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MODULATION OF INTERLEUKIN-17 BY TUMOR NECROSIS FACTOR-ALPHA DURING THE IMMUNE RESPONSE TO *BORRELIA BURGDORFERI*

by

Velinka Medić

A Thesis Submitted in

Partial Fulfillment of the

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ABSTRACT

MODULATION OF INTERLEUKIN-17 BY TUMOR NECROSIS FACTOR-ALPHA DURING THE IMMUNE RESPONSE TO *BORRELIA BURGDORFERI*

by

Velinka Medić

The University of Wisconsin-Milwaukee, 2013
Under the Supervision of Professor Dean Nardelli

Arthritis is one of the main complications of late-stage Lyme borreliosis. Cytokines play an important role in the persistent inflammation that is elicited by the causative agent of disease, *Borrelia burgdorferi*. Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine that is induced by *B. burgdorferi* and that has been implicated in development of arthritis. However, in a mouse model of Lyme arthritis, treatment with anti-TNF-α antibody increased the severity of disease. By contrast, injection of recombinant TNF-α ameliorated arthritis in this model. These findings suggested that other factors might be involved during the development of Lyme arthritis. One possible factor may be interleukin-17 (IL-17), a pro-inflammatory cytokine mainly produced by T helper 17 cells. IL-17 has been implicated in the development of Lyme arthritis. Treatment with anti-IL-17 antibodies reduces the inflammatory outcome in animal models of disease. However, it is not known how TNF-α affects the response of IL-17 to *B. burgdorferi*. The hypothesis of this thesis is that TNF-α will regulate the immune response to *B. burgdorferi* infection by decreasing IL-17 production. Modulation of IL-17 during the immune response *to B. burgdorferi* was analyzed in wild-type and interleukin-10 (IL-10)-deficient mice treated with anti-TNF-α antibodies. We found that anti-

TNF-α antibody treatment increased paw inflammation in both wild-type and IL-10 deficient mice. In addition, cell cultures from *B. burgdorferi*-infected, wild-type mice treated with anti-TNF-α antibodies typically produced a greater amount of IL-17 than untreated controls, reasoning that TNF-α decreases IL-17 levels following infection. Additional treatment of anti-TNF-α antibody-treated mice with anti-IL-17 antibodies lessened the severity of paw swelling. Our results indicate that TNF-α can play a protective role during the immune response to *B. burgdorferi* infection by restraining the production of IL-17.

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LIST OF ABBREVIATIONS

Ab: Antibody

Bb: *Borrelia burgdorferi*

BSK: Barbour-Stoenner-Kelly

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

NF-κB: nuclear factor kappa-light-chain-enchancer of activated B cells

OspA: outer surface protein A

OspC: outer surface protein C

PBS: phosphate buffered saline

PBMC: peripheral blood mononuclear cell

RA: rheumatoid arthritis

SEM: standard error of the mean

Th1: T helper type 1

Th17: T helper type 17

TNF-α: tumor necrosis factor-alpha

Treg: regulatory T

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This thesis is dedicated to my daughter, Teodora, and my niece, Ana.

CHAPTER 1: INTRODUCTION

I. Lyme Borreliosis

Zoonotic pathogens have become a threat to human health, despite increased

awareness and surveillance. The most common tick-borne disease in the United

States is Lyme borreliosis (Centers for Disease Control and Prevention [CDC]), a

multi-symptom disease caused by excessive inflammation elicited by *Borrelia*

burgdorferi, the pathogenic spirochete. The number of cases of Lyme borreliosis

has continued to increase annually, with 30,000 reported cases in 2012 (CDC).

However, in 2013 it was reported that the actual incidence of disease is 10 times

higher than reported cases, reaching 300,000 per year (CDC). Lyme borreliosis is

endemic to the East Coast and Great Lakes regions and northern California. This

geographic pattern, which is expanding, is a result of the combined presence of

suitable tick vectors and prevalence of infected ticks and animal reservoirs.

B. burgdorferi sensu lato is a complex of 19 genospecies (Rudenko *et al.*, 2011).

Some of these species are pathogens that cause the development of Lyme

borreliosis. These pathogenic strains have different geographic ranges and cause

different disease manifestations. For example, *B. burgdorferi sensu stricto* is

responsible for causing Lyme borreliosis in the United States. This species is well

known for inducing the development of Lyme arthritis in later-stage infection. By

contrast, *B. garinii* and *B. afzelii* are responsible for causing Lyme borreliosis in

Europe, and, in later stages of disease, typically cause more severe effects of the

skin and neurological system than *B. burgdorferi.* These pathogens are deficient

in the genes necessary for synthesis of amino acids and fatty acids (Aguero-Rosenfeld *et al.*, 2005), and, therefore, depend on host cells and tissues and immune evasion for survival.

The progression of Lyme borreliosis is often divided into three stages: early localized disease, early disseminated disease, and late disease (Steere *et al.*, 1989). The main clinical manifestation of early disease that is caused by *B. burgdorferi* is a bull's eye-shaped rash called erythema migrans (EM). EM is present in about 70% of cases of infection (Bacon *et al.*, 2008) and, in endemic areas, it is considered to be diagnostic (Aguero-Rosenfeld *et al.*, 2005; Wormser *et al.*, 2006). EM develops at the site of the tick bite approximately 7 to 14 days after infection and usually is accompanied by non-specific, flu-like symptoms (O'Connor, 2010). In early disseminated disease, in which *B. burgdorferi* organisms migrate from the skin throughout the body, multiple EM lesions can be present, as well as cranial nerve palsy, meningitis, and, rarely, carditis. During this stage, fever, fatigue, and headache are also common. The most commonly manifested complication of late disease in the United States is arthritis, but neurological symptoms such as mood and memory disorders, fatigue, and nerve pain may also be observed. By contrast, encephalomyelitis and acrodermatitis chronica atrophicans (a skin rash that can lead to skin atrophy and polyneuropathy) are common late symptoms of Lyme borreliosis in Europe (Stanek *et al.*, 1996), which is a result of infection with pathogenic species other than *B. burgdorferi,* such as *B. afzelii* and *B. garinii.*

II. Transmission and Dissemination of *B. burgdorferi*

Complex interactions between the microbes, vectors, and animal reservoirs lead to transmission of *B. burgdorferi*. The transmission of *B. burgdorferi* to humans depends on the infected deer tick *Ixodes scapularis*, which is found in the eastern and midwestern parts of United States, and *I. pacificus*, which is found on the Pacific coast. In order to understand the transmission of *B. burgdorferi*, it is important to describe the life cycle of *I. scapularis*. The life cycle of the tick ranges from two to four years, and it is divided into four stages: egg, larva, nymph, and adult. When *I. scapularis* ticks hatch, they are free of bacteria. At the first feeding on an infected animal reservoir, *B. burgdorferi* are transmitted into the larvae. After molting to the nymph stage, infected *I. scapularis* feed on another animal reservoir and transmit the bacteria to them, which sustains the presence of the bacteria in the environment. During the nymph stage, *I. scapularis* may also feed on humans, transmitting the bacteria to them. It has been suggested that humans are accidental or "dead-end" hosts (Radolf *et al.*, 2012). Ticks feed on white-footed mice, which are considered the main animal reservoir (Mather *et al.*, 1989), and deer, which are less competent reservoirs for *B. burgdorferi* (Telford *et al.*, 1988). Other animal reservoirs include squirrels, hares, and various other small mammals, as well as birds and lizards (Halperin, 2011). In addition, seasons that correspond to the tick life cycle are also important in the transmission of Lyme borreliosis. Summer months are when larvae hatch and feed on infected animal reservoirs, as well as when nymphs also feed. In these months, there is an

increase in the number of reported Lyme borreliosis cases (Bacon *et al.*, 2008). Overall, the prevalence of Lyme borreliosis is dependent on a combination of the feeding habits of these ticks, the proportion of ticks infected with *B. burgdorferi*, and the presence of animal reservoirs.

The effects of host and tick factors on *B. burgdorferi* are especially important in transmission of the spirochete. There is a change in expression of various outer-surface proteins (Osp) of *B. burgdorferi*, mainly OspA and OspC, during the tick feedings. In the infected, but unfed, tick, bacteria are contained in the midgut. OspA levels are upregulated during colonization of tick midgut, and it has been established that OspA plays a role in adhesion to the epithelial cells of ticks by binding to a protein named "tick receptor for OspA" (TROSPA) (Pal *et al.*, 2004). During feeding, there is a downregulation of OspA, causing detachment of *B. burgdorferi* from TROSPA and migration to the salivary gland. Concomitantly, there is an upregulation of OspC, which can bind tick salivary protein 15 (Salp15) (Ramamoorthi *et al.*, 2005). It has been demonstrated that Salp15 is needed for *B. burgdorferi* infection. It inhibits CD4$^+$ T cell activation, and its attachment to the bacteria allows dissemination into the mammalian host (Samuels, 2010).

After infection with *B. burgdorferi*, the spirochetes disseminate from the skin into the blood and various organs. To enter specific organs, they have to evade host defenses and induce the breakdown of extracellular matrix (ECM) components of tissues. Other Osp proteins that are produced by the bacteria ensure successful

infection and survival in the hosts. When *B. burgdorferi* enters the mammalian host, it disseminates and survives by evading the immune response. OspEF-related proteins (Erps) bind to complement factor H and complement factor H-like 1 protein (FHL-1) to inhibit the activity of the alternative complement pathway (Antonara *et al.*, 2011). In addition, *B. burgdorferi* does not produce secreted proteases. Instead, it has to rely on inducing host factors that result in tissue destruction and dissemination into tissues. For example, *B. burgdorferi* induces production of plasminogen and matrix metalloproteinase-9 (MMP-9), which cause ECM degradation (Heilpern *et al.*, 2009). Similarly, adhesion molecules, including outer-membrane surface proteins (OMPs), integrin binding protein p66 (Coburn *et al.*, 1999), the fibronectin binding protein Bbk32 (Probert and Johnson, 1998), and decorin-binding proteins A and B (Hagman *et al.*, 1998), have been shown to aid in dissemination within the host, particularly to sites of abundant connective tissue. Collectively, these findings demonstrate that different bacterial, tick, and host factors for are responsible for successful transmission, establishment of infection, and dissemination.

III. Diagnosis and Treatment of Lyme Borreliosis

The diagnosis of Lyme borreliosis is dependent on a combination of laboratory testing and observing clinical manifestations. The CDC requires confirmed cases of Lyme borreliosis to meet the following criteria: physician-confirmed EM, or positive serological testing with manifestations of cardiac, neurological, or arthritic symptoms (CDC). Aside from EM, the clinical manifestations are not specific.

However, diagnosis based on EM alone is often difficult. EM does not develop in every infected patient, and EM may occasionally be confused with rash, eczema, and insect bites (Puius and Kalish, 2007). In addition, EM lesions can be located on a part of the body that is difficult to observe, and the rash can be obscured by certain skin tones and the presence of hair. As a result, diagnosis of Lyme borreliosis only on the basis of observing clinical symptoms can be difficult. However, observing EM in an endemic area is often sufficient for a positive diagnosis.

Additionally, serological testing is frequently used to diagnose Lyme borreliosis. A two-step process is used for laboratory diagnosis: a screening enzyme-linked immunosorbent assay (ELISA), followed by a confirmatory Western blot. One major disadvantage of this approach is an inability to distinguish between current and past infection, since patients can be seropositive for years (Marques, 2010). Similarly, detection of *B. burgdorferi*-specific antibodies in the early stage of disease can be challenging, since the sensitivity and specificity of serological testing are inadequate at that stage (Shapiro and Gerber, 2000). Furthermore, cross-reactive antibodies from other spirochetal infections such as syphilis, relapsing fever, or leptospirosis, or from autoimmune diseases such as systemic lupus erythematosus, can yield false positives. These issues demonstrate the complexity of diagnosing Lyme borreliosis and the need for better diagnostic markers.

In 2006, the Infectious Diseases Society of America updated the stage-dependent guidelines for treatment of Lyme disease (Wormser *et al.*, 2006). For adults at an early stage of Lyme disease with no neurological complications, doxycycline, amoxicillin, or cefuroxime axetil are recommended for 10-21 days. During the late stage of Lyme disease, patients with Lyme arthritis can be treated with the same antibiotics, but administered for 28 days. If arthritis is not resolved by the end of the first treatment, a second dose of antibiotics is administered for another 4 weeks (Shapiro and Gerber, 2000; Wormser *et al.*, 2006).

However, some patients can still present with arthritic symptoms even after a second dose of antibiotics. At this point, non-steroidal anti-inflammatory agents and corticosteroids are used to counter the high levels of pro-inflammatory cytokines found in patients (Shin *et al.*, 2007). In addition, disease-modifying anti-rheumatic drugs may be used to combat synovial destruction.

There is an ongoing debate in the field of Lyme arthritis research as to whether patients treated with several doses of antibiotics continue to be infected with *B. burgdorferi*. This is particularly due to the inability to isolate live bacteria from synovial fluid of patients that suffer from late stage Lyme arthritis (Carlson *et al.*, 1999; Shin *et al.*, 2007). Different hypotheses have been proposed to explain the cause of continued synovitis in these patients in the absence of bacteria. Steere and Glickstein (2004) proposed the following hypotheses for this persistent arthritis: (1) *B. burgdorferi* might reside in a location that is not easily accessible to

antibiotics; (2) bacterial antigens, rather than live microbes, may have a continued presence in the synovial fluid and tissues; (3) the response to *B. burgdorferi* may cause autoimmunity due to T-cell epitope mimicry, and (4) infection may cause bystander activation of inflammatory cells. Recently, the "amber hypothesis" was proposed, postulating that antigens can become trapped in connective tissue (as if they were in amber), and then get released into the joint, eliciting a strong immune response (Wormser *et al.*, 2012). This has been proposed as a possible explanation for why active infection is not observed in patients with antibiotic-resistant Lyme arthritis (Wormser *et al.*, 2012). This theory questions the persistent use of antibiotics in treatment of late-stage Lyme disease because antigens, rather than live microbes, would elicit the inflammatory immune response. Therefore, non-recommended treatments for Lyme arthritis need to be approached with caution, and other targets, such as the immune response, need to be considered for therapy.

IV. Lyme Arthritis

Various cells and cytokines have been implicated in the development of Lyme arthritis. Strong innate and adaptive immune responses are activated during the inflammatory process against *B. burgdorferi*; however, the exact mechanisms of Lyme arthritis are not fully understood. It has been demonstrated that during the innate response, neutrophils (Brown *et al.*, 2003) and macrophages (Du Chateau *et al.*, 1996) are activated in the synovial tissue. Similarly, there is an influx of complement proteins, as well as pro-inflammatory chemokines and cytokines, into

the synovial fluid of patients with Lyme arthritis (Shin *et al.*, 2007). These innate factors lead to an inflammatory T cell-mediated response that often results in arthritis.

i. Animal Models of Lyme Arthritis

Animal models are used to study the immunopathogenic mechanisms of Lyme arthritis. The selection of an animal model depends on the purpose of the study because no animal model is able to combine all of the inflammatory events presented in humans with Lyme arthritis (Nardelli *et al.*, 2008b).

Infection of experimental mice with *B. burgdorferi* is used to study various features of Lyme arthritis. C3H/HeJ mice are the most commonly used mice for this disease model. Induction of Lyme arthritis is achieved by feeding of infected ticks or by injection of *B. burgdorferi* into the paws or between shoulder blades of the mice. Distinctive features of this model include synovitis that develops and resolves in 3-6 weeks (Barthold *et al.*, 1992) and that does not lead to cartilage or bone erosion (Barthold *et al.*, 1992; Nardelli *et al.*, 2010). In addition, this model exhibits a strong response by innate immune cells (Barthold *et al.*, 1996, Nardelli *et al.*, 2010). Contrary to this animal model, in humans, Lyme arthritis develops months to years after infection, and both innate and adaptive immune responses are important in this progression. Therefore, use of C3H mice in the study of Lyme arthritis may be best used to investigate the innate immune response following infection.

Using the infection model of disease, wild-type C57BL/6 mice are resistant to development of severe Lyme arthritis (Barthold *et al.*, 1992, Ma *et al.*, 1998). When C57BL/6 mice are injected with high doses of bacteria (up 2 x 10^5), they show minimal paw swelling, while C3H mice show severe paw swelling (Ma *et al.*, 1998). However, infected C57BL/6 mice have a similar number of spirochetes as infected C3H mice, even though they develop mild arthritis (Ma *et al.*, 1998). It has been proposed that differences in susceptibility between these two strains are linked to interleukin-10 (IL-10) production and its role in the immune response (Brown *et al.*, 1999, Ganapamo *et al.*, 2000). C57BL/6 macrophages stimulated with *B. burgdorferi* produce more IL-10 than stimulated cells from C3H mice (Brown *et al.*, 1999). Furthermore, recombinant IL-10 down-regulates pro-inflammatory cytokine production by bone-marrow derived macrophages, while treatment with anti-IL-10 antibody increases levels of pro-inflammatory cytokines produced by these macrophages (Gautam *et al.*, 2012). These studies demonstrate the basic differences between C3H and C57BL/6 mice and the involvement of the anti-inflammatory cytokine IL-10 in regulating the immune response in these mice following infection with *B. burgdorferi*.

Involvement of IL-10 in regulating Lyme arthritis has led researchers to utilize IL-10-deficient mice on both the C57BL/6 and C3H backgrounds to study the disease. C57BL/6 IL-10-deficient mice infected with *B. burgdorferi* have increased levels of paw swelling (Brown *et al.*, 1999; Hansen *et al.*, 2013), immune cell infiltrates (Sonderegger *et al.*, 2012) and arthritis (Brown *et al.*, 1999; Hansen *et al.*, 2013)

while maintaining lower numbers of spirochetes than infected wild-type mice (Brown *et al.*, 1999; Sonderegger *et al.*, 2012). Sonderegger *et al.* (2012) noted the importance of adaptive immunity in this model, as there is direct involvement of T cells following infection (Sonderegger *et al.*, 2012). Therefore, infected IL-10-deficient mice may be a feasible model to study the involvement of the adaptive immune response in development of later stage Lyme arthritis, which is observed in humans (Sonderegger *et al.*, 2012; Hansen *et al.,* 2013).

ii. T Cell Mediation of Lyme Arthritis

While innate immunity is involved in causing joint inflammation during early infection, T cells– more specifically, T helper type 1 (Th1) cells– are involved in the development of later-stage Lyme arthritis. Th1 cells produce an important pro-inflammatory cytokine, interferon-gamma (IFN-γ), which has been associated with the development of Lyme arthritis (Yssel *et al.*, 1991). However, several groups were able to elicit Lyme arthritis in IFN-γ-deficient mice (Brown and Reiner, 1999; Christopherson *et al.*, 2003; Burchill *et al.*, 2003). These studies demonstrate that Th1 cells are not solely responsible for development of Lyme arthritis. Recently, a subset of T helper cells which produce interleukin-17 (IL-17), T helper type 17 (Th17 cells), has been proposed to also play an important role in the immunopathogenesis of Lyme arthritis, particularly in later disease (Nardelli *et al.*, 2008b).

What factors support the hypothesis that IL-17-producing cells are involved in Lyme arthritis development? *B. burgdorferi* induces the production of IL-17 from cells of mice (Knauer *et al.*, 2006) and humans with Lyme arthritis (Infante-Duarte *et al.*, 2000). In addition, treatment with anti-IL-17 antibody in *Borrelia*-vaccinated and -infected mice prevented development of arthritis (Burchill *et al.*, 2003; Nardelli, *et al.* 2004, 2005, 2008a). Also, Th17 cells have been found in the synovial fluid of Lyme arthritis patients (Codolo *et al.*, 2008, 2013; Shen *et al.,* 2010). Furthermore, infected, arthritic mice produce increased amounts of IL-17, which can be reduced by antibodies to various Th17-cell-related cytokines (Amlong *et al.* 2006; Kotloski *et al.* 2008; Nardelli *et al.,* 2008b). This evidence shows that *B. burgdorferi* is able to elicit an IL-17 response that may contribute to the development of Lyme arthritis.

Other support for a potential role of IL-17-producing cells in the development of Lyme arthritis comes from experimental models of rheumatoid arthritis (RA) because of its similarities in pathology and clinical symptoms to Lyme arthritis. For example, in collagen-induced arthritis, it has been shown that IL-17 causes bone resorption and cartilage destruction (Lubberts *et al.*, 2001, 2002). Furthermore, increased levels of IL-17 mRNA were noted in the synovia and lymph nodes in mice using a model of adjuvant arthritis (Bush *et al.*, 2001). Similarly, Lubberts (2008) reported that IL-17-induced expression of NF-κB, which was necessary for osteoclastogenesis and bone resorption. These studies indicate the involvement

of IL-17 in development in several models of arthritis, supporting the concept that it may also be involved in the development of Lyme arthritis.

iii. TNF-α and Lyme Arthritis

Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine, produced by activated T cells and macrophages, that has been shown to play a role in rheumatoid arthritis pathogenesis (Katz *et al.*, 2001). In addition, TNF-α plays a role in the immune response to *B. burgdorferi* and affects the pathogenesis of Lyme arthritis. Peripheral blood mononuclear cells (PBMCs) (Defosse and Johnson, 1992) and macrophages (Ma and Weis, 1993) produce TNF-α when incubated with *B. burgdorferi*. Additionally, synovial fluid from patients with Lyme arthritis was found to have higher levels of TNF-α than the synovial fluid of non-arthritic patients (Defosse and Johnson, 1992). Currently, there are five FDA-approved TNF-α inhibitors used as therapies for rheumatoid arthritis (Dinarello 2005). Therefore, it is possible that TNF-α may be a viable target for the inhibition or prevention of Lyme arthritis.

The role of these TNF-α inhibitors is to neutralize the cytokine, preventing binding to its receptors. However, about 30% to 50% of rheumatoid arthritis patients receiving TNF-α inhibitor therapy do not see improvements (Rubbert-Roth and Finckh, 2009). Surprisingly, however, Christopherson *et al.* (2003) demonstrated that neutralization of TNF-α actually increased the severity of arthritis in a mouse model of Lyme arthritis, while treatment with recombinant TNF-α ameliorated

disease. Another protective property of TNF-α in Lyme arthritis was demonstrated by Yrjänainen *et al.* (2007). Administration of antibodies to TNF-α did not ameliorate arthritic swelling; in addition, when neutralization of TNF-α was coupled with ceftriaxone therapy, the number of spirochetes actually increased (Yrjänainen *et al.*, 2007). Both of these studies provide caution about the use of anti-TNF-α antibody for Lyme arthritis and highlight the need for further studies to elucidate the mechanism of TNF-α in its pathogenesis. While blocking TNF-α may be therapeutic for rheumatoid arthritis, it may not be for Lyme arthritis. This demonstrates that TNF-α may influence additional factors involved in the inflammatory response to *B. burgdorferi*.

The pro-inflammatory cytokine IL-17 may be one such factor. The interactions between IL-17 and TNF-α are complex and cause various downstream inflammatory effects. Jovanovic *et al.* (1998) found that IL-17 can induce the production of TNF-α in human macrophages. In addition, in human-TNF-α transgenic mice, blocking IL-17 production led to a decreased number of osteoclasts and played a protective role in TNF-α-mediated arthritis (Zwerina *et al.,* 2012). This study also demonstrated that IL-17 played a role not only in the inflammation of the joints, but also in joint destruction. Furthermore, IL-17 is able to induce the production of TNF-α and interleukin-1-beta (IL-1β) in the serum of rheumatoid arthritis patients (Chen *et al.*, 2011). Also, IL-17 was able to stimulate macrophages to release TNF-α along with IL-1β *in vitro* (Jovanovic *et al.*, 1998). Additionally, IL-17 and TNF-α were shown by Katz *et al.* (2001) to act

synergistically in promoting inflammation. Addition of TNF-α and IL-17 to fibroblasts led to secretion of both interleukin-6 and interleukin-8. The additive effect of these two cytokines was also shown in cultures of rheumatoid arthritis synoviocytes, in which their actions induced hypoxia inducible factor 1 alpha, which is found in high levels in patients (Hot *et al.*, 2012).

While many studies show that IL-17 influences the production of TNF-α, others show that TNF-α influences the activity of IL-17. Iwamoto *et al.* (2007) showed that TNF-α assists in differentiation of mature dendritic cells that stimulate a Th17 response. Also, use of the TNF-α inhibitor pentoxifylline to treat an animal model of rheumatoid arthritis reduces IL-17 levels in joint tissues. However, this treatment did not affect IL-17 levels in the periodontal-associated aspects of the experimental disease (Queiroz-Junior *et al.*, 2013). Similar to this, Chen *et al.* (2011) showed that rheumatoid arthritis patients who responded to anti-TNF-α therapy produced fewer Th17 cells and IL-17 than untreated patients. However, patients who did not respond to treatment produced more Th17 cells and IL-17 (Chen *et al.,* 2011). However, Koenders *et al.* (2006) showed that IL-17 acts independently of TNF-α in an *in vivo* rheumatoid arthritis model.

These findings show the complex relationship between TNF-α and IL-17. In some, but not all, contexts, blocking TNF-α reduces IL-17 production. The role of TNF-α in inducing IL-17-mediated Lyme arthritis is not fully known.

V. Borreliacidal Antibody Response

B. burgdorferi infection stimulates production of antibodies with highly specific killing activity against the spirochete. Borreliacidal antibodies specific to the outer surface proteins (Callister *et al.,* 1994) cause the killing of the bacteria by activation of complement. Schmitz *et al.* (1990) demonstrated that injection of immune sera into infected mice protected against swelling, joint destruction and infection of tissues. Similarly, Lovrich *et al.* (1993) demonstrated that serum from infected hamsters was able to kill *B. burgdorferi in vitro*. Production of these borreliacidal antibodies can be used to determine protection against *B. burgdorferi*. It was shown that the presence of borreliacidal antibodies correlates with elimination of *B. burgdorferi* from tissues (Schmitz *et al.*, 1991; Creson *et al.*, 1996), while the total antibody response is sustained for a longer amount of time (Padilla *et al.*, 1996). Collectively, these studies show that borreliacidal antibody production is important for protection against *B. burgdorferi*.

Different outer-surface proteins, including OspA, OspB and OspC, were identified as immunogens that produce borreliacidal antibodies. A recombinant OspA vaccine was made available on the market in 1998 but was removed after 4 years. Both of the clinical trials for the recombinant OspA vaccine (Sigal *et al.*, 1998; Steere *et al.*, 1998) required two boosters, underlining the importance of maintaining a strong humoral immune response in the protection against *B. burgdorferi,* which is the basis for developing effective Lyme borreliosis vaccines. Prior to this, Padilla *et al.* (1996) demonstrated that borreliacidal antibody titers

waned quickly after vaccination. By determining the immune mechanisms that influence microbial clearance through borreliacidal antibodies, additional factors that induce protection can be identified.

VI. HYPOTHESIS AND SPECIFIC AIMS

Arthritis is one of the major complications of late stage Lyme borreliosis. Cytokines play an important role in mediation of inflammation elicited by *B. burgdorferi* infection. Specifically, IL-17 has been implicated in development of Lyme arthritis (Infante-Duarte *et al.*, 2000; Burchill et al., 2003; Nardelli et al., 2004, 2005, 2008a; Knauer *et al.*, 2006; Hansen et al., 2013). However, the role of the pro-inflammatory cytokine TNF-α remains to be further elucidated. Incubation of peripheral blood mononuclear cells (Defosse and Johnson, 1992) and macrophages (Ma and Weis, 1993) with *B. burgdorferi* induce production of TNF-α. However, a protective role for TNF-α in Lyme arthritis was demonstrated (Christopherson *et al.*, 2003; Yrjänainen *et al.*, 2007). Studies of rheumatoid arthritis and psoriasis indicate that TNF-α can regulate IL-17 (Moran *et al.,* 2009; Chen *et al.*, 2011), while others indicate an independent relationship between TNF-α and IL-17 in arthritis development (Koenders et al., 2006; Iwamoto *et al.*, 2007). However, the effect of TNF-α on IL-17 production during *B. burgdorferi* infection has not been explored. The objective of this research is to elucidate the role of TNF-α and IL-17 in the inflammatory process of Lyme arthritis. The hypothesis of this thesis is that TNF-α will regulate the immune response to *B. burgdorferi* infection by decreasing IL-17 production.

The following specific aims of this proposal are:

1. **Determine the effect of TNF-α on IL-17 in the immune response to *B. burgdorferi* infection.** The working hypothesis of this aim is that TNF-α will decrease IL-17 production following *B. burgdorferi* infection.

2. **Determine the effects of TNF-α on the borreliacidal antibody response following infection with *B. burgdorferi*.** The working hypothesis of this aim is that anti-TNF-α treatment of *B. burgdorferi*-infected mice will inhibit borreliacidal antibody production, which will be reversed by also blocking IL-17.

CHAPTER 2: MATERIALS AND METHODS

I. Mice

Female and male, 6 to-12 week old wild-type and IL-10-deficient C57BL/6 mice were obtained from Dr. J.-A. Lyons (University of Wisconsin-Milwaukee). These mice were housed and cared for in the animal facility at the University of Wisconsin-Milwaukee. Food and water were provided *ad libitum*. The protocols and guidelines were reviewed and approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee.

II. Infection of Mice with *B. burgdorferi*

Borrelia burgdorferi strain 297 was provided by S. M. Callister (Gundersen Lutheran Medical Center, La Crosse, WI). *B. burgdorferi* organisms were grown in Barbour-Stoenner-Kelly (BSK) medium for 2-4 days at 34ºC. Mice were anesthetized with isoflurane in a nose-to-mouth cup and infected subcutaneously with 10^6 spirochetes (0.05 ml) into the hind paws.

III. Administration of Anti-Cytokine Antibodies

Anti-IL-17 and isotype control antibodies were purchased from Affymetrix eBioscience (San Diego, CA), while anti-TNF-α and isotype control antibodies were purchased from R & D Systems (Minneapolis, MN). Antibodies were reconstituted in phosphate buffered saline (PBS) to achieve a concentration of 2.5 μg/ml. One day after infection, two groups of 5 infected mice were anesthetized with isoflurane and then injected in the hind paw with 2.5 μg of TNF-α antibody.

One of these two anti-TNF-α antibody-treated groups was simultaneously injected with 2.5 µg of anti-IL-17 antibody, while the other was simultaneously injected with an isotype control antibody. A third group of infected mice was injected with anti-IL-17 antibody and an isotype control antibody. An additional group of infected mice was injected with isotype control antibodies. These antibody treatments were administered for four consecutive days following the initial treatment.

IV. Assessment of Inflammation

Mice were anesthetized with isoflurane in a nose-to-mouth cup. Using a Vernier caliper, hind paws were measured for thickness and width, and the average of these two measurements was calculated as the "mean paw value". Swelling of the hind paws was measured prior to injections on the day of infection and at the time of sacrifice, 8 days post infection. The measurements of hind paw swelling on day 8 after infection were compared to the measurements of paw thickness prior to infection. Since the different groups of mice did not have equal values of initial paw thickness, the overall increase in swelling from the day of infection to day 8 after infection was also determined.

V. Cell Culture

Spleens were collected and used for *in vitro* culture studies to assess production of IL-17. Single-cell suspensions were obtained by passing the tissue through a 100-µm nylon cell strainer into Roswell Park Memorial Institute 1640 (RPMI) medium. 2×10^6 spleen cells in RPMI medium were incubated at 37°C in 5% CO_2,

with or without 10^5 *B. burgdorferi* 297 organisms in BSK medium. Supernatants were collected after 18 and 24 hours of incubation.

VI. *In Vitro* Administration of Anti-Cytokine Antibodies

In another study, wild-type and IL-10-deficient C57BL/6 mice were infected subcutaneously with 10^6 spirochetes (0.05 ml) in the hind paws. Non-infected control mice received an injection of BSK medium. Ten days after infection, mice were sacrificed, and single-cell suspensions were prepared from spleens, as described previously. 2 x 10^6 spleen cells in RPMI medium were incubated at 37°C in 5% CO_2, with or without 10^5 *B. burgdorferi* 297 organisms in BSK medium in a total volume of 1 ml. Additionally, cells were incubated with isotype-control antibodies or with anti-TNF-α antibodies, anti-IL-17 antibodies, or both anti-TNF-α and anti-IL-17 antibodies. Antibodies were added to wells in concentrations of 2.5 µg/ml, 5 µg/ml, or 10 µg/ml. Cells from non-infected mice were incubated with or without spirochetes and with or without 2.5 µg/ml anti-TNF-α antibodies, anti-IL-17 antibodies, or both anti-TNF-α and anti-IL-17 antibodies. Supernatants were collected after 24 hours of incubation.

VII. ELISA

Supernatants were analyzed for IL-17 production using the Mouse IL-17A (homodimer) ELISA Ready-SET-Go! Kit (Affymetrix eBioscience) according to the manufacturer's instructions. Samples were analyzed in duplicates and plates were read at 450nm.

VIII. Borreliacidal Antibody Assay

Serum was collected from mice for the assessment of borreliacidal antibodies. Equal amounts of serum were pooled within a group. Each serum sample was heat inactivated in a 56°C water bath for 30 minutes, diluted 1:20 in BSK medium, and then serially diluted to 1:40,960. Subsequently, 10^5 *B. burgdorferi* strain 297 organisms in 50 µL of BSK medium were added to the diluted wild-type serum, and 10^4 *B. burgdorferi* strain 297 organisms were added to the diluted IL-10-deficient serum. Then, 10 µL of guinea pig complement were added, and the samples were incubated for 16 hours at 32°C. After incubation, samples were examined for motile *B. burgdorferi* (at least 30 fields per sample) using darkfield microscopy. The borreliacidal antibody titer was the inverse of the first dilution that showed no live organisms. Controls included heat-inactivated serum from naïve mice incubated with *B. burgdorferi* and guinea pig complement, *B. burgdorferi* incubated with guinea pig complement, and *B. burgdorferi* incubated in BSK medium.

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. Paw inflammation after antibody treatment of *B. burgdorferi*-infected mice.

B. burgdorferi-infected wild-type and IL-10-deficient mice were treated for four consecutive days after infection with anti-TNF-α antibodies, anti-IL-17 antibodies, or both anti-TNF-α and anti-IL-17 antibodies. Paws were measured prior to infection and at the time of sacrifice (day 8 after infection). The hind paws of infected but untreated wild-type mice were larger on day 8 after infection compare to baseline levels. This increase was not significant, however (P = 0.07; data not shown). By contrast, the hind paws of infected wild-type mice treated with anti-TNF-α antibodies were significantly larger on day 8 after infection compared to baseline levels (P = 0.006; data not shown). In addition, no significant differences in hind paw swelling were observed between days 0 and 8 of infection in wild-type mice treated with anti-IL-17 antibodies, alone or also with anti-TNF-α antibodies (data not shown).

We then measured the overall change in hind paw swelling in infected wild-type mice by subtracting measurements at day 8 from baseline values. The change in paw swelling of infected, anti-TNF-α antibody-treated mice was slightly, but not significantly, greater than the overall change of swelling in infected but untreated mice (Fig. 1). Additionally, the overall change in swelling in anti-IL-17 antibody-treated mice was slightly, but insignificantly, less than the control group. However, the overall change in paw swelling in anti-TNF-α antibody-treated mice was significantly greater than the degree of swelling in anti-IL-17 antibody-treated mice

(P = 0.045). Administration of antibodies against both IL-17 and TNF-α did not significantly change the magnitude of paw swelling compared to infected mice treated with anti-IL-17 antibodies (Fig. 1).
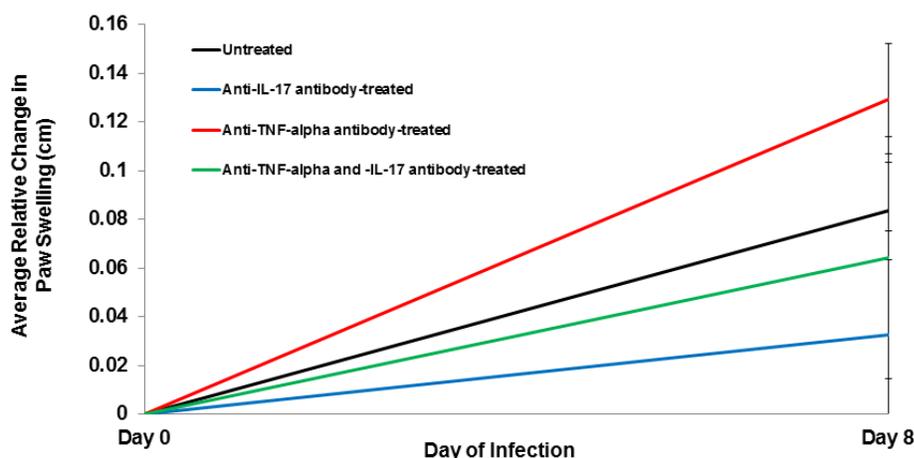

Figure 1. Overall change in paw swelling following treatment of *B. burgdorferi*-infected wild-type mice. Mice were infected with *B. burgdorferi* and then injected with antibodies to TNF-α, IL-17, both cytokines, or neither cytokine for four days. Treatment with TNF-α antibodies slightly, but not significantly, increased paw swelling in mice that were otherwise not treated or treated with anti-IL-17 antibodies. Values indicate means ± SEM. Groups consisted of 4-5 mice each.

In contrast to infected wild-type mice, all antibody-treated groups of *B. burgdorferi*-infected IL-10-deficient mice exhibited larger paws at day 8 after infection compared to baseline levels. At day 8 after infection, significantly larger paws were exhibited by infected IL-10-deficient mice that were untreated (P = 0.0008) or treated with anti-TNF-α (P = 1 x 10^{-6}) or anti-IL-17 (P = 6 x 10^{-5}) antibodies, compared to their baseline paw thickness (data not shown). Infected IL-10-deficient mice treated with both antibodies exhibited larger paws by day 8 after infection. However, this increase was not significant (P = 0.06; data not shown).

Compared to the overall change in paw swelling of infected but untreated IL-10-deficient mice, a significantly greater overall change in swelling was observed in

mice treated with anti-TNF-α (P = 0.0001) or anti-IL-17 (P = 0.01) antibodies (Fig.

2). However, the overall change in paw swelling in infected mice treated with anti-

IL-17 antibodies was not different than the change in paw swelling by mice treated
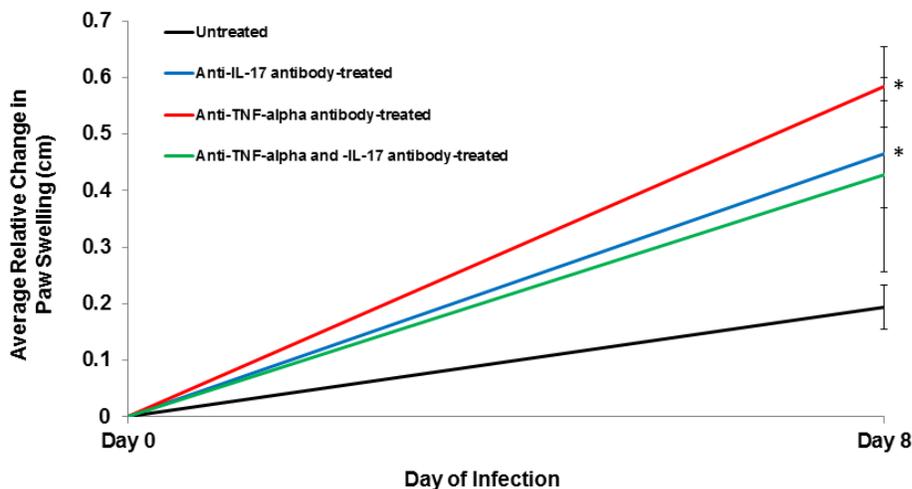
with antibodies to both IL-17 and TNF-α.

Figure 2. Overall change in paw swelling following treatment of *B. burgdorferi*-infected IL-10-deficient mice. Mice were infected with *B. burgdorferi* and then injected with antibodies to TNF-α, IL-17, both cytokines, or neither cytokine for four days. Treatment with anti-TNF-α or anti-IL-17 antibodies resulted in a significantly greater overall change in paw swelling compared to untreated controls. However, additional treatment with anti-TNF-α did not result in swelling changes in anti-IL-17 antibody-treated mice. Values indicate means ± SEM. Groups consisted of 4-5 mice each. *, P ≥ 0.05.

II. Effects of anti-cytokine antibodies on IL-17 production in *B. burgdorferi*-infected, wild-type mice.

To assess the involvement of TNF-α on production of IL-17 during *B. burgdorferi*

infection, C57BL/6 wild-type mice were treated with anti-TNF-α and/or anti-IL-17

antibodies for four consecutive days after infection. Eight days after infection,

spleen cells were obtained and incubated in the absence (Fig. 3) or presence (Fig.

4) of *B. burgdorferi* for 18 hours. When incubated for 18 hours, unstimulated spleen

cells from infected, anti-TNF-α antibody-treated wild-type mice produced

significantly greater amounts of IL-17 than unstimulated spleen cells from untreated, infected mice (56 pg/ml vs. 30 pg/ml; P = 0.05; Fig. 3). Surprisingly, unstimulated spleen cells from infected, anti-IL-17 antibody-treated wild-type mice produced significantly more IL-17 than unstimulated spleen cells from untreated, infected mice (68 pg/ml vs. 30 pg/ml; P = 0.005; Fig. 3). Unstimulated spleen cells from infected mice injected with antibodies against both IL-17 and TNF-α produced less IL-17 than unstimulated cells from infected, anti-IL-17 antibody treated mice (45 pg/ml vs. 68 pg/ml). However, this decrease in IL-17 production due to administration of anti-TNF-α antibodies did not achieve statistical significance (Fig. 3).

Figure 3. Production of IL-17 by unstimulated spleen cells from *B. burgdorferi*-infected, antibody (Ab)-treated wild-type mice after 18 hours of incubation. Eight days after infection, spleen cells from Ab-treated, wild-type mice were incubated for 18 hours in the absence of *B. burgdorferi*. Unstimulated cells from anti-TNF-α and anti-IL-17 Ab-treated mice produced significantly more IL-17 than unstimulated cells from untreated, infected mice. Additional treatment with anti-TNF-α Ab did not affect IL-17 production by cells of anti-IL-17 Ab-treated mice. Values indicate means ± SEM. Groups consisted of 4-5 mice each. *, P ≥ 0.05.

Additionally, cells from these groups of antibody-treated, *B. burgdorferi*-infected wild-type mice were stimulated *in vitro* with *B. burgdorferi* for 18 hours. Stimulation of spleen cells from infected, anti-TNF-α antibody-treated wild-type mice induced

slightly, but insignificantly, more IL-17 than stimulated spleen cells from infected but untreated mice (67 pg/ml vs. 45 pg/ml; P = 0.08; Fig. 4). *B. burgdorferi*-stimulated spleen cells harvested from infected, anti-IL-17 antibody-treated mice produced similar amounts of IL-17 than cells from infected but untreated mice (42 pg/ml vs. 45 pg/ml; Fig. 4). In addition, stimulated cells from infected mice treated with anti-IL-17 antibody produced similar amounts of IL-17 than cells from infected mice treated with both anti-IL-17 and anti-TNF-α antibodies (42 pg/ml vs. 48 pg/ml; Fig. 4).
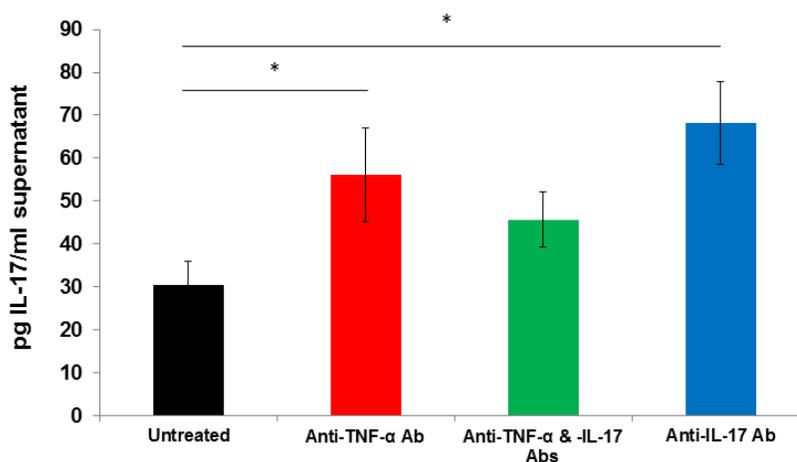

Figure 4. Production of IL-17 by *B. burgdorferi*-stimulated spleen cells from *B. burgdorferi*-infected, Ab-treated wild-type mice after 18 hours of incubation. Eight days after infection, spleen cells from Ab-treated, wild-type mice were incubated for 18 hours in the presence of *B. burgdorferi*. Stimulated cells from anti-TNF-α Ab-treated mice produced slightly, but not significantly, more IL-17 than stimulated cells from untreated, infected mice. Anti-TNF-α Ab treatment did not affect IL-17 production by cells from mice treated with anti-IL-17 Abs. Values indicate means ± SEM. Groups consisted of 4-5 mice each.

The effects of *in vitro* stimulation with *B. burgdorferi*, for 18 hours, on IL-17 production by these cells were then compared. Incubation of cells from infected, untreated wild-type mice with *B. burgdorferi* slightly, but insignificantly, increased production of IL-17 (Fig. 5). Similar findings were observed among stimulated cells obtained from infected mice that were treated with anti-TNF-α antibodies (Fig. 5).

By contrast, stimulation of cells from infected, anti-IL-17 antibody-treated mice significantly decreased IL-17 production (Fig. 5).
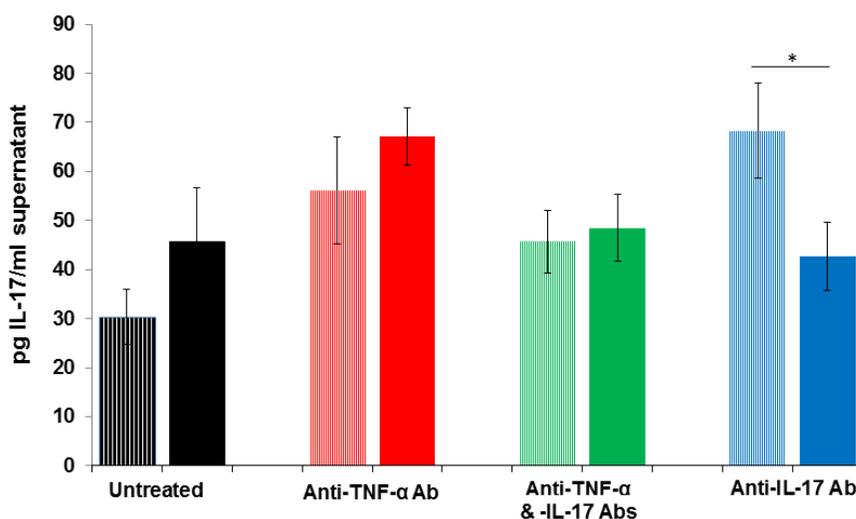
Figure 5. Effect of *in vitro* stimulation with *B. burgdorferi* for 18 hours on production of IL-17 from infected mice treated with anti-cytokine Abs. Stimulation with *B. burgdorferi* (darker bars) caused a slight, but insignificant, increase in IL-17 production by spleen cells obtained from infected mice that were untreated or treated with anti-TNF-α Abs. By contrast, stimulation caused a significant decrease in IL-17 production by cells obtained from infected mice treated with anti-IL-17 Abs. Values indicate means ± SEM. Groups consisted of 4-5 mice each. *, $P \geq 0.05$.

Spleen cells were also incubated with or without *B. burgdorferi* for 24 hours. Unstimulated cells from infected, anti-TNF-α antibody-treated mice produced slightly, but insignificantly, less IL-17 than spleen cells from infected but untreated wild-type mice (28 pg/ml vs. 44 pg/ml; $P = 0.13$; Fig. 6). However, unstimulated cells from infected, anti-IL-17 antibody-treated mice produced significantly less IL-17 than unstimulated cells from infected but untreated mice (21 pg/ml vs. 44 pg/ml; $P = 0.02$; Fig. 6). However, addition of anti-TNF-α antibody to infected, anti-IL-17 antibody-treated mice did not significantly affect IL-17 production by spleen cells incubated in the absence of *B. burgdorferi* for 24 hours (32 pg/ml vs. 21 pg/ml; Fig. 6).

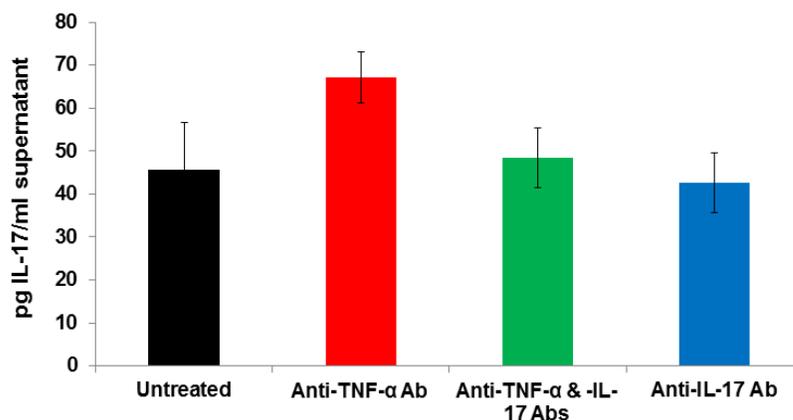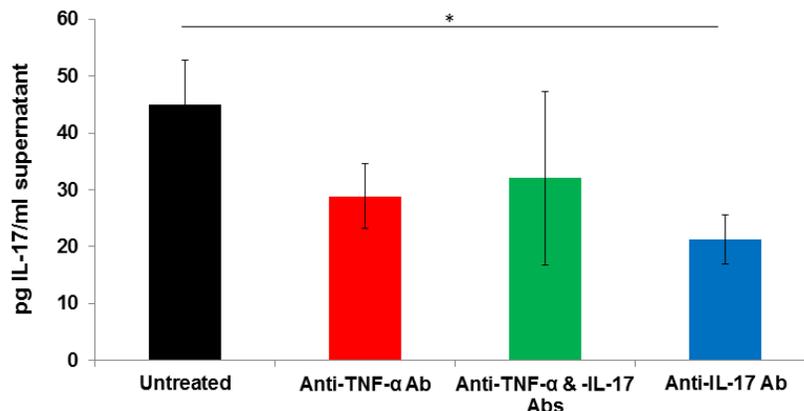**Figure 6. Production of IL-17 by unstimulated spleen cells from *B. burgdorferi*-infected, Ab-treated wild-type mice after 24 hours of incubation.** Eight days after infection, spleen cells from Ab-treated, wild-type mice were incubated for 24 hours in the absence of *B. burgdorferi*. Unstimulated cells from anti-TNF-α Ab-treated mice produced a similar amount of IL-17 than unstimulated cells from untreated, infected mice. By contrast, cells from anti-IL-17 Ab-treated mice produced significantly less IL-17 than cells from control mice. The level of IL-17 produced by cells from anti-IL-17 Ab-treated mice was comparable to that of cells from anti-IL-17 and -TNFα Ab-treated mice. Values indicate means ± SEM. Groups consisted of 4-5 mice each. *, $P \geq 0.05$.

After 24 hours of incubation, *B. burgdorferi*-stimulated spleen cells from infected, anti-TNF-α antibody-treated mice produced similar amounts of IL-17 as stimulated cells from infected but untreated mice (18 pg/ml vs. 26 pg/ml; $P = 0.36$; Fig. 7). Also, stimulated cells from infected, anti-IL-17 antibody-treated mice produced less, although statistically similar, amounts of IL-17 as stimulated cells from infected but untreated mice (9 pg/ml vs. 26 pg/ml; $P = 0.13$; Fig 7). Interestingly, stimulated cells harvested from infected mice treated with both anti-IL-17 and anti-TNF-α antibodies did not produce detectable levels of IL-17. However, this difference was not statistically different from stimulated spleen cells from infected, anti-IL-17 antibody-treated mice (Fig. 7).

Figure 7. Production of IL-17 by *B. burgdorferi*-stimulated spleen cells from *B. burgdorferi*-infected, Ab-treated wild-type mice after 24 hours of incubation. Eight days after infection, spleen cells from Ab-treated, wild-type mice were incubated for 24 hours in the presence of *B. burgdorferi*. Stimulated cells from anti-TNF-α and anti-IL-17 Ab-treated mice produced a similar amount of IL-17 as unstimulated cells from untreated, infected mice. By contrast, cells from dual-Ab-treated mice produced undetectable amounts of IL-17. While this was significantly less compared to cells of untreated or anti-TNF-α Ab-treated mice, it was not significantly different compared to cells of IL-17 Ab-treated mice. Values indicate means ± SEM. Groups consisted of 4-5 mice each. *, $P \geq 0.05$.

The effects of *in vitro* stimulation with *B. burgdorferi*, for 24 hours, on IL-17 production by these cells were then compared. Incubation of cells from infected, untreated wild-type mice with *B. burgdorferi* decreased production of IL-17 by cells from all groups of treated mice (Fig. 8). However, a statistically significant decrease in IL-17 production was observed following stimulation of cells only from infected mice treated with anti-TNF-α and anti-IL-17 antibodies. (Fig. 8).
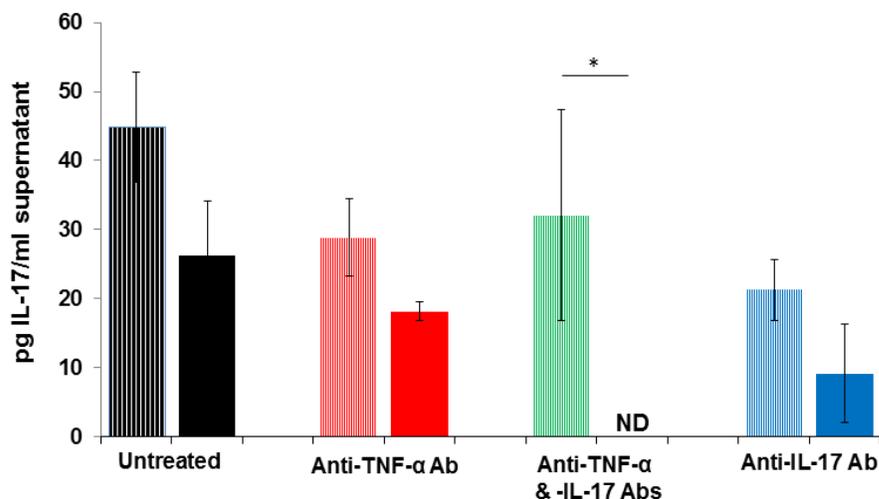
Figure 8. Effect of *in vitro* stimulation with *B. burgdorferi* for 24 hours on production of IL-17 from infected mice treated with anti-cytokine Abs. Stimulation with *B. burgdorferi* (darker bars) for 24 hours caused a decrease in IL-17 production by spleen cells obtained from infected mice treated with anti-cytokine Abs. A statistically significant decrease in IL-17 production was observed following stimulation of cells from dual-Ab-treated mice. Values indicate means ± SEM. Groups consisted of 4-5 mice each. *, $P \geq 0.05$.

III. Effects of anti-cytokine antibodies on IL-17 production in *B. burgdorferi*-infected, IL-10-deficient mice.

The effect of TNF-α on IL-17 production following *B. burgdorferi* infection in the absence of IL-10 was then determined. *B. burgdorferi*-infected, IL-10-deficient mice were treated for four consecutive days with anti-TNF-α antibody and/or anti-IL-17 antibodies. Spleen cells were collected eight days after infection and incubated with or without *B. burgdorferi*. Eighteen hours after incubation, unstimulated spleen cells obtained from all groups of mice produced low or undetectable levels of IL-17 (data not shown). By contrast, stimulated spleen cells from anti-TNF-α antibody-treated mice produced significantly greater amounts of IL-17 than stimulated cells from non-treated mice after 18 hours of stimulation with *B. burgdorferi* (27 pg/ml vs. 0.39 pg/ml; P = 0.02; Fig. 9). Stimulated cells from

infected, IL-10-deficient mice treated with anti-IL-17 antibodies produced an undetectable amount of IL-17 after 18 hours of incubation. Additional treatment of these mice with anti-TNF-α antibodies did not lead to a change in IL-17 production following stimulation for 18 hours (Fig. 9).
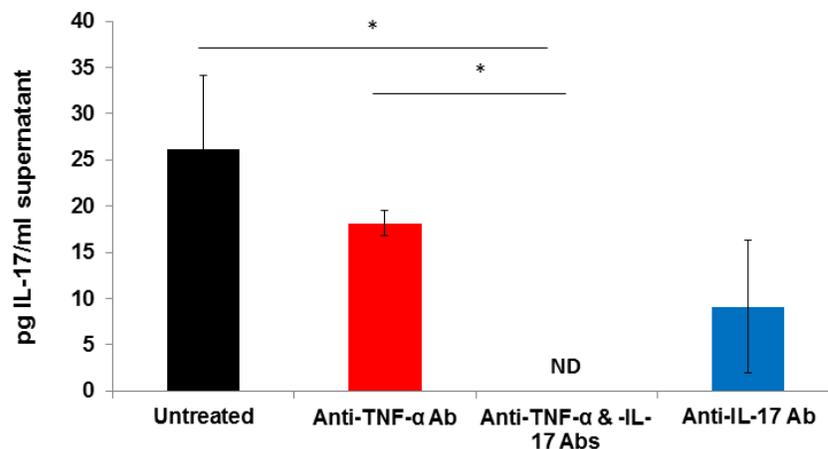

Figure 9. Production of IL-17 by *B. burgdorferi*-stimulated spleen cells from *B. burgdorferi*-infected, Ab-treated IL-10-deficient mice after 18 hours of incubation. Eight days after infection, spleen cells from Ab-treated, IL-10-deficient mice were incubated for 18 hours in the presence of *B. burgdorferi*. Stimulated cells from anti-TNF-α Ab-treated mice produced significantly, more IL-17 than stimulated cells from untreated, infected mice. Values indicate means ± SEM. Groups consisted of 4-5 mice each. *, $P \geq 0.05$.

Cells from these mice were also incubated in the presence or absence of *B. burgdorferi* for 24 hours (Fig. 10). Unstimulated cells from infected, IL-10-deficient mice treated with anti-TNF-α antibody produced IL-17 after 24 hours of incubation. However, these levels were equal to IL-17 production by unstimulated cells from untreated mice (27 pg/ml vs. 25 pg/ml; $P = 0.82$; Fig. 10). Similarly, unstimulated cells collected from infected, anti-IL-17 antibody-treated mice

Figure 10. Production of IL-17 by unstimulated spleen cells from *B. burgdorferi*-infected, Ab-treated IL-10-deficient mice after 24 hours of incubation. Eight days after infection, spleen cells from Ab-treated, IL-10-deficient mice were incubated for 24 hours in the absence of *B. burgdorferi*. No differences in IL-17 production were observed among cells from any of the Ab-treated mice. Values indicate means ± SEM. Groups consisted of 4-5 mice each.

produced a similar amount of IL-17 as unstimulated cells from infected, untreated mice (24 pg/ml vs. 25 pg/ml; $P = 0.92$; Fig. 10). Additionally, treatment of infected mice with antibodies against both IL-17 and TNF-α did not affect the production of IL-17 from stimulated spleen cells compared to infected, anti-IL-17 antibody-treated mice (23 pg/ml vs. 24 pg/ml; $P = 0.87$; Fig. 10). Overall, unstimulated cells from infected, IL-10 deficient mice produced consistent levels of IL-17 between all four different treatment groups after incubation for 24 hours.

Additionally, production of IL-17 was not significantly different between spleen cells from the untreated and single-antibody-treated groups of infected, IL-10-deficient mice after incubation with *B. burgdorferi* for 24 hours (Fig. 11).
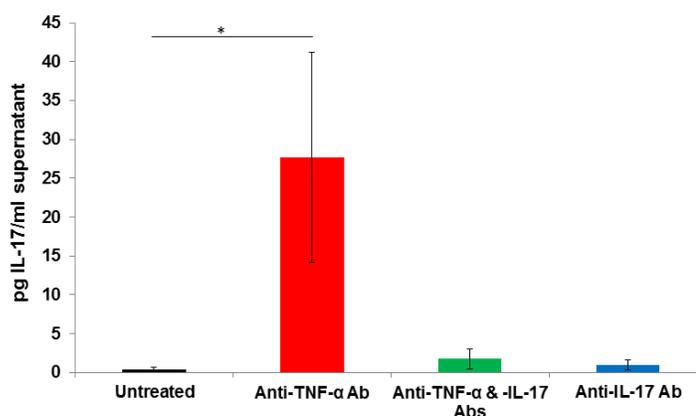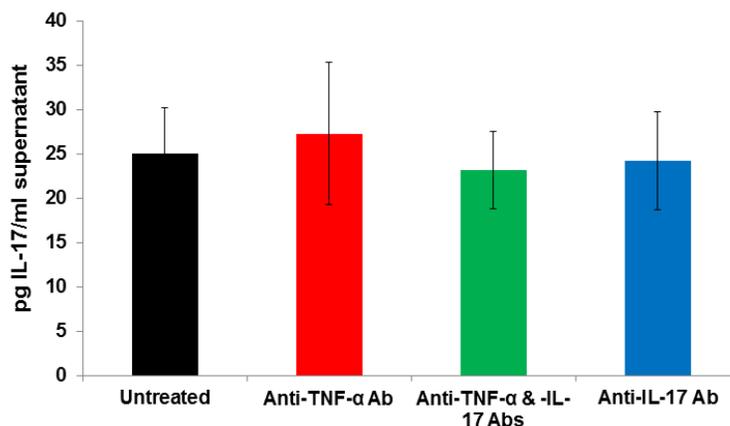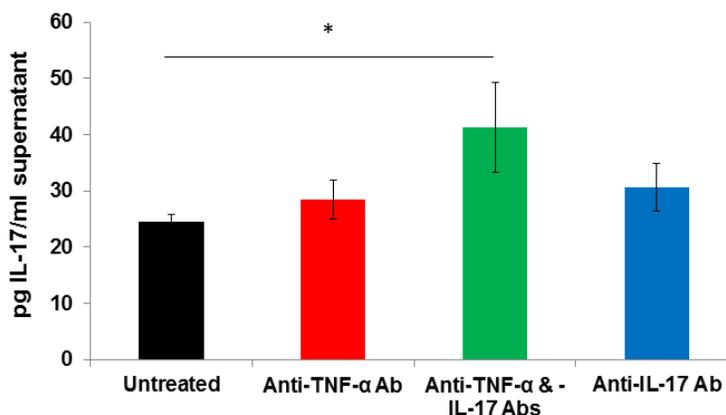
Figure 11. Production of IL-17 by *B. burgdorferi*-stimulated spleen cells from *B. burgdorferi*-infected, Ab-treated IL-10-deficient mice after 24 hours of incubation. Eight days after infection, spleen cells from Ab-treated, IL-10-deficient mice were incubated for 24 hours in the presence of *B. burgdorferi*. Cells from anti-TNF-α Ab-treated mice produced a similar amount of IL-17 as cells from untreated mice. Although the amount of IL-17 produced by cells of dual-Ab-treated mice was significantly greater than controls, it was similar to that of cells from anti-IL-17 Ab-treated mice. Values indicate means ± SEM. Groups consisted of 4-5 mice each. *, P ≥ 0.05.

Stimulated spleen cells that were obtained from *B. burgdorferi*-infected mice that were treated with anti-TNF-α antibodies produced similar amounts of IL-17 as did cells from infected, untreated mice (25 pg/ml vs. 23 pg/ml; Fig. 11). Likewise, stimulated cells from infected, IL-10-deficient mice treated with anti-IL-17 antibody produced similar levels of IL-17 as spleen cells from infected, untreated mice (Fig. 11). Injection of anti-TNF-α and anti-IL-17 antibodies to infected IL-10-deficient mice did not significantly alter the production of IL-17 by spleen cells compared to that of infected mice treated with only anti-IL-17 antibody (Fig. 11).

IV*. Production of IL-17 by wild-type cells following *in vitro* antibody treatment.

Spleen cells were harvested from *B. burgdorferi*-infected wild-type mice 10 days after infection. The cells were incubated with the spirochete and with antibodies to

TNF-α and/or IL-17 for 24 hours to provide information about the effect of TNF-α on IL-17 production without the variables associated with treatment *in vivo*. We first treated stimulated spleen cells obtained from wild-type, *B. burgdorferi*-infected mice with 2.5 µg of antibodies for 24 hours. Cells that were incubated with 2.5 µg of anti-TNF-α antibody produced significantly more IL-17 than non-treated cells (32 pg/ml vs. 17 pg/ml; P = 0.037; Fig. 12). In contrast, cells incubated with 2.5 µg of anti-IL-17 antibody produced a similar amount of IL-17 as untreated cells (Fig. 12). Furthermore, treatment with both anti-IL-17 and anti-TNF-α antibodies did not affect production of IL-17 compared to cells treated with anti-IL-17 antibody alone (Fig. 12).

Figure 12. Effect of 2.5 µg *in vitro* anti-cytokine Abs on production of IL-17 in wild-type cells. Wild-type mice were infected with *B. burgdorferi*. Eight days later, spleen cells were incubated with 2.5 µg of anti-TNF-α Ab, anti-IL-17 Ab, or both anti-TNF-α and antil-IL-17 Ab for 24 hours. Cells treated with anti-TNF-α Ab produced significantly more IL-17 than untreated cells. However, anti-TNF-α Ab treatment did not affect IL-17 production by cells treated with anti-IL-17 Ab. Values indicate means ± SEM. Groups consisted of 4-5 mice each. *, P ≥ 0.05.

We then treated stimulated spleen cells from wild-type *B. burgdorferi*-infected mice with a greater (5 µg) concentration of antibodies and measured the production of IL-17 (Fig. 13). Treatment with anti-TNF-α antibody did not affect IL-17 production

compared to non-treated cells (Fig. 13). Similarly, treatment of cells with anti-IL-17 antibody did not change IL-17 levels in comparison to non-treated cells (Fig. 13). However, cells that were treated with both anti-IL-17 and anti-TNF-α antibodies produced significantly greater levels of IL-17 than cells treated with just anti-17 antibody (35 pg/ml vs 25 pg/ml; P = 0.005; Fig. 13).

Figure 13. Effect of 5 μg *in vitro* anti-cytokine Abs on production of IL-17 in wild-type cells cells. Wild-type mice were infected with *B. burgdorferi*. Eight days later, spleen cells were incubated with 5 μg of anti-TNF-α Ab, anti-IL-17 Ab, or both Abs for 24 hours. Anti-TNF-α Ab treatment of IL-17 Ab-treated cells caused a significant increase in IL-17 production compared to cells treated with anti-IL-17 Ab alone. Values indicate means ± SEM. Groups consisted of 4-5 mice each. *, P ≥ 0.05.

In addition, a concentration of 10 μg of each antibody was administered to the spleen cells harvested from infected wild-type mice. However, cells from all four groups produced similar amounts of IL-17 (Fig. 14). Spleen cells incubated with anti-TNF-α antibody did not affect production of IL-17 compared to untreated cells. Similarly, anti-IL-17 antibody-treated cells produced similar amounts of IL-17 as non-treated cells. Additionally, anti-IL-17 antibody-treated cells produced similar amounts of IL-17 as cells treated with antibodies against both IL-17 and TNF-α (Fig. 14).
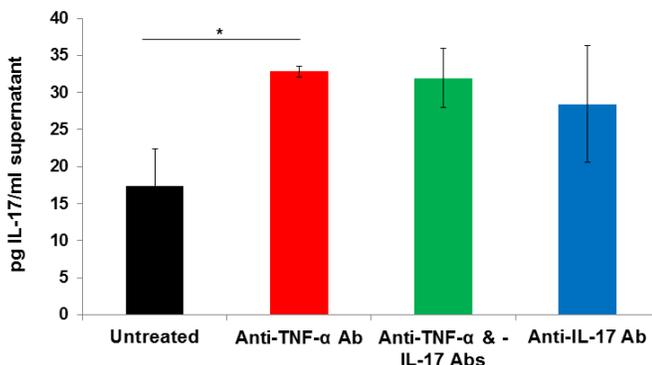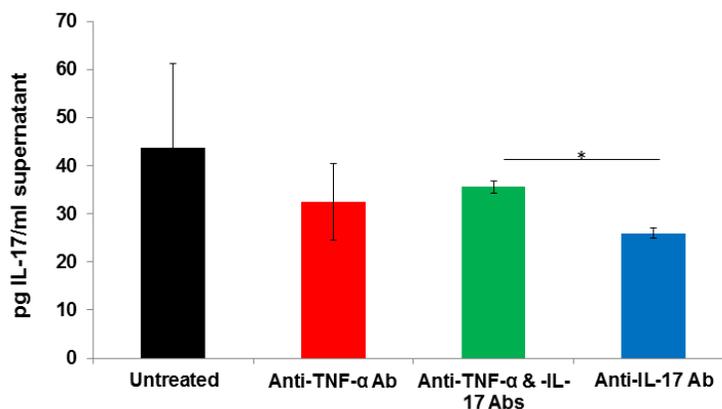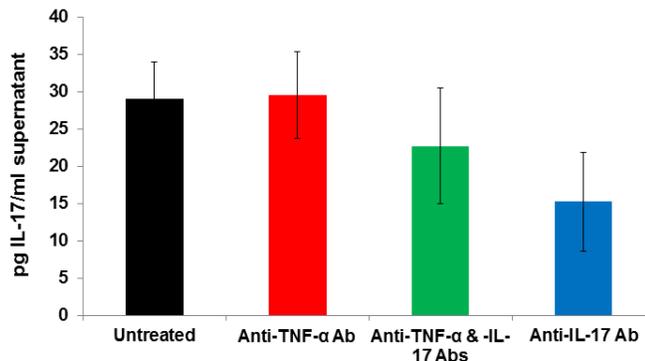
Figure 14. Effect of 10 µg *in vitro* anti-cytokine Abs on production of IL-17 in wild-type cells. Wild-type mice were infected with *B. burgdorferi*. Eight days later, spleen cells were incubated with 10 µg of anti-TNF-α Ab, anti-IL-17 Ab, or both anti-TNF-α and antil-IL-17 Ab for 24 hours. Anti-TNF-α Ab treatment had no effect on IL-17 production at this concentration. Values indicate means ± SEM. Groups consisted of 4-5 mice each. *, P ≥ 0.05.

V. Production of IL-17 by IL-10-deficient cells following *in vitro* antibody treatment.

Spleen cells were harvested from *B. burgdorferi*-infected, IL-10-deficient mice 10 days after infection. The cells were incubated with the spirochete and with antibodies to TNF-α and/or IL-17 for 24 hours to provide information about the effect of TNF-α on IL-17 production without the variables associated with treatment *in vivo*. Spleen cells harvested from infected IL-10-deficient mice and incubated with 2.5 µg anti-TNF-α antibody produced a similar amount of IL-17 as untreated cells (84 pg/ml vs. 88 pg/ml; Fig. 15). By contrast, spleen cells treated with 2.5 µg of anti-IL-17 antibody produced a significantly smaller amount of IL-17 than non-treated cells (43 pg/ml vs. 84 pg/ml; $P = 0.0004$; Fig. 15). Contrary to that which was observed in wild-type cells, treatment with both antibodies led to an even greater decrease in IL-17 production (29 pg/ml). However, this decrease was not statistically different than IL-17 production from anti-IL-17 antibody-treated cells ($P = 0.12$; Fig. 15).

Figure 15. Effect of 2.5 μg *in vitro* anti-cytokine Abs on production of IL-17 in IL-10-deficient cells. IL-10-deficient mice were infected with *B. burgdorferi*. Eight days later, spleen cells were incubated with 2.5 μg of anti-TNF-α Ab, anti-IL-17 Ab, or both anti-TNF-α and anti-IL-17 Ab for 24 hours. Cells treated with anti-IL-17 Ab produced significantly less IL-17 than untreated cells, but cells treated with anti-TNF-α Ab alone had no effect. Anti-TNF-α Ab treatment did not affect IL-17 production by cells also treated with anti-IL-17 Ab. Values indicate means ± SEM. Groups consisted of 4-5 mice each. *, $P \geq 0.05$.

We then treated stimulated spleen cells from IL-10-deficient, *B. burgdorferi*-infected mice with a greater (5 μg) concentration of antibodies and measured the production of IL-17 (Fig. 16). Spleen cells that were harvested from infected mice and treated with anti-TNF-α antibody produced similar amounts of IL-17 as untreated cells from infected mice (Fig. 16). As expected, cells treated with anti-IL-17 antibody produced significantly less IL-17 than non-treated cells (30 pg/ml vs. 73 pg/ml; $P = 0.009$; Fig. 16). However, addition of anti-TNF-α antibodies to cells treated with anti-IL-17 antibodies did not affect IL-17 production (28 pg/ml vs. 30 pg/ml; $P = 0.90$; Fig. 16).

Figure 16. Effect of 5 µg *in vitro* anti-cytokine Abs on production of IL-17 in IL-10-deficient cells. IL-10-deficient mice were infected with *B. burgdorferi*. Eight days later, spleen cells were incubated with 5 µg of anti-TNF-α Ab, anti-IL-17 Ab, or both anti-TNF-α and anti-IL-17 Ab for 24 hours. Cells treated with anti-IL-17 Ab, with or without anti-TNF-α Ab, produced significantly less IL-17 than untreated cells. Cells treated with anti-TNF-α Ab alone had no effect on IL-17 production. Values indicate means ± SEM. Groups consisted of 4-5 mice each. *, P ≥ 0.05.

In addition, 10 µg of antibodies were administered to spleen cells collected from *B. burgdorferi*-infected, IL-10-deficient mice (Fig. 17). Spleen cells incubated with anti-TNF-α antibody produced similar amounts of IL-17 as non-treated cells (P = 0.70; Fig. 17). As expected, treatment of cells with anti-IL-17 antibody significantly decreased IL-17 production compared to untreated cells (24 pg/ml vs. 69 pg/ml; P = 0.002; Fig. 17). However, the production of IL-17 by anti-IL-17 antibody-treated cells was similar to the production of IL-17 by cells treated with both anti-TNF-α and anti-IL-17 antibodies (P = 0.50).
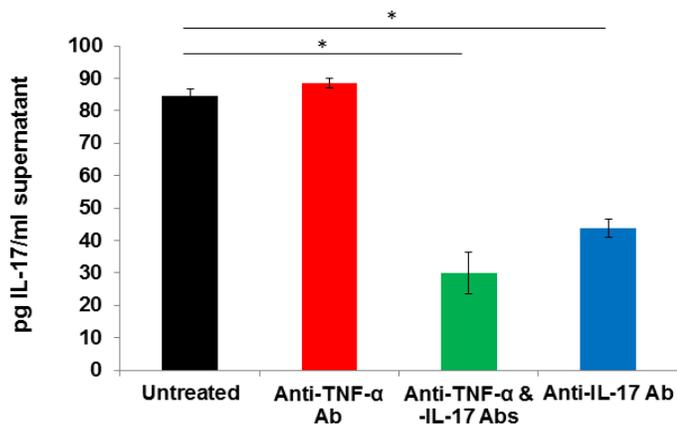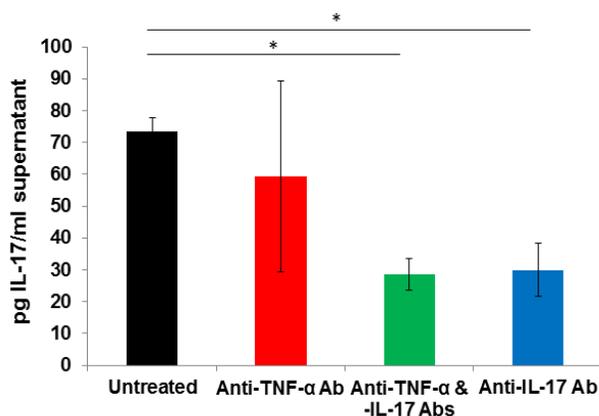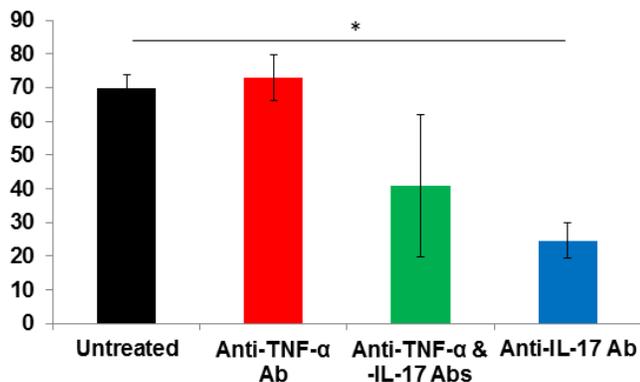
Figure 17. Effect of 10 µg *in vitro* anti-cytokine Abs on production of IL-17 in IL-10-deficient cells. IL-10-deficient mice were infected with *B. burgdorferi*. Eight days later, spleen cells were incubated with 10 µg of anti-TNF-α Ab, anti-IL-17 Ab, or both anti-TNF-α and antil-IL-17 Ab for 24 hours. Cells treated with anti-IL-17 Ab produced significantly less IL-17 than untreated cells. Cells treated with anti-TNF-α Ab alone, or in addition to anti-IL-17 Ab, had no effect on IL-17 production. Values indicate means ± SEM. Groups consisted of 4-5 mice each. *, P ≥ 0.05.

Overall, spleen cells from *B. burgdorferi*-infected, IL-10-deficient mice produced an increased amount of IL-17 than spleen cells from infected wild-type mice. Treatment of spleen cells from infected, IL-10 deficient mice with 2.5 µg or 10 µg of anti-TNF-α antibody induced a significantly greater amount of IL-17 than treatment of corresponding wild-type cells (P = 5 x 10^{-6} and 0.009; Fig. 18 and data not shown, respectively). However, these increases were also observed among untreated cells (Fig. 18 and data not shown). In contrast, no differences were observed in production of IL-17 by wild-type and IL-10-deficient cells after incubation with anti-IL-17 antibodies or with both anti-IL-17 and anti-TNF-α antibodies (Fig. 18 and data not shown). No changes in IL-17 production were observed between wild-type and IL-10-deficient cells treated with 5 µg of antibody (data not shown).
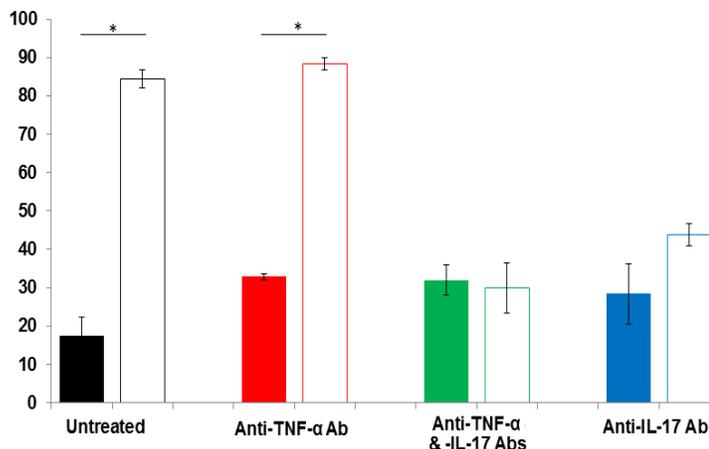
Figure 18. Effect of IL-10 deficiency on IL-17 production. Untreated or anti-TNF-α Ab-treated cells from IL-10-deficient mice (open bars) produce significantly more IL-17 than wild-type counterparts (closed bars) when incubated with 2.5 µg Ab. No difference in IL-17 production was observed when cultures contained anti-IL-17 antibody. Values indicate means ± SEM. Groups consisted of 4-5 mice each. *, $P \geq 0.05$.

Interestingly, within all four treated groups, there were no significant differences in production of IL-17 with an increased amount of their respective antibodies (data not shown). Surprisingly, treatment of spleen cells with increased concentrations of anti-IL-17 antibodies did not significantly reduce IL-17 levels in either IL-10 deficient or wild-type mice, although trends toward less production were observed (data not shown). Spleen cells from infected wild-type or IL-10 deficient mice, when incubated with anti-TNF-α antibodies, exhibited no dose-response in IL-17 production, even when anti-IL-17 antibodies were also added (data not shown).

VI. Borreliacidal antibody titers.

The purpose of this experiment was to evaluate the killing activity of serum antibodies directed against *B. burgdorferi* in wild-type and IL-10-deficient mice treated with anti-TNF-α and/or anti-IL-17 antibodies. A one-fold greater difference was noted in borreliacidal antibody titers of infected, anti-TNF-α antibody-treated

mice than infected, untreated mice (Fig. 19A). Sera from infected mice treated with both anti-TNF-α and anti-IL-17 antibodies possessed a one-fold greater titer of borreliacidal antibodies than infected mice that received antibodies to IL-17 alone (Fig. 19A).
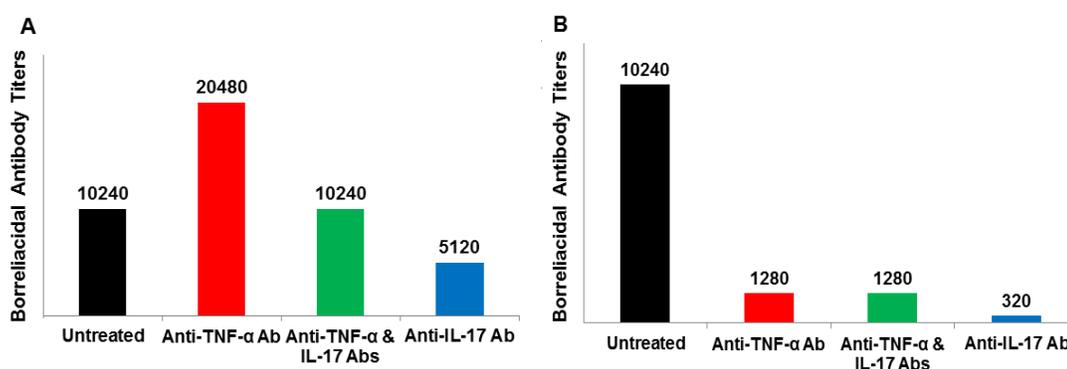
Figure 19. Borreliacidal antibody production. Borreliacidal antibody titers from serum of wild-type (A) and IL-10-deficient (B) mice. Mice were infected with *B. burgdorferi* and were untreated or treated with anti-TNF-α Ab, anti-IL-17 Ab, or both anti-TNF-α and anti-IL-17 Abs for 4 days following infection. Sera were collected on day 8 after infection. Groups consisted of 4-5 mice each.

By contrast, serum from infected, anti-TNF-α antibody-treated, IL-10-deficient mice had a three-fold less borreliacidal antibody titer than serum from infected, untreated IL-10-deficient mice (Fig. 19B). In addition, the sera from infected IL-10-deficient mice treated with both anti-IL-17 and anti-TNF-α antibodies exhibited a two-fold higher borreliacidal antibody titer than the sera from mice treated with anti-IL-17 antibody alone (Fig. 19B).

CHAPTER 4: DISCUSSION

This thesis describes the effect of TNF-α on IL-17 production during the immune response to *B. burgdorferi* infection in wild-type and IL-10 deficient C57BL/6 mice. The central hypothesis was that TNF-α will regulate the immune response to *B. burgdorferi* infection by decreasing IL-17 production. To analyze the effect of TNF-α on IL-17 in a mouse infection model of Lyme arthritis, *B. burgdorferi*-infected mice were treated with anti-TNF-α antibodies to quantify *in vitro* IL-17 production, paw swelling, and borreliacidal antibody production. Additionally, isolated spleen cells from infected mice were treated *in vitro* with anti-TNF-α antibodies to measure the amount of IL-17 produced. The following two specific aims were tested: (1) determine the effect of TNF-α on IL-17 in the immune response to *B. burgdorferi* infection and (2) determine the effects of TNF-α on the borreliacidal antibody response following infection with *B. burgdorferi.*

During the immune response to *B. burgdorferi,* macrophages and neutrophils are activated to produce inflammatory cytokines including IL-1β, TNF-α, and IL-6 that, in turn, activate an inflammatory cascade with an aim to kill the pathogen. This interaction further yields production of IL-12 that is necessary for survival of Th1 cells and IL-6 that is needed for Th17 cells. T cells then continue to perpetuate this positive feedback loop by releasing TNF-α, IL-17, and other cytokines. Research suggests that IL-17 regulates inflammation by acting on TNF-α (Fossiez *et al.*, 1996; Jovanovic *et al.*, 1998; Zwerina *et al.*, 2012). While these studies indicate that IL-17 affects TNF-α production, other research demonstrates that, actually,

TNF-α mediates IL-17 production (Iwamoto *et al.*, 2007, Chen *et al.*, 2001). What is known is that both TNF-α (Yssel et al., 1991) and IL-17 (Codolo *et al.*, 2008, 2013) are produced in response to *B. burgdorferi* infection in humans, and that IL-17 contributes to Lyme arthritis development in mice (Burchill *et al.* 2003; Hansen *et al.* 2013). Surprisingly, treatment with anti-TNF-α antibodies to IFN-γ-deficient, *Borrelia*-vaccinated and -infected mice aggravated arthritis in mice (Christopherson *et al.*, 2003). Therefore, we set out to determine if TNF-α would have same protective role in a standard infection model of Lyme arthritis and, importantly, if TNF-α would induce IL-17 production, leading to inflammation.

In this study, we show that TNF-α plays a protective role during inflammation elicited by *B. burgdorferi* infection in C57BL/6 mice. Additionally, TNF-α affects IL-17 production. These findings were evident when anti-TNF-α antibodies were injected into *B. burgdorferi*-infected wild-type mice for four consecutive days, and the mice presented with increased (although not significant statistically) swelling of the hind paws compared to untreated control mice (data not shown and Fig. 1). Furthermore, IL-17 production significantly increased in infected mice when they were treated with anti-TNF-α antibodies (Fig. 3). Additionally, greater amounts of IL-17 were measured from spleen cells that were obtained from infected, wild-type mice and that were treated *in vitro* with anti-TNF-α antibodies (Fig. 12). Importantly, compared to anti-TNF-α antibody-treated mice, both paw swelling and IL-17 production were reduced when mice were additionally injected with anti-IL-17 antibodies (Figs. 1 and 3).

These results that TNF-α protects against inflammation early after infection with *B. burgdorferi* support and extend the findings by Christopherson, *et al.* (2003). These researchers showed that *Borrelia*-vaccinated and -infected, IFN-γ-deficient, C57BL/6 mice treated with anti-TNF-α antibodies had increased paw swelling and severe arthritis. They also showed that paws from infected mice injected with recombinant TNF-α displayed decreased swelling and amelioration of arthritis (Christopherson *et al.*, 2003). It is important to note that they obtained these results using the *Borrelia*-vaccination and -challenge model arthritis. In contrast, we used a standard infection model in C57BL/6 mice, which are well known to be resistant to developing Lyme arthritis. It is possible that one mechanism for this resistance is that TNF-α is produced after infection with *B. burgdorferi* and acts in part to reduce the IL-17 response. In addition, we saw an even greater effect of anti-TNF-α antibody in promoting paw inflammation in IL-10-deficient mice. This is important because this mouse model has recently been suggested as a good model for the study of Lyme disease in humans (Sonderegger *et al.*, 2012). Furthermore, Yrjänainen *et al.* (2007) demonstrated that treatment of infected mice with anti-TNF-α antibodies and ceftriaxone actually increases the number of spirochetes in tissues. It is reasonable to assume that the increased bacterial load could lead to increased paw swelling and arthritis development. In addition, although we did not assess pathology here, the increased paw swelling and IL-17 levels in IL-10-deficient mice, compared to wild-type mice, are consistent with moderate-to-severe arthritis (Hansen *et al.*, 2013).

What role does TNF-α play during inflammation? The protective role of TNF-α is not uncommon during infections elicited by pathogens, including *B. burgdorferi* (Christopherson *et al.*, 2003), *Mycobacterium bovis* (Olleros *et al.*, 2002) and *Listeria* (Grivennikov *et al.*, 2005). In studies of RA, some patients that receive anti-TNF-α therapy actually have increased Th17 cytokines (Chen *et al.*, 2011). Similarly, certain patients that are treated for RA with TNF-α inhibitors may develop psoriasis skin lesions (Kary *et al.*, 2006, De Gannes *et al.*, 2007). Ma *et al.* (2010) analyzed the mechanism of action in development of psoriasis with treatment of TNF-α antagonists in an animal model of disease, and found that inhibition of TNF-α increased IL-17 and interleukin-22 compared to a control treatment. Therefore, production of the pro-inflammatory cytokine TNF-α may provide a counter-intuitive role in diseases such as early Lyme borreliosis. It is possible that early production of TNF-α acts to limit the growth and spread of *B. burgdorferi* for a period of time, with the result being that the IL-17 response does not develop immediately.

However, in contrast, other studies showed a decrease in the amount of IL-17 following inhibition of TNF-α. Moran *et al.* (2009) reported that treatment with anti-TNF-α antibodies significantly decreased production of IL-17 in RA patients classified as responders to TNF-α therapy. Chen *et al.* (2011) also reported a decrease in Th17 cytokines from responders treated with TNF-α inhibitors. Likewise, administration of etanercept (a soluble TNF-α receptor approved for treatment of RA) to psoriasis patients decreased the number of Th17 cells (Zaba

et al., 2007). Furthermore, uveitis treatment with anti-TNF-α antibodies affected CD4 T cells to significantly decrease IL-17 production (Sugita *et al.*, 2012). Therefore, the effect of TNF-α on IL-17 production likely varies, depending on the disease model studied. It is possible that infection-induced disease may cause a different effect on IL-17 than that which is seen during autoimmune diseases.

The varying effect of TNF-α on IL-17 production can be extended to include regulatory T (Treg) cells, which are widely known to be related in development to Th17 cells. While treatment with anti-TNF-α antibody in an animal model of psoriasis lowered the frequency of Treg cells (Ma *et al.*, 2010) in humans with RA, it was also shown that treatment with adalimumab (a monoclonal TNF-α antibody approved for treatment of RA) increased Treg cells by decreasing Th17 cells through inhibition of IL-6 (McGovern *et al.*, 2012). However, the Treg cell increase was not observed in patients taking etanercept. The correlation between Treg populations in adalimumab- and etanercept-treated groups was also assessed in the risk for development of tuberculosis. Patients treated with adalimumab showed 7-17 times greater risk of developing tuberculosis infection than patients treated with etanercept, which the authors suggest is possible reactivation of latent infection (Tubach *et al.*, 2009). Since Lyme disease is caused by a bacterium that also may cause chronic disease, use of anti-TNF-α antibodies needs to be treated with caution.

In our study, eight days after *B. burgdorferi*-infection, no differences in relative paw swelling were measured between untreated wild-type mice and mice injected with anti-IL-17 antibodies for four consecutive days (Fig. 1). These results seem to suggest that IL-17 is not involved in the immune response during *B. burgdorferi* infection, contrary to numerous findings (Burchill *et al.* 2003; Nardelli, *et al.* 2004, 2005, 2008; Kotloski *et al.* 2008; Hansen *et al.*, 2013). After 18 hours of incubation, there was an unexpected increase in IL-17 production from spleen cells collected from infected, anti-IL-17 antibody treated mice (Fig. 3). However, as expected, 24 hours after incubation, cells harvested from infected, anti-IL-17 antibody-treated mice produced significantly less IL-17 than cells from infected, untreated controls (Figs. 6). In addition, *in vitro* treatments with anti-IL-17 antibody slightly decreased IL-17 production by wild-type cells when compared to the amounts of IL-17 from spleen cells harvested from infected but untreated mice (Figs. 14 and 15). A possible explanation why we did not see a decrease in paw swelling with anti-IL-17 antibody treatment is that we treated mice for four days and assessed swelling at only two time points, before infection and 8 days after infection. Other studies treated infected wild-type C57BL/6 mice with anti-IL-17 antibodies for 7 days and measured paw swelling every other day. For example, in a previous study (Hansen *et al.*, 2013), treatment with anti-IL-17 antibodies was administered for 7 days. Statistically less swelling was shown in *B. burgdorferi*-infected, anti-IL-17 antibody-treated mice than infected, untreated wild-type mice at day 6 after infection. However, as we show here, this difference was not seen at day 8 (Hansen *et al.*

2013). Therefore, it is likely that IL-17 is involved in immune response during *B. burgdorferi* infection, even though no significance is demonstrated in this study.

How does IL-17 play a role in joint destruction? Under normal conditions, bone structure depends on the continuous balance of osteoblasts and osteoclasts for bone remodeling and resorption. During arthritic conditions, osteoclastogenesis is increased, while production of osteoblasts is decreased, leading to bone erosion. IL-17 has been implicated in promoting osteoclastogenesis (Zwerina *et al.*, 2012) through increased production of IL-1β. Additionally, IL-17 is a powerful inducer of inflammatory cell migration and invasion of the joint tissue (Hot *et al.*, 2012). Furthermore, IL-17 acts to stop collagen synthesis of bones and synovial tissue, along with inhibiting prostaglandin synthesis by cartilage cells (Chaubad *et al.*, 2001). All of these studies point to the direct involvement of IL-17 in bone destruction in arthritis. The increase in IL-17 following administration of antibodies to TNF-α after *B. burgdorferi* infection provide a possible mechanism for the protection given by TNF-α. While stimulation of PBMCs from healthy patients with TNF-α did not stimulate production of IL-17 (Ziolkowska *et al.*, 2000), we found that generally, anti-TNF-α antibody treatment increases IL-17 production in wild-type mice (Figs. 3, 4, and 12). Overall, the amounts of IL-17 in three different treatment groups of infected mice were higher for an incubation period of 18 hours than 24 hours (Figs. 3, 4, 6, and 7). Interestingly, treatment of mice with both anti-IL-17 and anti-TNF-α antibodies induced undetectable levels of IL-17 in stimulated wild-type cells (Fig. 7).

What contributes to this decrease? Literature suggests that TNF-α and IL-17 can work synergistically. For instance, addition of both TNF-α and IL-17 increased IL-1β and IL-6 (Katz *et al.*, 2001) production. It also has been demonstrated that both IL-17 (Shalom-Barak *et al.*, 1998) and TNF-α (Englaro *et al.*, 1999) mediate inflammation through the NF-κB pathway which, in turn, produces more inflammatory cytokines, including IL-6 and IL-8 (Katz *et al.*, 2001), that form a positive feedback loop. Similarly, the NF-κB pathway is activated during osteoclastogenesis and bone resorption (Zwerina *et al.*, 2012). Considering that Th17 cells produce both IL-17 and TNF-α, with TNF-α possibly causing positive feedback on Th17 cell production, it seems reasonable that inhibition of both of them may lower IL-17 levels considerably. It is important to emphasize that our study included a treatment group with both anti-IL-17 and anti-TNF-α antibodies, and the expected results were a further decrease in IL-17 than what would be seen with anti-IL-17 antibody treatment alone. This usually did not occur. This possibly could be due to an already strong neutralizing effect of the anti-IL-17 antibody. In support of this, using 2.5 µg of anti-IL-17 antibody has been shown to be effective in preventing arthritis after infection with *B. burgdorferi* (Burchill *et al.* 2003; Nardelli *et al.* 2004; Hansen *et al.* 2013). However, at what stage of disease TNF-α takes on the protective role in *B. burgdorferi* infection remains to be determined.

Susceptibility to Lyme arthritis differs by mouse strains. Wild-type C57BL/6 mice are arthritis-resistant, and C3H/HeJ mice are arthritis-susceptible. However,

Brown *et al.* (1999) demonstrated that C57BL/6 mice that are deficient in the anti-inflammatory cytokine IL-10 are arthritis-susceptible. This study also showed that macrophages derived from wild-type C57BL/6 mice produce more IL-10 than do C3H-derived macrophages in response to *B. burgdorferi* (Brown *et al.*, 1999). Furthermore, *in vitro* stimulation of PMBCs from healthy rhesus monkeys and humans with *B. burgdorferi* resulted in production of IL-10 and a decrease in IL-12 (Giambartolomei *et al.*, 1998). The higher production of IL-10 in arthritis-resistant mice provides a possible mechanism for the bacteria to survive in the host long enough to cause disease. Our results indicate more ankle swelling in infected, IL-10-deficient C57BL/6 mice compared to infected wild-type mice (Figs. 1 and 2). These results were also reported in a previous study (Hansen *et al.*, 2013) and by others (Brown *et al.*, 1999; Crandall *et al.*, 2006). It has been shown that reduced levels of IL-10 substantially contribute to arthritis susceptibility in mice otherwise known to be arthritis-resistant by increased production of IL-17 (Hansen *et al.*, 2013).

Overall, in our study, infected IL-10-deficient mice treated *in vivo* with anti-TNF-α antibodies produced more IL-17 than untreated wild-type mice when cells were incubated for 18 hours (Fig. 5). *In vitro* treatment of spleen cells with 2.5 µg and 10 µg of anti-TNF-α also caused increases in IL-17 production in between IL-10-deficient and wild-type mice (Fig. 18 and data not shown). However, these differences were not seen at concentrations of 5 µg, or in anti-IL-17 or dual-antibody treated groups (Fig. 18 and data not shown). It is reassuring that our *in*

vitro data shows consistent statistical significances in IL-17 production of IL-10-deficient mice between untreated and anti-IL-17 antibody treated groups at all three concentrations (Figs. 15, 16, and 17). These results support *in vivo* findings in a previous study (Hansen *et al.*, 2013) and implicate IL-17 in development of Lyme arthritis. In another study, bone-marrow derived macrophages cultured with *B. burgdorferi* and treated with anti-IL-10 antibodies produced increased levels of pro-inflammatory cytokines, while addition of recombinant IL-10 decreased IFN-γ, IL-6 and TNF-α (Gautam *et al.*, 2012). Therefore, it is likely that IL-10 controls multiple inflammatory pathways implicated in development of Lyme arthritis.

How are IL-10 and TNF-α regulated in Lyme arthritis? Literature suggests that IL-10 and TNF-α have an inverse relationship in disease. For example, treatment of synovial fluid mononuclear cells with anti-IL-10 antibodies increased *in vitro* production of TNF-α, while addition of recombinant IL-10 to the cells decreased the amount of TNF-α (Yin *et al.*, 1997). Additionally, when normal blood was incubated with *B. burgdorferi*, production of IL-10 was increased, whereas TNF-α was decreased (Diterich *et al.*, 2001). Furthermore, addition of recombinant IL-10 to cultures of a human monocytic cell line incubated with spirochetes decreased production of TNF-α (Murthy *et al.*, 2000). However, treatment of these cells with anti-IL-10 antibodies did not lower the amount of TNF-α produced (Murthy *et al.*, 2000). IL-10 is known to down-regulate the NF-κB pathway (Lentsch *et al.*, 1997), while TNF-α up-regulates the NF-κB pathway (Englaro *et al.*, 1999). Macrophages stimulated with *B. burgdorferi* showed an increase in NF-κB after stimulation with

B. burgdorferi, but when recombinant IL-10 was added, this response was lowered (Gautam *et al.*, 2011). These studies indicate that IL-10 can regulate the influence of TNF-α during *B. burgdorferi* infection and, possibly, Lyme arthritis. We showed that in the absence of IL-10, blocking TNF-α, more often than not, had little effect on the production of IL-17. By contrast, in the presence of IL-10, blocking TNF-α occasionally increased the production of IL-17. Therefore, IL-10 may regulate the interaction of TNF-α and IL-17 following *B. burgdorferi* infection. We also showed that a lack of IL-10 in *B. burgdorferi*-infected mice causes an extremely strong IL-17 response (Hansen *et al.*, 2013). This may explain why blocking TNF-α *in vivo* (Fig. 10 and 11) or *in vitro* (Fig. 15, 16, and 17) did not consistently result in a change in IL-17 production by cells of infected, IL-10-deficient mice.

Previously, it was shown that recombinant TNF-α can increase the borreliacidal antibody response in *B. burgdorferi*-vaccinated and -infected, IFN-γ-deficient mice (Christopherson *et al.*, 2003). Furthermore, injection of anti-TNF-α antibodies to these mice significantly reduced borreliacidal antibody titers (Christopherson *et al*., 2003). To determine if TNF-α affected the borreliacidal antibody response in wild-type and IL-10-deficient mice infected with *B. burgdorferi*, serum from was collected and analyzed by a borreliacidal antibody assay. Contrary to the results of Christopherson, *et al.* (2003), the borreliacidal antibody response in infected wild-type mice was increased when these mice were treated with anti-TNF-α antibody. However, this increase was only one-fold different (Fig. 19A), and therefore was likely not significant. Administration of anti-TNF-α antibodies also

caused a one-fold increase in the borreliacidal antibody titers observed in infected wild-type mice treated with anti-IL-17 antibodies (Fig. 19A). These conflicting results may be due to the disease model used. *Borrelia*-vaccinated and -infected, IFN-γ-deficient C57BL/6 mice exhibit severe arthritis, while infected wild-type C57BL/6 mice exhibit mild inflammation. To support this, we showed that infected IL-10-deficient C57BL/6 mice (which are moderately-to-severely arthritic) showed a three-fold decrease in borreliacidal antibody titers (10240 to 1280) due to anti-TNF-α antibody treatment (Fig. 19B). These results are similar to those of Christopherson *et al.* (2003), which showed decreased borreliacidal titers in mice infected with *B. burgdorferi* and treated with anti-TNF-α. Infected IL-10-deficient mice also showed a five-fold decrease (10240 to 320) due to anti-IL-17 antibody treatment (Fig. 19B), indicating that TNF-α and IL-17 each promote borreliacidal activity in IL-10-deficient mice. Additionally, we showed a similar titer for both infected but untreated wild-type and IL-10-deficient mice (Figs. 19A and 19B). Contrary to our studies, Brown *et al.* (1999) showed differences between borreliacidal titers in wild-type and IL-10-deficient C57BL/6 mice. However, it is important to note that these borreliacidal antibodies were analyzed four weeks after infection, while we quantified borreliacidal titers eight days after infection. It is highly likely that extended infection could alter the borreliacidal response and magnify differences between the two strains of mice.

CHAPTER 5: CONCLUSION AND FUTURE DIRECTIONS

In conclusion, this thesis addressed two specific aims. Specific Aim 1 was to determine the effect of TNF-α on IL-17 in the immune response to *B. burgdorferi* infection. The working hypothesis of this specific aim was that TNF-α would decrease IL-17 production following *B. burgdorferi* infection. This hypothesis was partially supported by the data presented in this study. Briefly, it was shown that anti-TNF-α antibody treatment of *B. burgdorferi*-infected wild-type mice increased the IL-17 levels produced by spleen cells. By contrast, this effect was not consistently observed in IL-10-deficient mice, although we did show that unstimulated cells from infected, anti-TNF-α antibody-treated mice produced a relatively large amount of IL-17. Overall, these data indicate that TNF-α may play a protective role during *B. burgdorferi* infection in C57BL/6 mice by suppressing the production of IL-17.

Specific Aim 2 addressed effects of TNF-α and IL-17 on the borreliacidal antibody response following infection with *B. burgdorferi*. The working hypothesis for this aim stated that anti-TNF-α treatment of *B. burgdorferi*-infected mice would inhibit borreliacidal antibody production, which would be reversed by also blocking IL-17. This hypothesis was also partially supported. Our data indicated that in infected wild-type mice, treatment with anti-TNF-α antibody had a minimal effect on increasing borreliacidal antibody titers. Additionally treating these mice with anti-IL-17 antibodies minimally reduced this production relative to anti-TNF-α antibody-treated mice, but minimally increased production compared to anti-IL-17 antibody-

treated mice. However, in infected IL-10-deficient mice, treatment with anti-TNF-α antibodies did, indeed, reduce borreliacidal antibody titers. However, addition blockage of IL-17 further reduced these titers, rather than reversed the effects of anti-TNF-α antibody treatment.

These conclusions additionally support the literature that suggests that TNF-α plays a protective role in *B. burgdorferi* infection. We provide a potential mechanism for this protection: inhibiting the production of IL-17, which has been shown to contribute to the development of Lyme arthritis in mouse models of disease (Burchill, *et al.* 2003; Nardelli *et al.* 2004, 2005, 2008; Hansen *et al.* 2013). However, other findings provide a warning about the use of anti-TNF-α therapy in treating Lyme arthritis because these treatments (1) may stimulate persisting infection (Yrjänainen *et al.*, 2007), (2) increase IL-17 levels (Chen *et al.*, 2011) in other systems, and (3) affect production of Treg cells (Ma *et al.*, 2010; McGovern *et al.*, 2012) which may or may not be desirable in the context of infection-mediated inflammation.

In conclusion, we show that TNF-α can play a protective role during *B. burgdorferi* infection and provide support that this protection involves suppressing IL-17 production. Future studies that require investigation include examining the effect of IL-17 on TNF-α production during *B. burgdorferi* infection. Additionally, the relationship between IL-17 and TNF-α will be studied in the early and later stages of *B. burgdorferi* infection, since the effects of TNF-α vary based on the disease

system, as well as the phase of disease at which it is considered (Grivennikov *et al.*, 2005).

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