage Infection

Patients with neuroborreliosis infection, and some patients with PTLDS, can develop cognitive abnormalities. Magnetic resonance imaging and pathology reports from patient tissues demonstrate that deficits in cognition may be associated with encephalopathy or encephalomyelitis (23, 123). Typical cognitive abnormalities include difficulty with verbal fluency, impaired short-term memory, and generally impaired ability to process information. These cognitive deficits are usually accompanied by fatigue, sleep disturbances, and depressed mood (123). To date, we have not identified any studies that have investigated behavioral changes in Bb infected mice. Thus, we sought to evaluate basic changes in nociception, movement/mobility, and visual memory in our model of neurological LD. To test these parameters, we utilized the Von Frey pain test, OF testing, and the NORT test (Figure 3.10 a-d, Figure 3.11). Based on the Von Frey pain test, Bb infected mice required less stimulus to induce a hind-leg withdrawal response (Figure 3.10a), suggesting increased nociception in the periphery, a phenomenon that is consistent with clinical manifestations of neuropathy in patients (124). The OF test revealed that Bb infected mice were in a “high mobility state”, as defined by a rotational displacement of the body that resulted in >25% surface area displacement, less often than sham-infected controls. In total, the Bb infected animals spent less cumulative time in the high mobility state (Figure 3.10 b).

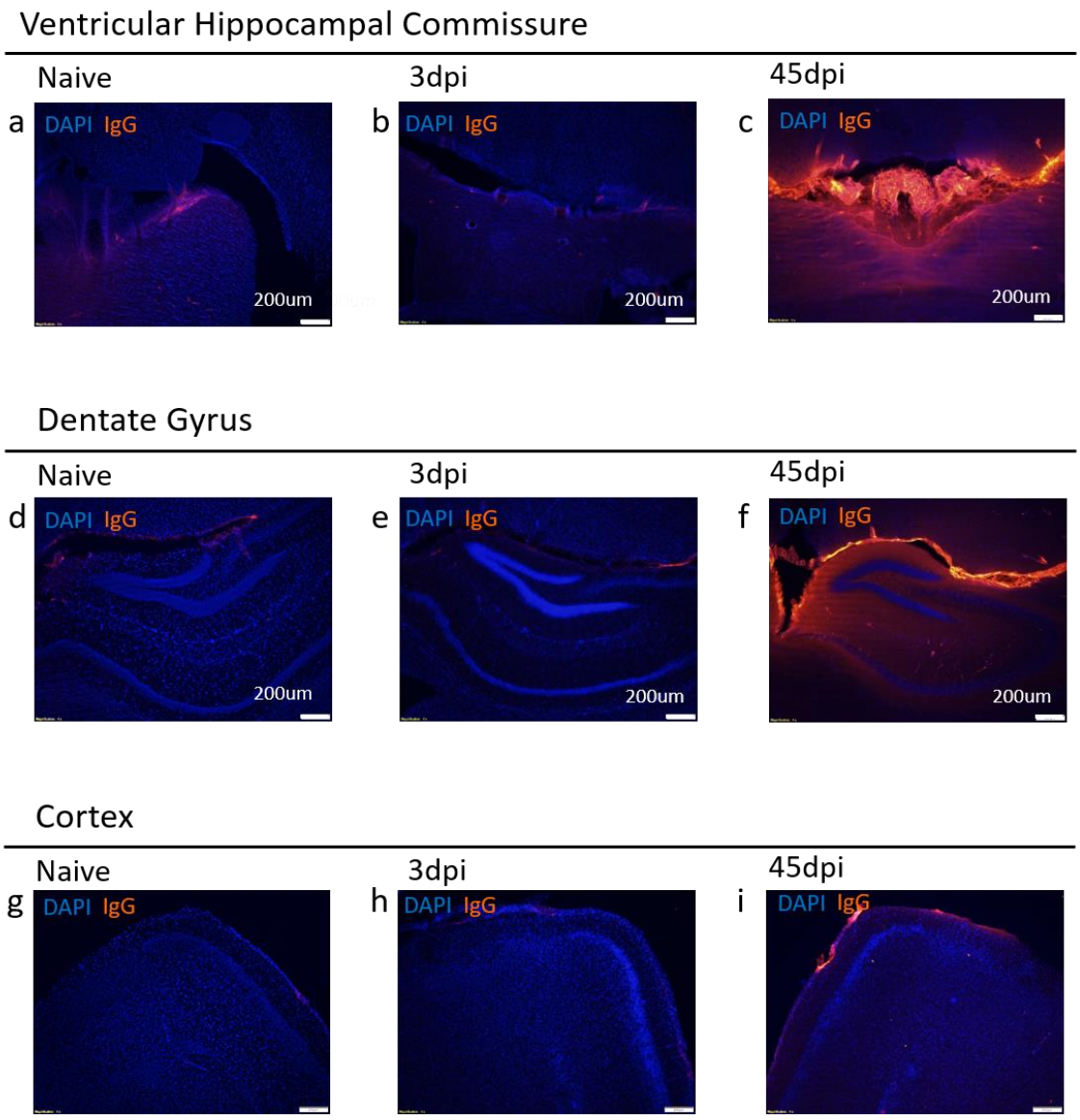

Figure 3.7 IgG deposition in the brain and ventricles during late-stage Bb infection. Epifluorescent images of brain sections from the (**a-c**) ventricular hippocampal commisure, (**d-f**) dentate gyrus, or (**g-i**) cortex, in mice that were (**a,d,g**) naïve, (**b,e,h**) infected by Bb for 3 days, or (**c,f,i**) infected by Bb for 45 days. Cells were stained with DAPI nuclear stain (blue), or IgG (orange); n=1 per group.

With respect to movement, Bb infected mice travelled less total distance, and had a lower average velocity when moving than sham controls (Figure 3.10 c). Surprisingly, neither groups demonstrated any differences in their ability to discern novel objects, as measured by the discrimination index, and recognition index (124) (Figure 3.10 d). The behavioral testing that we performed demonstrates that, in our mouse model, long-term Bb infection can result in increased nociception, and decreased mobility/movement activity. Importantly, our negative result in the NORT does not rule out the possibility that other cognitive abnormalities exist.

3.5 Discussion

We have used a CSF-tropic strain of Bb that is common in the United States to induce intradermal Bb infection in an LD-susceptible mouse strain. Within 45 dpi, CNS tissues and associated lymph nodes were culture positive for Bb. Confocal imaging demonstrated that Bb spirochetes in the dura had a tendency to localize near or in blood vessels, but not lymphatic vessels, suggesting a possible preference for blood borne dissemination into the CNS. We observed that a low frequency of B and T lymphocytes were detectable in the brain and dura of infected and control mice. In particular, CD4+ T cells in the brain increased in frequency and total number during early infection, and they persisted as late as 45 dpi, suggesting that CD4+ T cells may play a long-term role in Bb immunity in the brain. Behavioral testing demonstrated that, similar to patients, mice that were infected long-term by Bb exhibited decreased threshold to pain, impaired mobility, and decreased movement. Collectively these observations suggest that our

mouse model may be appropriate for understanding the etiologies that underlie LD and its associated neuroimmunological complications

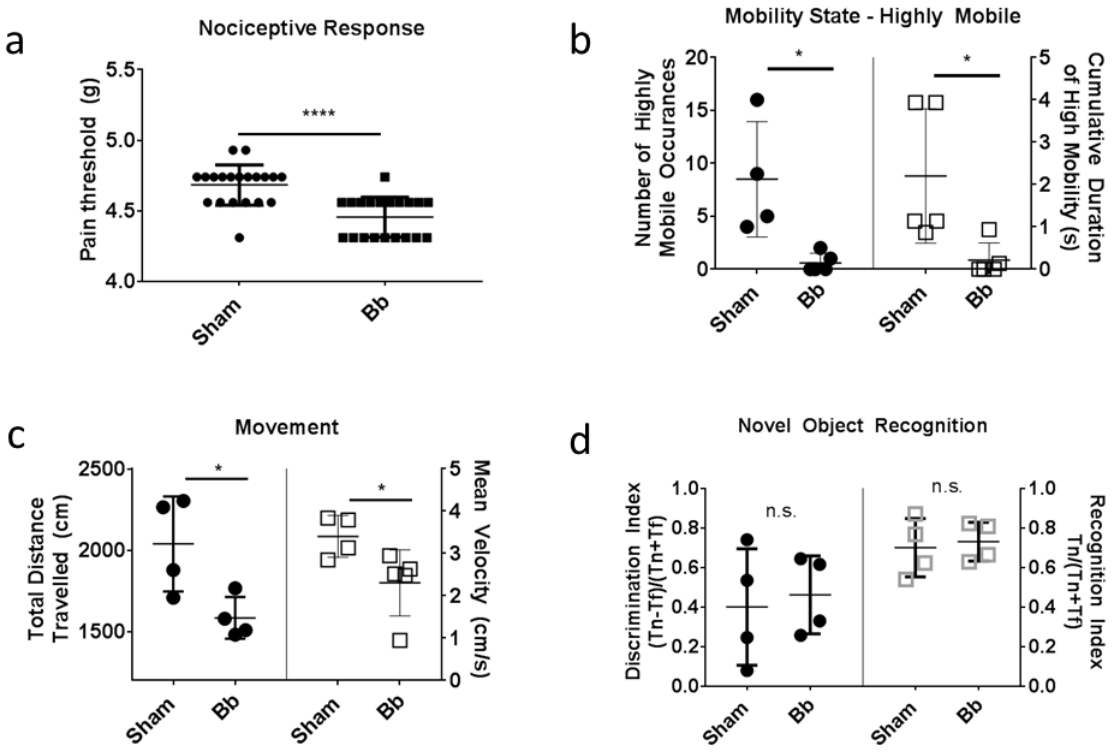

Figure 3.8 Behavioral changes in Bb-infected mice 45 dpi. (a) Changes in nociceptive response to Von Frey filaments in Bb vs Sham treated mice at 45 dpi; Each data point represents the withdrawal response from each of two hind-legs; All mice were tested as described in methods, and the Von Frey test was repeated two times for each group of n=5; (**b**) OF results of Bb infection-induced changes in number of highly mobile occurrences (left), and cumulative duration of time spent in highly mobile state (right); mobility threshold is defined as >25% displacement per second, as described in methods; n=4 animals per group ; (**c**) OF results of Bb infection-induced changes in total distance travelled (left), and mean velocity (right); n=4 animals per group; (**d**) NORT results quantifying changes in discrimination index (left) and recognition index (right); n=4 animals per treatment group; * P <0.05, **** P <0.0001, n.s. not significant, two-tailed unpaired t-test. Error bars represent standard deviation.

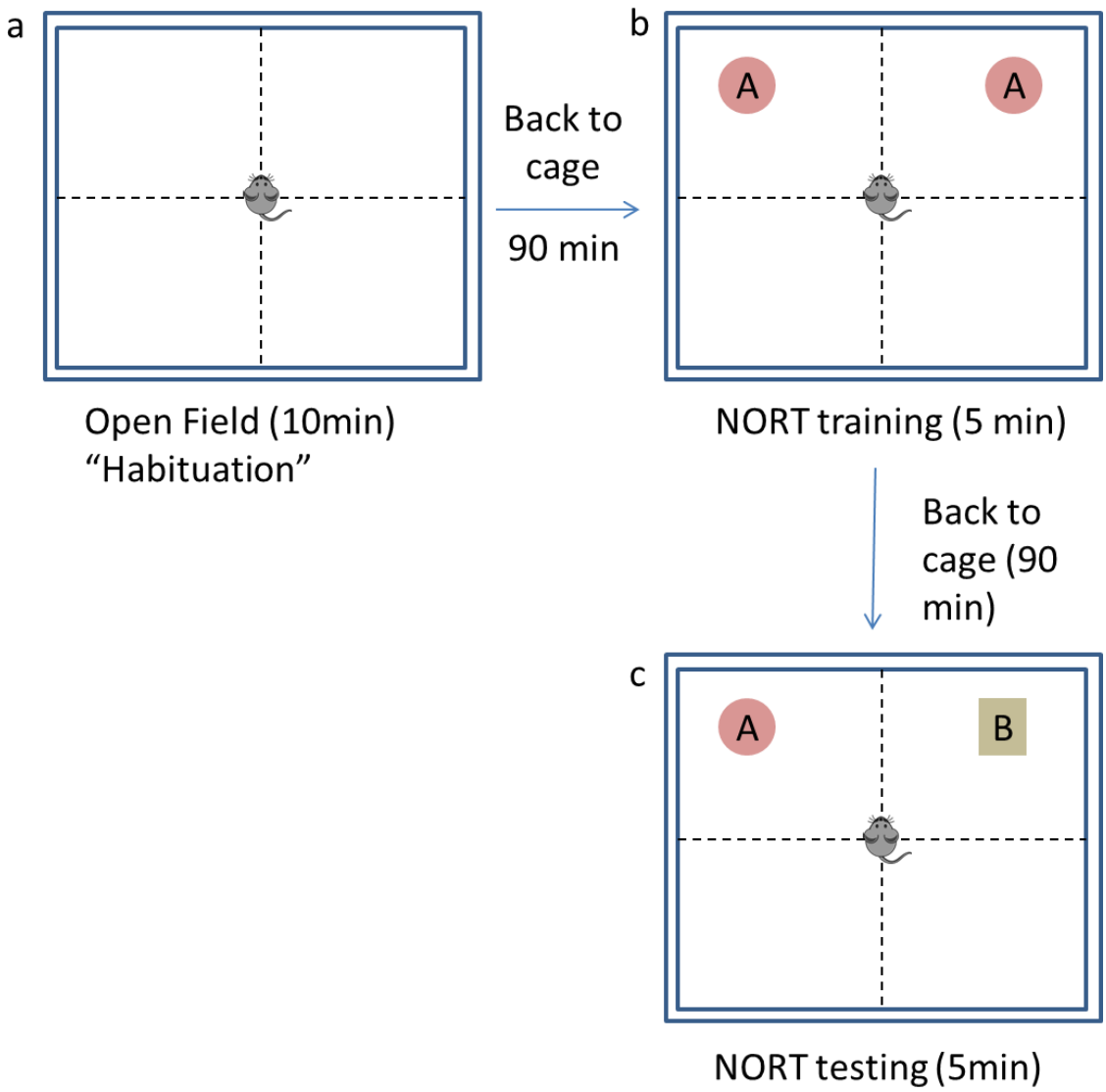

Figure 3.9 Schematic diagram of OF testing and NORT. (a) Habituation phase of OF testing, lasting 10 minutes, followed by a 90 minute rest interval; (**b**) Training phase of NORT with two identical objects, lasting 5 minutes, followed by a 90 minute rest interval; (**c**) Testing phase of NORT with one familiar object (pink circle A) and one novel object (tan square B), lasting 5 minutes.

4. SUMMARY

4.1 LD and its Post-infectious Complications

LD has a growing incidence in the United States, and Bbsl pathogens are emerging that can cause zoonotic infection, and may be infectious in humans (1-4, 6, 7). The myriad of symptoms that can arise after acute Bb infection provide evidence that Bb affects human physiology on a multisystem level. Although many individuals may recover from the symptoms that result from Bb infection, up to 20% of individuals that receive antibiotic treatment after late-stage disseminated infection do not find relief from their symptoms, resulting in RLA and/or PTLDS (29, 268, 269).

As we have described in detail, RLA is primarily characterized by persistent pain and joint inflammation, despite antibiotic therapy after Bb infection (31). Evidence suggests that TLR2 receptors and antigen presentation machinery belonging to the HLA-DR group play a role in responding to Bb infection, and some alleles or polymorphisms of these molecules are associated with increased probability of developing RLA (141, 142). Specifically, RLA is associated with HLA-DRB1 and HLA-DRB5 alleles, and at least one polymorphism (Arg753Gln) in the TLR-2 receptor. γδ T cells and DCs appear to facilitate and support RLA through non-classical antigen presentation via CD1 group 1 (CD1a-c) molecules, and Fas:FasL interactions (109, 141, 142, 145). CD1a-c molecules are regulated by IL-1β, which depends on cleavage induced activation by caspase-1. The combination of caspase-1 activity, and IL-1β production influence expression of CD1a-

c, and associated Bb antigen presentation via CD1a-c to CD1a-c ligands, that may include the γδ TCR (109). FasL expression by γδ T cells is primarily influenced by CD4+ T cell-secreted IL-2, a molecule that also upregulates CD25 on γδ T cells (105). The combination of two-way signaling between γδ T cells and DCs results in the production of proinflammatory cytokines that include IL-6, IL-12, IFN-γ, TNF-α, and IL-17 (108) (Fig 2.3). Ultimately, the γδ T cell response to Bb induces inflammation, presumably to clear Bb infection. In the case of RLA, it seems that the response to Bb infection is long-lasting, resulting in autoimmunity. The impetus for long-lasting immune responses may include persistent infection and/or permanently aberrant immune alterations due to the initial response to Bb infection, based on immunogenetics.

Like RLA, PTLDS is a post-infectious complication of LD that is thought to have autoimmune etiologies (122, 252, 253). Although patients with PTLDS can also exhibit symptoms of RLA, PTLDS includes persistent neurologic, neuropsychiatric, and neurocognitive deficits (23, 26, 29, 123, 270). The precise etiology of PTLDS is unknown, and to date, there are no FDA approved drugs for the treatment of PTLDS. The paucity of information about PTLDS is partly due to its relatively recent recognition as a legitimate syndrome (270), and the absence of an animal model. Typically, RMs have been used to model neurological LD in an experimental setting, but because of the high cost and relatively slow reproductive rate of RMs, our knowledge of the mechanisms that Bb utilizes to cause or facilitate neurological LD is especially limited. A mouse model of neuroborreliosis would be particularly useful, because it would allow

investigators to perform studies that could elucidate the pathogenic mechanisms that Bb utilizes to establish neuroborreliosis, without necessitating the use of the cost-prohibitive RM. Another advantage of a mouse model of neuroborreliosis, is that it could provide a test model for inducing PTLDS, and understanding its underlying etiologies. A single mouse model of neuroborreliosis was previously published, however the approach for establishing infection relied on intracerebroventricular inoculation of Bb into the CSF, and was therefore questionable with respect to the natural mechanism of pathogenesis (110). The natural route of infection is through a tick bite, which could not penetrate the skull, or the ventricles of the brain. It remains unclear why previous investigators did not use Bb 297 to induce CNS infection via the intradermal route. Perhaps they did and were unsuccessful. Overall, the mouse model of neuroborreliosis that we developed in the experiments that we described above, is the first mouse model of neuroborreliosis that replicates Bb infection of the CNS using an approach that has relevance to the natural vector.

4.2 Persistent Bb Infection in the Dura and Brain

For the first time, we have demonstrated that a common North American strain of Bb, Bb 297, is capable of establishing infection as early as 5 dpi and persisting in the CNS of a mouse as late as 45 days after intradermal infection (Figure 3.1 a-e, Fig 3.2 a-f, Table 3.1 – Table 3.4). These observations bring into question whether similar mechanisms may be at play in humans that have neurological LD, or PTLDS. In our experiments, most of the Bb that we observed in the dura were in the dural blood vessels (Figure 3.1

d,e, Figure 3.2 c,d) or the dural cortex (not shown), but not in the lymphatic vessels of the dura (Figure 3.1b). In every case, when we observed Bb adhering to blood vessels in the dura, the vessel diameter was always less than 100um (Figure 3.1 c). In mice, arteries and veins can range in diameter, depending on their location, and function, ranging from ~100um to ~2mm. Arterioles and venules, the next lowest hierarchy of blood vessels, are typically between 30um-100um in diameter, and at the capillary level, the luminal diameter is just large enough for erythrocytes to travel through, approximating 8-10 um. Although there are many differences between arterial and venous blood vessels, with respect to our studies, the most important difference is that veins and venules have much lower pressure than arteries or arterioles, and consequently the vessel walls experience lower sheer forces, thus providing better anchoring points for pathogens. Also, venules that begin immediately after capillaries, called post-capillary venules, are much more permeable than arterioles, and they are well described sites where leukocytes can roll, adhere, and extravasate into peripheral tissues (271-273). In other studies, it has been shown convincingly that Bb has a tendency to adhere to and extravasate through post-capillary venules in the periphery (256, 274). Because we did not observe spirochetes in any blood vessels that were large enough to be considered arteries or veins, arterioles or venules were the only candidates that could explain which type of blood vessels Bb had a tendency to adhere to (Figure 3.1, Figure 3.2). Although we did not perform any immunostaining that could distinguish between arterial or venous blood vessels, of the remaining candidates, the most logical and likely candidates are post-capillary venules,

because of their well-characterized function in supporting leukocyte and Bb adherence/extravasation into tissues.

We were surprised that Bb was not detected in any of the lymphatics in the dura, because others have provided indirect evidence that Bb disseminates and infects lymph nodes in a pattern that is consistent with the directionality of lymphatic flow (78), suggesting to us that Bb may disseminate through the lymphatic system. Contrary to our expectations, in our model, Bb was not observed in the lymphatics of the dura. Instead, it was apparent that Bb had a tendency to associate with blood vessels in the dura. There are a few possible explanations for the absence of Bb in the dural lymphatic vasculature. First, in the studies where Bb was indirectly shown to disseminate through the lymphatic vasculature, the strain of Bb used was strain N40, a strain that has been shown to induce lymphoadenopathy (78). In our model, we used Bb strain 297, a strain that was originally isolated from the CSF of a patient in New York (111). Importantly, in our experiments, unlike the group that used strain N40, we injected Bb in the dorsal thoracic midline, a region where multiple lymphatic drainage routes exist. Baumgarth et al administered Bb infection in the foot pad, an anatomical location where there is a single lymphatic pathway from the infection site to the downstream lymph nodes (78). Thus, although our data demonstrate that Bb may utilize multiple pathways for dissemination, they also don't reject the validity of the observations reported by Baumgarth et al (78), because we used fundamentally different approaches to induce infection. It is also possible that strain 297 may utilize different mechanisms for establishing pathogenesis

than strain N40, and these mechanisms may contribute to the different phenotypes that can manifest, for example, the lymphoadenopathy that is observed in response to infection by strain N40 versus CNS infection that is observed after infection by strain 297. Furthermore, the results with strain N40 were obtained using C57/BL6 mice that have substantially different immunogenetics than C3H/HeN mice that were used in our studies. Thus, in our model, the absence of Bb in the lymphatic vessels could be due to strain dependent differences either in Bb or the strain of mouse used. Another important possibility is that in our studies Bb may have originally disseminated into the dura by way of transmigration from the CSF through the dural lymphatics, and that at the later time points when we performed immunostaining and imaging, the Bb had migrated through the dural cortex toward dural blood vessels. Although this is possible, it seems unlikely that Bb would migrate toward and into blood vessels, because blood carries IgM antibodies that can destroy Bb after the onset of adaptive immunity to Bb infection (typically less than 1 week) (275). A much more plausible explanation is that Bb disseminated through the blood, not the lymphatics, because of the relative directionality of blood and lymph flow in the dura. Although our current understanding of lymph flow directionality in the dura is incomplete, all existing studies show that the dural lymphatic vessels transport some portion of interstitial fluid and CSF out of the CNS downstream into the DCLNs (101). If this is true, then Bb would be most likely to disseminate from the blood into the cortex of the dura, and then remain in the cortex thus evading clearance by high molecular weight antibodies such as IgM. The latter explanation would be most consistent with our results.

With respect to our investigation of the brain, similar to our results in the dura, we observed that infection with Bb 297 resulted in culture positivity at 5dpi and 45dpi. At both time points, however, less than half of the tissues were culture positive (Table 3.1). The phenomenon that bacteria can be viable, but not culturable (VBNC) has been explored in detail in multiple pathogens (276-278). In the case of Bb, an emerging body of evidence suggests that unculturable spirochetes may be in an altered metabolic state that is characterized by low production of ATP, a metabolic state that is commonly observed in Bb spirochetes that colonize the mid-gut of unfed/unengorged ticks (5). Remarkably, Bb spirochetes that are in stationary phase seem to have phenotypic differences that allow them to persist in spite of multiple antibiotic treatments, possibly due to alterations in their metabolic profiles (279, 280). In our experiments, we did not have evidence that any spirochetes were present in the culture negative brains, however, the presence of metabolically-altered, VBNC Bb, represents a likely possibility that may reconcile the differences that we saw within groups of mice that were infected with Bb.

We are also interested in determining where in the brain Bb may reside. Although our experiments demonstrated that Bb is culturable, and therefore resides somewhere in the brain, we do not have sufficient evidence to determine the anatomical location that Bb may be establishing infection in the brain. There are at least 9, non-exclusive possibilities that describe where Bb could be persisting in the brain. Bb may be 1) in the brain parenchyma, 2) adhering to the blood vessels that comprise the BBB, 3) Adhering

to the perivascular Virchow-Robin spaces between the blood vessels and the parenchyma, 4) adhering to ependymal cells of the CP, 5) adhering to blood vessels that supply the CP, 6) Floating freely in the CSF, 7) in the pia mater or the vessels that supply the pia mater (we did not remove the pia before culturing brains), 8) inside any of the cells that form the substructures of the brain described above (ie intracellular infection), 9) Any series of permutations resulting from options 1-8. Based on the number of possibilities described above, it is clearly out of the scope of this study to address these questions, however, future studies may be directed to this end. Speculatively, we predict that at least one of these possibilities is accurate, and that it is likely that more than one of these possibilities happen simultaneously. Specifically, we are in favor of options 3-6, for the reasons described below.

The blood vessels that supply the choroid plexus do not have the same tightly regulated barrier function as the BBB, and as a result, interstitial fluids freely leave the capillary beds and circulate into the perivascular spaces of the CP (94). The Ependymal cells that line the CP are connected by tight junctions, and with the aid of ion and aquaporin channels, they selectively regulate which components of interstitial fluids can travel through the basolateral surface of the blood-CSF barrier, into the CSF (95). We suggest that the relative permeability of the blood vessels that supply the CP, makes them good targets for Bb to adhere to, and/or extravasate through. Much like the dura, where we have demonstrated that spirochetes can persist, ependymal cells express collagen, and the surrounding matrix contains decorin, both proteins that Bb has tropisms for (118,

281). If Bb spirochetes can access ependymal cells by extravasating through the blood vessels of the CP, this is another area where the microenvironment seems suitable for Bb to persist. From there, Bb could either end its route of dissemination, or it could continue on through the brain. If it continued through the brain, it would have to get through the blood-CSF barrier that is regulated by ependymal cells of the CP. Bb could compromise the blood-CSF barrier by receptor mediated endocytosis (transcellular), or it could compromise the barrier by going around ependymal cells and degrading the CP (paracellular). If Bb utilized receptor-mediated pathways, we would expect to see Bb circulating in the CSF. If tight-junction proteins were degraded, we would still expect to see Bb in the CSF, but we would also expect that inappropriate constituents of the interstitial fluid would diffuse into the CSF, and therefore be detectable. Finally, if Bb entered the CSF it would almost definitely be in Virchow-Robin spaces of the parenchyma, because CSF circulates through these spaces as part of its function to clear interstitial fluids (94). Collectively, the theoretical mechanism described above, corresponds to the aforementioned possibilities 3-6. Because we have optimized the immunostaining protocol for imaging Bb and blood vessels in the dura, we are confident that we can use similar protocols to immunostain Bb in the brain, thus providing information about anatomical localization, and mechanisms that Bb may utilize to infect the CNS. Also, because CSF can be sampled, we should theoretically be able to determine whether Bb penetrated the blood-CSF barrier by transcellular (CSF constituents relatively unaltered), or paracellular (CSF constituents altered) routes. We will likely address these questions in the near future.

We were surprised to observe differences in culture positivity of the R-DCLN and L-DCLN at the 5 dpi time point (Table 3.1). Our assumptions going into the experiments were that CSF drainage into downstream peripheral lymph nodes, whether coming from the dural lymphatics or the cribiform plate or both, would be distributed without overt preference for a particular DCLN. After all, in the mouse, the R-DCLN and L-DCLN are within millimeters of each other, a relatively short distance for lymph to travel. The observation that all R-DCLN were culture positive, yet only 1/4 of L-DCLN were positive, suggests that there may something unique about how Bb disseminates from the infection site in the dorsal thoracic midline. We think there are at least two possible explanations for this observed phenomenon. First, it may be the case that the lymphatic vessels at the site of infection drain lymph in such a way that lymph resistance is lowest in the direction of the R-DCLN. Differences in lymph pressures within the lymphatic vessels could be achieved by selective shunting mechanisms at the level of the L-DCLN, or the afferent vessels upstream of it. In parallel, resistance in the lymphatic vessels leading to the R-DCLN could be reduced by any combination of mechanisms that regulate dilation of the afferent vessels upstream of the R-DCLN. Second, it may be the case that Bb disseminates into the CNS independent of lymph drainage at the infection site, and that once in the CSF, selective lymph drainage mechanisms via the cribiform plate or the dural lymphatics direct CSF drainage toward the R-DCLN. Because the lymph axis between the CSF and the DCLN is still not well-understood, there are likely other mechanisms, yet to be discovered, that may explain the phenomenon that we

observed. Importantly, our data suggest that with respect DCLNs, Bb does not disseminate through the blood, because if it did, there would be no differences in Bb culture positivity between the R-DCLN, or the L-DCLN, and as we have shown, this is clearly not the case.

4.3 Neuroimmunology of LD

The emerging paradigm in neuroimmunology is that the CNS is not necessarily immune privileged. In fact, it has been shown that lymphocytes are present in the dura, and can accumulate in the dural lymphatics, if the draining DCLNs are ligated (101). Consistent with this emerging paradigm, we have identified multiple subsets of B cells and T cells, albeit few in number, in the brain and dura under conditions of sham treatment with BSK-II medium or Bb infection (Figure 3.5a-f, Figure 3.6a-g). Strikingly, we observed an initial increase in the number and frequency of CD4+ T cells in the brain during early infection with Bb, and CD4+ T cell populations remained elevated in the brain as late as 45 dpi (Figure 3.5d). We also observed an increase in T cell dependent B2 B cells in the brain at 5 dpi, but the differences between Sham and Bb infection were not significant at 45 dpi (Figure 3.5b). This observation is consistent with reports that Bb tends to promote T cell-independent antibody production, a B cell function that is not supported by B2 B cells (78, 86). Thus, it is possible that B2 B cells that were observed at 5 dpi did not receive the signals necessary for survival, that they migrated to other secondary immune organs such as the lymph nodes, or that they moved to ectopic germinal centers. Another distinct possibility is that the B cells observed in the brain at 5 dpi, became activated and

changed the surface expression of their phenotypic markers. For example, if B2 B cells became activated by a subset of CD4+ T cells, they would ultimately mature into $MHCII^-$, $CD138^+$ plasma cells that produced IgG antibody (282). Although we did not test for CD138 as a surface marker, the data that we obtained from our immunoglobulin experiments in the brain (Figure 3.8a-i) demonstrate that in the CP and in certain areas of the hippocampus, there is a substantial increase in IgG at 45 dpi, that is not observed at 5 dpi. It is not surprising that IgG was undetectable at 5 dpi, because 5 dpi would be too soon for the mouse to seroconvert from IgM to a Bb-specific IgG response, as this can take up to 4 weeks (267). Thus, implicit in our results, is the indication that the IgG observed in the brain at 45 dpi was specific to Bb infection. The overall neuroimmunological picture demonstrates that persistent Bb infection in the brain induces a measurable neuroimmune response that is characterized by CD4+ T cells, B2 B cells, and IgG antibody in the brain. We are interested to know whether any Th1 or Th17 associated cytokines are produced by CD4+ T cells that were detected in the brains of Bb infected mice, at both of the time points that we tested.

B1 B cells represent a distinct subset of natural IgM secreting cells that come from a different cell lineage than conventional B2 B cells. B1 cells are subdivided into two subclasses, based on CD5 expression. The CD5+ group called B1a is generated early in life, and the CD5- group, called B1b arrives later than B1a and can increase in response to infection. The majority of IgM that is produced by B1a seems to be “natural antibody” that serves two functions. The first function of B1a-secreted natural antibody is to

opsonize pathogens either specifically, or non-specifically (283, 284). The second function of natural antibody is to bind self-antigens that are displayed by abnormal, injured, or dying cells, a function that is thought to fulfill a role in housekeeping, by marking unwanted cells for destruction (285). In contrast, the B1b subset seems to be primarily involved in T-independent IgM production of specific antibody against bacterial pathogens that include Borrelia hermsii, a relapsing fever spirochete that is related to Bb (286). Generally speaking, B1 cells originate in the fetal liver or bone marrow and rest in either the lymphoid tissues such as the spleen or lymph nodes, or they move to body cavities such as the peritoneum or pleural spaces where they function as memory B cells (287). Prior to our investigation, the role of B1 cells has not been explored in neuroborreliosis, however, there is a growing body of evidence that B1 cells are present in the brain during neurologic disorders, although their roles are not well understood. For example, in a mouse model of MS, called EAE, depletion of B1a cells after disease onset decreased demyelination, but depletion of B1a during the induction phase of EAE increased the incidence of EAE (288), suggesting that the timing and duration of a B1a response may determine whether it is helpful, or harmful to the individual. To date, there have not been any reports of B1b cells in the CNS. Whether the B1 cells that we observed in our model play a role in the outcome or progression of neuroborreliosis remains to be determined. Because, B1s predominantly produce IgM in response to pathogens, we would expect that if they had a role in responding to neuroborreliosis, IgM would be detectable somewhere in the CNS. Identifying the exact anatomical location of B1 cells within the CNS would help us to

determine where to look for IgM, and to design behavioral or neurophysiological experiments to assess what role(s) B1 cells or their associated immunoglobulins may have in response to Bb infection. Answering these questions could elucidate mechanisms specific to the neuroimmune response to Bb, or other related infections of the CNS that are implicated in autoimmunity.

We were surprised that there was a subset of B10 B cells that were that were detected transiently in the dura of Bb infected mice at 5 dpi (Figure 3.6g). B10 B cells have not previously been reported in the CNS, however, in other tissues they have been shown to have anti-inflammatory / immune suppressive function that is mediated by the secretion of IL-10 (261, 262). Although we did not specifically test for the production of IL-10 in the dura, the phenotypic surface markers of B10 ($CD45^{high}$, $CD3^{-}$, $CD19^{+}$, $CD5^{+}$, $CD1d^{+}$) were present. Assuming that the presence of B10 B cells in the dura was not irrelevant, we hypothesize that they may be part of a normal response to CNS infection, designed to limit the magnitude of inflammation that can occur in the CNS, thus providing a failsafe that inhibits inflammation-induced damage in response to infection. The other possibility is that B10 B cells are not part of a normal immune response to CNS infection, and that Bb subverts the immune system by activating B10 B cells, reducing inflammation, and consequently surviving in the niche environment of the dura. Loss or gain of function studies revolving around the respective absence or upregulation of B10 B cells would be helpful in clarifying the role that they may play in response to CNS infection by Bb or other CNS-tropic pathogens.

4.4 Altered Behavior

The behavioral experiments that we performed in Bb-infected mice were among the first behavioral studies in experimental LD infection. We expected that mice infected with Bb 297 for 45 days would display some of the behavioral deficits that are present in humans that have LD, such as musculoskeletal pain and peripheral neuropathy, as well as changes in cognition (23, 123, 289). We hypothesized that Bb infected mice would exhibit a decreased pain threshold, deficits in tasks related to motor function, increased anxiety behaviors, and impairment in long-term memory. Consistent with our expectations, Bb-infected mice showed increased nociception (decreased pain threshold) to external stimuli in the rear hind-paw, as determined by the Von Frey test (Figure 3.7a). Although the differences in the pain response only varied by one step size in the weight of the Von Frey filament, they were nonetheless statistically significant and consistent with the results of other behavioral tests that we performed.

The OF test serves as a general screening parameter for changes in movement, mobility, and anxiety behaviors (126, 127). Anxiety behavior is usually assessed by changes in exploratory behavior in the new cage, specifically total distance travelled over a period of time (290). There are two factors that can be a stimulus for anxiety in the OF test, 1) social isolation that results from physical separation from littermates, and 2) the stress caused by a brightly lit, novel environment (290-292). We expected that all three parameters, movement, mobility, and anxiety, would be altered in Bb-infected mice. Our results from the OF test demonstrated that Bb-infected mice exhibited overall decreases

in mobility relative to sham controls, as defined by the frequency (number of high mobility events that occurred), and cumulative duration of the highly mobile events (Figure 3.7b, also see methods, section 3.2). We also observed that Bb-infected mice moved with a slower average velocity, and that the total distance travelled was relatively less than sham controls (Figure 3.7c). Collectively, the OF data demonstrate that there are unequivocal differences in mobility and movement, however the interpretation of anxiety behavior is less clear. Although anxiety behavior is typically quantified by exploration and total distance travelled, the results that we obtained could be confounded by general increases in peripheral neuropathy and nociception, as we observed in the Von Frey test (Figure 3.7a). It is possible that Bb-infected mice travelled slower, and thus explored the cage less because of increased musculoskeletal or joint pain in the periphery. Another possibility is that the mice had anxiety in addition to peripheral pain. The limitation of our approach is that we were not able to definitively determine whether anxiety played a role in the results of the OF test, and this is something that should be addressed moving forward, possibly by implementing different behavioral tests.

The purpose of the NORT test, was to determine whether Bb-infected mice experienced changes in visual memory impairment. The principle of the test is that mice with intact memory will spend more time exploring a novel object, than an object that is otherwise familiar (293). We expected that Bb-infected mice would show some impairment in their ability to discern the novel object from the familiar object, presumably due to Bb-related cognitive deficits in visual memory. Our results demonstrated that Bb-infected mice

showed no differences in their preference for a novel object, as compared to sham controls (Figure 3.7d). Preference for the novel object was measured by the discrimination index, and recognition index, as described (293). A major challenge in designing NORT experiments is determining the resting interval between the training period, when mice spend time with two identical objects, and the testing period, when mice are re-exposed to the familiar object from the training period in addition to a novel object. We chose a resting interval of 90 minutes because this retention period is relatively long, and corresponds to brain function related to the hippocampus, whereas a short resting interval would measure function related to the perirhinal cortex (293-295). We hypothesized that the hippocampus was the more likely target of Bb-related inflammation because the hippocampus if often affected in tertiary syphilis, an infection caused by a spirochete that is a close cousin of Bb, Treponema pallidum (296), and because we observed IgG deposition in the areas near the hippocampus of Bb infected mice (Figure 3.9 c,f). Although our results demonstrated no differences in visual memory between Bb-infected mice and sham controls, we cannot rule out the possibility that shorter, or longer retention intervals would produce different results. Furthermore, the NORT is simply one measure of memory, and it does not assess changes in other memory-related parameters such in spatial or visuospatial memory. Other tests that may be appropriate for testing spatial or visuospatial memory would include the Morris water maze, or the novel object location test (NOLT), respectively (297, 298).

4.5 Limitations of This Study

One of the inherent challenges of culturing tissues ex-vivo is preventing contamination of the tissues by common microbes that may be in the lab. This is especially difficult when the tissues are coming from organisms that have abundant commensal biota within and around many of their cavities, that under normal circumstances the immune system prevents from growing. It is practically impossible to keep these tissues sterile, so the next best approach is to use aseptic technique, and this is the approach that we used. In our experience, out of 5 tissues harvested, anywhere from 0-2 samples would become contaminated upon culture for spirochetes. Contamination was evident based on dark field microscopic visualization of bacteria with cocci or bacilli morphology. When contamination was observed, the samples were omitted from the study, and this is why the total n-values for each tissue vary (Table 3.1). We are not aware of alternative approaches that would reduce the probability of contaminating samples without introducing lurking variables, thus occasional contamination may simply be an acceptable limitation of our approach.

The immunoglobulin data that we presented (Figure 3.9 a-i) suggests that IgG may accumulate in the brains of Bb infected mice, at 45 dpi. The main limitation of these experiments is that each group had an n=1. Although the differences in IgG deposition between Bb infection at 45 dpi, and Bb infection at 5 dpi are substantial, if we are to state the data conclusively, the experiments will need to be replicated with higher n-values, and perhaps quantified by analytical imaging software such as ImageJ. It is also

important to note that at the time the experiments were performed, although we did serum block the tissues, we did not Fc block prior to immunostaining. It is unlikely that omitting the Fc blocking step would result in such a substantial difference in immunostaining, however, it is still an important control that will be necessarily in maintaining the rigor of our experiments, thus giving us greater confidence in the conclusions that we draw.

A typical question and criticism that we have received from peers is “why hasn’t anyone else been able to establish this model?” It is a good question, because for many years independent efforts to establish a mouse model of neuroborreliosis have been unsuccessful. It is difficult to know why others were not successful in developing the model, because their approaches were not published, so it is not really possible to assess or criticize their methodology. What we have observed is that in the literature, investigators rarely use Bb 297, but often use Bb B31. We reasoned that this could possibly account for the difference between the results that we obtained, and the negative results that others obtained. Based on our assessment using immunostaining, neither B31 Bb, nor a GFP variant of B31 Bb resulted in infection in the brain, or its associated perivascular / periventricular tissues (data not shown). A limitation in our approach was that we did not attempt to culture the brains of B31-infected animals. Although we think that our immunostaining approach would have revealed Bb infection in the brain, we would be more confident in our conclusions if we had cultured the brain,

dura, and DCLNs of B31 infected animals, as we did for 297 infection. Currently we are performing experiments to address this limitation.

We used a tracer-based assay with 10KD FITC-Dextran to determine whether the BBB was compromised (Figure 3.3 a-c). Based on our results, it was evident that there were no differences in tracer signal between mice infected with Bb 297 and sham treated mice. Although there was little variability in this data, and the n-values were appropriate, we are concerned that the methodology that we used may have diluted an already low signal. The methods relied on tissue homogenization, formamide-based tracer extraction, and multiple rounds of centrifugation and removal of supernatant. This methodology would likely be appropriate for circumstances where there is a significant breach in the BBB, for example traumatic brain injury, or ischemic stroke, but it is unclear how sensitive this approach was. A better approach may have been to use intravital microscopy to assess the diffusion of tracer through brain or pial vessels of interest in real time. Indeed, we did consider the intravital approach, but we were initially restricted in our resources, and although we did eventually obtain funding to purchase the necessary equipment, the bureaucratic hierarchy of decision making delayed our acquisition of the equipment until the funding expired. For us, this was a seminal lesson on diseconomies of scale, and the major limitations of overly bureaucratic administrative chains that ultimately invalidate the purpose of medical research, that is, to do research.

In all of our experiments, prior to tissue collection, we perfused animals transcardially to remove all blood from their blood vessels. The rationale for this procedure was to prevent any spirochetes that may have been circulating in the blood, from producing a false positive in any of the CNS tissues during culture. Likewise, we wanted to prevent leukocytes that were circulating in the blood from giving us the false impression that they were in the brain or dura. We also perfused the animals prior to performing immunostaining in the dura. We think that this approach was conservative, rational, and justified for the purpose of cell culture, and flow cytometry, but it may have been a disadvantage for immunostaining. It is possible that more borrelia may have been adhering to blood vessels in the dura, and that the sheer forces produced by perfusions may have displaced spirochetes that would have otherwise been detectable with immunostaining. Moving forward, we will probably not perfuse mice prior to immunostaining, so we can get a better idea of whether perfusions underestimate the number of spirochetes in the CNS.

Finally, with respect to isolating leukocytes from the brain, we used Percoll gradients to separate leukocytes from brain myelin, based on the differences in their respective densities. Overall this approach is effective, but relatively inefficient, as many leukocytes can be lost in the process, and sometimes there are too few leukocytes isolated to obtain the subsets of interest. A better approach would be to use one of many cell sorting technologies to isolate leukocytes based on the expression of common

leukocyte markers, such as CD45. Had we used this approach, we may have seen less variability in our flow cytometric data (Figure 3.4-Figure 3.8).

4.6 Concluding Thoughts and Future Directions

We have demonstrated that in C3H/HeN mice, intradermal infection with Bb 297 results in persistent infection in the meninges, alterations in B cell and T cell populations in the CNS, and behavioral deficits related to nociception, motor function and mobility. The observations that we made occurred most notably at 45 dpi, suggesting that our novel approach may have established an appropriate mouse model for late-stage Bb infection, ultimately resulting in neurological LD. We expect that this model may be quite important in future investigations, as it may elucidate the underlying mechanisms that facilitate or set the stage for either disease remission and clearance, or persistent illness, as is characterized by the collective syndromes of RLA and/or PTLDS.

Of the many important questions that remains, one of them is "what other behavioral deficits exist in these mice?" We are interested in answering this question, because the results of other behavioral tests may give us clues as to which regions of the CNS are most affected by Bb infection. For example, if we repeat NORT experiments using shorter and longer resting intervals between tests, we may be able to determine whether the perirhinal cortex or hippocampus are affected in our model of neurological LD. As we discussed above, the NOLT test and the Morris water maze are also behavioral tests of interest, as they relate to visuospatial and spatial memory/learning, respectively. Other

behavioral tests that may provide valuable information include the balance beam, tail flick test, elevated plus maze, forced swim test, and sociability test, as described in Table 4.1.

As we described earlier, it will be useful to repeat the immunoglobulin experiments in the brain, and in the dura, with higher n-values, because these experiments could give us reliable information about the quality and magnitude of the humoral response in the CNS, in response to infection. In the future, we would like to perform these experiments using IgG, and specific IgG subclasses that include IgG1-IgG4, as each subclass can provide information about the type of B cell that is producing antibody. We are also interested in probing for differences in IgM production, to determine whether the B1 B cells that we observed in our model play an active role in antibody production. Performing immunostaining or ELISA tests for other IgG isotypes such as IgA and IgE may also provide new information about the function(s) of secreted antibodies in neuroimmune responses. On a related note, B cell depletion therapies as could help us understand the respective contributions that B cells and T cells make to the behavioral phenotype that we observed during Bb infection. Finally, cytokine analyses of the brain and dura would guide us in understanding whether a particular cytokine profile is associated with neuroborreliosis. We expect that either a Th1 or a Th17 cytokine bias is likely, due to the growing body of evidence that these types of cytokines can be associated with neuropathologies.

We are especially interested in determining where in the brain compartment Bb may reside, and which mechanism(s) it uses to establish pathogenesis. As we described above, there are at least 9 possibilities that encompass how Bb may enter different compartments of the brain. It will be critical to utilize tissue culture, at early time points so we may determine when exactly after infection is Bb first detectable in the brain. Immunostaining and imagining will also provide important information about anatomical location(s) that Bb has a tropism(s) for. Our expectation is that Bb enters the CSF from the blood via the CP, resulting in persistent infection and inflammation in the brain. Determining the specific mechanisms that Bb utilizes to subvert the BBB and/or the blood-CSF barrier could provide new insights about how to target and prevent Bb from establishing neurological infection.

It will also be important to know whether our neurological LD mouse model could model post-infectious complications of LD, such as RLA or PTLDS. An approach that we are interested in, is determining whether the current "standard of care" antibiotic therapy with oral doxycycline, or intravenous ceftriaxone has any effect on Bb infection burden in the CNS, alterations in leukocyte populations in the CNS, or behavioral deficits. If we were to observe that antibiotic treatment did not effectively reverse the alterations that are associated with late-stage Bb infection, our mouse model would likely be appropriate for modeling RLA and/or PTLDS. These are particularly important questions, because answering them will help us to determine whether the current standards of care really are the best possible modalities of treatment. For example, if we

were to observe that none of the mice recovered with the current antibiotic treatment guidelines, we would be justified in trying other antibiotic therapies to determine efficacy of treatment for optimal outcomes. Alternatively, if we observed that only some mice recovered, mechanistic studies to explain these phenomena would be warranted, and could potentially explain why only some people develop post-infectious complications of LD, and/or other diseases.

Our novel mouse model of neurological LD brings to light many other questions that could provide valuable insights about the relationship between the Bb spirochete, and its host(s). Other subjects of interest include determining what effect immunogenetics may have on the outcome of Bb infection, whether the timing of antibiotic therapy affects disease outcome, and whether sex differences may play a role in disease progression or pathophysiology.

It appears that by answering a few simple questions about Bb infection, we have found ourselves surrounded by many new questions of greater depth, and an array of possibilities. It truly is an exciting time to be in science.

Table 4.1 Additional behavioral tests. Summary of additional behavioral tests of interest in mice with neuroborreliosis

Behavioral Test	NOLT	Morriz water maze	Balance beam	Tail flick	Elevated plus maze	Forced swim	Sociability test
Parameter Tested	Visuospatial memory/learning	Spatial memory/learning	Motor coordination	Pain tolerance	Anxiety	Anxiety / depression	Social preference

5. REFERENCES

1. Dworkin MS, Schwan TG, Anderson DE, Jr., & Borchardt SM (2008) Tick-borne relapsing fever. *Infectious disease clinics of North America* 22(3):449-468, viii.

2. Krause PJ, *et al.* (2014) Borrelia miyamotoi sensu lato seroreactivity and seroprevalence in the northeastern United States. *Emerging infectious diseases* 20(7):1183-1190.

3. Rudenko N, *et al.* (2008) Detection of Borrelia bissettii in cardiac valve tissue of a patient with endocarditis and aortic valve stenosis in the Czech Republic. *Journal of clinical microbiology* 46(10):3540-3543.

4. Ryffel K, Peter O, Rutti B, Suard A, & Dayer E (1999) Scored antibody reactivity determined by immunoblotting shows an association between clinical manifestations and presence of Borrelia burgdorferi sensu stricto, B. garinii, B. afzelii, and B. Valaisiana in humans. *Journal of clinical microbiology* 37(12):4086-4092.

5. Schotthoefer AM & Frost HM (2015) Ecology and Epidemiology of Lyme Borreliosis. *Clinics in laboratory medicine* 35(4):723-743.

6. Molloy PJ, *et al.* (2015) Borrelia miyamotoi Disease in the Northeastern United States: A Case Series. *Annals of internal medicine* 163(2):91-98.

7. Pritt BS, *et al.* (2016) Identification of a novel pathogenic Borrelia species causing Lyme borreliosis with unusually high spirochaetaemia: a descriptive study. *The Lancet. Infectious diseases*.

8. Steere AC, *et al.* (1977) Lyme arthritis: an epidemic of oligoarticular arthritis in children and adults in three connecticut communities. *Arthritis and rheumatism* 20(1):7-17.

9. Ackermann R (1983) [Chronic erythema migrans and tick-transmitted meningopolyneuritis (Garin-Bujadoux-Bannwarth): Borrelia infections?]. *Deutsche medizinische Wochenschrift* 108(15):577-580.

10. Burgdorfer W, *et al.* (1982) Lyme disease-a tick-borne spirochetosis? *Science* 216(4552):1317-1319.

11. Steere AC, Malawista SE, Newman JH, Spieler PN, & Bartenhagen NH (1980) Antibiotic therapy in Lyme disease. *Annals of internal medicine* 93(1):1-8.

12. Steere AC, Broderick TF, & Malawista SE (1978) Erythema chronicum migrans and Lyme arthritis: epidemiologic evidence for a tick vector. *American journal of epidemiology* 108(4):312-321.

13. Ackermann R, *et al.* (1984) Ixodes ricinus spirochete and European erythema chronicum migrans disease. *The Yale journal of biology and medicine* 57(4):573-580.

14. Burgdorfer W, Lane RS, Barbour AG, Gresbrink RA, & Anderson JR (1985) The western black-legged tick, Ixodes pacificus: a vector of Borrelia burgdorferi. *The American journal of tropical medicine and hygiene* 34(5):925-930.

15. Rauter C & Hartung T (2005) Prevalence of Borrelia burgdorferi sensu lato genospecies in Ixodes ricinus ticks in Europe: a metaanalysis. *Applied and environmental microbiology* 71(11):7203-7216.

16. Piesman J, Mather TN, Sinsky RJ, & Spielman A (1987) Duration of tick attachment and Borrelia burgdorferi transmission. *Journal of clinical microbiology* 25(3):557-558.

17. Strle F, *et al.* (1996) European Lyme borreliosis: 231 culture-confirmed cases involving patients with erythema migrans. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 23(1):61-65.

18. Hynote ED, Mervine PC, & Stricker RB (2012) Clinical evidence for rapid transmission of Lyme disease following a tickbite. *Diagnostic microbiology and infectious disease* 72(2):188-192.

19. Cook MJ (2015) Lyme borreliosis: a review of data on transmission time after tick attachment. *International journal of general medicine* 8:1-8.

20. Bockenstedt LK & Wormser GP (2014) Review: unraveling Lyme disease. *Arthritis & rheumatology* 66(9):2313-2323.

21. Shapiro ED (2014) Clinical practice. Lyme disease. *The New England journal of medicine* 370(18):1724-1731.

22. Steere AC, Schoen RT, & Taylor E (1987) The clinical evolution of Lyme arthritis. *Annals of internal medicine* 107(5):725-731.

23. Fallon BA, *et al.* (2009) Regional cerebral blood flow and metabolic rate in persistent Lyme encephalopathy. *Archives of general psychiatry* 66(5):554-563.

24. Luft BJ, *et al.* (1992) Invasion of the central nervous system by Borrelia burgdorferi in acute disseminated infection. *Jama* 267(10):1364-1367.

25. Halperin JJ (2014) Nervous system Lyme disease. *Handbook of clinical neurology* 121:1473-1483.

26. Fallon BA & Nields JA (1994) Lyme disease: a neuropsychiatric illness. *The American journal of psychiatry* 151(11):1571-1583.

27. Kaplan RF & Jones-Woodward L (1997) Lyme encephalopathy: a neuropsychological perspective. *Seminars in neurology* 17(1):31-37.

28. Keilp JG, *et al.* (2006) WAIS-III and WMS-III performance in chronic Lyme disease. *Journal of the International Neuropsychological Society : JINS* 12(1):119-129.

29. Aucott JN (2015) Posttreatment Lyme disease syndrome. *Infectious disease clinics of North America* 29(2):309-323.

30. Kalish RA, Leong JM, & Steere AC (1993) Association of treatment-resistant chronic Lyme arthritis with HLA-DR4 and antibody reactivity to OspA and OspB of Borrelia burgdorferi. *Infection and immunity* 61(7):2774-2779.

31. Divan A, Budd RC, Tobin RP, & Newell-Rogers MK (2015) gammadelta T Cells and dendritic cells in refractory Lyme arthritis. *Journal of leukocyte biology* 97(4):653-663.

32. Nelder MP, *et al.* (2016) Human pathogens associated with the blacklegged tick Ixodes scapularis: a systematic review. *Parasites & vectors* 9(1):265.

33. Aliota MT, *et al.* (2014) The prevalence of zoonotic tick-borne pathogens in Ixodes scapularis collected in the Hudson Valley, New York State. *Vector borne and zoonotic diseases* 14(4):245-250.

34. Belongia EA (2002) Epidemiology and impact of coinfections acquired from Ixodes ticks. *Vector borne and zoonotic diseases* 2(4):265-273.

35. Mitchell PD, Reed KD, & Hofkes JM (1996) Immunoserologic evidence of coinfection with Borrelia burgdorferi, Babesia microti, and human granulocytic Ehrlichia species in residents of Wisconsin and Minnesota. *Journal of clinical microbiology* 34(3):724-727.

36. Diuk-Wasser MA, Vannier E, & Krause PJ (2016) Coinfection by Ixodes Tick-Borne Pathogens: Ecological, Epidemiological, and Clinical Consequences. *Trends in parasitology* 32(1):30-42.

37. Grab DJ, Nyarko E, Barat NC, Nikolskaia OV, & Dumler JS (2007) Anaplasma phagocytophilum-Borrelia burgdorferi coinfection enhances chemokine, cytokine, and matrix metalloprotease expression by human brain microvascular endothelial cells. *Clinical and vaccine immunology : CVI* 14(11):1420-1424.

38. Hongo I & Bloch KC (2006) Ehrlichia infection of the central nervous system. *Current treatment options in neurology* 8(3):179-184.

39. Hinckley AF, *et al.* (2014) Lyme disease testing by large commercial laboratories in the United States. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 59(5):676-681.

40. Wormser GP, *et al.* (2006) The clinical assessment, treatment, and prevention of lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 43(9):1089-1134.

41. Thomas RJ, Dumler JS, & Carlyon JA (2009) Current management of human granulocytic anaplasmosis, human monocytic ehrlichiosis and Ehrlichia ewingii ehrlichiosis. *Expert review of anti-infective therapy* 7(6):709-722.

42. Abusaada K, Ajmal S, & Hughes L (2016) Successful Treatment of Human Monocytic Ehrlichiosis with Rifampin. *Cureus* 8(1):e444.

43. Halperin JJ (2015) Nervous system lyme disease. *Current infectious disease reports* 17(1):445.

44. Aguero-Rosenfeld ME, Wang G, Schwartz I, & Wormser GP (2005) Diagnosis of lyme borreliosis. *Clinical microbiology reviews* 18(3):484-509.

45. Rebman AW, Crowder LA, Kirkpatrick A, & Aucott JN (2015) Characteristics of seroconversion and implications for diagnosis of post-treatment Lyme disease syndrome: acute and convalescent serology among a prospective cohort of early Lyme disease patients. *Clinical rheumatology* 34(3):585-589.

46. Brown SL, Hansen SL, & Langone JJ (1999) Role of serology in the diagnosis of Lyme disease. *Jama* 282(1):62-66.

47. Dressler F, Whalen JA, Reinhardt BN, & Steere AC (1993) Western blotting in the serodiagnosis of Lyme disease. *The Journal of infectious diseases* 167(2):392-400.

48. Nowakowski J, *et al.* (2001) Laboratory diagnostic techniques for patients with early Lyme disease associated with erythema migrans: a comparison of different techniques. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 33(12):2023-2027.

49. Donahue JG, Piesman J, & Spielman A (1987) Reservoir competence of white-footed mice for Lyme disease spirochetes. *The American journal of tropical medicine and hygiene* 36(1):92-96.

50. Voordouw MJ, Lachish S, & Dolan MC (2015) The lyme disease pathogen has no effect on the survival of its rodent reservoir host. *PloS one* 10(2):e0118265.

51. Radolf JD, Caimano MJ, Stevenson B, & Hu LT (2012) Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes. *Nature reviews. Microbiology* 10(2):87-99.

52. Richter D & Matuschka FR (2006) Modulatory effect of cattle on risk for lyme disease. *Emerging infectious diseases* 12(12):1919-1923.

53. Stefancikova A, Adaszek L, Pet'ko B, Winiarczyk S, & Dudinak V (2008) Serological evidence of Borrelia burgdorferi sensu lato in horses and cattle from Poland and diagnostic problems of Lyme borreliosis. *Annals of agricultural and environmental medicine : AAEM* 15(1):37-43.

54. Ebani VV, Bertelloni F, Pinzauti P, & Cerri D (2012) Seroprevalence of Leptospira spp. and Borrelia burgdorferi sensu lato in Italian horses. *Annals of agricultural and environmental medicine : AAEM* 19(2):237-240.

55. Funk RA, *et al.* (2016) Seroprevalence of Borrelia burgdorferi in Horses Presented for Coggins Testing in Southwest Virginia and Change in Positive Test Results Approximately 1 Year Later. *Journal of veterinary internal medicine / American College of Veterinary Internal Medicine*.

56. Imai DM, *et al.* (2011) Lyme neuroborreliosis in 2 horses. *Veterinary pathology* 48(6):1151-1157.

57. Krupka I & Straubinger RK (2010) Lyme borreliosis in dogs and cats: background, diagnosis, treatment and prevention of infections with Borrelia burgdorferi sensu stricto. *The Veterinary clinics of North America. Small animal practice* 40(6):1103-1119.

58. Magnarelli LA, Anderson JF, Kaufmann AF, Lieberman LL, & Whitney GD (1985) Borreliosis in dogs from southern Connecticut. *Journal of the American Veterinary Medical Association* 186(9):955-959.

59. Krimer PM, *et al.* (2011) Molecular and pathological investigations of the central nervous system in Borrelia burgdorferi-infected dogs. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc* 23(4):757-763.

60. Appel MJ, *et al.* (1993) Experimental Lyme disease in dogs produces arthritis and persistent infection. *The Journal of infectious diseases* 167(3):651-664.

61. Straubinger RK, Summers BA, Chang YF, & Appel MJ (1997) Persistence of Borrelia burgdorferi in experimentally infected dogs after antibiotic treatment. *Journal of clinical microbiology* 35(1):111-116.

62. Summers BA, *et al.* (2005) Histopathological studies of experimental lyme disease in the dog. *Journal of comparative pathology* 133(1):1-13.

63. LaFleur RL, *et al.* (2010) One-year duration of immunity induced by vaccination with a canine Lyme disease bacterin. *Clinical and vaccine immunology : CVI* 17(5):870-874.

64. LaFleur RL, *et al.* (2015) Vaccination with the ospA- and ospB-Negative Borrelia burgdorferi Strain 50772 Provides Significant Protection against Canine Lyme Disease. *Clinical and vaccine immunology : CVI* 22(7):836-839.

65. LaFleur RL, *et al.* (2009) Bacterin that induces anti-OspA and anti-OspC borreliacidal antibodies provides a high level of protection against canine Lyme disease. *Clinical and vaccine immunology : CVI* 16(2):253-259.

66. Callister SM, LaFleur RL, Jobe DA, Lovrich SD, & Wasmoen TL (2015) Antibody responses to Borrelia burgdorferi outer surface proteins C and F in experimentally infected Beagle dogs. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc* 27(4):526-530.

67. Pachner AR, Gelderblom H, & Cadavid D (2001) The rhesus model of Lyme neuroborreliosis. *Immunological reviews* 183:186-204.

68. Bai Y, *et al.* (2004) Spinal cord involvement in the nonhuman primate model of Lyme disease. *Laboratory investigation; a journal of technical methods and pathology* 84(2):160-172.

69. Rupprecht TA, Koedel U, Fingerle V, & Pfister HW (2008) The pathogenesis of lyme neuroborreliosis: from infection to inflammation. *Molecular medicine* 14(3-4):205-212.

70. Ramesh G, Santana-Gould L, Inglis FM, England JD, & Philipp MT (2013) The Lyme disease spirochete Borrelia burgdorferi induces inflammation and apoptosis in cells from dorsal root ganglia. *Journal of neuroinflammation* 10:88.

71. Cadavid D, O'Neill T, Schaefer H, & Pachner AR (2000) Localization of Borrelia burgdorferi in the nervous system and other organs in a nonhuman primate model of lyme disease. *Laboratory investigation; a journal of technical methods and pathology* 80(7):1043-1054.

72. Fedorova N, *et al.* (2014) Remarkable diversity of tick or mammalian-associated Borreliae in the metropolitan San Francisco Bay Area, California. *Ticks and tick-borne diseases* 5(6):951-961.

73. Matuschka FR, *et al.* (1996) Risk of urban Lyme disease enhanced by the presence of rats. *The Journal of infectious diseases* 174(5):1108-1111.

74. Moody KD & Barthold SW (1991) Relative infectivity of Borrelia burgdorferi in Lewis rats by various routes of inoculation. *The American journal of tropical medicine and hygiene* 44(2):135-139.

75. Brissette CA, Houdek HM, Floden AM, & Rosenberger TA (2012) Acetate supplementation reduces microglia activation and brain interleukin-1beta levels in a rat model of Lyme neuroborreliosis. *Journal of neuroinflammation* 9:249.

76. Valencia C, Serrano A, & Guerrero A (1993) [Experimental model of Lyme disease in rats]. *Enfermedades infecciosas y microbiologia clinica* 11(2):65-69.

77. Akins DR, Bourell KW, Caimano MJ, Norgard MV, & Radolf JD (1998) A new animal model for studying Lyme disease spirochetes in a mammalian host-adapted state. *The Journal of clinical investigation* 101(10):2240-2250.

78. Tunev SS, *et al.* (2011) Lymphoadenopathy during lyme borreliosis is caused by spirochete migration-induced specific B cell activation. *PLoS pathogens* 7(5):e1002066.

79. Barthold SW, Beck DS, Hansen GM, Terwilliger GA, & Moody KD (1990) Lyme borreliosis in selected strains and ages of laboratory mice. *The Journal of infectious diseases* 162(1):133-138.

80. Brinster RL, *et al.* (1981) Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. *Cell* 27(1 Pt 2):223-231.

81. Jaenisch R (1975) Infection of mouse blastocysts with SV40 DNA: normal development of the infected embryos and persistence of SV40-specific DNA sequences in the adult animals. *Cold Spring Harbor symposia on quantitative biology* 39 Pt 1:375-380.

82. Jaenisch R & Mintz B (1974) Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. *Proceedings of the National Academy of Sciences of the United States of America* 71(4):1250-1254.

83. Levine JF, Wilson ML, & Spielman A (1985) Mice as reservoirs of the Lyme disease spirochete. *The American journal of tropical medicine and hygiene* 34(2):355-360.

84. Magnarelli LA, Anderson JF, & Chappell WA (1984) Geographic distribution of humans, raccoons, and white-footed mice with antibodies to Lyme disease spirochetes in Connecticut. *The Yale journal of biology and medicine* 57(4):619-626.

85. Barthold SW, Persing DH, Armstrong AL, & Peeples RA (1991) Kinetics of Borrelia burgdorferi dissemination and evolution of disease after intradermal inoculation of mice. *The American journal of pathology* 139(2):263-273.

86. Hastey CJ, Elsner RA, Barthold SW, & Baumgarth N (2012) Delays and diversions mark the development of B cell responses to Borrelia burgdorferi infection. *Journal of immunology* 188(11):5612-5622.

87. Hastey CJ, Ochoa J, Olsen KJ, Barthold SW, & Baumgarth N (2014) MyD88- and TRIF-independent induction of type I interferon drives naive B cell accumulation but not loss of lymph node architecture in Lyme disease. *Infection and immunity* 82(4):1548-1558.

88. Barthold SW, de Souza MS, Janotka JL, Smith AL, & Persing DH (1993) Chronic Lyme borreliosis in the laboratory mouse. *The American journal of pathology* 143(3):959-971.

89. Fikrig E, *et al.* (1994) Sera from patients with chronic Lyme disease protect mice from Lyme borreliosis. *The Journal of infectious diseases* 169(3):568-574.

90. Melkus MW, *et al.* (2006) Humanized mice mount specific adaptive and innate immune responses to EBV and TSST-1. *Nature medicine* 12(11):1316-1322.

91. Pearson T, Greiner DL, & Shultz LD (2008) Creation of "humanized" mice to study human immunity. *Current protocols in immunology* Chapter 15:Unit 15 21.

92. Traggiai E, *et al.* (2004) Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science* 304(5667):104-107.

93. Ramer PC, Chijioke O, Meixlsperger S, Leung CS, & Munz C (2011) Mice with human immune system components as in vivo models for infections with human pathogens. *Immunology and cell biology* 89(3):408-416.

94. Jessen NA, Munk AS, Lundgaard I, & Nedergaard M (2015) The Glymphatic System: A Beginner's Guide. *Neurochemical research* 40(12):2583-2599.

95. Brown PD, Davies SL, Speake T, & Millar ID (2004) Molecular mechanisms of cerebrospinal fluid production. *Neuroscience* 129(4):957-970.

96. Thrane AS, Rangroo Thrane V, & Nedergaard M (2014) Drowning stars: reassessing the role of astrocytes in brain edema. *Trends in neurosciences* 37(11):620-628.

97. Iliff JJ, *et al.* (2012) A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid beta. *Science translational medicine* 4(147):147ra111.

98. Sakka L, Coll G, & Chazal J (2011) Anatomy and physiology of cerebrospinal fluid. *European annals of otorhinolaryngology, head and neck diseases* 128(6):309-316.

99. Johnston M, Zakharov A, Papaiconomou C, Salmasi G, & Armstrong D (2004) Evidence of connections between cerebrospinal fluid and nasal lymphatic vessels in humans, non-human primates and other mammalian species. *Cerebrospinal fluid research* 1(1):2.

100. Walter BA, Valera VA, Takahashi S, & Ushiki T (2006) The olfactory route for cerebrospinal fluid drainage into the peripheral lymphatic system. *Neuropathology and applied neurobiology* 32(4):388-396.

101. Louveau A, *et al.* (2015) Structural and functional features of central nervous system lymphatic vessels. *Nature* 523(7560):337-341.

102. Centers for Disease C & Prevention (2013) Three sudden cardiac deaths associated with Lyme carditis - United States, November 2012-July 2013. *MMWR. Morbidity and mortality weekly report* 62(49):993-996.

103. Bockenstedt LK, Gonzalez DG, Haberman AM, & Belperron AA (2012) Spirochete antigens persist near cartilage after murine Lyme borreliosis therapy. *The Journal of clinical investigation* 122(7):2652-2660.

104. Collins C, Shi C, Russell JQ, Fortner KA, & Budd RC (2008) Activation of gamma delta T cells by Borrelia burgdorferi is indirect via a TLR- and caspase-dependent pathway. *Journal of immunology* 181(4):2392-2398.

105. Collins C, *et al.* (2005) Lyme arthritis synovial gammadelta T cells instruct dendritic cells via fas ligand. *Journal of immunology* 175(9):5656-5665.

106. Imai DM, Feng S, Hodzic E, & Barthold SW (2013) Dynamics of connective-tissue localization during chronic Borrelia burgdorferi infection. *Laboratory investigation; a journal of technical methods and pathology* 93(8):900-910.

107. Liu N, Montgomery RR, Barthold SW, & Bockenstedt LK (2004) Myeloid differentiation antigen 88 deficiency impairs pathogen clearance but does not alter inflammation in Borrelia burgdorferi-infected mice. *Infection and immunity* 72(6):3195-3203.

108. Shi C, *et al.* (2011) Reduced immune response to Borrelia burgdorferi in the absence of gammadelta T cells. *Infection and immunity* 79(10):3940-3946.

109. Yakimchuk K, *et al.* (2011) Borrelia burgdorferi infection regulates CD1 expression in human cells and tissues via IL1-beta. *European journal of immunology* 41(3):694-705.

110. Li L, Narayan K, Pak E, & Pachner AR (2006) Intrathecal antibody production in a mouse model of Lyme neuroborreliosis. *Journal of neuroimmunology* 173(1-2):56-68.

111. Steere AC, *et al.* (1983) The spirochetal etiology of Lyme disease. *The New England journal of medicine* 308(13):733-740.

112. Anguita J, *et al.* (2000) Borrelia burgdorferi gene expression in vivo and spirochete pathogenicity. *Infection and immunity* 68(3):1222-1230.

113. Moody KD, Barthold SW, & Terwilliger GA (1990) Lyme borreliosis in laboratory animals: effect of host species and in vitro passage of Borrelia burgdorferi. *The American journal of tropical medicine and hygiene* 43(1):87-92.

114. Cabello FC, Godfrey HP, & Newman SA (2007) Hidden in plain sight: Borrelia burgdorferi and the extracellular matrix. *Trends in microbiology* 15(8):350-354.

115. Guo BP, Norris SJ, Rosenberg LC, & Hook M (1995) Adherence of Borrelia burgdorferi to the proteoglycan decorin. *Infection and immunity* 63(9):3467-3472.

116. Scholzen T, *et al.* (1994) The murine decorin. Complete cDNA cloning, genomic organization, chromosomal assignment, and expression during organogenesis and tissue differentiation. *The Journal of biological chemistry* 269(45):28270-28281.

117. Wadhwa S, *et al.* (2007) Impaired posterior frontal sutural fusion in the biglycan/decorin double deficient mice. *Bone* 40(4):861-866.

118. Kappler J, *et al.* (1998) Developmental regulation of decorin expression in postnatal rat brain. *Brain research* 793(1-2):328-332.

119. Li L, Mendis N, Trigui H, Oliver JD, & Faucher SP (2014) The importance of the viable but non-culturable state in human bacterial pathogens. *Frontiers in microbiology* 5:258.

120. Amor S, Puentes F, Baker D, & van der Valk P (2010) Inflammation in neurodegenerative diseases. *Immunology* 129(2):154-169.

121. Ubogu EE (2015) Inflammatory neuropathies: pathology, molecular markers and targets for specific therapeutic intervention. *Acta neuropathologica* 130(4):445-468.

122. Chandra A, *et al.* (2010) Anti-neural antibody reactivity in patients with a history of Lyme borreliosis and persistent symptoms. *Brain, behavior, and immunity* 24(6):1018-1024.

123. Fallon BA, Levin ES, Schweitzer PJ, & Hardesty D (2010) Inflammation and central nervous system Lyme disease. *Neurobiology of disease* 37(3):534-541.

124. Hansen K, Crone C, & Kristoferitsch W (2013) Lyme neuroborreliosis. *Handbook of clinical neurology* 115:559-575.

125. Chaplan SR, Bach FW, Pogrel JW, Chung JM, & Yaksh TL (1994) Quantitative assessment of tactile allodynia in the rat paw. *Journal of neuroscience methods* 53(1):55-63.

126. Van Meer P & Raber J (2005) Mouse behavioural analysis in systems biology. *The Biochemical journal* 389(Pt 3):593-610.

127. Wilson RC, Vacek T, Lanier DL, & Dewsbury DA (1976) Open-field behavior in muroid rodents. *Behavioral biology* 17(4):495-506.

128. Leger M, *et al.* (2013) Object recognition test in mice. *Nature protocols* 8(12):2531-2537.

129. Stanek G & Strle F (2003) Lyme borreliosis. *Lancet* 362(9396):1639-1647.

130. Marchal C, *et al.* (2011) Antialarmin effect of tick saliva during the transmission of Lyme disease. *Infection and immunity* 79(2):774-785.

131. Shih CM & Spielman A (1993) Accelerated transmission of Lyme disease spirochetes by partially fed vector ticks. *Journal of clinical microbiology* 31(11):2878-2881.

132. Baker PJ (2008) Perspectives on "chronic Lyme disease". *The American journal of medicine* 121(7):562-564.

133. Embers ME, *et al.* (2012) Persistence of Borrelia burgdorferi in rhesus macaques following antibiotic treatment of disseminated infection. *PloS one* 7(1):e29914.

134. Feder HM, Jr., *et al.* (2007) A critical appraisal of "chronic Lyme disease". *The New England journal of medicine* 357(14):1422-1430.

135. Hodzic E, Imai D, Feng S, & Barthold SW (2014) Resurgence of persisting non-cultivable Borrelia burgdorferi following antibiotic treatment in mice. *PloS one* 9(1):e86907.

136. Crowder LA, Yedlin VA, Weinstein ER, Kortte KB, & Aucott JN (2014) Lyme disease and post-treatment Lyme disease syndrome: the neglected disease in our own backyard. *Public health* 128(9):784-791.

137. Halperin JJ (2014) Lyme disease: neurology, neurobiology, and behavior. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 58(9):1267-1272.

138. Lantos PM, Auwaerter PG, & Wormser GP (2014) A systematic review of Borrelia burgdorferi morphologic variants does not support a role in chronic Lyme disease. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 58(5):663-671.

139. Lantos PM & Wormser GP (2014) Chronic coinfections in patients diagnosed with chronic lyme disease: a systematic review. *The American journal of medicine* 127(11):1105-1110.

140. Stricker RB & Johnson L (2013) Chronic Lyme disease: liberation from Lyme denialism. *The American journal of medicine* 126(8):e13-14.

141. Schroder NW, *et al.* (2005) Heterozygous Arg753Gln polymorphism of human TLR-2 impairs immune activation by Borrelia burgdorferi and protects from late stage Lyme disease. *Journal of immunology* 175(4):2534-2540.

142. Steere AC, *et al.* (2006) Antibiotic-refractory Lyme arthritis is associated with HLA-DR molecules that bind a Borrelia burgdorferi peptide. *The Journal of experimental medicine* 203(4):961-971.

143. Marques A (2008) Chronic Lyme disease: a review. *Infectious disease clinics of North America* 22(2):341-360, vii-viii.

144. Nocton JJ & Steere AC (1995) Lyme disease. *Advances in internal medicine* 40:69-117.

145. Shi C, *et al.* (2006) Fas ligand deficiency impairs host inflammatory response against infection with the spirochete Borrelia burgdorferi. *Infection and immunity* 74(2):1156-1160.

146. Vincent MS, *et al.* (1998) Lyme arthritis synovial gamma delta T cells respond to Borrelia burgdorferi lipoproteins and lipidated hexapeptides. *Journal of immunology* 161(10):5762-5771.

147. Brenner MB, *et al.* (1986) Identification of a putative second T-cell receptor. *Nature* 322(6075):145-149.

148. Hong NA, *et al.* (1997) A targeted mutation at the T-cell receptor alpha/delta locus impairs T-cell development and reveals the presence of the nearby antiapoptosis gene Dad1. *Molecular and cellular biology* 17(4):2151-2157.

149. Haas W, Kaufman S, & Martinez C (1990) The development and function of gamma delta T cells. *Immunology today* 11(10):340-343.

150. Theodorou I, *et al.* (1994) Recombination pattern of the TCR gamma locus in human peripheral T-cell lymphomas. *The Journal of pathology* 174(4):233-242.

151. Cui Y, Kang L, Cui L, & He W (2009) Human gammadelta T cell recognition of lipid A is predominately presented by CD1b or CD1c on dendritic cells. *Biology direct* 4:47.

152. Sugita M & Brenner MB (2000) T lymphocyte recognition of human group 1 CD1 molecules: implications for innate and acquired immunity. *Seminars in immunology* 12(6):511-516.

153. Russano AM, *et al.* (2007) CD1-restricted recognition of exogenous and self-lipid antigens by duodenal gammadelta+ T lymphocytes. *Journal of immunology* 178(6):3620-3626.

154. Uldrich AP, *et al.* (2013) CD1d-lipid antigen recognition by the gammadelta TCR. *Nature immunology* 14(11):1137-1145.

155. Blink SE, *et al.* (2014) gammadelta T cell subsets play opposing roles in regulating experimental autoimmune encephalomyelitis. *Cellular immunology* 290(1):39-51.

156. Hu C, *et al.* (2012) Antigen-presenting effects of effector memory Vgamma9Vdelta2 T cells in rheumatoid arthritis. *Cellular & molecular immunology* 9(3):245-254.

157. Olive C, Gatenby PA, & Serjeantson SW (1992) Molecular characterization of the V gamma 9 T cell receptor repertoire expressed in patients with rheumatoid arthritis. *European journal of immunology* 22(11):2901-2906.

158. Olive C, Gatenby PA, & Serjeantson SW (1994) Persistence of gamma/delta T cell oligoclonality in the peripheral blood of rheumatoid arthritis patients. *Immunology and cell biology* 72(1):7-11.

159. Liu W, Moussawi M, Roberts B, Boyson JE, & Huber SA (2013) Cross-regulation of T regulatory-cell response after coxsackievirus B3 infection by NKT and gammadelta T cells in the mouse. *The American journal of pathology* 183(2):441-449.

160. Modlin RL, *et al.* (1989) Lymphocytes bearing antigen-specific gamma delta T-cell receptors accumulate in human infectious disease lesions. *Nature* 339(6225):544-548.

161. Plattner BL, Doyle RT, & Hostetter JM (2009) Gamma-delta T cell subsets are differentially associated with granuloma development and organization in a bovine model of mycobacterial disease. *International journal of experimental pathology* 90(6):587-597.

162. van der Heyde HC, *et al.* (1996) Expansion of the gammadelta T cell subset in vivo during bloodstage malaria in B cell-deficient mice. *Journal of leukocyte biology* 60(2):221-229.

163. Yanez DM, Batchelder J, van der Heyde HC, Manning DD, & Weidanz WP (1999) Gamma delta T-cell function in pathogenesis of cerebral malaria in mice

infected with Plasmodium berghei ANKA. *Infection and immunity* 67(1):446-448.

164. Vincent MS, *et al.* (1996) Apoptosis of Fashigh CD4+ synovial T cells by borrelia-reactive Fas-ligand(high) gamma delta T cells in Lyme arthritis. *The Journal of experimental medicine* 184(6):2109-2117.

165. Hacker G, *et al.* (1992) V delta 1+ subset of human gamma delta T cells responds to ligands expressed by EBV-infected Burkitt lymphoma cells and transformed B lymphocytes. *Journal of immunology* 149(12):3984-3989.

166. Orsini DL, *et al.* (1993) A subset of V delta 1+ T cells proliferates in response to Epstein-Barr virus-transformed B cell lines in vitro. *Scandinavian journal of immunology* 38(4):335-340.

167. Orsini DL, *et al.* (1994) Functional and molecular characterization of B cell-responsive V delta 1+ gamma delta T cells. *European journal of immunology* 24(12):3199-3204.

168. Devilder MC, Allain S, Dousset C, Bonneville M, & Scotet E (2009) Early triggering of exclusive IFN-gamma responses of human Vgamma9Vdelta2 T cells by TLR-activated myeloid and plasmacytoid dendritic cells. *Journal of immunology* 183(6):3625-3633.

169. Fang H, *et al.* (2010) gammadelta T cells promote the maturation of dendritic cells during West Nile virus infection. *FEMS immunology and medical microbiology* 59(1):71-80.

170. Inoue S, *et al.* (2012) Enhancement of dendritic cell activation via CD40 ligand-expressing gammadelta T cells is responsible for protective immunity to Plasmodium parasites. *Proceedings of the National Academy of Sciences of the United States of America* 109(30):12129-12134.

171. Xu R, *et al.* (2010) Complement C5a regulates IL-17 by affecting the crosstalk between DC and gammadelta T cells in CLP-induced sepsis. *European journal of immunology* 40(4):1079-1088.

172. Chakour R, *et al.* (2009) A new function of the Fas-FasL pathway in macrophage activation. *Journal of leukocyte biology* 86(1):81-90.

173. Lettau M, Paulsen M, Kabelitz D, & Janssen O (2009) FasL expression and reverse signalling. *Results and problems in cell differentiation* 49:49-61.

174. Ramaswamy M, Cleland SY, Cruz AC, & Siegel RM (2009) Many checkpoints on the road to cell death: regulation of Fas-FasL interactions and Fas signaling in peripheral immune responses. *Results and problems in cell differentiation* 49:17-47.

175. Rust C, *et al.* (1992) Phenotypical and functional characterization of small intestinal TcR gamma delta + T cells in coeliac disease. *Scandinavian journal of immunology* 35(4):459-468.

176. Strasser A, Jost PJ, & Nagata S (2009) The many roles of FAS receptor signaling in the immune system. *Immunity* 30(2):180-192.

177. Hengartner MO (2000) The biochemistry of apoptosis. *Nature* 407(6805):770-776.

178. Krammer PH (2000) CD95's deadly mission in the immune system. *Nature* 407(6805):789-795.

179. Shu HB, Halpin DR, & Goeddel DV (1997) Casper is a FADD- and caspase-related inducer of apoptosis. *Immunity* 6(6):751-763.

180. Willems F, *et al.* (2000) Expression of c-FLIP(L) and resistance to CD95-mediated apoptosis of monocyte-derived dendritic cells: inhibition by bisindolylmaleimide. *Blood* 95(11):3478-3482.

181. Ashany D, Savir A, Bhardwaj N, & Elkon KB (1999) Dendritic cells are resistant to apoptosis through the Fas (CD95/APO-1) pathway. *Journal of immunology* 163(10):5303-5311.

182. Lens SM, *et al.* (2002) The caspase 8 inhibitor c-FLIP(L) modulates T-cell receptor-induced proliferation but not activation-induced cell death of lymphocytes. *Molecular and cellular biology* 22(15):5419-5433.

183. Ahn JH, *et al.* (2001) Non-apoptotic signaling pathways activated by soluble Fas ligand in serum-starved human fibroblasts. Mitogen-activated protein kinases and NF-kappaB-dependent gene expression. *The Journal of biological chemistry* 276(50):47100-47106.

184. Chinnaiyan AM & Dixit VM (1996) The cell-death machine. *Current biology : CB* 6(5):555-562.

185. Chinnaiyan AM, O'Rourke K, Tewari M, & Dixit VM (1995) FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* 81(4):505-512.

186. Hu WH, Johnson H, & Shu HB (2000) Activation of NF-kappaB by FADD, Casper, and caspase-8. *The Journal of biological chemistry* 275(15):10838-10844.

187. Kataoka T, *et al.* (2000) The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways. *Current biology : CB* 10(11):640-648.

188. Lee EW, Seo J, Jeong M, Lee S, & Song J (2012) The roles of FADD in extrinsic apoptosis and necroptosis. *BMB reports* 45(9):496-508.

189. Safa AR (2012) c-FLIP, a master anti-apoptotic regulator. *Experimental oncology* 34(3):176-184.

190. Hirschfeld M, *et al.* (1999) Cutting edge: inflammatory signaling by Borrelia burgdorferi lipoproteins is mediated by toll-like receptor 2. *Journal of immunology* 163(5):2382-2386.

191. Akira S, Hoshino K, & Kaisho T (2000) The role of Toll-like receptors and MyD88 in innate immune responses. *Journal of endotoxin research* 6(5):383-387.

192. Medzhitov R, Preston-Hurlburt P, & Janeway CA, Jr. (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 388(6640):394-397.

193. Rock FL, Hardiman G, Timans JC, Kastelein RA, & Bazan JF (1998) A family of human receptors structurally related to Drosophila Toll. *Proceedings of the National Academy of Sciences of the United States of America* 95(2):588-593.

194. Deetz CO, *et al.* (2006) Gamma interferon secretion by human Vgamma2Vdelta2 T cells after stimulation with antibody against the T-cell receptor plus the Toll-Like receptor 2 agonist Pam3Cys. *Infection and immunity* 74(8):4505-4511.

195. Pietschmann K, *et al.* (2009) Toll-like receptor expression and function in subsets of human gammadelta T lymphocytes. *Scandinavian journal of immunology* 70(3):245-255.

196. Komai-Koma M, Jones L, Ogg GS, Xu D, & Liew FY (2004) TLR2 is expressed on activated T cells as a costimulatory receptor. *Proceedings of the National Academy of Sciences of the United States of America* 101(9):3029-3034.

197. Dar AA, Patil RS, & Chiplunkar SV (2014) Insights into the Relationship between Toll Like Receptors and Gamma Delta T Cell Responses. *Frontiers in immunology* 5:366.

198. Jameson J, Witherden D, & Havran WL (2003) T-cell effector mechanisms: gammadelta and CD1d-restricted subsets. *Current opinion in immunology* 15(3):349-353.

199. Lees RK, Ferrero I, & MacDonald HR (2001) Tissue-specific segregation of TCRgamma delta+ NKT cells according to phenotype TCR repertoire and activation status: parallels with TCR alphabeta+NKT cells. *European journal of immunology* 31(10):2901-2909.

200. De Libero G & Mori L (2007) Structure and biology of self lipid antigens. *Current topics in microbiology and immunology* 314:51-72.

201. Kumar H, Belperron A, Barthold SW, & Bockenstedt LK (2000) Cutting edge: CD1d deficiency impairs murine host defense against the spirochete, Borrelia burgdorferi. *Journal of immunology* 165(9):4797-4801.

202. Belperron AA, Dailey CM, & Bockenstedt LK (2005) Infection-induced marginal zone B cell production of Borrelia hermsii-specific antibody is impaired in the absence of CD1d. *Journal of immunology* 174(9):5681-5686.

203. Tupin E, *et al.* (2008) NKT cells prevent chronic joint inflammation after infection with Borrelia burgdorferi. *Proceedings of the National Academy of Sciences of the United States of America* 105(50):19863-19868.

204. Porcelli S, *et al.* (1989) Recognition of cluster of differentiation 1 antigens by human CD4-CD8-cytolytic T lymphocytes. *Nature* 341(6241):447-450.

205. Porcelli S, Morita CT, & Brenner MB (1992) CD1b restricts the response of human CD4-8- T lymphocytes to a microbial antigen. *Nature* 360(6404):593-597.

206. Roura-Mir C, *et al.* (2005) Mycobacterium tuberculosis regulates CD1 antigen presentation pathways through TLR-2. *Journal of immunology* 175(3):1758-1766.

207. Leslie DS, *et al.* (2002) CD1-mediated gamma/delta T cell maturation of dendritic cells. *The Journal of experimental medicine* 196(12):1575-1584.

208. Spada FM, *et al.* (2000) Self-recognition of CD1 by gamma/delta T cells: implications for innate immunity. *The Journal of experimental medicine* 191(6):937-948.

209. Alnemri ES, *et al.* (1996) Human ICE/CED-3 protease nomenclature. *Cell* 87(2):171.

210. Sehra S & Dent AL (2006) Caspase function and the immune system. *Critical reviews in immunology* 26(2):133-148.

211. Koenig A, *et al.* (2014) The c-FLIPL cleavage product p43FLIP promotes activation of extracellular signal-regulated kinase (ERK), nuclear factor kappaB (NF-kappaB), and caspase-8 and T cell survival. *The Journal of biological chemistry* 289(2):1183-1191.

212. Thornberry NA, *et al.* (1992) A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature* 356(6372):768-774.

213. Eder C (2009) Mechanisms of interleukin-1beta release. *Immunobiology* 214(7):543-553.

214. Oosting M, *et al.* (2012) Murine Borrelia arthritis is highly dependent on ASC and caspase-1, but independent of NLRP3. *Arthritis research & therapy* 14(6):R247.

215. van de Veerdonk FL, Netea MG, Dinarello CA, & Joosten LA (2011) Inflammasome activation and IL-1beta and IL-18 processing during infection. *Trends in immunology* 32(3):110-116.

216. Barczyk A, Pierzchala W, & Sozanska E (2003) Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respiratory medicine* 97(6):726-733.

217. Duerr RH, *et al.* (2006) A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* 314(5804):1461-1463.

218. Kirkham BW, *et al.* (2006) Synovial membrane cytokine expression is predictive of joint damage progression in rheumatoid arthritis: a two-year prospective study (the DAMAGE study cohort). *Arthritis and rheumatism* 54(4):1122-1131.

219. Krueger GG, *et al.* (2007) A human interleukin-12/23 monoclonal antibody for the treatment of psoriasis. *The New England journal of medicine* 356(6):580-592.

220. Matusevicius D, *et al.* (1999) Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Multiple sclerosis* 5(2):101-104.

221. Molet S, *et al.* (2001) IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *The Journal of allergy and clinical immunology* 108(3):430-438.

222. Korn T, Bettelli E, Oukka M, & Kuchroo VK (2009) IL-17 and Th17 Cells. *Annual review of immunology* 27:485-517.

223. Maddur MS, Miossec P, Kaveri SV, & Bayry J (2012) Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies. *The American journal of pathology* 181(1):8-18.

224. Park H, *et al.* (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nature immunology* 6(11):1133-1141.

225. Genovese MC, *et al.* (2010) LY2439821, a humanized anti-interleukin-17 monoclonal antibody, in the treatment of patients with rheumatoid arthritis: A phase I randomized, double-blind, placebo-controlled, proof-of-concept study. *Arthritis and rheumatism* 62(4):929-939.

226. Martin DA, *et al.* (2013) A phase Ib multiple ascending dose study evaluating safety, pharmacokinetics, and early clinical response of brodalumab, a human anti-IL-17R antibody, in methotrexate-resistant rheumatoid arthritis. *Arthritis research & therapy* 15(5):R164.

227. Codolo G, *et al.* (2008) Borrelia burgdorferi NapA-driven Th17 cell inflammation in lyme arthritis. *Arthritis and rheumatism* 58(11):3609-3617.

228. Caccamo N, *et al.* (2011) Differentiation, phenotype, and function of interleukin-17-producing human Vgamma9Vdelta2 T cells. *Blood* 118(1):129-138.

229. Cho JS, *et al.* (2010) IL-17 is essential for host defense against cutaneous Staphylococcus aureus infection in mice. *The Journal of clinical investigation* 120(5):1762-1773.

230. Shibata K, Yamada H, Hara H, Kishihara K, & Yoshikai Y (2007) Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after Escherichia coli infection via IL-17 production. *Journal of immunology* 178(7):4466-4472.

231. Sutton CE, Mielke LA, & Mills KH (2012) IL-17-producing gammadelta T cells and innate lymphoid cells. *European journal of immunology* 42(9):2221-2231.

232. Lockhart E, Green AM, & Flynn JL (2006) IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. *Journal of immunology* 177(7):4662-4669.

233. Hiromatsu K, *et al.* (1992) A protective role of gamma/delta T cells in primary infection with Listeria monocytogenes in mice. *The Journal of experimental medicine* 175(1):49-56.

234. Kaufmann SH & Ladel CH (1994) Role of T cell subsets in immunity against intracellular bacteria: experimental infections of knock-out mice with Listeria monocytogenes and Mycobacterium bovis BCG. *Immunobiology* 191(4-5):509-519.

235. Mixter PF, Camerini V, Stone BJ, Miller VL, & Kronenberg M (1994) Mouse T lymphocytes that express a gamma delta T-cell antigen receptor contribute to resistance to Salmonella infection in vivo. *Infection and immunity* 62(10):4618-4621.

236. Rosat JP, MacDonald HR, & Louis JA (1993) A role for gamma delta + T cells during experimental infection of mice with Leishmania major. *Journal of immunology* 150(2):550-555.

237. Tsuji M, *et al.* (1994) Gamma delta T cells contribute to immunity against the liver stages of malaria in alpha beta T-cell-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* 91(1):345-349.

238. Balbi B, Moller DR, Kirby M, Holroyd KJ, & Crystal RG (1990) Increased numbers of T lymphocytes with gamma delta-positive antigen receptors in a subgroup of individuals with pulmonary sarcoidosis. *The Journal of clinical investigation* 85(5):1353-1361.

239. Brennan FM, *et al.* (1988) T cells expressing gamma delta chain receptors in rheumatoid arthritis. *Journal of autoimmunity* 1(4):319-326.

240. Ferreira LM (2013) Gammadelta T cells: innately adaptive immune cells? *International reviews of immunology* 32(3):223-248.

241. Poquet Y, *et al.* (1996) Human gamma delta T cells in tuberculosis. *Research in immunology* 147(8-9):542-549.

242. Szereday L, Baliko Z, & Szekeres-Bartho J (2008) The role of Vdelta2+T-cells in patients with active Mycobacterium tuberculosis infection and tuberculin anergy. *The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease* 12(3):262-268.

243. Havlir DV, Ellner JJ, Chervenak KA, & Boom WH (1991) Selective expansion of human gamma delta T cells by monocytes infected with live Mycobacterium tuberculosis. *The Journal of clinical investigation* 87(2):729-733.

244. Shen Y, *et al.* (2002) Adaptive immune response of Vgamma2Vdelta2+ T cells during mycobacterial infections. *Science* 295(5563):2255-2258.

245. Meraviglia S, El Daker S, Dieli F, Martini F, & Martino A (2011) gammadelta T cells cross-link innate and adaptive immunity in Mycobacterium tuberculosis infection. *Clinical & developmental immunology* 2011:587315.

246. Dieli F, *et al.* (2003) Differentiation of effector/memory Vdelta2 T cells and migratory routes in lymph nodes or inflammatory sites. *The Journal of experimental medicine* 198(3):391-397.

247. Granucci F, Feau S, Angeli V, Trottein F, & Ricciardi-Castagnoli P (2003) Early IL-2 production by mouse dendritic cells is the result of microbial-induced priming. *Journal of immunology* 170(10):5075-5081.

248. Meuer SC, *et al.* (1982) Cellular origin of interleukin 2 (IL 2) in man: evidence for stimulus-restricted IL 2 production by T4+ and T8+ T lymphocytes. *Journal of immunology* 129(3):1076-1079.

249. Zelante T, Fric J, Wong AY, & Ricciardi-Castagnoli P (2012) Interleukin-2 production by dendritic cells and its immuno-regulatory functions. *Frontiers in immunology* 3:161.

250. Steere AC & Angelis SM (2006) Therapy for Lyme arthritis: strategies for the treatment of antibiotic-refractory arthritis. *Arthritis and rheumatism* 54(10):3079-3086.

251. Forrester JD, *et al.* (2014) Notes from the field: update on Lyme carditis, groups at high risk, and frequency of associated sudden cardiac death--United States. *MMWR. Morbidity and mortality weekly report* 63(43):982-983.

252. Alaedini A & Latov N (2005) Antibodies against OspA epitopes of Borrelia burgdorferi cross-react with neural tissue. *Journal of neuroimmunology* 159(1-2):192-195.

253. Hodzic E, Feng S, Holden K, Freet KJ, & Barthold SW (2008) Persistence of Borrelia burgdorferi following antibiotic treatment in mice. *Antimicrobial agents and chemotherapy* 52(5):1728-1736.

254. Wlodarczyk A, Lobner M, Cedile O, & Owens T (2014) Comparison of microglia and infiltrating CD11c(+) cells as antigen presenting cells for T cell proliferation and cytokine response. *Journal of neuroinflammation* 11:57.

255. de Souza MS, *et al.* (1993) Variant responses of mice to Borrelia burgdorferi depending on the site of intradermal inoculation. *Infection and immunity* 61(10):4493-4497.

256. Ebady R, *et al.* (2016) Biomechanics of Borrelia burgdorferi Vascular Interactions. *Cell reports* 16(10):2593-2604.

257. Drouin EE, *et al.* (2013) A novel human autoantigen, endothelial cell growth factor, is a target of T and B cell responses in patients with Lyme disease. *Arthritis and rheumatism* 65(1):186-196.

258. Iliopoulou BP, Guerau-de-Arellano M, & Huber BT (2009) HLA-DR alleles determine responsiveness to Borrelia burgdorferi antigens in a mouse model of self-perpetuating arthritis. *Arthritis and rheumatism* 60(12):3831-3840.

259. Wang Q, *et al.* (2017) Immunogenic HLA-DR-Presented Self-Peptides Identified Directly from Clinical Samples of Synovial Tissue, Synovial Fluid, or Peripheral Blood in Patients with Rheumatoid Arthritis or Lyme Arthritis. *Journal of proteome research* 16(1):122-136.

260. Garraud O, *et al.* (2012) Revisiting the B-cell compartment in mouse and humans: more than one B-cell subset exists in the marginal zone and beyond. *BMC immunology* 13:63.

261. Tedder TF (2015) B10 cells: a functionally defined regulatory B cell subset. *Journal of immunology* 194(4):1395-1401.

262. Watanabe R, *et al.* (2010) Regulatory B cells (B10 cells) have a suppressive role in murine lupus: CD19 and B10 cell deficiency exacerbates systemic autoimmunity. *Journal of immunology* 184(9):4801-4809.

263. Avasarala JR, Cross AH, & Trotter JL (2001) Oligoclonal band number as a marker for prognosis in multiple sclerosis. *Archives of neurology* 58(12):2044-2045.

264. Hersh DS, *et al.* (2016) Pathologic deposition of non-amyloid immunoglobulin in the brain leading to mass effect and neurological deficits. *Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia* 30:143-145.

265. Link H (1972) Oligoclonal immunoglobulin G in multiple sclerosis brains. *Journal of the neurological sciences* 16(1):103-114.

266. Singh VK & Fudenberg HH (1989) Increase of immunoglobulin G3 subclass is related to brain autoantibody in Alzheimer's disease but not in Down's syndrome. *Autoimmunity* 3(2):95-101.

267. DePietropaolo DL, Powers JH, Gill JM, & Foy AJ (2006) Diagnosis of Lyme disease. *Delaware medical journal* 78(1):11-18.

268. Akin E, Aversa J, & Steere AC (2001) Expression of adhesion molecules in synovia of patients with treatment-resistant lyme arthritis. *Infection and immunity* 69(3):1774-1780.

269. Goldings EA & Jericho J (1986) Lyme disease. *Clinics in rheumatic diseases* 12(2):343-367.

270. Wormser GP (2006) Hematogenous dissemination in early Lyme disease. *Wiener klinische Wochenschrift* 118(21-22):634-637.

271. Atherton A & Born GV (1972) Quantitative investigations of the adhesiveness of circulating polymorphonuclear leucocytes to blood vessel walls. *The Journal of physiology* 222(2):447-474.

272. Farr AG & De Bruyn PP (1975) The mode of lymphocyte migration through postcapillary venule endothelium in lymph node. *The American journal of anatomy* 143(1):59-92.

273. Schmid-Schonbein GW, Usami S, Skalak R, & Chien S (1980) The interaction of leukocytes and erythrocytes in capillary and postcapillary vessels. *Microvascular research* 19(1):45-70.

274. Kumar D, *et al.* (2015) Intravital Imaging of Vascular Transmigration by the Lyme Spirochete: Requirement for the Integrin Binding Residues of the B. burgdorferi P66 Protein. *PLoS pathogens* 11(12):e1005333.

275. Barthold SW, Feng S, Bockenstedt LK, Fikrig E, & Feen K (1997) Protective and arthritis-resolving activity in sera of mice infected with Borrelia burgdorferi. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 25 Suppl 1:S9-17.

276. Duncan S, Glover LA, Killham K, & Prosser JI (1994) Luminescence-based detection of activity of starved and viable but nonculturable bacteria. *Applied and environmental microbiology* 60(4):1308-1316.

277. Oliver JD (2010) Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS microbiology reviews* 34(4):415-425.

278. Xu HS, *et al.* (1982) Survival and viability of nonculturableEscherichia coli andVibrio cholerae in the estuarine and marine environment. *Microbial ecology* 8(4):313-323.

279. Feng J, Zhang S, Shi W, & Zhang Y (2016) Ceftriaxone Pulse Dosing Fails to Eradicate Biofilm-Like Microcolony B. burgdorferi Persisters Which Are Sterilized by Daptomycin/ Doxycycline/Cefuroxime without Pulse Dosing. *Frontiers in microbiology* 7:1744.

280. Sharma B, Brown AV, Matluck NE, Hu LT, & Lewis K (2015) Borrelia burgdorferi, the Causative Agent of Lyme Disease, Forms Drug-Tolerant Persister Cells. *Antimicrobial agents and chemotherapy* 59(8):4616-4624.

281. Urabe N, *et al.* (2002) Basement membrane type IV collagen molecules in the choroid plexus, pia mater and capillaries in the mouse brain. *Archives of histology and cytology* 65(2):133-143.

282. Piskurich JF, *et al.* (2000) BLIMP-I mediates extinction of major histocompatibility class II transactivator expression in plasma cells. *Nature immunology* 1(6):526-532.

283. Choi YS & Baumgarth N (2008) Dual role for B-1a cells in immunity to influenza virus infection. *The Journal of experimental medicine* 205(13):3053-3064.

284. Cole LE, *et al.* (2009) Antigen-specific B-1a antibodies induced by Francisella tularensis LPS provide long-term protection against F. tularensis LVS challenge. *Proceedings of the National Academy of Sciences of the United States of America* 106(11):4343-4348.

285. Vas J, Gronwall C, & Silverman GJ (2013) Fundamental roles of the innate-like repertoire of natural antibodies in immune homeostasis. *Frontiers in immunology* 4:4.

286. Alugupalli KR, *et al.* (2003) The resolution of relapsing fever borreliosis requires IgM and is concurrent with expansion of B1b lymphocytes. *Journal of immunology* 170(7):3819-3827.

287. Baumgarth N (2016) B-1 Cell Heterogeneity and the Regulation of Natural and Antigen-Induced IgM Production. *Frontiers in immunology* 7:324.

288. Peterson LK, Tsunoda I, & Fujinami RS (2008) Role of CD5+ B-1 cells in EAE pathogenesis. *Autoimmunity* 41(5):353-362.

289. Hansen ES, *et al.* (2013) Interleukin-10 (IL-10) inhibits Borrelia burgdorferi-induced IL-17 production and attenuates IL-17-mediated Lyme arthritis. *Infection and immunity* 81(12):4421-4430.

290. Bailey KR & Crawley JN (2009) Anxiety-Related Behaviors in Mice. *Methods of Behavior Analysis in Neuroscience*, Frontiers in Neuroscience, ed Buccafusco JJBoca Raton (FL)), 2nd Ed.

291. Bidner S & Finnegan M (1989) Femoral fractures in Paget's disease. *Journal of orthopaedic trauma* 3(4):317-322.

292. File SE (1980) The use of social interaction as a method for detecting anxiolytic activity of chlordiazepoxide-like drugs. *Journal of neuroscience methods* 2(3):219-238.

293. Antunes M & Biala G (2012) The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cognitive processing* 13(2):93-110.

294. Baxter MG (2010) "I've seen it all before": explaining age-related impairments in object recognition. Theoretical comment on Burke et al. (2010). *Behavioral neuroscience* 124(5):706-709.

295. Reger ML, Hovda DA, & Giza CC (2009) Ontogeny of Rat Recognition Memory measured by the novel object recognition task. *Developmental psychobiology* 51(8):672-678.

296. Miklossy J (2012) Chronic or late lyme neuroborreliosis: analysis of evidence compared to chronic or late neurosyphilis. *The open neurology journal* 6:146-157.

297. Vogel-Ciernia A & Wood MA (2014) Examining object location and object recognition memory in mice. *Current protocols in neuroscience* 69:8 31 31-17.

298. Vorhees CV & Williams MT (2006) Morris water maze: procedures for assessing spatial and related forms of learning and memory. *Nature protocols* 1(2):848-858