ROLE OF INTERLEUKIN-17 IN THE ADAPTIVE IMMUNE RESPONSE IN LYME ARTHRITIS

by

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A Thesis Submitted in

Partial Fulfillment of the

Requirements for the Degree of

Master of Science

in Biomedical Sciences

at

The University of Wisconsin-Milwaukee

August 2012

ABSTRACT

ROLE OF INTERLEUKIN-17 IN THE ADAPTIVE

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The University of Wisconsin-Milwaukee, 2012

Under the Supervision of Dr. Dean T. Nardelli

Lyme arthritis is a devastating symptom of Lyme borreliosis that causes severe inflammation of the synovial joints. Interleukin-17 (IL-17) plays a role in the pathogenesis of various arthritides, including, possibly, Lyme arthritis, by causing the expression of genes involved in the production of inflammatory cytokines by synoviocytes. However, the cellular sources of IL-17 in the context of *Borrelia burgdorferi* infection are unknown, as are the effects of these cells on the development of arthritis and stimulation of humoral immunity through the production of borreliacidal antibodies. Using multiple models of Lyme arthritis, the hypothesis that IL-17 produced by CD4+ cells contributes to the inflammation associated with *B. burgdorferi* infection and is associated with an increase in borreliacidal antibody production was tested. Serum IL-17 levels were increased during *Borrelia* infection, especially at the time of peak swelling. Neutralization of interferon-gamma increased these IL-17 levels, and the addition of anti-CD4 antibodies to cultures of stimulated cells isolated during peak swelling reduced IL-17 levels. In addition, a role for IL-10 in the production of IL-17 was demonstrated. Furthermore, increased IL-17 was associated with an increase of

borreliacidal antibodies. These findings suggest CD4+ cells (possibly T cells) contribute to the production of IL-17 following infection with *B. burgdorferi* and that levels of this cytokine may affect borreliacidal antibody production.

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LIST OF ABBREVIATIONS

αCD4: anti-CD4

ANOVA: analysis of variance

Bb: *Borrelia burgdorferi*

BSK: Barbour-Stoenner-Kelly

CIA: collagen-induced arthritis

ELISA: enzyme-linked immunosorbent assay

EM: erythema migrans

IFN-γ: interferon-gamma

IL-1β: interleukin-1-beta

IL-6: interleukin-6

IL-10: interleukin-10

IL-17: interleukin-17

IL-21: interleukin-21

IL-23: interleukin-23

IL-35: interleukin-35

KO: knock-out

NK: natural killer

OspA: outer surface protein A

OspC: outer surface protein C

PBS: phosphate buffered saline

SEM: standard error of the mean

TGF-β: transforming growth factor-beta

Th1: T helper type 1

Th17: T helper type 17

TNF-α: tumor necrosis factor-alpha

T_{reg}: regulatory T

WT: wild-type

ACKNOWLEDGEMENTS

I want to express my gratitude to Dr. Dean Nardelli, my major professor, for his continuous attention, guidance, insight, and support during this research and the preparation of this thesis.

It is a pleasure for me to thank my committee members, Dr. Jeri-Anne Lyons, Dr. Jennifer Doll, and Dr. Janis Eells, for their guidance and support.

I offer many thanks to the numerous people who assisted in this research project. Specifically, I would like to thank my fellow lab member, Velinka Medić, for all of her help. Additionally, this project would be impossible without Jennifer Nemke, Dr. Berri Forman, and the many animal caretakers in the Animal Resource Center.

Lastly, I would like to thank all my family and friends who helped me get through this process by providing unending patience and love throughout this project.

CHAPTER 1: INTRODUCTION

I. Background

1. Lyme Borreliosis

Lyme borreliosis is the most common tick-borne disease in the United States, affecting tens of thousands of Americans infected with the spirochete *Borrelia burgdorferi* annually (1). It is a multi-stage, multi-symptom disease that is often misdiagnosed due to its vague clinical presentation. The main manifestations of Lyme borreliosis involve the neurological, cardiovascular, and musculoskeletal systems, with symptoms increasing in severity in the absence of antimicrobial therapy. However, a small proportion of genetically predisposed individuals continue to exhibit symptoms despite receiving, in accordance with guidelines established by experts in the field, antimicrobial therapy considered sufficient for clearing infection (2, 3). The cause of these persistent, chronic symptoms is a source of great contention in the field; among the most frequently considered theories are survival of the microbe in connective tissues, retention of antigenic microbial fragments, and infection-induced autoimmunity (4). Regardless, the pathology of Lyme borreliosis is caused by the immune response to infection, as *B. burgdorferi* does not secrete toxins or other products that induce tissue damage. Therefore, in the absence of effective antimicrobial treatment, anti-inflammatory therapeutic interventions would be a significant means for reducing morbidity of Lyme borreliosis.

Lyme borreliosis is endemic to several regions of the United States, including the Northeast, Mid-Atlantic, Upper Midwest, and Far West. Lyme borreliosis is transmitted by the bite of the *Ixodes* tick and is endemic to regions in which these tick vectors, as well as their animal reservoirs, live. In the northeastern and upper midwestern United States, *B. burgdorferi* is transmitted to humans by *I. scapularis.* These ticks become infected with the spirochete when they take a blood meal from infected small rodents (typically mice) and other mammalian hosts, such as deer and foxes. In the western United States, lizards occasionally serve as a reservoir for the bacteria (5) and are transmitted via *I. pacificus*. Moreover, birds have been identified as carriers of *B. burgdorferi*, providing a means for the bacteria to spread farther distances (6). Transmission most commonly occurs during the nymphal stage of tick development, when they are relatively small and are difficult to observe (2). The greatest onset of illness occurs between the months of May and August, when ticks have reached this stage of development. Though relatively rare, adult ticks are also capable of causing infection (2). In endemic areas, up to 30% of *Ixodes* ticks may be infected (7). An infected tick must be attached to its host for several days in order to transmit *B. burgdorferi* effectively (8). Unfortunately, patients diagnosed with Lyme borreliosis often do not recall having been bitten by a tick.

2. Stages of Disease, Diagnosis, and Therapeutic Measures

Untreated Lyme borreliosis is characterized by three phases: early infection, disseminated infection, and late infection. Most early symptoms of Lyme

borreliosis are typically vague, presenting as flu-like symptoms such as fever, headache, fatigue, and joint and muscle aches one to two weeks after the bite of an infected tick. However, diagnosis in this early stage is facilitated greatly by a skin lesion called erythema migrans (EM). EM is a painless, flat rash that radiates from the site of the tick bite and often exhibits central clearing, resembling a bull's-eye. Approximately 70% of infected individuals develop EM and, when it is observed in an endemic area or following a known tick bite, it is usually considered diagnostic (2). However, due to the rash's varied appearance and occasionally inconspicuous anatomical location, EM is often not observed by the patient or physician. Moreover, since the other symptoms of this early phase are constitutional, a diagnosis of Lyme borreliosis frequently is not considered and the infection progresses without treatment.

The second stage of Lyme borreliosis involves dissemination throughout the body weeks to months after infection and manifests in more severe symptoms. Secondary EM lesions are commonly observed at locations distal from the site of infection (2). Atrioventricular block, as well as neurological abnormalities such as numbness or tingling of the extremities, visual disturbances, and partial paralysis of facial muscles (9, 10), are commonly observed. Patients frequently develop more pronounced musculoskeletal symptoms, including pain and swelling of the joints and tendons (2). These symptoms may be exacerbated in late, persistent Lyme borreliosis, which occurs months to years after the initial onset of disease. Patients may experience profound neurological symptoms, including memory loss, fluctuations in mood, and peripheral neuropathy. In addition, 60% of

infected individuals at this stage exhibit Lyme arthritis, which is characterized by severe inflammation of the synovial tissues, ligaments, and tendons (2). Despite antimicrobial treatment, some individuals develop a chronic, severe, destructive arthropathy characterized by erosion of cartilage and bone (11).

Although frequently severe, most of the symptoms of Lyme borreliosis are shared by numerous other conditions and, therefore, often confound physicians and patients. Difficulties in diagnosis are compounded by the fact that suspected cases must fit several criteria before they are accepted as actual cases of disease. For example, in the absence of serodiagnostic confirmation, the EM lesion must be at least 5 centimeters in diameter and be accompanied by at least one late manifestation of disease (12). Current laboratory standards call for serodiagnostic confirmation using a two-tiered system: a screening enzyme-linked immunosorbent assay (ELISA), followed by a confirmatory Western blot that is deemed positive based on observation of immunoglobulin M or immunoglobulin G antibodies against a certain number of borrelial antigens (3). The infection can be cleared with antibiotics; the current standard of treatment is two to three weeks of oral doxycyline (3). However, in cases of prolonged infection or more severe symptoms, intravenous ceftriaxone may be prescribed (3). Subsequent or longer treatments may be needed if the symptoms persist beyond the initial course of antibiotics. Delayed treatment increases the likelihood of developing chronic symptoms, such as Lyme arthritis.

3. Lyme Arthritis

Lyme arthritis is among the most common clinical manifestations of Lyme borreliosis, affecting approximately 60% of individuals infected with *B. burgdorferi* (2). In humans, intermittent episodes of arthritis typically begin in the weeks or months following infection and may continue for years. Antibiotic treatment typically leads to successful resolution of disease; however, for a small subset of patients, arthritis persists despite several courses of antibiotics and negative polymerase chain reaction results from synovial tissue and fluid (13). These patients, who continue to have proliferative synovitis, are said to have antibiotic-refractory, or slow-resolving, arthritis (13). In addition to slower resolution of joint inflammation, patients with antibiotic-refractory Lyme arthritis also have higher levels of pro-inflammatory cytokines and lower levels of anti-inflammatory cytokines in the synovial fluid during the entire course of disease, including after treatment, when compared to patients with antibiotic-responsive Lyme arthritis (14, 15).

Despite a well-defined clinical course, the immune mechanisms responsible for the development and persistence of Lyme arthritis are not fully understood. As a result, very few treatment options exist. After antibiotic therapy has been used to clear the spirochete infection, the current recommended therapy is the use of non-steroidal anti-inflammatory agents (3). Understanding the immune mechanisms involved during the course of arthritis, as well as during the course of bacterial infection, is essential for the development of novel treatments.

4. Innate Immune Events of Lyme Arthritis

Several innate immune cells are responsible for both the inflammatory response observed in early-stage disease and the initial containment of *B. burgdorferi*. Due to the microbe's ability to bind to various connective tissues (11, 16), an inflammatory response is often observed in sites such as the skin, ligaments, tendons, and synovial tissues. Large numbers of macrophages and dendritic cells, but not neutrophils, are present in the primary EM rash, which is the location of initial infection in the skin (17). Interaction of macrophages with *B. burgdorferi* has been shown to cause the production of several inflammatory molecules, including interleukin-1-beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and nitric oxide (18). Macrophages are likely activated via Toll-like receptor 2, which binds to the lipoproteins found on the surface of *B. burgdorferi* (19). In addition, macrophages and dendritic cells are significant activators of the adaptive immune response to *B. burgdorferi*.

In addition, neutrophils play a major role in the inflammatory response to *B. burgdorferi*. Activation of these cells by *B. burgdorferi* also causes the production of IL-1β and TNF-α (3, 20). Importantly, these cells are found in large numbers in the synovial tissues of infected patients (4) and are known producers of the inflammatory cytokine interleukin-17 (IL-17) (21). Moreover, natural killer (NK) cells, through the production of interferon-gamma (IFN-γ), have been shown to contribute to pathology in both C3H and C57BL/6 mice (22, 23), the most

commonly used animal models for Lyme arthritis. However, NK cells may not be completely required for disease to develop, since their removal in infected mice did not reduce the severity of arthritis (22, 23).

These innate inflammatory mediators play a significant role in the initial response to the spirochete and the early events of Lyme arthritis. It is imperative to identify novel therapies to minimize inflammation at this early stage without disrupting the ability of the immune response to contain the infection.

5. T Cell Involvement in Lyme Arthritis

While innate immunity is involved in early pathology, the later-stage events observed in humans, typically months to years after infection, involve events of adaptive immunity. Synovial lesions in patients with Lyme arthritis contain T helper cells with B cells intermixed (24). Due to their predominant presence in synovial lesions, T cells have long been investigated as a contributor to Lyme arthritis. Traditionally, later-stage Lyme arthritis has been considered to be caused by the inflammatory effects of type 1 T helper (Th1) cells and their signature cytokine, IFN-γ (25, 26, 27). Increased numbers of Th1 cells and levels of IFN-γ have been observed in the synovial tissue, synovial fluid, and peripheral blood of humans with Lyme arthritis (28, 29, 30). Th1 cells and IFN-γ are also present in experimental mice that develop Lyme arthritis after infection with *B. burgdorferi* (25).

However, studies using mice genetically deficient in IFN-γ, its receptor, or its signaling pathway have demonstrated that the cytokine is not absolutely required for the development of Lyme arthritis (29, 30, 31). Still, T helper cells are involved in disease, since animals depleted of CD4+ T cells before infection with *B. burgdorferi* develop minimal inflammatory changes (32). Conversely, transfer of *Borrelia*-primed CD4+ T cells into recipient mice increases pathology following infection (33). Collectively, these results suggest that CD4+ T cell-associated inflammatory factors in addition to IFN-γ may contribute to the pathogenesis of Lyme arthritis.

Another subset of helper T cell, type 17 T helper (Th17) cells, are characterized by production of the inflammatory cytokine IL-17 (34) and may also contribute to the development of Lyme arthritis. Production of IL-17 is stimulated by borrelial lipoproteins (35) and induces other cells to generate a cascade of inflammatory mediators capable of damaging host tissue, including that observed in chronic arthritis. The inflammatory capacity of IL-17 is demonstrated by its stimulation of IL-6, IL-1β, TNF-α, and prostaglandins from various cell types, as well as by its role in contributing to collagenase activity and osteoclast formation (36). IL-17 is an important mediator of several arthritides, including rheumatoid arthritis (37), juvenile idiopathic arthritis (38), antigen-induced arthritis (39), and collagen-induced arthritis (CIA) (40). Th17 cells play a major role in the development of autoimmunity, in part due their production of interleukin-21 (IL-21), which may act in a positive feedback loop to maintain a Th17 response (41). Th17 cells also

play a significant role in the immune response against fungal and bacterial pathogens (42).

Research has suggested that Th17 cells play a potentially pathogenic role following infection with *B. burgdorferi*. Neutralization of IL-17 in *B. burgdorferi*-infected mice reduces paw swelling and prevents arthritis (43). In addition, simultaneous neutralization of IL-6 and transforming growth factor-β (TGF-β), the major differentiation factors for Th17 cells *in vitro* (44), protects mice from Lyme arthritis (45). Moreover, borrelial antigens induce dendritic cells to secrete interleukin-23 (IL-23) (46), the major survival factor for Th17 cells (44). Neutralization of IL-23 prevents the development of Lyme arthritis in *B. burgdorferi*-infected mice and reduces the production of IL-17 from stimulated immune cells (47). Furthermore, borrelial lipoproteins stimulate the *in vitro* production of IL-17 from T cells isolated from the synovia of humans with Lyme arthritis (48), and Th17 cells have been isolated directly from such individuals (28). However, the direct role of these cells in mediating pathology has not been determined. Significantly, it was shown that neutralization of IL-17 in a mouse model of Lyme arthritis, in addition to preventing the development of arthritis, also resulted in an increase in the number of CD4+CD25+ T cells with immunoregulatory function (49).

Current research suggests a role for not just one, but several types of T cells in Lyme arthritis. As a result, interactions between the cell types may be involved in the development of disease. For example, Th1 and Th17 cells display an

antagonistic relationship (50). Through the production of IFN-γ, Th1 cells inhibit the proliferation of Th17 cells (50). Likewise, Th17 cells inhibit Th1 cells via the production of IL-17 (50). However, despite the ability to counter-regulate each other, Th17 and Th1 cells can also display a cooperative relationship. Th17 cells can develop into a Th1-like cell (51) and have been shown to produce IFN-γ in response to an infectious stimulus (52). The relationship between these two cell types may influence the cytokine profile that leads to the development of arthritis. This altered cytokine profile occurs in CIA-resistant mice, which have a higher ratio of IL-17 to IFN-γ than CIA-susceptible mice (53).

The developmental relationships of these T cells are not straightforward. For instance, Th1 cells also influence the development of regulatory T (T_{reg}) cells. IFN-γ, produced by Th1 cells, is involved in the conversion of CD4+CD25- helper T cells into CD4+Foxp3+ T_{reg} cells via an increase in Foxp3 expression (54). Also, Th17 cells may produce interleukin-10 (IL-10), an anti-inflammatory cytokine typically produced by T_{reg} cells (52). Taken together, these findings suggest an intertwined role of multiple T cell types in the development of Lyme arthritis that may be dependent on the cytokine profile expressed following infection with *B. burgdorferi*.

6. Animal Models of Lyme Arthritis

Traditionally, Lyme arthritis has been studied using C3H mice, which are considered genetically “arthritis susceptible” (55) following needle infection with

particularly virulent strains of *B. burgdorferi* (56). In this model, the arthritis that ensues manifests days after infection and primarily involves inflammatory events of innate immunity. However, Lyme arthritis in humans develops weeks to years following infection, suggesting that adaptive immunity plays a significant role in disease. In support of this, synovial fluid of human Lyme arthritis patients contains CD4+ T cells specific for the likely arthrogenic borrelial antigen outer surface protein A (OspA) (57). This finding provides support that cells of adaptive immunity–specifically, T cells–are significant contributors to human Lyme arthritis. A murine model of Lyme arthritis–the *Borrelia*-vaccination and -infection model–incorporates the activity of primed and activated T cells in inducing disease, which is observed in humans with later-stage Lyme arthritis.

Mice are vaccinated with heat-killed *B. burgdorferi* in aluminum hydroxide in order to prime T cells. Three weeks later, they are infected with a heterologous strain of *Borrelia* that is capable of inducing activation of vaccine-primed T cells while evading the protective antibody response generated by vaccination. This model exhibits an antigen-specific reaction to viable Lyme spirochetes (58, 59) and is dependent on CD4+ T cells (33). Infection with homologous spirochetes induces arthritis if infection occurs prior to development of protective antibodies (59). However, infection with a heterologous strain allows for a degree of pathology more suitable for recognition of the effects of immune modulators such as anti-cytokine antibodies. A homologous infection strain can also cause arthritis following vaccination, as long as infection occurs after the vaccine-induced protective antibody response has declined sufficiently. However, this may take as

long as two months (60) and could vary between individual animals. In addition to reflecting events of adaptive immunity, another benefit of this model is that different combinations of Lyme spirochetes can be used for vaccination and infection. Also, this model can induce arthritis in C57BL/6 mice, which are considered “genetically resistant” to developing arthritis following infection with *B. burgdorferi*, as observed in the *Borrelia*-infection model (23).

7. Borreliacidal Antibody Response

An antibody response is required for eliminating *B. burgdorferi* and resolving the inflammatory response following infection. In particular, antibodies with the ability to specifically recognize and stimulate killing of the infecting borrelial strain–borreliacidal antibodies–are considered vital to the clearance of the spirochete (61). Early studies showed that injection of serum from *B. burgdorferi*-infected rabbits was able to protect against establishment of infection in hamsters (62). It was then shown that injection of *Borrelia*-immune serum after infection protected hamsters from developing arthritis (63). Stimulation of high levels of borreliacidal antibodies that are maintained for long periods of time are required for effective vaccines against Lyme borreliosis (64). In the mid-1990s, human trials of Lyme vaccines required patients to receive three doses of OspA (65, 66). While the vaccine was effective against infection during the observation period of the study, concerns were raised about the vaccine’s ability to prevent late-stage manifestations of disease. The reports of these human trials did not describe for how long high borreliacidal antibody titers lasted, and this uncertainty may have

contributed to the removal of the Lyme vaccine from the marketplace (64). A reliable assay to detect borreliacidal antibody activity is well established (67).

Borreliacidal antibodies against an antigen differ from total antibodies against that antigen in important ways. Padilla, *et al.* (60) showed that humans vaccinated with OspA produced borreliacidal antibodies that waned within weeks. However, the titers of total antibodies directed against OspA remained high for months (60). This difference highlights the importance of identifying factors that sustain borreliacidal antibody production, as life-long protective immunity does not develop after *B. burgdorferi* infection. It has also been shown that borreliacidal antibody titers decrease as arthritis resolves, indicating a possible relationship between arthritis-inducing inflammatory cytokines and the antibody response (61). In addition, levels of borreliacidal antibodies decrease as *B. burgdorferi* is cleared from tissues, suggesting that borreliacidal antibodies may serve as an indicator of the state of infection (68). This suggests that immune factors which increase the borreliacidal antibody response may be useful as a therapy against infection with *B. burgdorferi*.

II. Hypothesis and Specific Aims

Arthritis is a major symptom of Lyme disease, causing inflammation and, in some cases, degradation of the synovial joints. A role for IL-17 in the development of experimental Lyme arthritis has been established. IL-17 also plays a role in the pathogenesis of various arthritides by stimulating the expression of genes

involved in the production of inflammatory cytokines, specifically in synoviocytes. However, while Th17 cells have been shown to produce IL-17 in response to exposure with *B. burgdorferi in vitro*, it is currently unknown whether T cells are the primary cellular source of IL-17 that mediates inflammation. In addition, the effects of IL-17 modulation on the stimulation of humoral immunity require further examination. The overall objective of this study is to elucidate the contributions of IL-17-producing CD4+ cells in the development of *B. burgdorferi*-induced arthritis and IL-17 in the stimulation of the borreliacidal antibody response. Identifying sources of IL-17 in Lyme arthritic mice may help reveal immune mechanisms that drive the pathogenesis of disease and may lead to identification of novel therapeutic targets. Furthermore, understanding the effect of IL-17-producing cells on induction of borreliacidal antibodies is essential for the development of safe vaccines that provide protection against *B. burgdorferi* infection.

The hypothesis of this thesis is: *IL-17 produced by CD4+ cells contributes to the inflammation associated with* B. burgdorferi *infection and is associated with an increase in borreliacidal antibody production.*

The following specific aims are pursued to test this hypothesis:

1. **Establish CD4+ cells as a source of IL-17 following infection with *B. burgdorferi*.** The working hypothesis of this aim is: *CD4+ cells are a significant source of IL-17 during* B. burgdorferi*-induced inflammation.*

2. **Establish a relationship between IL-17 levels and borreliacidal antibody production.** The working hypothesis of this aim is: *Increases in IL-17 are associated with an increase in borreliacidal antibody titers following infection with* B. burgdorferi.

CHAPTER 2: MATERIALS AND METHODS

I. Mice

Male, 6-to-12 week old wild-type C3H/HeN mice were purchased from Charles River Laboratories (Wilmington, MA). Male and female, 6-to-12-week old, IL-10-deficient or wild-type C57BL/6 mice were obtained from J.-A. Lyons (University of Wisconsin-Milwaukee). All mice were housed in micro-isolator cages in a humidity- and temperature-controlled environment under a 12-hour light/dark schedule at the University of Wisconsin-Milwaukee animal facility. Food and water were provided *ad libitum*. Experimental protocols were reviewed and approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee.

II. Vaccination of Mice

Borrelia bissettii (formerly *B. burgdorferi* strain C-1-11) organisms were provided by S. M. Callister (Gundersen Lutheran Medical Center, La Crosse, WI). These organisms were heat-inactivated by incubating for 30 minutes in a 56℃ water bath. Successful inactivation was determined by observing a lack of motility under dark-field microscopy. In addition, these organisms failed to grow in Barbour-Stoenner-Kelly (BSK) medium at 34℃ for 7 d ays. The heat-inactivated organisms were then washed 4 times by centrifuging with phosphate-buffered saline (PBS; 7000*g*, 10 minutes), followed by resuspension in PBS. The heat-inactivated organisms were then combined with 1% aluminum hydroxide to create a 4 x 10^6 organisms/mL vaccine preparation. Mice were injected

subcutaneously in each inguinal region with 0.25 mL vaccine preparation while under anesthesia with isoflurane in a nose-and-mouth cup.

III. Infection of Mice

B. burgdorferi strain 297 organisms were provided by S. M. Callister (Gundersen Lutheran Medical Center, La Crosse, WI). Organisms were grown at 34℃ for 3-6 days in BSK medium. Twenty-one days after vaccination, mice were injected subcutaneously in each hind paw with 10^6 viable *B. burgdorferi* strain 297 organisms in 0.05 mL BSK medium while anesthetized with isoflurane in a nose-and-mouth cup. Control groups were created by sham-infecting vaccinated mice (injecting with BSK medium alone) or injecting vaccinated mice with heat-killed *B. burgdorferi* strain 297 organisms in BSK medium. In other studies, non-vaccinated mice were infected with *B. burgdorferi* strain 297 in a similar manner. Control groups were created by sham-infecting non-vaccinated mice (injecting with BSK medium alone).

IV. Antibody Treatment

Monoclonal antibody specific for mouse IFN-γ (clone 37895) was purchased from R & D Systems (Minneapolis, MN). The antibodies were resuspended in PBS to achieve a concentration of 50 µg/mL. One hour after infection, mice were injected with 2.5 µg (0.05 mL) anti-IFN-γ antibody subcutaneously in each hind paw while anesthetized with isoflurane in a nose-and-mouth cup. Mice were then injected

daily for 7 days. Control mice were injected similarly with either an isotype control antibody in PBS or PBS alone.

V. Assessment of Inflammation

Hind paw swelling was assessed by measuring the width and thickness of the tibiortarsal joints using a digital caliper (Marathon). These measurements were averaged to provide a measure for each paw. Hind paw swelling was measured immediately prior to infection with *B. burgdorferi* (or injection with BSK medium) and then every other day thereafter. Measurements were obtained prior to injection of antibodies, isotype control antibodies, or PBS.

VI. Cell Culture

On days 8 or 10 after infection (the days of peak hind paw swelling), inguinal and popliteal lymph nodes and/or spleens were harvested from *B. bissettii*-vaccinated mice injected with live *B*. *burgdorferi* 297, heat-killed *B. burgdorferi* 297, or BSK medium. Single-cell suspensions were generated by passing cells through a nylon mesh screen into cold Dulbecco's Modified Eagle's Medium or Roswell Park Memorial Institute 1640 medium. 1×10^6 cells were incubated at 37℃ in 5% CO_2 with or without 1×10^5 viable *B. burgdorferi* strain 297 organisms and with or without 5 µg (5µL) anti-mouse CD4 antibodies (clone GK1.5; eBioscience, San Diego, CA). Supernatants were collected after 6 and 24 hours of incubation. Controls consisted of cells incubated without antibodies or organisms.

VII. ELISA

Blood was collected by intracardiac puncture on days 8, 10, or 12 after infection. Levels of serum IL-17 and IFN-γ were determined using IL-17A and IFN-γ ELISA Ready-Set-Go! Kits (eBioscience, San Diego, CA) according to the manufacturer's directions. Cytokine levels were also assessed from cell culture supernatants. The plates were read at 450nm and values were expressed as optical densities. Standard curves were created and used to calculate pg cytokine/mL for each sample. Cytokine levels not detectable by ELISA were given a value of 0.00 pg/mL for statistical purposes.

VIII. Borreliacidal Antibody Assay

Sera or cell culture supernatants were used to determine titers of borreliacidal antibodies. Samples of serum or supernatant were pooled within a group. Each pooled sample was diluted 1:20 in PBS, passed through a 0.2 µm filter, and then further diluted serially, 1:40 to 1:10,240, with PBS. The diluted specimens were heated at 56℃ for 30 minutes to inactivate complement and then cooled to 35℃. The complement-inactivated specimens were incubated with 10^4 viable *B. burgdorferi* 297 organisms and 20 µL of sterile guinea pig complement (Sigma-Aldrich; St. Louis, MO) in PBS for 24 hours. The presence of viable microbes was then observed in 20 fields using dark-field microscopy. The titer of borreliacidal antibody was considered the reciprocal of the final dilution in which motile *B. burgdorferi* was observed. Controls contained *B. burgdorferi* 297 and complement (without sample), *B. burgdorferi* 297 and PBS (without complement

or sample), and *B. burgdorferi* 297 and PBS added to a sample (without complement). A four-fold increase in borreliacidal antibody titer was considered significant.

IX. Statistics

The results of some studies were expressed as mean ± standard error of the mean (SEM) and analyzed using a two-tailed Student's *t*-Test to determine the level of significance. Other data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post-hoc test. All *P*-values were calculated with the alpha level set at 0.05 prior to initiation of experiments.

CHAPTER 3: RESULTS

I. Specific Aim 1: Establish CD4+ cells as a source of IL-17 following infection with *B. burgdorferi*.

1. Effects of anti-IFN-γ antibody administration on hind paw swelling

This study was performed to assess the requirement of IFN-γ for inflammation following *B. burgdorferi* infection. Non-vaccinated, *B. burgdorferi*-infected C3H mice were injected with anti-IFN-γ or isotype-control antibodies, and the swelling of the hind paws was measured throughout infection. Control mice were injected with BSK medium, followed by injections with PBS. The *B. burgdorferi*-infected mice administered isotype-control antibodies developed greater swelling of the hind paws than non-infected control mice. Swelling was observed on day 2 after infection, peaked at day 9, and gradually subsided (Figure 1). This swelling remained greater than that observed in control mice until day 17 after infection. In addition, *B. burgdorferi*-infected mice injected with anti-IFN-γ antibodies exhibited a pattern of hind paw swelling similar to that of infected mice injected with isotype control antibodies. An additional group of *B. burgdorferi*-infected mice injected daily with PBS alone developed swelling not significantly different than that of infected mice injected with isotype control antibodies (data not shown).

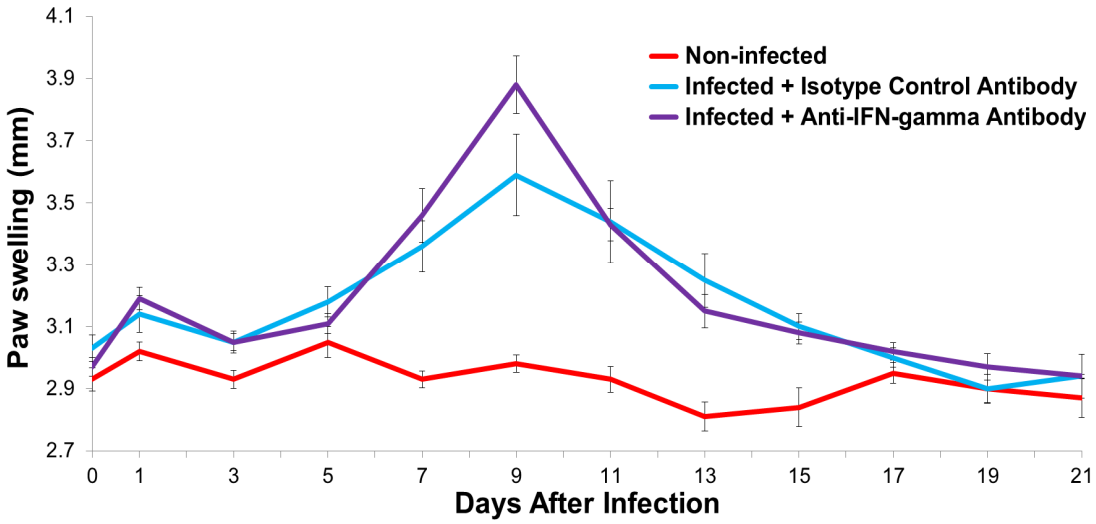

Figure 1. Administration of anti-IFN-γ antibody does not affect paw swelling in *B. burgdorferi*-infected C3H mice. Mice were infected with *B. burgdorferi* 297 and treated with anti-IFN-γ antibody (n=10) or an isotype control antibody (n=10) for 7 days. Non-infected control mice (n=10) were treated with PBS. Error bars indicate the SEM.

2. Serum levels of IL-17 following treatment with anti-IFN-γ antibodies

Th1 and Th17 cells are known to act antagonistically through IFN-γ and IL-17, respectively. To determine the effect of anti-IFN-γ antibody treatment on IL-17 production, sera were collected from *B. burgdorferi* 297-infected C3H mice treated with anti-IFN-γ or isotype control antibodies on days 7, 12, 21, and 47 after infection. The amounts of serum IL-17 in these mice were then assessed by ELISA. The levels of serum IL-17 in *B. burgdorferi*-infected mice injected with isotype control antibodies increased gradually from day 7 (15.5 pg/mL) to day 12 (28.1 pg/mL) to day 21 (47.5 pg/mL). By contrast, the levels of serum IL-17 in infected mice treated with anti-IFN-γ antibodies increased rapidly from day 7 (19.9 pg/mL) to day 12 (44.9 pg/mL), and then decreased by day 21 (35.6 pg/mL). Infected, anti-IFN-γ-antibody-treated mice had a significantly greater

amount of serum IL-17 than infected, non-treated mice at day 12 after infection (P=0.0006; Figure 2). The amount of serum IL-17 in non-infected controls gradually decreased following injections of BSK and PBS, and no significant amounts of serum IL-17 were detected in any group at day 47 after infection (data not shown).

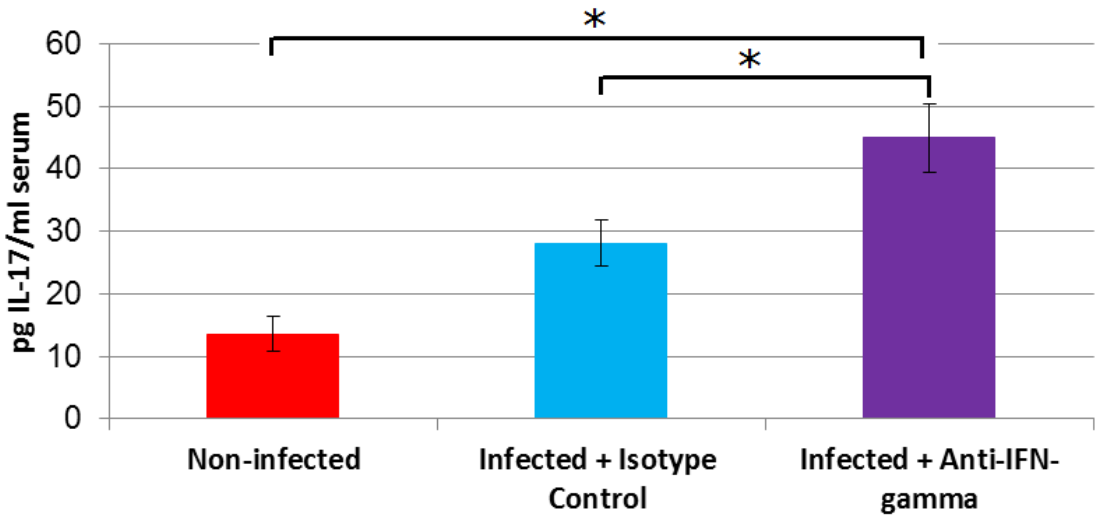

Figure 2. Concentrations of IL-17 in serum of non-infected C3H mice (n=5) and C3H mice infected with *B. burgdorferi* 297, with (n=5) or without (n=5) administration of anti-IFN-γ antibodies, at day 12 after infection. * denotes a significant difference (P<0.05). Error bars indicate the SEM.

3. Role of sustained infection on production of IL-17

The *Borrelia*-vaccination and -infection model of Lyme arthritis was used to investigate whether live *B. burgdorferi* infection, as opposed to a non-viable infection, is required for an IL-17 response associated with increased hind-paw swelling. C3H mice were vaccinated with *B. bissettii* and later injected with viable *B. burgdorferi* 297 organisms, heat-killed *B. burgdorferi* 297 organisms, or BSK medium. The swelling of their hind paws was measured. Vaccinated mice infected with live *B. burgdorferi* developed significantly greater paw swelling than

mice injected with dead *B. burgdorferi* or medium consistently by day 8 after infection (p=0.003; Figure 3). All mice were sacrificed at day 10 after these injections, near the presumed peak swelling time after infection.

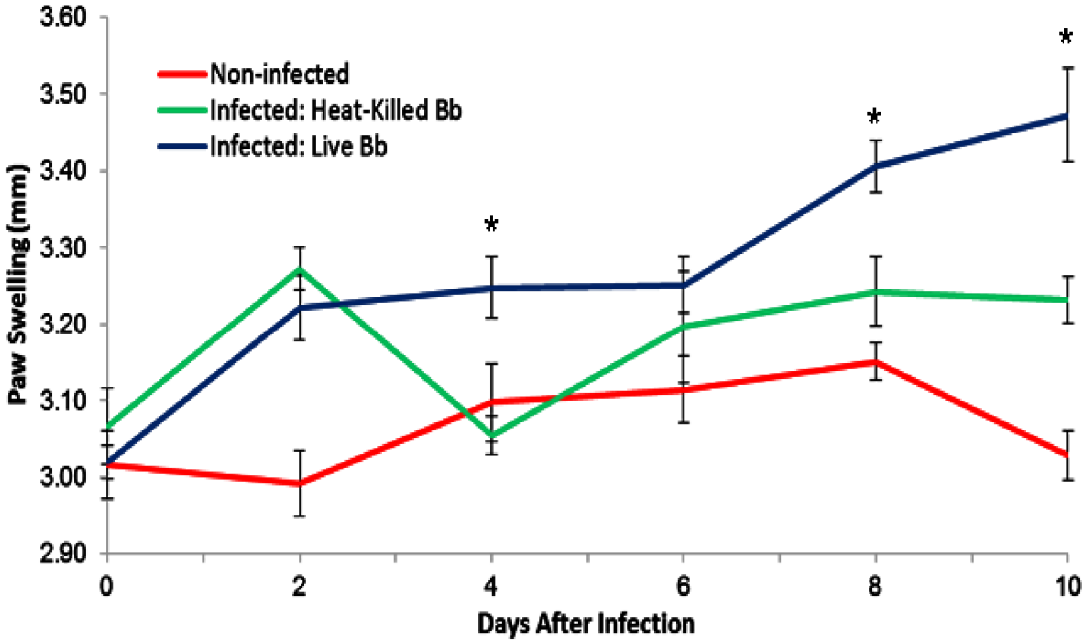

Figure 3. Hind paw swelling of *Borrelia*-vaccinated C3H mice injected with live (n=16) or heat-killed (n=12) *B. burgdorferi* 297 (Bb). A control group consisted of vaccinated mice that were not infected (n=8). * denotes a significant (P<0.05) difference in paw swelling. Error bars indicate SEM.

From these mice, cell culture supernatants of unseparated inguinal lymph nodes cells cultured with live *B. burgdorferi* 297 organisms were measured for IL-17 production. Upon *in vitro* stimulation with viable *B. burgdorferi* 297, cells harvested from *Borrelia*-vaccinated- and -infected mice produced less, but not statistically different, levels of IL-17 than stimulated cells obtained from *Borrelia*-vaccinated mice injected with non-viable spirochetes or BSK medium (Figure 4).

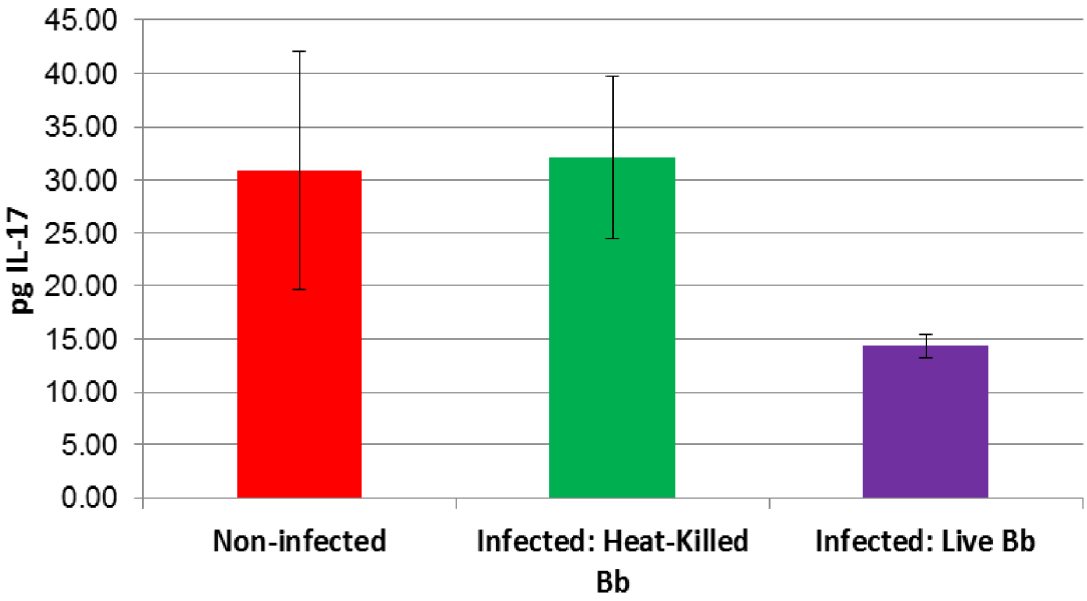

Figure 4. Concentrations of IL-17 from culture supernatants of lymph node cells from *Borrelia*-vaccinated C3H mice injected with live (n=3) or heat-killed (n=4) *B. burgdorferi* 297 (Bb) or BSK medium (n=2). Cells were obtained at day 10 after infection and restimulated with *B. burgdorferi* 297. Error bars indicate SEM.

4. Assessment of IL-17 production in different models of Lyme arthritis

Both the *Borrelia*-infection and *Borrelia*-vaccination and -infection models of Lyme arthritis were used to determine the relative abundance of IL-17 produced by spleen cells after infection with *B. burgdorferi*. To characterize these differences, the amounts of IL-17 produced by cells from wild-type C57BL/6 mice obtained at the peak of swelling in both models were determined. Spleen cells were collected at day 8 after infection and then incubated with *B. burgdorferi* 297 for 6 or 24 hours.

Cells obtained from naïve, wild-type mice produced non-detectable levels of IL-17 when cultured with *B. burgdorferi* 297 for 6 hours (Figure 5). Production of IL-17 by these cells increased after 24 hours, but the increase did not reach

statistical significance (P=0.08). Similarly, cells from *B. burgdorferi*-infected mice that were restimulated with the spirochete for 6 hours produced low levels of IL-17. No difference in IL-17 production was detected after 24 hours (Figure 5). When comparing IL-17 production between cells from naïve and infected mice after *in vitro* stimulation, there were no significant differences observed at either time point.

Cells obtained from *Borrelia*-vaccinated, wild-type mice, whether or not the mice were infected, produced no detectable IL-17 when cultured with *B. burgdorferi* 297 for 6 hours (Figure 5). However, upon *in vitro* stimulation with viable *B. burgdorferi* 297, cells from vaccinated, non-infected mice produced significantly more IL-17 after 24 hours (P=0.008; Figure 5). The amount of IL-17 produced from cells of naïve mice, non-vaccinated but infected mice, and vaccinated-and-infected mice after 24-hour restimulation was significantly lower than the amount of IL-17 produced from cells of vaccinated, non-infected mice (P=0.008).

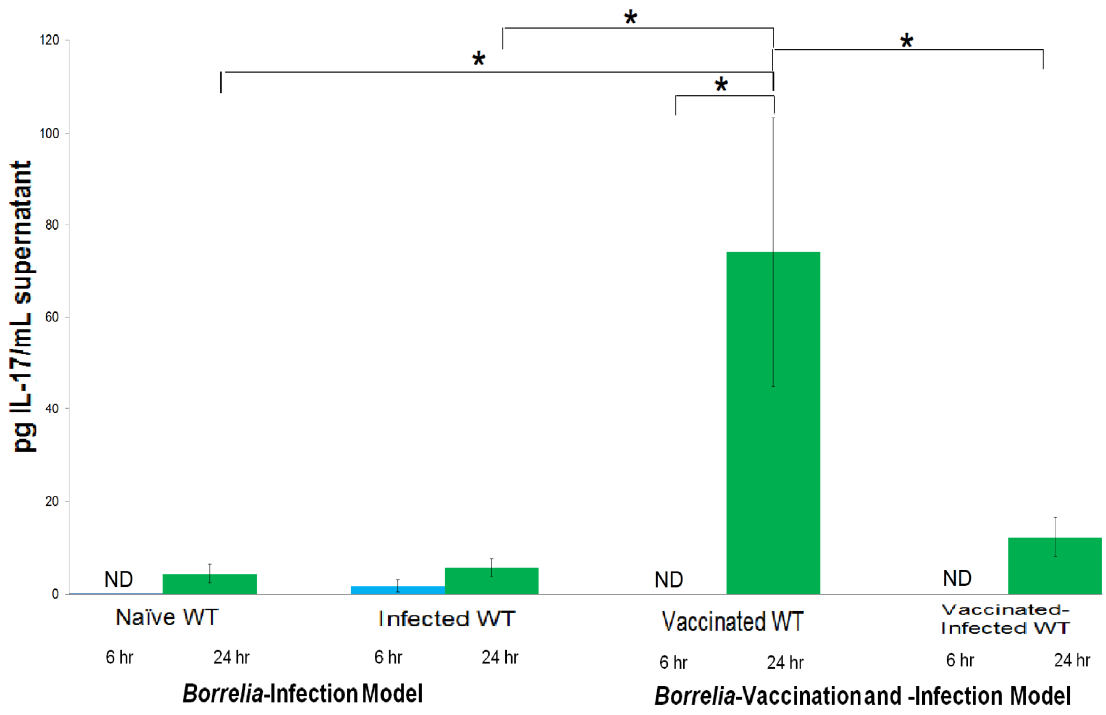

Figure 5. Concentrations of IL-17 from culture supernatants of spleen cells obtained from naïve (n=7), *Borrelia*-infected (n=8), *Borrelia*-vaccinated (n=6), and *Borrelia*-vaccinated and –infected (n=6) wild-type (WT) C57BL/6 mice. Cells were obtained at peak swelling (8 days after the time of infection) and then stimulated with *B. burgdorferi in vitro* for 6 or 24 hours. * denotes a significant difference (P<0.05). ND, not detected. Error bars indicate SEM.

5. Assessment of Borrelia*-induced IL-17 production in models of Lyme arthritis in IL-10-deficient mice*

The role of the anti-inflammatory cytokine IL-10 on the production of IL-17 in the *Borrelia*-infection and *Borrelia*-vaccination and -infection models of Lyme arthritis was characterized. Spleen cells were collected from IL-10-deficient mice of both models at day 8 after the day of infection and then incubated with *B. burgdorferi* 297 for 6 or 24 hours.

Spleen cells from naïve, IL-10 knock-out (KO) mice produced low levels of IL-17 following incubation with *B. burgdorferi* 297 for 6 hours (Figure 6). No significant

increase was observed after 24 hours of incubation. By contrast, IL-17 production by spleen cells of *Borrelia*-infected IL-10 KO mice increased significantly, by 1300% (P=0.005), between 6 and 24 hours of restimulation with *B. burgdorferi* 297 (Figure 6). In addition, stimulated spleen cells from infected mice produced greater amounts of IL-17 than stimulated cells from naïve mice at each time point. The increases at both time points, however, were not significant.

Cells obtained from *Borrelia*-vaccinated, non-infected IL-10 KO mice produced low amounts of IL-17 after 6 hours of stimulation with *B. burgdorferi* 297 (Figure 6). A highly significant increase of 3600% (P=0.002) in IL-17 production was observed from these cells after 24 hours of incubation. .However, no differences in IL-17 production were observed between these groups after 24 hours of incubation with the spirochete.

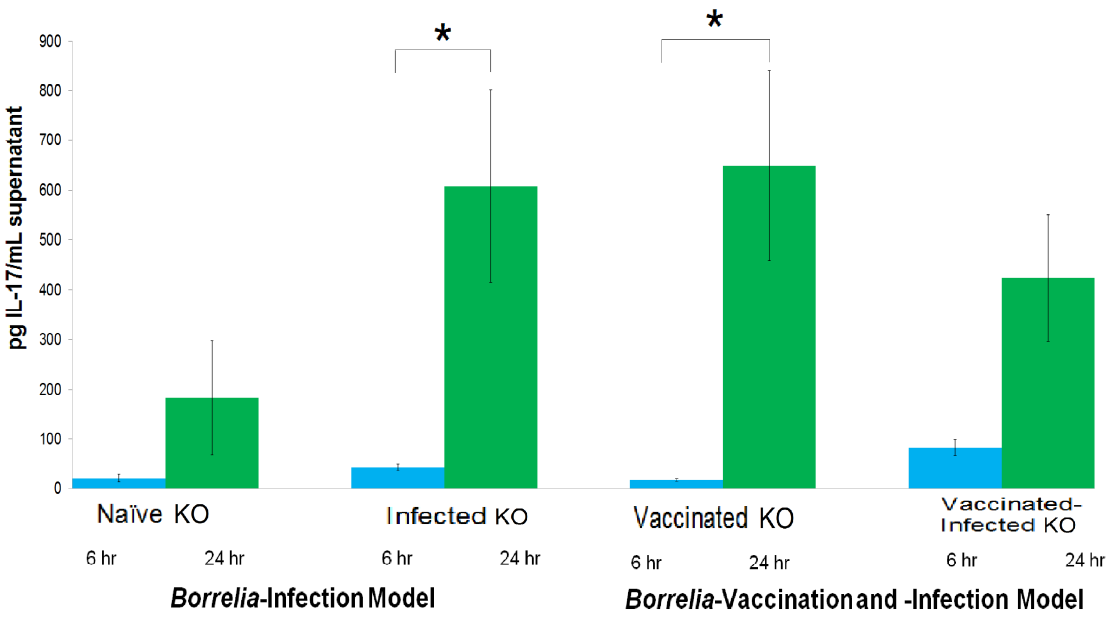

Figure 6. Concentrations of IL-17 from culture supernatants of spleen cells obtained from naïve (n=6), *Borrelia*-infected (n=8), *Borrelia*-vaccinated (n=10), and *Borrelia*-vaccinated and –infected (n=10) IL-10 knock-out (KO) C57BL/6 mice. Cells were obtained at peak swelling (8 days after the time of infection) and then stimulated with *B. burgdorferi in vitro* for 6 or 24 hours. * denotes a significant difference (P<0.05). Error bars indicate SEM.

6. Effect of IL-10 deficiency on IL-17 production in models of Lyme arthritis

Using the data described above, the effect of IL-10 deficiency on IL-17 production during the peak phase of swelling following *B. burgdorferi* infection was assessed. In both models of infection (non-vaccinated but *Borrelia*-infected; *Borrelia*-vaccinated and -infected) and both control groups (non-vaccinated and non-infected [naïve]; *Borrelia*-vaccinated but non-infected), spleen cells of IL-10 KO mice produced more IL-17 when exposed to *B. burgdorferi* 297 *in vitro* than cells of wild-type mice. Naïve IL-10 KO cells displayed higher levels of IL-17 after 6 and 24 hours of incubation (Figure 7), with significantly higher levels detected after the 6-hour incubation (P=0.05). Similar results were observed from the cells of *Borrelia*-infected IL-10 KO mice, with significant increases of IL-17 production

at 6 hours (2400%, P=0.0003) and at 24 hours (10,700%, P=0.02) compared to cells of infected wild-type mice (Figure 7).

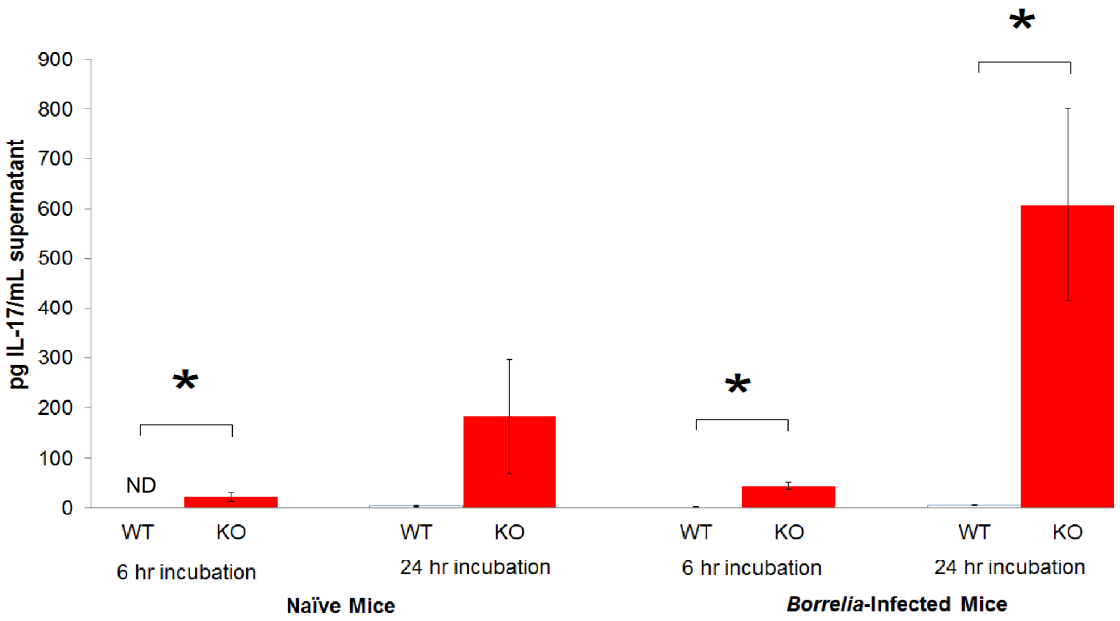

Figure 7. Concentrations of IL-17 from culture supernatants of spleen cells obtained from wild-type (WT) and IL-10-deficient (KO), naïve (n=7) and *Borrelia*-infected (n=8) C57BL/6 mice. Cells were obtained at peak swelling (day 8 after the time of infection) and were stimulated with *B. burgdorferi in vitro* for 6 or 24 hours. * denotes a significant difference (P<0.05). ND, not detected. Error bars indicate SEM.

Similarly, significantly greater levels of IL-17 were produced by stimulated spleen cells of *Borrelia*-vaccinated, non-infected IL-10 KO mice after 6 hours (P=0.001) and 24 hours (770%, P=0.02) of incubation compared to stimulated cells of wild-type mice (Figure 8). Also, stimulated spleen cells from *Borrelia*-vaccinated and -infected IL-10 KO mice produced significantly more IL-17 after 6 hours (P=0.0005) and 24 hours (3300%, P=0.01) of incubation compared to cells from wild-type mice.

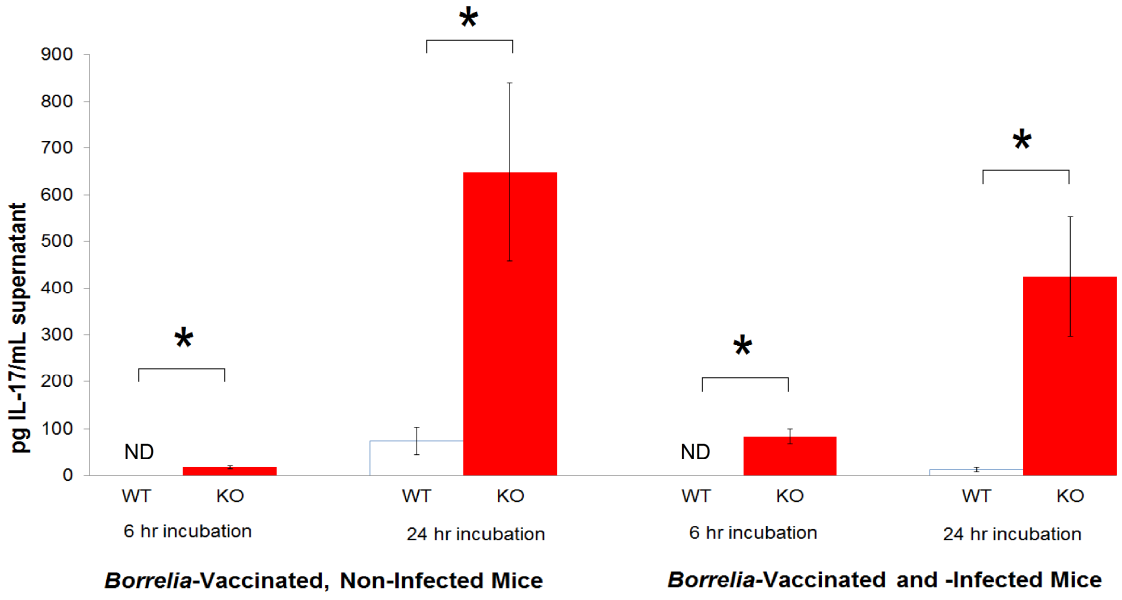

Figure 8. Concentrations of IL-17 from culture supernatants of spleen cells obtained from wild-type (WT) and IL-10-deficient (KO), *Borrelia*-vaccinated (n=8) and *Borrelia*-vaccinated and –infected (n=8) C57BL/6 mice. Cells were obtained at peak swelling (day 8 after the time of infection) and were stimulated with *B. burgdorferi in vitro* for 6 or 24 hours. * denotes a significant difference (P<0.05). ND, not detected. Error bars indicate SEM.

7. Anti-CD4 antibody modulation of IL-17 in models of Lyme arthritis

Here, the contribution of CD4-expressing cells to the production of IL-17 in the *Borrelia*-infection and *Borrelia*-vaccination and -infection models of Lyme arthritis was assessed. Spleen cells were harvested at the peak of swelling from wild-type and IL-10-deficient mice and incubated for 6 and 24 hours with *B. burgdorferi*, with or without anti-CD4 antibodies. Data from cells incubated without anti-CD4 antibody have been shown previously (Figures 5-8).

No differences in IL-17 production due to anti-CD4 antibodies were observed among stimulated cells from naïve, wild-type mice at either 6 or 24 hours of incubation (Figure 9). By contrast, after 24 hours of incubation with anti-CD4

antibodies, stimulated cells from non-vaccinated, *B. burgdorferi*-infected wild-type mice produced 86% less IL-17 than stimulated cells not exposed to antibodies (Figure 9); this difference was significant (P=0.03). These results were not observed after 6 hours of incubation. Also, after 24 hours of incubation with anti-CD4 antibodies, stimulated spleen cells from *Borrelia*-vaccinated, non-infected wild type mice produced 44% less IL-17 than cells not incubated with antibodies. However, this decrease was not significant (P=0.10; Figure 9). No changes in IL-17 production were detected following anti-CD4 antibody treatment of stimulated cells obtained from *Borrelia*-vaccinated and -infected wild-type mice at either time point.

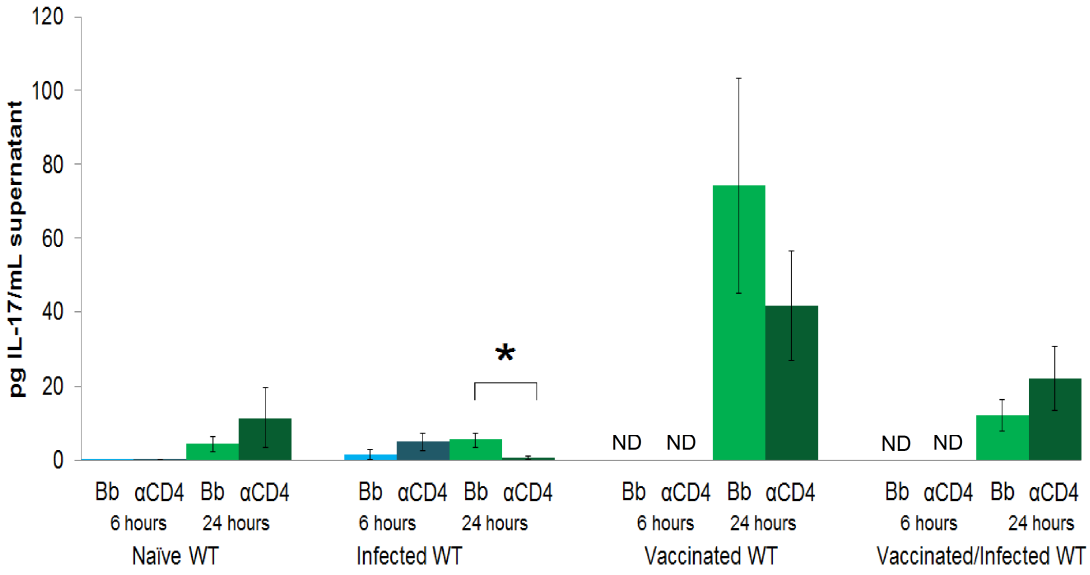

Figure 9. Concentrations of IL-17 from culture supernatants of spleen cells from naïve (n=6), *Borrelia*-infected (n=8), *Borrelia*-vaccinated (n=6), and *Borrelia*-vaccinated and –infected (n=6) wild-type (WT) C57BL/6 mice. Cells were obtained at peak swelling (day 8 after the time of infection) and then stimulated with *B. burgdorferi* 297 (Bb), with or without anti-CD4 antibody (αCD4). * denotes a significant difference (P<0.05). ND, not detected. Error bars indicate SEM.

The effects of anti-CD4 antibodies on IL-17 production were also assessed among stimulated spleen cells obtained from IL-10 KO mice. No differences in IL-17 production due to anti-CD4 antibodies were observed among stimulated cells from naïve IL-10 KO mice, *Borrelia*-infected IL-10 KO mice, or *Borrelia*-vaccinated, non-infected IL-10 KO mice after either 6 or 24 hours of incubation (Figure 10). By contrast, a statistically significant decrease (56%, P=0.006) in IL-17 production was observed among stimulated, anti-CD4 antibody-treated cells from *Borrelia*-vaccinated and -infected IL-10 KO mice at 6 hours of incubation. However, no differences in IL-17 production were exhibited at 24 hours of incubation.

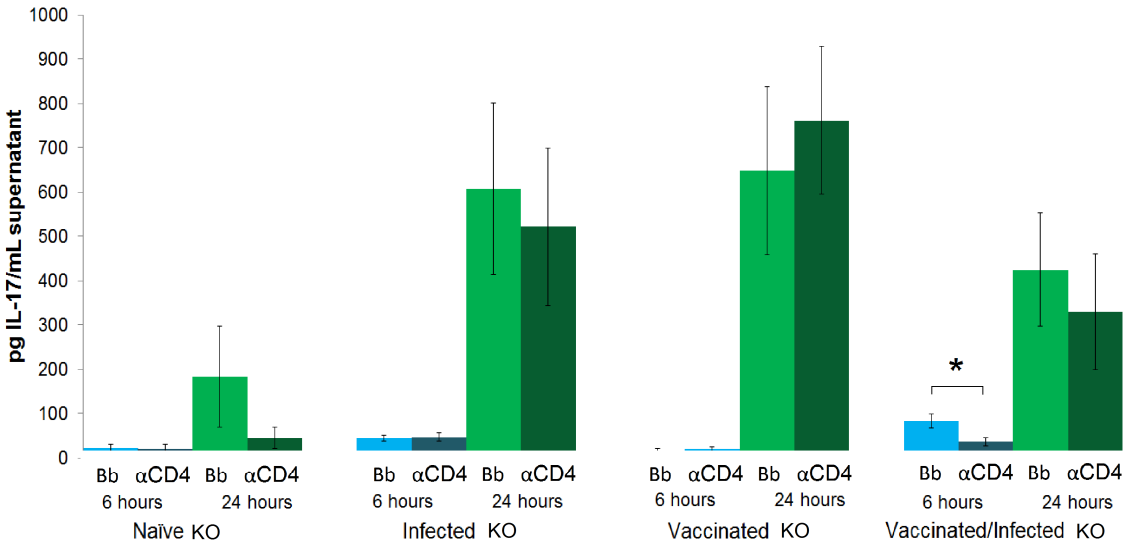

Figure 10. Concentrations of IL-17 from culture supernatants of spleen cells from naïve (n=6), *Borrelia*-infected (n=8), *Borrelia*-vaccinated (n=10), and *Borrelia*-vaccinated and –infected (n=10) IL-10 deficient (KO) C57BL/6 mice. Cells were obtained at peak swelling (day 8 after the time of infection) and then stimulated with *B. burgdorferi* 297 (Bb), with or without anti-CD4 antibody (αCD4). * denotes a significant difference (P<0.05). Error bars indicate SEM.

II. Specific Aim 2: Establish a relationship between IL-17 levels and borreliacidal antibody production.

1. Borreliacidal antibody titers following sustained infection with B. burgdorferi

The data presented previously indicate that stimulation of lymph node cells from *Borrelia*-vaccinated C3H mice infected with live *B. burgdorferi* 297 resulted in less IL-17 production than stimulation of cells from *Borrelia*-vaccinated mice injected with non-viable spirochetes. Therefore, borreliacidal antibody titers were assessed from cell culture supernatants in order to examine increased microbe killing *in vitro* as a possible reason for these unexpected results. Lymph node cells from *Borrelia*-vaccinated, non-infected mice, incubated with *B. burgdorferi* 297 *in vitro*, produced a borreliacidal antibody titer of 640 against *B. burgdorferi* 297 (Figure 11). By contrast, cells from *Borrelia*-vaccinated mice injected with viable or heat-killed *B. burgdorferi* 297 both produced significantly higher titers of borreliacidal antibody, which exceeded the detection ability of the assay (≥ 10,240).

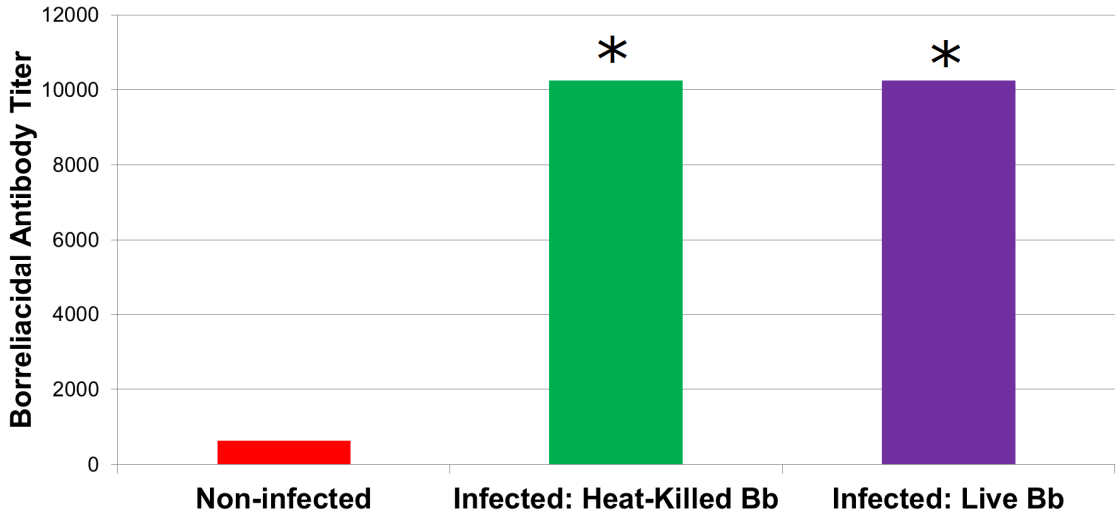

Figure 11. Borreliacidal antibody titers from culture supernatants of lymph node cells from *Borrelia*-vaccinated C3H mice injected with live (n=8) or heat-killed (n=6) *B. burgdorferi* 297 or BSK medium (n=4). Cells were obtained at the peak of swelling (day 10 after the time of injections) and stimulated with *B. burgdorferi* 297 *in vitro* for 24 hours. * denotes a significantly greater (4-fold) titer than that of cells from non-infected mice.

2. Effect of IL-10 on borreliacidal antibody titers in models of Lyme arthritis

Hind paws of IL-10 KO, C57BL/6 mice developed greater swelling after infection with *B. burgdorferi* than their wild-type counterparts (data not shown). The cells of these mice also had a greater IL-17-producing capacity following *in vitro* exposure to the spirochete than wild-type cells (Figures 5-8). Borreliacidal antibody titers were measured from the sera of these mice at the peak of paw swelling to examine the effect of IL-10 on a possible mechanism responsible for clearing infection. As expected, naïve, wild-type mice possessed a low level of borreliacidal antibody against *B. burgdorferi* 297 (titer, 10; Figure 12). Absence of IL-10 caused a significant (8-fold) increase in titers. However, these titers were still very low. Absence of IL-10 slightly, but insignificantly, increased the borreliacidal antibody titers in non-vaccinated, *B. burgdorferi* 297-infected mice (titer, 40) compared to wild-type mice (titer, 20). Lack of IL-10 in *Borrelia*-

vaccinated, non-infected mice significantly increased the borreliacidal antibody titers (titer, ≥10,240) against *B. burgdorferi* 297 compared to wild-type mice (titer, 640). The titers of borreliacidal antibody in *Borrelia*-vaccinated and -infected mice, regardless of strain, exceeded the levels of detection in the assay (≥10,240).

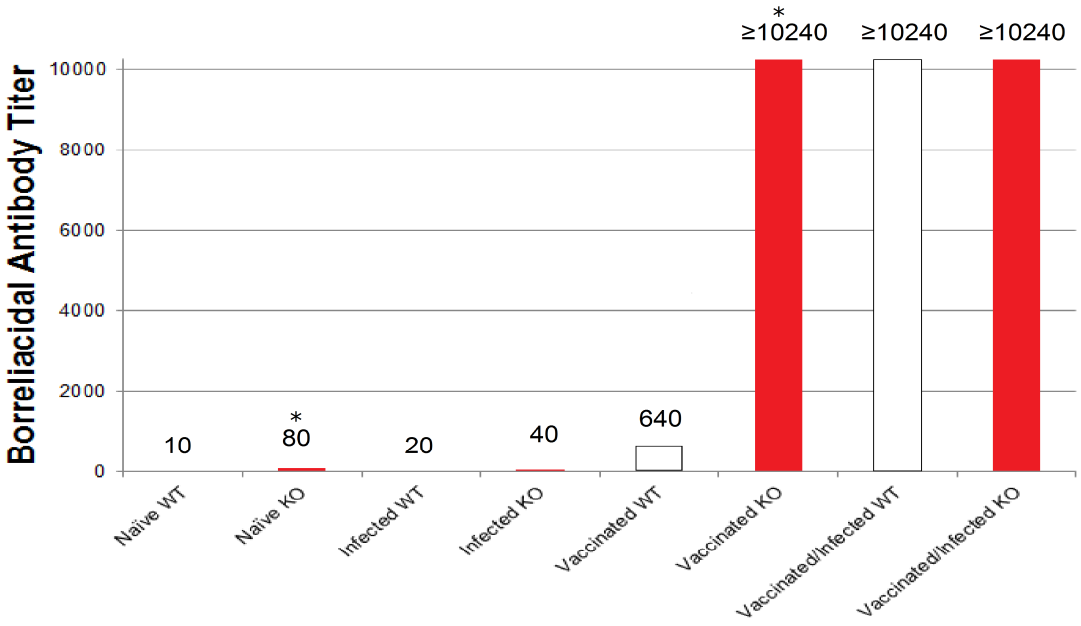

Figure 12. Borreliacidal antibody titers obtained from sera of naïve (n=4), *Borrelia*-infected (n=4), *Borrelia*-vaccinated (n=4), and *Borrelia*-vaccinated and -infected (n=4) wild-type (WT) and IL-10-deficient (KO) C57BL/6 mice at the peak of hind paw swelling (day 8 after the time of infection). * denotes a significantly greater (4-fold) titer than wild-type counterparts.

CHAPTER 4: DISCUSSION

This thesis contributes to knowledge of how IL-17 is involved in the inflammatory response to *B. burgdorferi*. IL-17 is a pro-inflammatory cytokine that is produced in response to *B. burgdorferi* infection (35) and is associated with various forms of arthritis (37-40). Recent studies have also characterized the role of IL-17 and related cytokines in the development of Lyme arthritis, leading to the hypothesis that Th17 cells are a potential contributor to disease (45). In addition, since IL-17 is produced by other cell types in addition to Th17 cells (21, 69), the contribution of potential Th17 cells to the production of IL-17 in response to *B. burgdorferi* is unknown. Also, whether IL-17 is associated with the protective borreliacidal antibody response has not been fully examined. The objective of this thesis was to elucidate the contributions of IL-17-producing CD4+ cells in the development of *B. burgdorferi*-induced arthritis and IL-17 in the stimulation of the borreliacidal antibody response. The following specific aims were pursued to test this hypothesis: (1) Establish CD4+ cells as a source of IL-17 following infection with *B. burgdorferi*; (2) Establish a relationship between IL-17 levels and borreliacidal antibody production.

I. Specific Aim 1: Establish CD4+ cells as a source of IL-17 following infection with *B. burgdorferi.*

Th17 and Th1 cells have been shown to have an antagonistic relationship though the effects of IL-17 and IFN-γ, respectively (50). In order to explore the role of IL-17 in a reduced Th1 environment during Lyme arthritis, mice were infected with

B. burgdorferi 297 and then injected with anti-IFN-γ antibody. These mice developed similar degrees of paw swelling than *B. burgdorferi*-infected, isotype antibody-treated controls (Figure 1). However, a greater degree of peak paw swelling was observed, although this difference was not statistically significant (Figure 1). Additionally, the serum of *Borrelia*-infected mice treated with anti-IFN-γ antibody contained greater levels of IL-17 than non-treated, infected controls (Figure 2). These results are consistent with previous findings in which mice genetically deficient in IFN-γ, its receptor, or its signaling pathway can develop Lyme arthritis (29, 30, 31), which demonstrates that IFN-γ is not the sole cytokine responsible for the development of pathology. Furthermore, these findings provide additional support for the antagonistic relationship between Th1 and Th17 cells (50). They also support findings in *Borrelia*-infected mice that demonstrated an increase in IFN-γ following treatment with anti-IL-17 antibodies (70). The results presented here are consistent with these data by displaying this inverse relationship, since anti-IFN-γ antibody treatment led to an increase in IL-17 production (Figure 2). Non-infected control mice also produced low levels of IL-17 (Figure 2). This is most likely due to the daily injections of PBS that these mice received. The repeated trauma would likely produce a mild inflammatory response, resulting in low levels of IL-17.

These data also fit with research establishing IL-17 as a contributor to Lyme arthritis using the *Borrelia*-vaccination and -infection model of disease, which reflects later-stage arthritis in humans through its significant involvement of CD4+

T cells (32, 33). Christopherson, *et al.* showed that *Borrelia*-vaccinated and -infected C57BL/6 mice lacking IFN-γ (i.e., the Th1 response) developed severe, destructive arthritis (30), pointing to the involvement of other inflammatory mechanisms. IL-17, which recently had been shown to be produced by helper T cells in response to *B. burgdorferi* (35), was investigated as a possible cause of Lyme arthritis. Treatment of *Borrelia*-vaccinated and -infected IFN-γ KO mice with anti-IL-17 antibodies at the time of infection for 11 days (43) or 7 days (49) prevented the development of arthritis. In addition, treatment of wild-type C57BL/6, *Borrelia*-vaccinated and -infected mice with anti-IL-17 antibodies reduced the severity of pathology (45), although not to the level observed in treated IFN-γ KO mice (43, 49). This shows that IFN-γ may be an important regulator of IL-17, possibly from T cells, in Lyme arthritis.

Following these earlier findings in the context of *B. burgdorferi* infection, Park, *et al.* (34) described the existence of a unique subpopulation of IL-17-producing CD4+ T cells (Th17 cells). Bettelli*, et al.* (44) then showed that the development of these cells from naïve T cells *in vitro* required a combination of IL-6 and TGF-β. Veldhoen, *et al.* extended this observation to show that TGF-β, in the presence of inflammatory mediators (IL-1β, IL-6, TNF-α), leads to Th17 differentiation (71). Also, survival of Th17 cells was shown to require stimulation with IL-23 (44). IL-23 is also required for full differentiation of Th17 cells (72) and is also induced by *B. burgdorferi* stimulation (46). Using the *Borrelia*-vaccination and -infection model in wild-type C57BL/6 mice, it was shown that neutralization

of IL-23 prevented arthritis and was required for IL-17 production *in vitro* (47). In addition, injection of infected mice with a combination of anti-IL-6 and anti-TFG-β antibodies reduced arthritic pathology (45). Codolo, *et al.* (48) provided direct evidence that Th17 cells are found in the joints of human Lyme arthritis patients. Specifically, neutrophil activating protein A of *B. burgdorferi* causes the release of IL-6, IL-1β, TGF-β, and IL-23 from various innate cells, which work together to stimulate synovial T cells to produce IL-17 (48). The findings presented here provide additional support that T cells are a possible source of IL-17 during the peak arthritic stage after infection with *B. burgdorferi*. However, direct evidence of a role for Th17 has not yet been shown.

Conflicting evidence exists for a role for Th17 cells in Lyme arthritis. Th17 cells were also found in the synovial fluid and tissues of humans with disease by Shen, *et al.* (28). However, they found the Th17 population to only comprise approximately 10% of the CD4+ T cell population in most patients (28). They did not examine the arthritic potential of these cells, but concluded that they may not contribute to disease. Consistent with this study, Oosting, *et al*. (73) showed that some humans possess a polymorphism of the IL-23 receptor that causes defective binding of the cytokine. Humans with this polymorphism who are also infected with *B. burgdorferi* produce less IL-17 than those without the polymorphism, but the severity of their arthritis does not differ (73). However, these data were collected with relatively low sample sizes in humans (28, 73).

The natural variation in humans, coupled with these low sample sizes, prevents absolute conclusions from being made.

In order to determine whether established *Borrelia* infection was required to stimulate this inflammatory cascade, vaccinated mice were challenged with either live or heat-killed *B. burgdorferi* 297. *Borrelia*-vaccinated mice infected with live organisms developed significantly greater swelling at peak disease (Figure 3). Unexpectedly, unseparated lymph node cells from these mice, following restimulation with *B. burgdorferi* 297 (the microbe of infection), produced less IL-17 than stimulated cells obtained from vaccinated mice injected with heat-killed organisms (Figure 4). These surprising results may have occurred due to differences in the immune response to primary versus secondary exposure to live organisms. The first exposure of cells from *Borrelia*-vaccinated mice injected with heat-killed spirochetes to live *B. burgdorferi* 297 occurred in culture. By contrast, lymph node cells from *Borrelia*-vaccinated, *B. burgdorferi* 297-infected mice had already been exposed to the live bacteria and were encountering it for the second time while in culture. Since secondary, *ex vivo* exposure decreased IL-17 production, it was thought that this restimulation may have caused a robust borreliacidal antibody response from B cells present in the unseparated lymph node cultures (examined in *Specific Aim 2* below). Rapid stimulation of borreliacidal antibodies may have cause the killing of the bacteria in culture, resulting in a reduced antigenic stimulus and less IL-17 production.

By contrast, Ganapamo *et al.* (74) investigated the cytokine response to *B. burgdorferi* strain JD1 infection by simulating lymph node cells of infected mice with the same isolate *in vitro*. However, these mice were not vaccinated before infection, unlike the mice used in these studies. It was previously shown that cells from *B. bissettii*-vaccinated, non-infected mice produced cytokines associated with Th17 cells when exposed to *B. burgdorferi* 297 for the first time *in vitro* (47, 75). These studies, with the data presented here, show that a secondary exposure to a live *B. burgdorferi* strain *in vitro*, using the *Borrelia*-vaccination and -infection model, likely do not yield cytokine results that reflect primary infection. However, this secondary exposure may allow for examination of the borreliacidal antibody response, given that B cells in these unseparated cell cultures may be highly activated.

To further explore the role of adaptive immune sources of IL-17, we examined the effects of the *Borrelia*-infection and *Borrelia*-vaccination and -infection models on the production of the cytokine. Researchers typically use the *Borrelia*-infection model of Lyme arthritis, in which C3H, “arthritis-susceptible” mice, are infected with *Borreliae* (56). This model, however, favors the events of innate immunity, which may not appropriately mimic the adaptive immune response involvement seen in humans with later-stage disease (70). The *Borrelia*-vaccination and -infection model addresses this issue by priming and activating T cells, resulting in a stronger adaptive immune response (70). Spleen cells harvested from mice at peak swelling were used to evaluate IL-17 production *in vitro*. Cells from naïve

and *Borrelia*-infected wild-type mice produced low levels of IL-17 upon incubation with live *B. burgdorferi* 297, with little change occurring between the 6 and 24 hour incubations (Figure 5). These results were expected, considering that the *Borrelia*-infection model may not fully reflect the involvement of IL-17 following *B. burgdorferi* infection (70). By contrast, cells from *Borrelia*-vaccinated, non-infected mice produced significantly higher levels of IL-17 after 24 hours in culture (Figure 5). These results are consistent with the rationale behind the use of the *Borrelia*-vaccination and -infection model, which primes T cells at vaccination and involves a greater adaptive immune response, especially the adaptive IL-17 response, upon infection with live organisms (70). Cells from *Borrelia*-vaccinated and -infected mice also produced higher, but not significantly greater, levels of IL-17 at the 24 hour incubation time point (Figure 5). These levels, however, were lower than those seen in the stimulated cells from *Borrelia*-vaccinated, non-infected mice. This again points to the possible differences in the development of borreliacidal antibodies occurring in primary versus secondary exposure. Regardless, cells from *Borrelia*-vaccinated, but non-infected mice had greater levels of IL-17 than cells from naïve, non-vaccinated, but *B. burgdorferi*-infected, and *Borrelia*-vaccinated and -infected mice, supporting the hypothesis that the *Borrelia*-vaccination and -infection model of disease better reflects the involvement of IL-17 in the response to the spirochete (70).

Additionally, IL-10-deficient mice on a C57BL/6 background have been proposed as another model of Lyme arthritis (76). This is due, in part, to the involvement of

T cells that lead to a sustained IFN-γ response, which reflects the persistent arthritis that occurs in humans (76). Wild-type C57BL/6 mice are typically resistant to arthritis and, generally, produce more IL-10 in response to *Borrelia* exposure (29). Conversely, susceptibility to developing Lyme arthritis has been linked to an early production of IL-10 after infection with *B. burgdorferi*, which regulates the effects of IFN-γ (74), macrophages (77), and macrophage signaling pathways (78) in the response to infection. IL-10 also has been shown to inhibit the production of IL-17 by Th17 cells (79). However, the effect of IL-10 on IL-17 production in Lyme arthritis has not been investigated.

Here, the production of IL-17 in the different models was compared. Cells from non-vaccinated, *Borrelia*-infected IL-10 KO mice produced highly increased levels of IL-17 between 6 and 24 hours of *in vitro* stimulation (Figure 6), a change that was not seen in wild-type counterparts (Figure 5). These IL-10 KO cells produced significantly more IL-17 than wild-type cells at both time points (Figure 7). Similarly, stimulated cells from *Borrelia*-vaccinated, non-infected IL-10 KO mice produced amounts of IL-17 which were significantly greater than wild-type counterparts at both time points (Figure 8).

In general, stimulated spleen cells from all IL-10 KO groups developed greater levels of IL-17 (Figure 8) and hind paw swelling (data not shown) than their wild-type counterparts. As expected, this indicates that the presence of IL-10, an anti-inflammatory cytokine, inhibits the production of IL-17. In support of these

findings, IL-10 directly inhibits the activity of Th17 cells and T cells that produce both IL-17 and IFN-γ in a model of colitis (79). In terms of Lyme arthritis, specifically, these findings are also consistent with those of others. *B. burgdorferi*-infected IL-10 KO mice retain more spirochetes in their tissues (80) and develop greater paw swelling (76) than infected wild-type mice. This lack of IL-10 was associated with increased levels of IFN-γ, and neutralization of this IFN-γ reduced inflammation (76). These researchers did not examine the effects of IL-10 deficiency on IL-17, however. Here, we show that IL-10 deficiency increases levels of IL-17 following infection with *B. burgdorferi*, and future studies will assess the contribution of IL-17 to arthritis in these mice. The findings here, which show an additional effect of IL-10 on inflammation caused by a known helper T cell cytokine, support the hypothesis that IL-17 formed in response to *B. burgdorferi* infection may be derived from CD4+ T cells.

To obtain additional support for this, CD4 was blocked in *in vitro* cultures of stimulated spleen cells obtained at peak swelling in different models of Lyme arthritis. This was done to assess the contribution of IL-17-secreting CD4+ cells (likely Th17 cells) in the different models of disease. Significant decreases in the production of IL-17 were induced following administration of an anti-CD4 antibody in stimulated cells from *Borrelia*-infected, wild-type mice (Figure 9). This exposure to live organisms likely resulted in an increased CD4+ T cell (potentially Th17 cell) response that was sensitive to the effects of the antibody. In addition, cells from vaccinated, but not infected, wild-type mice also trended toward a

decrease in IL-17 with exposure to anti-CD4 antibody, though this decrease was not statistically significant (Figure 9). A significant decrease in IL-17 following anti-CD4 antibody treatment was expected in this group, as the *in vitro* culture was these vaccinated cells' primary exposure to live *B. burgdorferi* 297. As anticipated, significant IL-17 production was observed following this activation of vaccine-primed cells by a heterologous strain. Therefore, it may be that the amount of anti-CD4 antibody used was not sufficient to fully counter the level of Th17 cell activation and IL-17 production. Still, however, these data do point to the possible presence of Th17 cells stimulated by *B. burgdorferi*. In addition, other IL-17-producing cells may be induced by *B. burgdorferi*, since addition of anti-CD4 antibodies to cultures did not completely abrogate IL-17 production.

Cells from *Borrelia*-vaccinated and -infected IL-10 KO mice did achieve a significant reduction in IL-17 in the presence of anti-CD4 antibody after 6 hours in culture (Figure 10). Considering that the lack of IL-10 in these cells could naturally skew towards a Th17 response after encountering antigen, these data are not surprising. In addition, since this decrease was not observed in cells from wild-type *Borrelia*-vaccinated and -infected mice (Figure 9), this may indicate just how powerful the effect of IL-10 is in inhibiting a Th17 response. However, no similar effect was observed in stimulated cells from non-vaccinated, *Borrelia*-infected IL-10 KO mice or *Borrelia*-vaccinated, non-infected IL-10 KO mice. This may have resulted from the substantial amount of IL-17 produced by these cells, which may have been too great to counter with the amount of anti-CD4 antibody

used. A large amount of cell activation seems to have occurred in both of these groups upon *in vitro* stimulation for 24 hours. As previously stated, the increase in IL-17 production by cells from the *Borrelia*-vaccinated, non-infected IL-10 KO mice may be due to the primary exposure of these cells to the *in vitro* antigen, *B. burgdorferi* 297. However, non-vaccinated, *Borrelia*-infected IL-10 KO mice were challenged with *B. burgdorferi* 297, yet still contained cells that produced significant IL-17 during *in vitro* exposure to *B. burgdorferi* 297 (Figure 10). This may provide additional support that the *Borrelia*-infection model of Lyme arthritis does not reflect the *in vivo* IL-17 response well. However, this also points to the possibility that prior vaccination with *B. bissettii* may prime mice to robustly produce borreliacidal antibody against *B. burgdorferi* 297 following infection with this latter strain. In support of this, less IL-17 was observed in restimulated cells from *Borrelia*-vaccinated and -infected mice IL-10 KO mice than from infected IL-10 KO mice that were not vaccinated (Figure 10). The effect of prior vaccination seems to affect the strength of the response to secondary antigen exposure. In any case, these data in IL-10 KO mice provide support that IL-17 may influence disease in Lyme arthritis. Also, Th17 cells may be a source of IL-17, though it is likely not the only source, as anti-CD4 antibody did not completely prevent production of IL-17. In future experiments, a dose-response experiment with anti-CD4 antibody will be performed to account for the considerable IL-17 levels seen in these groups.

In summary, the working hypothesis for this aim was *CD4+ cells are a significant source of IL-17 during* B. burgdorferi-*induced inflammation.* These data provide support to this hypothesis. Known relationships between Th1 and Th17 cells, as well as between IL-10 and Th17 cells, were demonstrated following infection with *B. burgdorferi*. Importantly, these relationships were demonstrated at the peak of swelling in different Lyme arthritis models. In addition, CD4-expressing cells were shown to be involved in the production of IL-17 at the peak of swelling. One possible confounding factor was the unexpectedly low amount of IL-17 produced following secondary exposure to *B. burgdorferi* 297. However, this is explored in Specific Aim 2 and is discussed below.

II. Specific Aim 2: Establish a relationship between IL-17 levels and borreliacidal antibody production.

A potent antibody response is required for protection against *B. burgdorferi* infection and the ensuing inflammation. Th17 cells have been shown to exert effects on the ability of B cells to produce antibody (81). For example, Th17 cells induce the production of autoantibodies that contribute to experimental arthritis (82). In addition, IL-21, a product of Th17 cells, contributes to the IL-6-mediated antibody response (83). In addition, IL-17 has been shown to stimulate class-switching in response to infection (84). Furthermore, *B. burgdorferi*-primed T cells have been shown to influence borreliacidal antibody production (85). However, the role of IL-17 in the antibody response to *B. burgdorferi* is not fully known.

As stated above, the effects of viable *B. burgdorferi* infection, as opposed to non-viable infection, on IL-17 production during peak swelling were investigated. Surprisingly, whole lymph node cells from *Borrelia*-vaccinated mice infected with live *B. burgdorferi* 297, restimulated with *B. burgdorferi* 297, produced less IL-17 than stimulated cells obtained from vaccinated mice injected with heat-killed organisms. This unexpected result may have occurred due to significant priming of borreliacidal antibody-producing B cells in vaccinated mice given a live infection. It was hypothesized that the *in vitro* exposure to *B. burgdorferi* stimulated a strong borreliacidal response in those vaccine-primed cells that had already interacted with *B. burgdorferi* 297 *in vivo*. These antibodies would have cleared the *in vitro* infection, removing the stimulus for IL-17 production. Since unseparated lymph node populations were used to assess cytokine levels in these mice, the association of IL-17 with development of the borreliacidal antibody response was investigated.

B. burgdorferi 297-stimulated cells from *Borrelia*-vaccinated, non-infected mice produced low levels of borreliacidal antibodies (Figure 11). Unexpectedly, supernatants of restimulated cells from *Borrelia*-vaccinated mice injected with either viable or heat-killed *B. burgdorferi* 297 revealed high levels of borreliacidal antibody production (Figure 11). Unfortunately, whether or not the titers differed between these groups could not be determined, as the titers exceeded the level of detection in the assay. While these antibodies are stimulated by the presence of heat-killed bacteria, these results may also indicate that live infection induced

the production of borreliacidal antibodies which prevented a robust IL-17 response in the *in vitro* system used here. Although stimulation of cells obtained from mice injected with heat-killed organisms also induced a high titer, an IL-17 response also developed. These cells may have initially produced borreliacidal antibodies only upon primary exposure to live organism *in vitro*, while the cells from mice infected with live organism had already been producing these antibodies *in vivo* for 10 days. If this is the case, it is possible that the IL-17 response developed prior to the significant production of protective antibodies, and that the prior existence of these antibodies inhibited the IL-17 response.

Th17 cells may also contribute to the development of borreliacidal antibodies. IL-6, a cytokine that supports Th17 cell differentiation (86), promotes the production of borreliacidal antibodies against immunodominant *Borrelia* epitopes *in vitro* (87, 88). In addition, borreliacidal antibody titers are increased following *in vitro* neutralization of IFN-γ (89), which appears to be antagonistic to IL-17 in Lyme arthritis. It is shown here that *Borrelia*-infected mice treated with anti-IFN-γ antibodies produced more IL-17 during peak swelling, which may suggest that the corresponding increase in IL-17 may also contribute to borreliacidal antibody production. By contrast, treatment of *Borrelia*-vaccinated and -infected mice with antibodies against combinations of IL-17, IL-6, and TGF-β yielded borreliacidal antibody titers that did not fully correlate with disease outcome (45). Therefore, the timing of borreliacidal antibody production and induction of IL-17 may coincide, and each may affect the other.

Here, lower titers of borreliacidal antibodies in naïve wild-type mice, compared to naïve IL-10 KO mice, were observed (Figure 12). The increased levels of antibodies observed in the serum of IL-10 KO mice, despite lack of previous exposure to the organism, demonstrate the significance of IL-10 in borreliacidal antibody production. IL-10 KO mice also produced significantly greater levels of borreliacidal antibodies than their wild-type counterparts in the *Borrelia*-vaccinated, non-infected group (Figure 12). These results are consistent with data demonstrating that large amounts of IL-10 suppress the proliferation and differentiation of certain types of B cells (90). Previous research has also shown that deficiency of IL-10 leads to increased borreliacidal antibody titers *in vivo* following infection with *B. burgdorferi* (80). However, these researchers reported that increased microbial clearance resulted from an enhanced innate immune response, rather than from the increased titers of borreliacidal antibodies (80). These findings are also consistent with our results. If IL-10 increases borreliacidal antibody production and IL-17 production, this IL-17 may also be produced by activated innate cells, including populations of neutrophils, dendritic cells, and/or natural killer cells. This is plausible, since it is shown here that cells other than CD4+ cells produce IL-17, as blocking CD4 did not completely inhibit production of the cytokine.

In summary, the working hypothesis for this aim was *increases in IL-17 are associated with an increase in borreliacidal antibody titers following infection with*

B. burgdorferi. The data shown here partially support this hypothesis; however, an association between IL-17 and borreliacidal antibodies induced following infection of mice with live or dead *B. burgdorferi* was unable to be established. This may be due to the fact that the borreliacidal antibody titers exceeded the detection ability of the assay. This detection ability can be remedied in the future by creating additional dilutions for examination. In addition, infected IL-10 KO mice produced greater amounts of borreliacidal antibody and possessed cells that produced greater amounts of IL-17 upon *in vitro* culture. While an association can be made between increased IL-17 and increased borreliacidal antibodies, it is likely that the genetic deficiency in IL-10 is the major contributor.

However, an association between IL-17 and borreliacidal antibody production may be observed in the wild-type mice used here. , *In vitro*-stimulated cells from *Borrelia*-vaccinated, non-infected wild-type mice produced larger amounts of IL-17 than stimulated naïve cells and stimulated cells from *Borrelia*-infected mice after 24 hours of incubation (Figure 5). These mice also had significantly higher serum borreliacidal antibody titers than either group (Figure 12). However, while *Borrelia*-vaccinated and -infected wild-type mice produced high borreliacidal antibody titers than other groups (Figure 12), stimulated, unseparated lymph node cells from these mice produced lower-than-expected amounts of IL-17 (Figure 5). This may be due to the presence of the borreliacidal antibodies in the *in vitro* cultures, showing that a relationship between borreliacidal antibodies and IL-17 production may exist. A future study to further examine this relationship

would be to administer anti-IL-17 antibodies to wild-type and IL-10 KO mice using both of these Lyme arthritis models, and then observe the borreliacidal antibody response. A previous investigation of this, using wild-type C57BL/6 mice, *B. burgdorferi* 297 as the vaccine strain, and *B. bissettii* as the infection strain, led inconclusive results pertaining to a relationship between borreliacidal antibody titers and the presence of Th17 cytokines (45). However, in that study, blocking IL-17 and the Th17-differentiating cytokines IL-6 and TGF-β completely inhibited the borreliacidal antibody response, demonstrating that Th17 cells and borreliacidal antibody production may be linked. Further studies are needed to further elucidate the relationship between IL-17 and the production of borreliacidal antibodies..

CHAPTER 5: CONCLUSION AND FUTURE DIRECTIONS

The hypothesis for this thesis was that IL-17 produced by CD4+ cells contributes to the inflammation associated with *B. burgdorferi* infection and is associated with an increase in borreliacidal antibody production. The first aim, to establish CD4+ cells as a source of IL-17 following infection with *B. burgdorferi*, was supported. Using different models of Lyme arthritis, the data presented here supported known relationships between Th1 and Th17 cells as well as between IL-10 and Th17 cells. Production of IL-17 increased following *B. burgdorferi* infection and was prominent at the peak of hind paw swelling. At this peak swelling period, IL-17 production was regulated by both IFN-γ and IL-10, and was produced, in part, by CD4+ cells. It is likely that Th17 cells are contributing to the development of Lyme arthritis through their production of IL-17. The second specific aim, which was to establish a relationship between IL-17 levels and borreliacidal antibody production, was partially supported. As levels of IL-17 increased in both wild-type mice and IL-10 KO mice infected with *B. burgdorferi*, an increase in the production of borreliacidal antibodies was observed. However, additional research is required to better characterize the role of IL-17 in this process.

Overall, these findings provide additional evidence to support consideration of inflammatory cytokines other than IFN-γ as contributors to Lyme arthritis following infection with *B. burgdorferi*. This may lead to new targets for therapy against Lyme arthritis. One future study to more directly examine the role of IL-17 following *B. burgdorferi* infection will be conducted by administering anti-IL-17

antibodies to wild-type and IL-10 KO mice using both the *Borrelia*-infection and *Borrelia*-vaccination and -infection Lyme arthritis models, and then observing the borreliacidal antibody response and joint histopathology that result. Future studies are also needed to determine the role of additional sources of IL-17, including neutrophils and γδ-T cells (21, 69), following borrelial infection. In addition, experiments are planned to determine the actual abundance of Th17 cells in *B. burgdorferi*-infected mice and to determine their contribution to Lyme arthritis.

However, recent findings show that T cell populations are not as well defined as is commonly presented, which confounds investigations pertaining to Th17 cells. Depending on the cytokine environment during development, Th17 cells are able to convert into both Th1- and T_{reg}-like cells (52). For example, some Th17 cells may also produce IFN-γ, giving them characteristics of Th1 cells. IL-17^{+}IFN-γ^{+} Th17 cells are more prominent in autoimmune diseases and, perhaps not surprisingly, suppress traditional IL-17^{+}IFN-γ^{-} Th17 cells (91). In addition, populations of Th17 cells can also produce IL-10 (52), thereby potentially regulating the immune response in the same manner as T_{reg} cells. Future studies will require consideration of these newly discovered T cell phenotypes in investigation of *B. burgdorferi* infection and Lyme arthritis. Importantly, this also opens a line of research into the modulation of T cells for therapy for, or prevention of, Lyme arthritis.

The findings presented here support a potential role for T_{reg} cells in the regulation of Lyme arthritis. Specifically, CD4+CD25+Foxp3+ T_{reg} cells are critical for maintaining tolerance and managing autoimmune diseases, in part, through secretion of IL-10 (92). Bettelli *et al.* (44) published the major finding that Th17 and CD4+CD25+Foxp3+ regulatory T cells are derived from a common precursor T cell, with the presence or absence of a certain inflammatory cytokine environment determining the outcome of differentiation. Prior to this finding, an association between IL-17 and presumed T_{reg} cells was described in the development and regulation of Lyme arthritis, using IFN-γ-deficient, *Borrelia*-vaccinated and -infected mice (49). Neutralization of IL-17 in these mice, in addition to preventing the development of arthritis, led to an increase in the numbers of CD4+CD25+ T cells in the local lymph nodes (49). Depletion of CD25+ cells in anti-IL-17 antibody-treated, *Borrelia*-vaccinated and -infected mice led to severe, destructive arthritis (49). In addition, transfer of CD4+CD25+ cells obtained from these anti-IL-17 antibody-treated mice into infected recipient mice protected against arthritis development (93). By contrast, transfer of CD4+CD25- T cells into infected recipients increased disease severity (93). Moreover, depletion of CD4+CD25+ T cells in *Borrelia*-vaccinated and -infected mice not administered anti-IL-17 antibodies did not affect arthritis (94), suggesting that the absence of IL-17 induced a distinct subpopulation of CD4+CD25+ T cells that protected against the induction of Lyme arthritis.

Additional data support a role for T_{reg} cells (and, by extension, Th17 cells), in Lyme arthritis. Mice deficient in CD28, a co-stimulatory molecule for T_{reg} cells, displayed intensified and chronic arthritis in response to infection with *B. burgdorferi* (95). Shorter duration to resolution of disease has also been associated with higher percentages of T_{reg} cells in patients with Lyme arthritis (28). By contrast, patients with antibiotic-refractory Lyme arthritis had lower percentages of T_{reg} cells and displayed an inability to resolve synovial inflammation (28). However, Kuo *et al.* demonstrated that administration of recombinant interleukin 35 (IL-35), another anti-inflammatory cytokine produced by T_{reg} cells, to *B. burgdorferi*-vaccinated and -infected mice actually increased the severity of Lyme arthritis and decreased borreliacidal antibody titers (96). Collectively, these findings point to T_{reg} cells as a significant factor in the regulation of Lyme arthritis. Since Th17 cells are closely related to these cells, investigation of both cell types is needed for a complete examination of the immune response to *B. burgdorferi* infection.

Due to the large increases in production of IL-17 after 24 hours of cell culture by the cells obtained at the peak of hind paw swelling shown here, it is also important to consider the production of the cytokine at earlier time points in the development of arthritis. Preliminary data indicate that IL-17 may play a significant role early in infection. Cells obtained from mice one day after infection produced significant levels of IL-17 (data not shown). These levels were reduced upon addition of anti-CD4 antibody, regardless of infection model (data not

shown). Incubation of stimulated cells with anti-CD4 antibody also reduced IFN-γ in these cells (data not shown), further demonstrating that both inflammatory cytokines may contribute to Lyme arthritis.

Understanding the immune mechanisms of Lyme arthritis, particularly the role of IL-17, is necessary for the development of safe vaccines for Lyme borreliosis. Currently, OspA and outer surface protein C (OspC) are the most commonly considered vaccine candidates for Lyme borreliosis. While OspA has been shown to induce borreliacidal antibody titers, it may also induce an autoimmune response (57). In fact, Croke *et al*. showed that vaccination of hamsters with OspA primed the animals for development of severe Lyme arthritis following infection with *B. burgdorferi* (97). These findings fueled concern regarding the safety of OspA vaccines in humans (64). The data presented here suggest that a successful vaccinate candidate needs to elicit high borreliacidal antibodies titers without inducing an IL-17 response in order to confer immunity without the induction of arthritis.

In conclusion, IL-17 has been shown to play a role in the development of Lyme arthritis. Here, a role for CD4+ cells as a major producer of IL-17 following infection with *B. burgdorferi* is supported. Additionally, a relationship between IL-17 and borreliacidal antibodies may exist. Future studies will be needed to more completely characterize the immune response to *B. burgdorferi* and the development of Lyme arthritis. However, the findings presented here have

significant implications for the development of both therapeutic interventions and safe, effective vaccines against Lyme borreliosis.

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