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Regulatory Mechanisms in *Borrelia Burgdorferi*-Induced Arthritis

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REGULATORY MECHANISMS IN *BORRELIA BURGDORFERI*-INDUCED ARTHRITIS

by

Emily S. Hansen

A Dissertation Submitted in
Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
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ABSTRACT

REGULATORY MECHANISMS IN *BORRELIA BURGDORFERI*-INDUCED ARTHRITIS

by

Emily Hansen

The University of Wisconsin-Milwaukee, 2016
Under the Supervision of Professor Dean T. Nardelli, PhD

Lyme arthritis is a common symptom of Lyme borreliosis that involves inflammation of the synovial joints. Elucidating the immune events involved in Lyme arthritis is complicated by the fact that not all individuals infected with *B. burgdorferi* develop arthritis. Additionally, Lyme arthritis manifests in different severities between affected individuals. It is known that an inflammatory response is initiated by *B. burgdorferi* infection and that inflammatory T cells contribute to the development of arthritis. However, the anti-inflammatory mechanisms that regulate the pathogenic T cells' response are not entirely understood. Here, the hypothesis that a dysregulated immune response results in an excessive inflammatory response and the development of arthritis following *B. burgdorferi* infection was tested. Interleukin-10 (IL-10) is involved in regulating the immune response during infection with *B. burgdorferi*. We demonstrate that IL-10 regulates the development of Lyme arthritis through inhibition of interleukin-17 (IL-17) production. We also demonstrate that IL-10 regulates the production of IL-17 by *Borrelia*-primed CD4⁺ cells early after interaction with Lyme spirochetes *in vitro*, and that infection of *Borrelia*-primed mice with *B. burgdorferi* leads to significant production of IL-17 that contributes to the development of severe arthritis. Further, we demonstrate that regulatory T (Treg) cell depletion prior to infection results in hind paw swelling and

the development of arthritis along with an increased *B. burgdorferi*-specific antibody response in an arthritis-resistant mouse model. We further demonstrate that Treg cells inhibit paw swelling and inflammatory cytokine production during the course of *B. burgdorferi* infection, but may not modulate severity of arthritis in established disease. Based on our findings, this suggests that Treg cells present prior to *B. burgdorferi* infection results in regulation of IL-17 by IL-10, thereby inhibiting pathology. Our findings identify novel regulatory mechanisms that may be responsible for resistance to Lyme arthritis, and suggest that modulation of Treg cells may prove useful in the development of new strategies for treatment and/or prevention of Lyme arthritis.

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LIST OF ABBREVIATIONS

α CD4: anti-CD4

ANOVA: analysis of variance

APC: allophycocyanin

APCs: antigen presenting cells

Bb: *Borrelia burgdorferi*

BSK: Barbour-Stoenner-Kelly

ConA: concanavalin A

CSF: cerebrospinal fluid

CTLA-4: cytotoxic T-lymphocyte-associated protein 4

DTx: diphtheria toxin

ELISA: enzyme-linked immunosorbent assay

EM: erythema migrans

FoxP3: forkhead box P3

FITC: fluorescein isothiocyanate

IFN- γ : interferon-gamma

IDO: indoleamine 2,3-dioxygenase

IL-1 β : interleukin-1-beta

IL-2: interleukin-2

IL-6: interleukin-6

IL-10: interleukin-10

IL-17: interleukin-17

IL-23: interleukin-23

IL-35: interleukin-35

IPEX: immune dysregulation, polyendocrinopathy, enteropathy, X-linked

KO: knock-out

MHC: major histocompatibility complex

NapA: neutrophil activation protein A

ND: not detected

NK: natural killer

OspA: outer surface protein A

Pam3Cys: tripalmitoyl-S-glyceryl-cysteine

PBS: phosphate buffered saline

PE: phycoerythrin

PCR: polymerase chain reaction

SEM: standard error of the mean

TGF- β : transforming growth factor-beta

Th1: T helper type 1

Th17: T helper type 17

TLR: toll-like receptor

TNF- α : tumor necrosis factor-alpha

Treg: regulatory T

WT: wild-type

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CHAPTER 1: INTRODUCTION

Lyme Borreliosis

Lyme borreliosis, often known as Lyme disease, is caused by infection with the spirochete *Borrelia burgdorferi*. *B. burgdorferi* is a microaerophilic organism approximately 20 μm long and 0.2-0.5 μm wide (Johnson *et al.* 1984). The bacteria are motile due to the presence of 7 to 10 periplasmic flagella, allowing the organism to travel throughout the host from the initial site of infection (Johnson *et al.* 1984). Sites such as the skin, ligaments, tendons, and synovial tissue are often affected, as the microbe is able to bind to collagen along with other components of the extracellular matrix (Barthold *et al.* 1993, Zambrano *et al.* 2004). *B. burgdorferi* infection can cause pathology of the neurological, cardiovascular, and musculoskeletal systems in humans. Lyme borreliosis is the most common tick-borne illness in the United States and Europe (Centers for Disease Control and Prevention 2015), with approximately 30,000 reports of Lyme borreliosis confirmed in the United States annually. However, due to difficulty in diagnosing Lyme borreliosis, a large number of cases go undiagnosed and unreported. Using clinical laboratory records of the number of people who have tested positive for Lyme borreliosis along with insurance company claims of diagnosis, it is estimated that approximately 300,000 cases occur annually (Nelson *et al.* 2015; Centers for Disease Control and Prevention 2015). The increased estimate suggests that Lyme borreliosis is a greater public health problem than previously thought.

Lyme borreliosis is endemic to several regions of the United States, including the Northeast, Mid-Atlantic, Upper Midwest, and Far West (Centers for Disease Control and

Prevention 2015). Endemic regions include areas where the infected *Ixodes* tick vectors and animal reservoirs, including small rodents (typically mice), deer, foxes, and other mammalian hosts, live. In the Northeastern and Upper Midwestern United States, *B. burgdorferi* is transmitted to humans by *I. scapularis*. Deer are particularly important for the spread of *B. burgdorferi*, as they are capable of hosting a large number of ticks and traveling longer distances than smaller hosts; however, they do not display any signs of disease (Stanek *et al.* 2012). In the Western United States, lizards occasionally serve as a reservoir, and the bacteria are transmitted via *I. pacificus* (Swei *et al.* 2011). Moreover, birds have been identified as carriers of *B. burgdorferi*, providing a means for the bacteria to spread farther distances (Anderson *et al.* 1986).

B. burgdorferi is transmitted to humans from ticks through injection of the bacteria into the skin during feeding (Stanek *et al.* 2012). Transmission most commonly occurs between the months of May and August, during the nymphal stage of tick development, when the ticks are relatively small and difficult to observe (Steere *et al.* 2004). Although rare, transmission by adult ticks can occur (Steere *et al.* 2004). In endemic areas, up to 25% of *Ixodes* ticks may be infected (Committee on Lyme Disease and Other Tick-Borne Diseases 2011), but the average risk of human infection in an endemic area is approximately 5% (Sood *et al.* 1997). This is due to the fact that an infected tick must be attached to its host for several days in order to transmit *B. burgdorferi* effectively (Bhate & Schwartz 2011a). Specifically, transmission most often occurs when the tick is attached between 48 and 72 hours (Vazquez *et al.* 2008). Patients diagnosed with

Lyme borreliosis often do not recall having been bitten by a tick, which leads to delayed treatment of the infection.

Untreated Lyme borreliosis progresses through three stages: early localized infection, early disseminated infection, and late persistent infection. The first stage of Lyme borreliosis occurs in the days to weeks following infection. During early localized infection, approximately 75% of individuals develop a rash called erythema migrans (EM) (Centers for Disease Control and Prevention 2015). EM is a flat, painless rash that spreads to an average diameter of 10-16 cm, occasionally with central clearing that exhibits a bulls-eye appearance (Bhate & Schwartz 2011a). Other symptoms of early localized infection are generally mild and include fatigue, fever, aches, and neuromuscular symptoms (Donta 2012). In the absence of EM, these vague symptoms may prevent a correct diagnosis, leading to disease progression.

The second stage, early disseminated disease, may occur in the weeks to months after a tick bite. Progression to this stage can be indicated by the presence of multiple EM lesions, which are typically observed in areas other than the site of the tick bite (Steere *et al.* 2004). Additionally, more severe symptoms may occur, including musculoskeletal pain and swelling, cardiac involvement exhibited by atrioventricular block, and neurological abnormalities such as numbness or tingling of the extremities, visual disturbances, and partial paralysis of facial muscles (Steere *et al.* 2004, Earl 2010, Halperin 2011). While these symptoms eventually resolve in many individuals (Halperin 2011), disease in some patients will continue to progress in the absence of treatment.

Late persistent infection, the third stage of disease, involves intensified neuropathies, cardiac involvement, and arthralgia that can occur months to years following infection. Profound neurological symptoms may include memory loss, fluctuations in mood, and peripheral neuropathy that can affect nearly every cranial nerve (Bhate and Schwartz 2011a). Cardiac manifestations may also intensify to include myocarditis and heart failure, which, although rare, has recently been identified as a cause of death (Centers for Disease Control and Prevention 2013). In addition, individuals at this stage may exhibit intense joint pain and swelling, occasionally with degradation of cartilage and erosion of the bone (Bhate and Schwartz 2011a). Of the many manifestations of Lyme borreliosis, arthritis is the most frequent clinical presentation (Steere *et al.* 1987).

Lyme borreliosis is treated using antimicrobial therapy. Generally, 2 to 3 weeks of oral doxycycline is used, but a more aggressive therapy of intravenous ceftriaxone for 2 to 4 weeks may be prescribed for patients with neurological or other later stage symptoms, or who do not respond to doxycycline (Bhate & Schwartz 2011b). However, some patients continue to experience symptoms after clearance of the infection by antibiotic therapy. These patients are considered to be free of active infection, as determined by negative polymerase chain reaction (PCR) results of synovial fluid (Steere & Angelis 2006). Additional rounds of antibiotic treatments may not improve symptoms (Klempner *et al.* 2001), so these patients may have limited treatment opportunities available. Non-steroidal anti-inflammatory agents are recommended to alleviate inflammation and pain associated with symptoms of antibiotic-refractory Lyme arthritis (Wormser *et al.* 2006).

Some patients who have exhausted these options resort to arthroscopic synovectomies, in which the synovial lining of the affected joints is removed (Schoen *et al.* 1991). The development of new treatment approaches is necessary for patients who continue to display symptoms after antimicrobial therapy in order to prevent invasive procedures and unwarranted continuation of antibiotic therapy.

Lyme Arthritis

The most common clinician presentation of Lyme borreliosis is Lyme arthritis, which presents as swelling of the synovial joints, typically the knee (Arvikar & Steere 2015). Lyme arthritis affects approximately 60% of individuals not treated for *B. burgdorferi* infection (Steere *et al.* 2004). Lyme arthritis involves intermittent inflammatory episodes of the synovial tissue, ligaments, and tendons that ensue weeks to months following infection, with each occurrence lasting approximately one week (Steere *et al.* 1987). These attacks typically occur every few months and may continue for years (Steere *et al.* 2004). Approximately 10% of untreated patients with Lyme arthritis will experience disease progression to a chronic, destructive arthritis that involves cartilage degradation and bone erosion (Steere *et al.* 1987). For most patients, antimicrobial therapy is sufficient to treat the infection and alleviate symptoms of Lyme arthritis. Others, however, continue to experience arthritic bouts despite multiple courses of antimicrobial therapy (Steere & Angelis 2006). Patients who continue to display proliferative synovitis despite receiving antibiotic therapy are said to have “antibiotic-refractory” or “slow-resolving” arthritis (Steere & Angelis 2006). Antibiotic-refractory arthritis is hypothesized to be the result of residual borrelial proteins or nonviable organisms that remain and

continue to elicit an immune response after the infection is treated, or to be the result of autoimmunity induced by *B. burgdorferi* infection (Wormser *et al.* 2012, Drouin *et al.* 2013). Although the cause of persistent symptoms of arthritis is not known, the immune events that contribute to Lyme arthritis have been studied.

Innate immune cells are responsible for the initial inflammatory response to *B. burgdorferi* and contribute to the development of Lyme arthritis. Innate immune cells, especially macrophages, often encounter the bacteria at the site of the tick bite (Steere *et al.* 2004). *B. burgdorferi* signaling occurs mainly through recognition of tripalmitoyl-S-glycerol-cysteine (Pam₃Cys)-modified lipoprotein portion of outer surface protein A (OspA) by toll-like receptor (TLR) 2 on macrophages (Yoder *et al.* 2003). Macrophages promote an inflammatory response through the production of cytokines, including interleukin-1-beta (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF- α) (Ma *et al.* 1994). Additionally, macrophages are important for antigen presentation and activation of the T cell response that leads to Lyme arthritis (DuChateau *et al.* 1999). Notably, the number of macrophages in the joint increases with the development of arthritis and decreases during resolution of disease (Lasky *et al.* 2015b). As a result, macrophages are important contributors to the inflammatory response following *B. burgdorferi* infection.

Neutrophils also play a major role in the inflammatory response to *B. burgdorferi*, as activation of these cells leads to the production of inflammatory cytokines, including IL-1 β , TNF- α , and interleukin-17 (IL-17), which are believed to contribute to the

development of Lyme arthritis (Wormser *et al.* 2006, Werner *et al.* 2011). Neutrophils are also capable of phagocytosing and killing spirochetes at both the initial site of infection and after dissemination (Benach *et al.* 1984), which may contribute to Lyme arthritis through recruitment of inflammatory cells. Synovial fluid samples from patients with Lyme arthritis contain large amounts of neutrophil chemotactic factors (Georgilis *et al.* 1991), and large numbers of neutrophils are found in the synovial tissues of patients infected with *B. burgdorferi* (Steere & Glickstein 2004). Importantly, infiltration of neutrophils into the joint tissue contributes to the induction of Lyme arthritis, likely through the recruitment of T cells (Brown *et al.* 2003). Collectively, these findings demonstrate the contribution of neutrophils to the pro-inflammatory environment that occurs in the development of Lyme arthritis.

While the innate immune response reacts to the initial infection, adaptive immunity is critical for the control of *B. burgdorferi* infection; however, it can also contribute to the development of Lyme arthritis. $\delta\gamma$ T cells are also important components of the immune response to *B. burgdorferi* infection. Like macrophages, $\delta\gamma$ T cells express TLR2 and are activated following binding of OspA (Collins *et al.* 2008). $\delta\gamma$ T cells activated following *B. burgdorferi* infection stimulate dendritic cells, which in turn, trigger pathways that induce adaptive immunity (Shi *et al.* 2011, Filgueira *et al.* 1996). Notably, mice deficient in $\delta\gamma$ T cells display decreased T and B cell responses following *B. burgdorferi* infection and an increased bacterial burden in the tissues (Shi *et al.* 2011). By activating additional adaptive immune cells, $\delta\gamma$ T cells may also contribute to the development of Lyme arthritis. $\delta\gamma$ T cells in the synovial fluid of patients with Lyme

arthritis proliferate in response to stimulation with *B. burgdorferi* (Glatzel *et al.* 2002).

Additionally, increased numbers of $\delta\gamma$ T cells are found within the joints of patients with antibiotic-refractory Lyme arthritis (Vincent *et al.* 1996). While other reports indicate that $\delta\gamma$ T cells do not directly contribute to severity of arthritis (Bockenstedt *et al.* 2003), they have been shown to contribute to the inflammatory response.

Other forms of T cells, specifically CD4⁺ T cells, have been shown to contribute to pathology in Lyme disease (McKisic *et al.* 2000). Later-stage Lyme arthritis is caused, in part, by the inflammatory effects of type 1 T helper (Th1) cells through the production of their signature cytokine, interferon-gamma (IFN- γ). Increased numbers of Th1 cells and levels of IFN- γ are present in mice after infection with *B. burgdorferi* (Dong *et al.* 1997, Keane-Myers & Nickell 1995). IFN- γ produced by CD4⁺ T cells is involved in the development of arthritis and increases the severity of disease in a mouse model (Sonderegger *et al.* 2012). In addition, increased numbers of Th1 cells and levels of IFN- γ have been observed in the synovial tissue, synovial fluid, and peripheral blood of patients with Lyme arthritis (Gross *et al.* 1998; Matyniak and Reiner 1995; Yssel *et al.* 1991; Shen *et al.* 2010). In fact, Th1 cells were found to comprise the majority of T cells in the synovial fluid of Lyme arthritis patients (Shen *et al.* 2010). Additionally, an increased Th1 response, as indicated by increased IFN- γ production, is associated with the development of antibiotic-refractory arthritis (Strle *et al.* 2012). These findings indicate that Th1 cells and IFN- γ are likely involved in the development of Lyme arthritis.

Other cells, however, also contribute to disease, as mice genetically deficient in IFN- γ , its receptor, or its signaling pathway have been shown to develop arthritis after *B. burgdorferi* infection (Brown and Reiner 1999; Christopherson *et al.* 2003). Notably, arthritis-resistant C57BL/6 mice developed severe, destructive arthritis in the absence of IFN- γ in a model of T cell-driven arthritis (Christopherson *et al.* 2003). Although the lack of IFN- γ does not prevent arthritis, only minimal inflammation is seen in animals depleted of CD4⁺ T cells before infection with *B. burgdorferi* (Lim *et al.* 1995a), suggesting that T helper cells are involved. In support of this, transfer of *Borrelia*-primed CD4⁺ T cells into recipient animals increases pathology following infection (Lim *et al.* 1995b). These reports suggest that CD4⁺ T cell-associated inflammatory factors in addition to IFN- γ may contribute to the pathogenesis of Lyme arthritis.

Another subset of helper T cell, the type 17 T helper (Th17) cell, is characterized by production of the inflammatory cytokine IL-17 (Park *et al.* 2005) and may also contribute to the development of Lyme arthritis. IL-17 is an important mediator of several arthritides, including rheumatoid arthritis (Chabaud *et al.* 2000), juvenile idiopathic arthritis (Aggarwal *et al.* 2008), antigen-induced arthritis (Pinto *et al.* 2010), and collagen-induced arthritis (Lubberts *et al.* 2004). While the arthritic potential of these cells has yet to be examined fully following *B. burgdorferi* infection, research suggests that Th17 cells may play a potentially pathogenic role in the immune response to the spirochete. Neutralization of IL-17 reduces paw swelling and prevents arthritis in a model of *B. burgdorferi*-induced arthritis in mice (Burchill *et al.* 2003; Nardelli *et al.*, 2004, Nardelli *et al.* 2008a). Additionally, borrelial proteins have been shown to

stimulate production of IL-17 *in vitro* from T cells collected from the synovial fluid of patients with Lyme arthritis (Infante-Duarte *et al.* 2000). Moreover, exposure to borrelial antigens causes dendritic cells to produce interleukin-23 (IL-23), a survival factor for Th17 cells (Stritesky *et al.* 2008). Neutralization of IL-23 has been shown to inhibit IL-17 production from *B. burgdorferi*-stimulated immune cells (Knauer *et al.* 2007) and prevents the development of Lyme arthritis in mice following infection with *B. burgdorferi* (Kotloski *et al.* 2008). Similarly, neutrophil-activating protein A (NapA) expressed by *B. burgdorferi* leads to production of IL-6 and transforming growth factor beta (TGF- β) by monocytes, which drive production of IL-17 (Codolo *et al.* 2008). Neutralization of IL-6 and TGF- β also prevented development of disease in a model of late-stage Lyme arthritis (Nardelli *et al.*, 2008).

Th17 cells typically comprise approximately 10% of the CD4⁺ T cell population in the synovial fluid of humans with Lyme arthritis (Codolo *et al.* 2008; Shen *et al.* 2010). While the arthritic potential of Th17 cells has yet to be examined fully, some reports have suggested that these cells may not contribute to disease. For example, C3H mice have low levels of IL-17 in the serum following *B. burgdorferi* infection, and administration of anti-IL-17 antibodies to these mice led to increased arthritis (Nardelli *et al.* 2010). Additionally, mice deficient in the IL-17 receptor A subunit developed the same degree of arthritis after infection as wild-type mice that possessed effective IL-17 signaling pathways (Lasky *et al.* 2015a). Likewise, a recent study found that mice deficient in IL-17 still develop arthritis in a model of late-stage Lyme arthritis (Kuo *et al.* 2016). In addition, peripheral blood mononuclear cells isolated from patients with a single

nucleotide polymorphism of the IL-23 receptor (Arg381Gln) produced less IL-17 in response to *B. burgdorferi* stimulation (Oosting *et al.* 2011). Despite reduced IL-17 production *in vitro*, no difference in severity of arthritis was observed between people with the polymorphism and those with the wild-type (WT) IL-23 receptor (Oosting *et al.* 2011). While the exact contribution of Th17 cells and IL-17 in Lyme arthritis remains uncertain, research suggests that in certain individuals, Th17 cells and IL-17 may contribute to the development of arthritis.

Th1 and Th17 cells have been investigated for their roles in *B. burgdorferi*-induced arthritis, and increased numbers and cytokines produced by these cells are often observed in disease. While an inflammatory immune response contributes to clearing *B. burgdorferi* infection, how this response leads to pathology in only some patients remains uncertain. Ineffective regulation of the adaptive immune response following *B. burgdorferi* infection may lead to an excessive inflammatory environment that results in arthritis. CD4⁺CD25⁺Foxp3⁺ regulatory T (Treg) cells regulate the inflammatory response by inhibiting Th1 and Th17 cells, in part, through production of interleukin-10 (IL-10) (Rubtsov *et al.* 2008). Both IL-10 and Treg cells have been proposed as regulators of inflammation in Lyme arthritis. IL-10-deficient mice develop more severe arthritis following *B. burgdorferi* infection than WT mice (Brown *et al.* 1999). Notably, CD4⁺ T cells have been identified as a major source of IL-10 within the joint tissue of *B. burgdorferi*-infected mice (Sonderegger *et al.* 2012). Additionally, administration of anti-IL-17 antibody in *B. burgdorferi*-infected IFN- γ -deficient mice results in an increase of CD4⁺CD25⁺ putative Treg cells and prevents the development of arthritis in a murine

model of Lyme arthritis (Nardelli *et al.* 2004). Adoptive transfer of this cell population to *B. burgdorferi*-infected mice prevents arthritis (Nardelli *et al.* 2005). A role for Treg cells in human disease has also been described. Patients with antibiotic-refractory Lyme arthritis who experience a longer duration of joint inflammation have fewer numbers of Treg cells in the synovial fluid than patients whose arthritis resolved following antibiotic therapy (Shen *et al.* 2010, Vudattu *et al.* 2013). Therefore, it is possible that a dysregulated immune response may result in more severe disease. Future studies regarding immune regulation in *B. burgdorferi*-induced arthritis will require consideration of these anti-inflammatory cells. Further characterization of the immune events involved in Lyme arthritis, specifically on the prevention or resolution of inflammation, is vital for identifying novel therapeutic treatments for the disease.

Regulatory T Cells

Treg cells were initially identified for their role in maintaining tolerance and controlling autoimmune disease (Sakaguchi *et al.* 1995); however, Treg cells are also important mediators of the immune response through inhibition of inflammation and prevention of tissue destruction. Treg cells comprise 5-10% of the peripheral T cell population (Baecher-Allan *et al.* 2001). Treg cells were previously identified by the expression of surface molecules CD4 and CD25 (Sakaguchi *et al.* 1995). However, activated effector T cells also express CD25 (Fazekas De St. Groth *et al.* 2004). Currently, expression of forkhead box P3 (Foxp3) is the most specific indicator of Treg cell phenotype (Fontenot *et al.* 2005). Foxp3 expression by Treg cells was first discovered in scurfy mice, as these mice display an overproliferation and dysregulation of CD4+ T cells.

Characterization of the genome of scurfy mice led to the identification of a genetic mutation in the *FoxP3* gene, which results in ineffective Treg cell development (Brunkow *et al.*, 2001). Similarly, a *Foxp3* gene mutation was found to result in the human autoimmune disease equivalent of scurfy known as immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) (Bennett *et al.* 2001). Additionally, Treg cells have been implicated in a variety of other diseases, with decreased suppressive function or reduced numbers occurring in multiple sclerosis (Viglietta *et al.* 2004), rheumatoid arthritis (Van amelsfort *et al.* 2004), myasthenia gravis (Gertel-Lapter *et al.* 2013), autoimmune hepatitis (Longhi *et al.* 2004), and polyglandular syndrome type 2 (Kriegel *et al.* 2004).

The mechanisms by which Treg cells modulate inflammation have not been fully elucidated; however, the major mechanisms appear to be inhibition of antigen presentation, direct suppression of effector cells, and production of anti-inflammatory cytokines. Treg cells can inhibit antigen-presenting cells (APCs) by directly binding to them, thereby preventing APCs from activating effector T cells (Cederborn *et al.* 2000, Misra *et al.* 2004). Specifically, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) expression by Treg cells induces a downregulation of costimulatory molecules on the surface of dendritic cells following binding, which prevents activation and proliferation of effector T cells (Misra *et al.* 2004). Increased CTLA-4 binding also leads to increased expression of indoleamine 2,3-dioxygenase (IDO) by dendritic cells (Schmidt *et al.* 2012). IDO degrades tryptophan to kynurenines, and tryptophan depletion leads to cell cycle arrest of effector T cells (Schmidt *et al.* 2012). Additionally, Treg cells can have

increase expression of CD27, which then binds to CD70 on the surface of dendritic cells (Dhainaut *et al.* 2015). Interaction between the receptors leads to downregulation of CD70, as interaction with CD27 suppresses CD70 transcription (Kuka *et al.* 2013). CD70 expression by APCs is required for effective Th1 cell priming, and a downregulation of the receptor leads to decreased Th1 cell activation (Dhainaut & Moser 2015). By inhibiting costimulatory molecules, Treg cells are able to render APCs ineffective, thereby reducing effector T cell activation.

In addition to inhibiting antigen presentation, Treg cells can directly suppress effector T cells. During infection, Treg cells migrate to the draining lymph nodes and encounter effector T cells (Zhang *et al.* 2009). Treg cells inhibit effector T cells through direct interaction between TGF- β 1 on their cell surface with TGF- β R on effector T cells (Nakamura *et al.* 2001). Treg cells also migrate to tissue sites, where they reduce the number of activated CD4⁺ and CD8⁺ T cells through direct killing by the release of granzyme A using the perforin/granzyme pathway (Grossman *et al.* 2004). It has also been suggested that Treg cells compete with effector T cells for interleukin-2 (IL-2) and other cytokines necessary for survival, which leads to apoptosis of effector T cells (Pandiyan *et al.* 2007). However, others have found that while IL-2 is required for Treg cell suppression of effector T cell proliferation, it does not lead to apoptosis (Szymczak-Workman *et al.* 2011, Vercoulen *et al.* 2009). Interestingly, IL-2-deficient mice display an overproliferation of both CD4⁺ and CD8⁺ T cells, which indicates that IL-2 is not required for effector T cell proliferation (Sadlack *et al.* 1993). Additionally, IL-2 consumption was found to have no effect on the suppressive abilities of human Treg

cells (Tran *et al.* 2009). Additionally, Treg consumption of IL-2 has been reported to promote Th17 cell differentiation with no effect on proliferation (Pandiyani *et al.* 2011). It has also been suggested that Treg cell consumption of IL-2 only affects effector T cell proliferation when IL-2 is extremely limited (de la Rosa *et al.* 2004). Collectively, these reports suggest that while inducing apoptosis of effector T cells is not a major mechanism of Treg cell-mediated suppression, it may occur under specific conditions to further suppress the effector T cell response.

Additionally, Treg cells exert suppression on effector T cells indirectly through production of anti-inflammatory cytokines. IL-10, the major cytokine produced by Treg cells, leads to decreased antigen presentation by downregulating major histocompatibility complex (MHC) class II expression on APCs (Chattopadhyay & Shevach 2013) and inhibits effector T cell activation via CD28 co-stimulation (Joss *et al.* 2000). IL-10 produced by Treg cells inhibits Th17 cell proliferation (Chaudhry *et al.* 2011) and leads to decreased production of IFN- γ by Th1 cells (Fiorentino *et al.* 1991, Sojka & Fowell 2011). However, Treg cells isolated from IL-10-deficient mice inhibit effector cells equally as well as those isolated from wild-type mice, indicating that IL-10 production is not the only method of cytokine suppression (Sojka & Fowell 2011, Thornton & Shevach 1998). Treg cells not only produce IL-10, but also TGF- β , which can inhibit the activation and differentiation of both Th1 and Th17 cells (Li *et al.* 2007). While IL-10 is not required for Treg cells to exhibit inhibitory effects, suppression by Treg cells is dependent on TGF- β . In fact, mice that were adoptively transferred Treg cells could not control inflammation following anti-TGF- β antibody treatment (Powrie *et*

et al. 1996). More recently, interleukin-35 (IL-35) has been identified as an essential cytokine for contact-independent suppression by Treg cells (Chaturvedi *et al.* 2011). IL-35 suppresses the proliferation of Th1 and Th17 cells and inhibits IFN- γ and IL-17 production by these cells, respectively (Niedbala *et al.* 2007, Nakano *et al.* 2014). Collectively, Treg cells utilize a variety of mechanisms to suppress effector T cells. The redundancy of these suppressive mechanisms suggests Treg cells play a vital role in regulating the immune response.

Immunoregulation in Spirochete Infection

Spirochetes encompass six genera of bacteria, some of which are pathogenic.

Pathogenic spirochetes include *Borrelia*, *Leptospira*, *Brachyspira*, and *Treponema*.

Spirochetes, like other bacterial infections, induce an inflammatory immune response.

T cells and the cytokines they produce are critical components in the immune response to bacterial infection (D'Elios *et al.* 2011) and are induced by spirochete infection. Mice infected with *B. burgdorferi* have greater levels of IFN- γ than uninfected mice (Ilipoulou *et al.* 2007). Likewise, humans infected with *B. burgdorferi* who develop arthritis have increased numbers of Th1 cells in the synovial fluid (Shen *et al.* 2010). Moreover, IL-17 levels are elevated in the cerebral spinal fluid (CSF) of patients with neuroborreliosis, suggesting a role for Th17 cells in the disease (Henningsson *et al.* 2011). Similarly, infection with *Leptospira interrogans*, which causes leptospirosis, leads to an increase in inflammatory CD4⁺ T cells in the spleen and lungs of infected mice (Pereira *et al.*, 1998) (Richer *et al.* 2015). Additionally, patients with leptospirosis have significantly greater percentages of Th1 cells compared to healthy controls (Volz *et al.* 2015). Infection with

Brachyspira hyodysenteriae, which causes gastroenteritis, also results in an upregulation of inflammatory T cells at the onset of disease in swine (Jergens *et al.* 2006) and leads to increased levels of T effector cells in swine (Hontecillas *et al.* 2005, Jonasson *et al.* 2004). Moreover, CD4+ T cells isolated from the lymph nodes of *B. hyodysenteriae*-infected mice produce IFN- γ following restimulation with the spirochete (Jergens *et al.* 2006; Hontecillas *et al.* 2003). Also, clearance of *Treponema pallidum* infection is mediated by Th1 cells through production of IFN- γ (Arroll *et al.* 1999). Patients with chronic periodontitis resulting from infection with *Treponema denticola* also have increased IFN- γ production and *Treponema*-specific CD4+ T cells (Shin *et al.* 2013). Together, the findings of these studies suggest that CD4+ T cells, especially Th1 cells, are important components of the immune response to spirochete infection.

However, research suggests that long-term spirochetal infection is associated with a reduced immune response. Patients with post-treatment Lyme borreliosis have lower numbers of Th1 cells compared to asymptomatic individuals (Jarefors *et al.* 2007), indicating that chronic Lyme borreliosis symptoms may result from an inadequate immune response to the infection. Additionally, following infection with *Brachyspira hodystenteriae*, pigs that develop dysentery had decreased CD4+ T cell numbers compared to those that did not develop disease (Jonasson *et al.* 2004). Decreased T cell responses are also observed in *Treoponema* infection, as *T. pallidum*-infected hamsters display atrophy of the thymus and a decreased T cell response during the first 14 days following infection (Bagasra *et al.* 1985). In addition, T cell blastogenesis is reduced during active infection, yet increases in disease remission (Pavia *et al.* 1976).

Also, cells isolated from patients infected with *Treponema* species displayed a decreased lymphocyte response to stimulation with concanavalin A (Con A) compared to healthy controls (From *et al.* 1976). Additionally, incubation of normal lymphocytes with the plasma of patients with syphilis before treatment reduced T cell proliferation following stimulation, while serum collected 2-6 months after treatment had no effect (From *et al.* 1976). These findings indicate that immune factors in the plasma during active infection are repressing the T cell response. Patients with syphilis that have reduced numbers of CD4+ T cells due to HIV infection are more likely to experience neurological involvement than those without HIV infection, suggesting that lack of an effector T cell response may lead to treponemal dissemination (Li *et al.* 2013). Together, these findings indicate that a reduced immune response during spirochetal infection may intensify the length or severity of disease.

CD4+ T cells are important for bacterial clearance, but when inflammation becomes excessive, it may lead to pathology. IL-10 has been described in spirochetal infection as an important mediator of the immune response to prevent pathogenesis. *B. burgdorferi*-infected C57BL/6 mice, which are resistant to the development of Lyme arthritis, have greater IL-10 production and decreased inflammatory cytokine production than arthritis-susceptible C3H/HeN mice (Brown *et al.* 1999). Increased IL-10 production is also associated with decreased inflammation and the prevention of severe disease in patients with leptospirosis (Volz *et al.* 2015). Moreover, higher levels of IL-10 correlated with decreased inflammatory cytokines and the disappearance of clinical symptoms in *B. hyodysenteriae* infection (Kruse *et al.* 2008). Collectively, these

findings suggest that IL-10 production is important for modulating disease severity in spirochete infection.

Treg cells prevent pathological changes, in part, through production of IL-10, and may contribute to immune regulation in spirochete infection. Mice deficient in CD28, a co-stimulatory molecule required for the differentiation of Treg cells, have 80% fewer Treg cells than WT mice (Tang *et al.* 2003). CD28-deficient mice display a longer, more chronic course of Lyme arthritis (Iliopoulou *et al.* 2007). Additionally, *B. burgdorferi*-primed and -infected IFN- γ -deficient mice treated with antibodies against IL-17 developed increased numbers of CD4+CD25+ T cells in the lymph nodes and did not develop arthritis. These were not observed in mice that did not receive anti-IL-17 antibodies (Nardelli *et al.* 2004). Moreover, *Borrelia*-primed and -infected mice that received CD4+CD25+ T cells isolated from infected mice treated with anti-IL-17 antibody did not develop arthritis; however, administration of CD4+CD25- cells led to pathology (Nardelli *et al.* 2005). Depletion of CD25-expressing cells in *B. burgdorferi*-infected mice treated with anti-IL-17 antibodies resulted in destructive arthritis (Nardelli *et al.* 2004); however, depletion of CD25-expressing cells in the same mouse model in which IL-17 was not neutralized had no effect on pathology (Nardelli *et al.* 2006).

Treg cells and Th17 cells develop from a common precursor in a reciprocal manner (Bettelli *et al.* 2006). Therefore, it is likely that the CD4+CD25+ T cell population induced following IL-17 neutralization consisted of true Treg cells. Additionally, patients with Lyme arthritis who fail to achieve resolution of disease following antibiotic treatment

were found to have fewer numbers of CD4+CD25+Foxp3+ Treg cells in the synovial fluid than those whose arthritis resolved (Shen *et al.* 2010). No differences were seen in the suppressive capacity of Treg cells between groups, nor were differences in the percentages of Th1 or Th17 cells observed (Shen *et al.* 2010). However, lower frequencies of Treg cells in the synovial fluid led to increased effector T cell proliferation and inflammatory cytokine production, which contribute to more severe arthritis in patients with antibiotic-refractory Lyme arthritis (Vudattu *et al.* 2013). Together, these findings demonstrate the importance of Treg cells in the ability to prevent pathology or resolve inflammation in Lyme arthritis.

Although research suggests that Treg cells may prevent pathology, downregulation of the immune response by these cells may also lead to advanced bacterial dissemination. In *T. pallidum* infection, increased IL-10 mRNA expression occurs in the skin at the site of infection and contributes to ineffective spirochete clearance (Wicher *et al.* 1998). Additionally, increased IL-10 production is associated with increased disease severity (Lusiak & Podwinska 2001). The TpF1 antigen of *Treponema* causes monocytes to release IL-10 and TGF- β , which promote Treg cell differentiation (Babolin *et al.* 2011). Accordingly, increased expression of TpF1 leads to increased numbers of Treg cells and enhanced Treg cell-associated cytokine production (Li *et al.* 2013, Babolin *et al.* 2011). T cells with suppressive functions induced by *T. palladium* infection are known to inhibit phagocytosis of spirochetes by macrophages (Tabor *et al.* 1984, Tabor *et al.* 1987), which may result in increased bacterial survival and dissemination. Specifically, patients with neurosyphilis have a greater number of Treg cells in the cerebrospinal fluid

than patients without neurological involvement (Li *et al.* 2013). Therefore, an enhanced Treg cell response during spirochete infection may promote survival of the organism and allow for its increased dissemination, leading to a longer or more severe course of disease.

In conclusion, spirochetes, including *B. burgdorferi*, induce an inflammatory response that assists in clearing the infection, but the inflammatory response can also result in pathology. Treg cells may regulate the immune response to spirochetal infection and limit pathology. However, the presence of Treg cells can inhibit the inflammatory response, resulting in increased bacterial persistence and dissemination. Current research suggests that a delicate balance between the pro-inflammatory response and anti-inflammatory response is vital to efficient bacterial clearance without leading to tissue damage. Further discoveries regarding the timing and degree of the Treg cell response will lead to a more complete understanding of the role of Treg cells during *B. burgdorferi* infection.

Hypothesis and Specific Aims

Arthritis is a common clinical symptom of Lyme disease. It is known that an inflammatory response is initiated by *B. burgdorferi* infection, and that inflammatory T cells contribute to the development of Lyme arthritis. However, the anti-inflammatory mechanisms that regulate the pathogenic T cell response are poorly understood. The overall objective of this dissertation is to identify novel regulatory mechanisms of the immune response to *B. burgdorferi*, which will provide the foundation for the development of effective therapeutic strategies for Lyme arthritis. The hypothesis of this

dissertation is: a dysregulated immune response results in an excessive inflammatory response and in the development of arthritis following *B. burgdorferi* infection. The hypothesis will be tested by addressing the following specific aims:

1. Determine the effect of IL-10 in *B. burgdorferi*-induced arthritis. The working hypothesis of this aim is: IL-10 regulates the inflammatory immune response in Lyme arthritis through inhibition of IL-17.
2. Determine the effect of IL-10 in a T cell-driven model of Lyme arthritis. The working hypothesis of this aim is: IL-10 reduces CD4⁺ cell-derived IL-17 production and attenuates Lyme arthritis.
3. Determine the contribution of existing Treg cells on the induction of arthritis following *B. burgdorferi* infection. The working hypothesis of this aim is: Treg cells prevent the onset of arthritis following infection with *B. burgdorferi*.
4. Determine the role of Treg cells on arthritis development during *B. burgdorferi* infection. The working hypothesis of this aim is: Treg cells present following *B. burgdorferi* infection reduce arthritis severity.

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CHAPTER 2: SPECIFIC AIM 1

IL-10 Inhibits *Borrelia burgdorferi*-Induced IL-17 Production and Attenuates IL-17-Mediated Lyme Arthritis

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Abstract

Previous studies have shown that cells and cytokines associated with interleukin-17 (IL-17)-driven inflammation are involved in the arthritic response to *Borrelia burgdorferi* infection. Here, we report that IL-17 is a contributing factor in the development of Lyme arthritis by showing that its production and histopathological effects are regulated by interleukin-10 (IL-10). Spleen cells obtained from *B. burgdorferi*-infected, “arthritis-resistant” wild-type C57BL/6 mice produced low levels of IL-17 following stimulation with the spirochete. By contrast, spleen cells obtained from infected, IL-10-deficient C57BL/6

mice produced a significant amount of IL-17 following stimulation with *B. burgdorferi*. These mice developed significant arthritis, including erosion of the bones in the ankle joints. We further show that treatment with antibody to IL-17 partially inhibited the significant hind paw swelling and histopathological changes observed in *B. burgdorferi*-infected, IL-10-deficient mice. Taken together, these findings provide additional evidence of a role for IL-17 in Lyme arthritis and reveal an additional regulatory target of IL-10 following borrelial infection.

Introduction

Borrelia burgdorferi, the causative agent of Lyme borreliosis, stimulates a complex series of inflammatory events that functions to eliminate the spirochete following infection but which also frequently leads to arthritis in humans (Steere *et al.* 2004). In addition, *B. burgdorferi* induces production of the anti-inflammatory cytokine interleukin-10 (IL-10) early after stimulation of host cells (Giambartolomei *et al.* 1998, Brown *et al.* 1999, Ganapamo *et al.* 2000), suggesting a possible mechanism of facilitating infection in the host. IL-10 also plays a central role in regulating the immune response during sustained infection with *B. burgdorferi*. Notably, a deficiency of IL-10 in otherwise “arthritis-resistant” C57BL/6 mice predisposes these animals to developing remarkable joint pathology following *B. burgdorferi*-infection (Brown *et al.* 1999). Despite exhibiting more severe arthritis, these *B. burgdorferi*-infected IL-10-deficient mice are more effective in eliminating the pathogen than are wild-type counterparts (Brown *et al.* 1999). Therefore, the dual outcomes associated with the inflammatory response to borrelial infection—robust anti-pathogen and arthritic responses—are inexorably linked to IL-10. As

additional immune mechanisms against *B. burgdorferi* continue to be identified, the extent to which IL-10 participates in these responses must be defined further.

Accumulating evidence supports the contribution of such novel mechanisms in the development of Lyme arthritis. The pathology of disease in humans is driven, in large part, by Th1 cells and their prototypical inflammatory cytokine, interferon-gamma (IFN- γ) (Yssel *et al.* 1991, Gross *et al.* 1998, Strle *et al.* 2012, Shen *et al.* 2010). However, studies in multiple animal models have shown that a Th1 response is not absolutely necessary for the development of arthritis after *B. burgdorferi* infection (Brown & Reiner 1999, Christopherson *et al.* 2003, Munson *et al.* 2004), while the inflammatory cytokine interleukin-17 (IL-17) contributes to disease (Burchill *et al.* 2003, Nardelli *et al.* 2004, Amlong *et al.* 2006, Nardelli *et al.* 2008b). Specifically, blocking IL-17 and various Th17-associated cytokines *in vivo* reduced the severity of arthritis observed in models of *Borrelia*-associated arthritis in the presence (Nardelli *et al.* 2008b, Nardelli *et al.* 2010-Kotloski *et al.* 2008) or absence (Burchill *et al.* 2003, Nardelli *et al.* 2004, Amlong *et al.* 2006) of IFN- γ . Importantly, Infante-Duarte, *et al.* described the capacity of synovial cells of human Lyme arthritis patients to produce IL-17 when stimulated with *B. burgdorferi* microbes or lipoproteins (Infante-Duarte *et al.* 2000). Specifically, the borrelial antigen neutrophil activating protein A (NapA) stimulates innate immune cells to provide a cytokine environment conducive to the development of Th17 cells within the joint (Codolo *et al.* 2008). In addition, NapA is retained in the synovia of humans with Lyme arthritis and maintains this Th17 response through its chemoattractant activity on various inflammatory cell types, including T cells that produce both IL-17 and IFN- γ

(Codolo *et al.* 2013). Therefore, IL-17 is a likely contributor to the pathogenesis of Lyme arthritis. Since IL-10 is a known modulator of the IL-17 response (Huber *et al.* 2011), it is necessary to determine the relationship of these cytokines during *B. burgdorferi* infection.

In this study, we show that IL-10 inhibits the production of IL-17 from *B. burgdorferi*-stimulated cells. In addition, we demonstrate that multiple cell types are likely involved in the IL-17 response following borrelial infection, and that the arthritis observed in infected IL-10-deficient mice develops, in part, due to the actions of IL-17. Collectively, these findings provide additional evidence that IL-17 contributes to the arthritis induced by *B. burgdorferi* and reveal an additional target of regulation by IL-10 in the context of borrelial infection.

Materials and Methods

Mice

Six-to-12-week old, wild-type and IL-10-deficient mice on the C57BL/6 background (originally obtained from Jackson Laboratories) were provided by J.-A. Lyons (University of Wisconsin-Milwaukee) and were housed at the University of Wisconsin-Milwaukee animal facility. The mice weighed 20-30 g each and were housed in a humidity-controlled environment at an ambient temperature of 21°C under a light-and-dark cycle of 12 h. Food and acidified water were provided *ad libitum*. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee for the University of Wisconsin-Milwaukee.

Organisms and Preparation

Virulent *B. burgdorferi* 297 organisms (isolated from human spinal fluid) in modified Barbour-Stoenner-Kelly (BSK) medium supplemented with 10% glycerol were provided by S. M. Callister (Gundersen Lutheran Medical Center, La Crosse, WI). Low-passage (<10) organisms were grown at 32°C until they reached a concentration of 10^7 microbes/ml, dispensed into 1.5-ml screw-cap tubes (Sarstedt, Newton, NC), and stored at -80°C. Prior to infection of mice, a 1-ml frozen suspension of spirochetes was thawed, added to 4 ml of BSK medium (Sigma-Aldrich, St. Louis, MO), and incubated at 34°C for 6 days. On the day of infection, microbes were visualized by dark-field microscopy for motility and enumerated using a Petroff-Hausser counting chamber.

Infection of mice

Naïve mice were anaesthetized with isoflurane in a nose-and-mouth cup and then injected subcutaneously in each hind paw with 10^6 viable *B. burgdorferi* strain 297 organisms in a volume of 50 µl BSK medium. Control groups consisted of naïve mice that were injected with BSK medium alone. Experimental and control groups with or without infection with *B. burgdorferi* contained 4 or 5 mice each.

Administration of anti-IL-17 antibody

Neutralizing anti-mouse IL-17A purified antibody (clone eBioMM17F3; IgG1) and mouse IgG1 purified isotype control antibody were purchased from eBioscience (San Diego, CA). The antibodies were resuspended in filter-sterilized (0.22 µm-pore-sized filter; Fisher Scientific, Waltham, MA) phosphate buffered saline (PBS) to achieve

concentrations of 50 µg/ml. Groups of *B. burgdorferi*-infected mice were injected within 3 hours after infection with 2.5 µg (50 µl) of anti-IL-17 antibody subcutaneously in each hind paw while anesthetized with isoflurane contained in a nose-and-mouth cup. Mice were then injected daily thereafter with anti-IL-17 antibody for 6 days. Groups of *B. burgdorferi*-infected control mice were injected with isotype control antibody in a similar manner.

Assessment of swelling

Edematous changes of the hind paws of mice were evaluated in order to provide a measure of inflammation following infection with *B. burgdorferi*. A digital caliper (Marathon, Richmond Hill, Ontario) with a sensitivity of 0.01 mm was used to measure the width and thickness of each tibiotarsal joint. The width and thickness measurements were averaged to provide the mean caliper value. Baseline swelling levels of each group were measured prior to infection, and the changes in swelling from these baseline levels were determined at 3, 6, and 8 days after infection.

Cell culture and ELISA

Spleens were harvested from 4 or 5 mice per group on days 1 or 8 after infection. Single-cell, whole-spleen suspensions of each group were generated by gently passing cells through a nylon mesh screen (BD Falcon, BD Biosciences, San Jose, CA) into cold RPMI 1640 medium supplemented with L-glutamine and 25 mM HEPES (Cellgro, Corning, Manassas, VA). 1×10^6 whole spleen cells were incubated at 37°C in 5% CO₂ with or without 1×10^5 viable *B. burgdorferi* organisms in BSK medium and with or

without 5 µg anti-mouse CD4 antibody (clone GK1.5; eBioscience) in PBS.

Supernatants were collected after 6 and 24 hours of incubation. Controls consisted of cells incubated with PBS but without antibodies, or with BSK medium but without organisms.

Levels of IL-17 present in the supernatants of cell cultures were assessed using the IL-17A ELISA Ready-Set-Go! Kit (eBioscience, San Diego, CA) and detected according to the manufacturer's directions. The plates were read at 450 nm and values were expressed as optical densities. Standard curves were created and used to calculate pg IL-17A/ml of each sample.

Borreliacidal Antibody Assay

Blood was obtained by intracardiac puncture 8 days after infection, and sera were used to determine titers of borreliacidal antibodies. Samples of equal volume were pooled within a group of 4 or 5 mice. Each pooled sample was diluted 1:20 in PBS, passed through a 0.22 µm filter, and then further diluted serially, 1:40 to 1:10,240, with PBS. The diluted specimens were heated at 56°C for 30 minutes to inactivate complement and then cooled to 35°C. The complement-inactivated specimens were incubated with 10⁴ viable *B. burgdorferi* strain 297 organisms and 20 µL of sterile guinea pig complement serum (Sigma-Alrich, St. Louis, MO; 50% hemolytic component ≥ 200 U/ml) in PBS for 24 hours. The presence of viable microbes was then observed in 20 fields using dark-field microscopy. The titer of borreliacidal antibody was considered the reciprocal of the final dilution in which motile *B. burgdorferi* were observed. Control

samples contained *B. burgdorferi* and complement alone; *B. burgdorferi* and PBS alone; and *B. burgdorferi* and PBS added to naïve, non-immune serum.

Preparation of tissues for histological examination

At eight days after infection, the different groups of 4 or 5 mice each were euthanized, and their hind paws were amputated above the knee joint. The paws were cryptically coded, fixed in 10% buffered zinc formalin, and then decalcified. Following decalcification, the paws were placed in tissue-embedding cassettes, embedded in paraffin, and cut into 6- μ m sections. The sections were placed on glass slides and stained with hematoxylin and eosin. An unbiased examination was performed by a board-certified pathologist (T. F. W.). The following scale was used to quantify the degree of histopathologic changes: 0, no change; 1, mild leukocytic infiltration of the subsynovial tissues; 2, moderate infiltration of the subsynovial tissues, synovium, and synovial space; 3, profuse leukocytic infiltration of subsynovial tissues, synovium, and synovial space; 4; profuse leukocytic infiltration of tissues with accompanying destruction of bone or cartilage.

Statistics

Data were analyzed by ANOVA, with the Tukey-Kramer post-hoc test used to examine pairs of means when a significant *F* ratio indicated reliable mean differences between the control and test groups. Paired data were analyzed using a two-tailed Student's *t*-Test. The alpha level was set at 0.05 prior to initiating the experiments. Data are

expressed as the means \pm standard error of the mean. Figures indicate a single experiment. Replicate experiments showed similar relationships.

Results

B. burgdorferi-induced IL-17 production is increased eight days after infection in the absence of IL-10

We measured the *in vitro* production of IL-17 by cells of wild-type and IL-10-deficient mice that were uninfected or infected with *B. burgdorferi* for eight days. Cells from wild-type mice, regardless of whether the mice were previously infected, produced negligible amounts of IL-17 after 6 hours of incubation with *B. burgdorferi* (less than 4 pg/ml; Fig. 1A). Production of IL-17 by these stimulated cells did not differ from that of unstimulated counterparts. In addition, stimulated cells from *B. burgdorferi*-infected wild-type mice produced slightly more IL-17 than stimulated cells from uninfected wild-type mice, but this increase was not significant. By contrast, *B. burgdorferi*-stimulated cells from uninfected and infected IL-10-deficient mice produced significantly more IL-17 than stimulated wild-type counterparts ($P \leq 0.05$; Fig. 1A). Production of IL-17 by stimulated IL-10-deficient cells also was significantly greater than that of unstimulated IL-10-deficient cells ($P \leq 0.05$; Fig. 1A). These increases in IL-17 production following *in vitro* stimulation were observed among cells of both uninfected and *B. burgdorferi*-infected IL-10-deficient mice. Additionally, stimulated cells from infected IL-10-deficient mice produced significantly more IL-17 than stimulated cells from uninfected IL-10-deficient mice ($P \leq 0.05$; Fig. 1A).

After 24 hours of *in vitro* stimulation with *B. burgdorferi*, cells from uninfected and infected wild-type mice produced low levels of IL-17 (less than 10 pg/ml; Fig. 1B). Production of IL-17 by wild-type cells stimulated *in vitro* was slightly, but not significantly, greater than that of unstimulated wild-type cells. In addition, the production of IL-17 by cells of infected wild-type mice did not differ from the production of IL-17 by cells of uninfected wild-type mice. In contrast, *B. burgdorferi*-stimulated cells of uninfected and infected IL-10-deficient mice produced more IL-17 than did stimulated wild-type counterparts (over 100 pg/ml and 600 pg/ml, respectively; Fig. 1B); however, only the latter cells exhibited a statistically significant increase ($P \leq 0.05$). Unstimulated cells from infected IL-10-deficient mice also produced significantly more IL-17 than their unstimulated counterparts from infected wild-type mice ($P \leq 0.05$; Figure 1B). In addition, stimulation of cells from infected IL-10-deficient mice caused a significant increase in IL-17 production compared to unstimulated conditions ($P \leq 0.05$; Figure 1B). Moreover, cells from infected IL-10-deficient mice produced significantly more IL-17 than cells from uninfected IL-10-deficient mice, regardless of whether the cells were stimulated *in vitro* ($P \leq 0.05$; Figure 1B).

Effects of early borrelial exposure on IL-17 production

Splenic cells obtained from wild-type mice infected with *B. burgdorferi* for one day produced a negligible amount of IL-17 (less than 4 pg/ml) when cultured with or without *B. burgdorferi* for six hours (Fig. 2A). By contrast, cells from uninfected wild-type mice produced significantly more IL-17 than their counterparts from infected wild-type animals, regardless of whether the cells were stimulated *in vitro* with the spirochete ($P \leq$

0.05; Figure 2A). Also, stimulated cells from uninfected wild-type mice produced significantly more IL-17 than stimulated cells from uninfected IL-10-deficient mice ($P \leq 0.05$; Figure 2A). *In vitro* stimulation did not cause a significant change in IL-17 production among cells of either uninfected or *B. burgdorferi*-infected wild-type mice. By contrast, *in vitro* exposure to *B. burgdorferi* for six hours caused a significant decrease in the production of IL-17 by cells of both uninfected and infected IL-10-deficient mice ($P \leq 0.05$; Figure 2A).

After 24 hours of incubation with or without *B. burgdorferi*, cells from uninfected wild-type mice produced significantly more IL-17 than did cells from wild-type mice that were infected with the spirochete for one day ($P \leq 0.05$; Figure 2B). Cells from uninfected wild-type mice also produced a significantly greater amount of IL-17 than cells from uninfected IL-10-deficient mice, regardless of *in vitro* stimulation ($P \leq 0.05$; Figure 2B). Exposure to *B. burgdorferi in vitro* for 24 hours caused a significant decrease in IL-17 production by cells of infected wild-type mice ($P \leq 0.05$; Figure 2B) but did not significantly affect cells from uninfected counterparts. In addition, stimulation with the spirochete for 24 hours did not affect IL-17 production by cells of uninfected or *B. burgdorferi*-infected IL-10-deficient mice.

IL-17 production following blockage of CD4 *in vitro*

Suspensions of splenic cells derived from wild-type and IL-10-deficient mice, either uninfected or infected with *B. burgdorferi* for eight days, were stimulated *in vitro* with spirochetes in the presence or absence of 5 μ g/ml anti-CD4 antibody for 6 and 24

hours. After 6 hours of incubation, no differences in IL-17 production were observed due to the presence of anti-CD4 antibody (data not shown).

After 24 hours of incubation, the presence of anti-CD4 antibody slightly enhanced the production of IL-17 by cells of uninfected wild-type mice (Fig. 3A). However, this increase in IL-17 production was not statistically significant. In contrast, treatment of cells from *B. burgdorferi*-infected wild-type mice with anti-CD4 antibody significantly reduced IL-17 production compared to untreated cells ($P \leq 0.05$; Fig. 3A). Treatment of cells from uninfected or infected IL-10-deficient mice with anti-CD4 antibody caused a decrease in IL-17 production that was not statistically significant (Fig. 3B).

Effects of anti-IL-17 antibody treatment on hind paw swelling

Wild-type and IL-10-deficient mice were infected in the hind paws with viable *B. burgdorferi* strain 297 organisms. One group of mice from each strain was administered anti-IL-17 antibody on the day of infection and then daily for 7 days, while the remaining group of mice received an isotype control antibody. The hind paws of *B. burgdorferi*-infected, untreated IL-10-deficient mice had increased swelling of the hind paws compared to those of infected, untreated wild-type mice up to 6 days after infection ($P \leq 0.05$; Fig. 4). The edematous changes in both groups decreased to levels below baseline measurements by day 8 after infection.

Treatment of *B. burgdorferi*-infected mice with antibody to IL-17 ameliorated the swelling observed in untreated mice. Administration of anti-IL-17 antibody significantly

reduced the edematous changes in *B. burgdorferi*-infected IL-10-deficient mice at 3 and 6 days after infection ($P \leq 0.05$; Fig. 4). By day 8 after infection, there were no significant differences in hind paw swelling between these groups. Similarly, treatment of *B. burgdorferi*-infected wild-type mice with anti-IL-17 antibody reduced hind-paw swelling; however, the effects of the antibody in these mice were delayed and milder. A significant decrease in paw swelling was only observed at day 6 after infection ($P \leq 0.05$; Fig. 4). Mice that were injected with BSK medium and then injected daily with anti-IL-17 or isotype control antibody did not show changes in hind paw swelling from baseline levels (data not shown).

Effect of IL-17 antibody administration on histopathology

Histopathological analysis of hind paws, including the tibiotarsal joints and knees, was performed on untreated or anti-IL-17 antibody-treated mice infected with *B. burgdorferi* in order to determine whether the presence of IL-10 regulates IL-17-mediated arthritis induced by the spirochete. *B. burgdorferi*-infected wild-type mice exhibited minimal histopathological changes on day 8 after infection, with only mild hyperplasia observed in some animals (Table 1). *B. burgdorferi*-infected wild-type mice that were administered anti-IL-17 antibody displayed no significant histopathological changes. The histopathological changes observed in untreated, infected wild-type mice were too mild to attribute the lack of pathology in treated animals to the anti-IL-17 antibody.

By contrast, the severity of pathology among untreated IL-10-deficient mice, particularly in the knee and perisynovium of the tibiotarsal joints, was significantly greater than that

of untreated wild-type mice ($P \leq 0.05$; Table 1). IL-10-deficient mice displayed moderate lymphocytic infiltration, as well as hyperplasia and hypertrophy, in the tibiotarsal tissues and knee 8 days after infection with *B. burgdorferi* (Table 1). In addition, these mice exhibited mild erosion of bones in the tibiotarsal joint. Treatment of *B. burgdorferi*-infected, IL-10-deficient mice with antibodies to IL-17 slightly reduced, but did not abrogate, cellular infiltration, hyperplasia, and hypertrophy of the synovium and perisynovium of the tibiotarsal joint (Table 1). However, treatment of infected IL-10-deficient mice with anti-IL-17 antibodies did significantly reduce the histopathological changes of the knee joints observed in infected, but untreated, IL-10-deficient mice ($P \leq 0.05$; Table 1).

Borreliacidal antibody titers

Borreliacidal antibody titers were obtained with sera from wild-type and IL-10-deficient mice that were uninfected or infected with *B. burgdorferi* for 8 days. The borreliacidal antibody titers were low (≤ 80 ; Fig. 5) in all groups. No significant differences were detected.

Discussion

In this report, we support and extend recent findings that IL-17 is a contributing factor in the development of Lyme arthritis by showing that its production and histopathological effects are regulated by IL-10. Spleen cells obtained from wild-type C57BL/6 mice, which are resistant to developing arthritis following infection with *B. burgdorferi* (Barthold *et al.* 1990), produced low levels of IL-17 following stimulation with the

spirochete. By contrast, spleen cells obtained from IL-10-deficient C57BL/6 mice, which develop moderate arthritis after borrelial infection (Brown *et al.* 1999), produced a significant amount of IL-17 at the time of arthritic manifestations following infection with *B. burgdorferi*. We further showed that the significant hind paw swelling and histopathological changes observed in *B. burgdorferi*-infected, IL-10-deficient mice were partially inhibited by administration of anti-IL-17 antibody. Collectively, these findings support our claims (Burchill *et al.* 2003, Nardelli *et al.* 2004, Amlong *et al.* 2006, Nardelli *et al.* 2008b, Nardelli *et al.* 2010, Kotloski *et al.* 2008, Nardelli *et al.* 2008a) and those of others (Codolo *et al.* 2008, Codolo *et al.* 2013) that IL-17 is involved in the development of arthritis following infection with *B. burgdorferi*. More importantly, they reveal a significant regulatory mechanism for an inflammatory pathway that is induced by borrelial infection (Infante-Duarte *et al.* 2000) and which is known to contribute to several other types of arthritis (Chabaud *et al.* 2000, Agarwal *et al.* 2008, Pinto *et al.* 2010, Lubberts *et al.* 2004).

The finding that IL-10 regulates the arthritic, IL-17-mediated response to *B. burgdorferi* adds to the known properties of the anti-inflammatory cytokine in controlling the immune response to, and pathological outcomes of, borrelial infection. Early investigations into the differences in pathology observed between C3H and C57BL/6 mice demonstrated that the effect of IL-10 is a key factor in determining disease outcome (Brown *et al.* 1999, Ganapamo *et al.* 2000). Until now, much of the investigation pertaining to the inflammatory targets of IL-10 focused on the Th1/IFN- γ response, which has long been known as a major inflammatory pathway of later-stage Lyme arthritis (Yssel *et al.* 1991,

Gross *et al.* 1998). For example, modulation of IL-10 *in vitro* reciprocally influenced IFN- γ production by stimulated synovial cells of Lyme arthritis patients (Yin *et al.* 1997). In addition, differences in IL-10-mediated regulation of IFN- γ accounted for the differences in arthritic severity exhibited by C3H and C57BL/6 mice (Brown *et al.* 1999, Ganapamo *et al.* 2000). In support of this, recent findings show that IL-10 plays a particularly important role in controlling the IFN- γ -driven arthritis caused, in large part, by CD4⁺ T cells in *B. burgdorferi*-infected mice (Sonderegger *et al.* 2012). Our current findings suggest that IL-10 additionally exerts its anti-inflammatory effects during *B. burgdorferi* infection by suppressing the production of IL-17, even in the presence of IFN- γ .

These findings are important because they provide additional support that IL-17 is elicited in response to *B. burgdorferi* and, ultimately, contributes to the pathology of Lyme arthritis. Consideration of IL-17 as an inflammatory mediator of Lyme arthritis stems from the observation that, while Th1-mediated inflammation is a major cause of pathology (Yssel *et al.* 1991, Gross *et al.* 1998, Strle *et al.* 2012, Shen *et al.* 2010), arthritis also develops independently of IFN- γ (Brown & Reiner 1999, Christopherson *et al.* 2003, Munson *et al.* 2004). Infante-Duarte, *et al.* first determined that stimulated cells of Lyme arthritis patients produce IL-17 (Infante-Duarte *et al.* 2000). Following this observation, a series of studies showed that various Th17-associated cytokines were associated with pathology in murine models of *Borrelia*-induced arthritis (Burchill *et al.* 2003, Nardelli *et al.* 2004, Amlong *et al.* 2006, Nardelli *et al.* 2008b, Nardelli *et al.* 2010, Kotloski *et al.* 2008), supporting claims of the arthritic potential of IL-17 in Lyme arthritis

(Nardelli *et al.* 2008a). Supporting this, NapA of *B. burgdorferi* stimulated innate cells to drive the development of Th17 cells that were present in the synovia of humans with Lyme arthritis (Codolo *et al.* 2008). NapA is a key antigen in the recruitment of inflammatory cells which, along with NapA itself, stimulate the production of IL-17 from different T cell populations (Codolo *et al.* 2013). However, Th17 cells were greatly outnumbered by Th1 cells in the synovia of humans with antibiotic-refractory or antibiotic-responsive Lyme arthritis, comprising approximately 5% of all CD4+ T cells (Shen *et al.* 2010). As a result, little, if any, contribution to disease was attributed to Th17 cells. Although some studies have demonstrated the reciprocal nature of Th1 and Th17 cells (Yoshimura *et al.* 2006), others have shown that these inflammatory pathways can function simultaneously and, occasionally, even be expressed simultaneously by an individual cell (Codolo *et al.* 2013, Zielinski *et al.* 2012). Our current findings support the assertion that IL-17 contributes to the pathology of Lyme arthritis.

Our findings also suggest that multiple cell types are responsible for the production of IL-17 in response to *B. burgdorferi*. Various cell types, including Th17 cells, produce the cytokine (Pappu *et al.* 2011). We showed that unseparated spleen cells from infected C57BL/6 wild-type mice, when incubated with *B. burgdorferi* and antibodies to CD4, produced significantly less IL-17 than did stimulated cells not additionally incubated with antibodies. However, the production of IL-17 by these latter cells was initially low. When we blocked CD4 in cultures of stimulated cells obtained from IL-10-deficient mice, we observed decreases in IL-17 production; however, these decreases were not

statistically significant. It is possible that the concentration of anti-CD4 antibody we used here was insufficient to counter the significant production of IL-17 by these IL-10-deficient cells. However, it is also probable that CD4⁺ and non-CD4⁺ cell populations produced IL-17 in this system. We are currently assessing the production of IL-17 by these cells in the presence of multiple doses of anti-CD4 antibody, and are characterizing IL-17-producing cell phenotypes during infection.

It is important also to note that the immune pathways associated with regulation of IL-17 are complex. For example, in support of our findings, opposing functions of IL-10 and various IL-17-producing cell types have been reported in other disease models, including infections (Frodermann *et al.* 2011, Gonzalez-Lombana *et al.* 2013) and, importantly, arthritis (Yang *et al.* 2012, Heo *et al.* 2010). However, cells that produce both cytokines exist (Zielinski *et al.* 2012, McGeachy *et al.* 2007), and IL-10-producing regulatory T (Treg) cells have also been shown to aid in the production of Th17 cells at certain periods of development (Chen *et al.* 2011). Moreover, the roles of Th17 cells and IL-17 in Lyme arthritis have been challenged by the finding that humans with a reduced capacity to bind the Th17 cell-survival cytokine interleukin-23 (IL-23) have reduced IL-17 production but do not exhibit reduced arthritis (Oosting *et al.* 2011). In addition, we showed that the Treg cell-associated cytokine interleukin-35 enhances, rather than inhibits, arthritis in a murine model of disease and only reduces IL-17 production slightly (Kuo *et al.* 2011). Taken together, these findings demonstrate the complexity of inflammatory and regulatory pathways involved in Lyme arthritis and necessitate their further investigation.

Our findings suggest that IL-10 regulates *B. burgdorferi*-mediated pathology, in part, by limiting the arthritic potential of IL-17. The data we present here are reminiscent of our findings linking IL-17 and putative Treg cells in a model of *Borrelia*-induced arthritis (Nardelli *et al.* 2004, Nardelli *et al.* 2005, Nardelli *et al.* 2006). We showed that blocking IL-17 in IFN- γ -deficient, *Borrelia*-vaccinated and -infected mice prevented pathology and increased the production of CD25-expressing CD4⁺ cells (Nardelli *et al.* 2004). Concomitantly injecting these mice with antibody to CD25 reduced this cell population and resulted in massive erosion of the ankle joints (Nardelli *et al.* 2004). Adoptive transfer of CD4⁺CD25⁺ cells, enriched following anti-IL-17 antibody treatment, into infected recipients completely prevented the development of arthritis (Nardelli *et al.* 2005). Importantly, we did not observe regulatory functions among CD4⁺CD25⁺ cells obtained from mice in which IL-17 was not blocked (Nardelli *et al.* 2006). Following these early experiments, Bettelli, *et al.* showed that Th17 cells and CD4⁺CD25⁺Foxp3⁺ Treg cells developed via inverse pathways from a common precursor cell (Bettelli *et al.* 2006). In light of our findings from nearly a decade ago, it may not be surprising that we observed a significant IL-17 response in IL-10-deficient, *B. burgdorferi*-infected mice, as CD4⁺CD25⁺Foxp3⁺ Treg cells function, in large part, through their production of IL-10 (Huber *et al.* 2011). In addition, it may not be surprising that anti-IL-17 antibody treatment did not completely abrogate arthritis in infected IL-10-deficient mice, since increased IL-17 production is likely just one of many effects of an absence of IL-10. In support of our hypothesis that Treg cells are key mediators of Lyme arthritis (Nardelli *et al.* 2004, Nardelli *et al.* 2008a, Nardelli *et al.* 2005, Nardelli *et al.* 2006), more recent

findings have shown that patients with persistent, antibiotic-refractory disease produce more Treg cells than those with disease that readily resolves with antimicrobial therapy (Shen *et al.* 2010). In addition, longer durations of antibiotic-refractory Lyme arthritis post-treatment are associated with lower frequencies of Treg cells (Shen *et al.* 2010, Vudattu *et al.* 2013). Collectively, these data imply that Lyme arthritis may be modulated by IL-10, which, in the context of borrelial infection, down-regulates inflammatory cytokines, including IL-17.

What is the importance of this alternative response to *B. burgdorferi*? We show that wild-type or IL-10-deficient cells harvested one day after infection, when subsequently stimulated with the spirochete, produce an equal or reduced amount of IL-17.

Additionally, cells from *B. burgdorferi*-infected wild-type mice produce less IL-17 than cells from uninfected mice. Collectively, these findings suggest a tendency toward an anti-inflammatory mechanism early after interaction with the spirochete. In support of this, early infection with *B. burgdorferi* results in an increase in IL-10 (Lazarus *et al.* 2008), suggesting that the resulting suppression of the inflammatory response may allow a successful infection to occur. Our findings suggest that initial exposure with the spirochete may cause an early delay or decrease in IL-17 that may not only assist in successful infection, but which may occur independently of IL-10. Suppression of IL-17 production following early exposure to *B. burgdorferi* may allow some of the initial infectious spirochetes to survive, possibly until the inflammatory response to borrelial antigens becomes more robust. Additional studies, particularly ones using skin samples of tick-infected mice, would be required to test this hypothesis. By contrast, the effects

of IL-10 on IL-17 production were more pronounced after one week of infection. Borrelial infection in the presence or absence of IL-10 may be associated with an inverse response of various inflammatory cytokines, including not only tumor necrosis factor- α and IFN- γ (Brown *et al.* 1999, Ganapamo *et al.* 2000, Ganapamo *et al.* 2000, Yin *et al.* 1997, Sonderegger *et al.* 2012, Lazarus *et al.* 2008, Murthy *et al.* 2000, Diterich *et al.* 2001, Crandall *et al.* 2006, Lazarus *et al.* 2006, Gautam *et al.* 2011), but, also, IL-17. If a certain threshold of immune suppression by IL-10 is not achieved, a significant, multi-faceted inflammatory response may ensue.

Our histopathological findings support the claim of multiple inflammatory pathways contributing to the development of Lyme arthritis. As expected, *B. burgdorferi*-infected, C57BL/6 wild-type mice exhibited mild paw swelling and minimal histopathological changes by day 8 after infection. Despite the mild hind paw swelling of these mice, administration of anti-IL-17 antibody significantly decreased the levels of edema by day six after infection. By contrast, *B. burgdorferi*-infected IL-10-deficient mice exhibited moderate swelling and histopathological changes of the hind paws. Treatment with antibody to IL-17 resulted in a significant reduction of swelling that was observed sooner and which was sustained for the duration of the experiment. Importantly, the overall histopathology of these treated mice was decreased, particularly in the knee and perisynovium of the tibiotarsal joint. However, some mice were free of pathology, while others still exhibited a degree of pathology. This supports the idea that IL-17 is a partial contributor to arthritis and, therefore, blocking only this cytokine would likely not be a panacea.

Finally, we found that after eight days of infection, IL-10 failed to significantly affect the levels of borreliacidal antibodies in the serum of *B. burgdorferi*-infected mice. A deficiency in IL-10 has been linked to reduced microbial loads and increased levels of *B. burgdorferi*-specific antibodies (Brown *et al.* 1999, Lazarus *et al.* 2006); however, these findings were observed after at least two weeks after infection. In addition, a robust innate immune response, rather than the borreliacidal antibody response, was implicated in the reduced levels of *B. burgdorferi* in the tissues of IL-10-deficient mice (Lazarus *et al.* 2006). Therefore, the lack of significant borreliacidal antibody activity in our IL-10 deficient mice may not be surprising. The apparent lack of an influence of IL-17 on borreliacidal antibody production in these mice may also not be surprising, considering our previous finding that blocking IL-17 in a model of *B. burgdorferi*-mediated arthritis failed to affect these titers (Amlong *et al.* 2006), and the recent finding that antibody production overall may not be affected directly by IL-17 (Shibui *et al.* 2012). We are further investigating the role of IL-17 in the early antibody response to *B. burgdorferi*.

In summary, we showed that IL-10 regulates an arthritic IL-17 response following infection with *B. burgdorferi*. These findings add to the known immunomodulatory functions of IL-10 in the context of borreliacidal infection, as well as support the growing evidence that IL-17 contributes to disease. These findings were observed using infected IL-10-deficient C57BL/6 mice, which has been described recently as a viable model to investigate elements of adaptive immunity not readily observed in traditional disease

models (Sonderegger *et al.* 2012). This sentiment echoes our previous claims that the use of models that exhibit the full array of immune events (including T cells) involved in the development of arthritis in humans is vital for a more complete understanding of the inflammatory events associated with Lyme arthritis in humans (Nardelli *et al.* 2010, Kotloski *et al.* 2008, Nardelli *et al.* 2008a). Here, we showed that this model also allows for investigating the role of IL-17 in *Borrelia*-mediated arthritis, which while shown to potentially contribute to human disease (Codolo *et al.* 2008, Colodo *et al.* 2013), has not been demonstrated in traditional animal models (Nardelli *et al.* 2010). More importantly, we provide additional evidence for considering an inflammatory pathway that may be targeted for the treatment of Lyme arthritis.

Acknowledgments

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Figure 1.

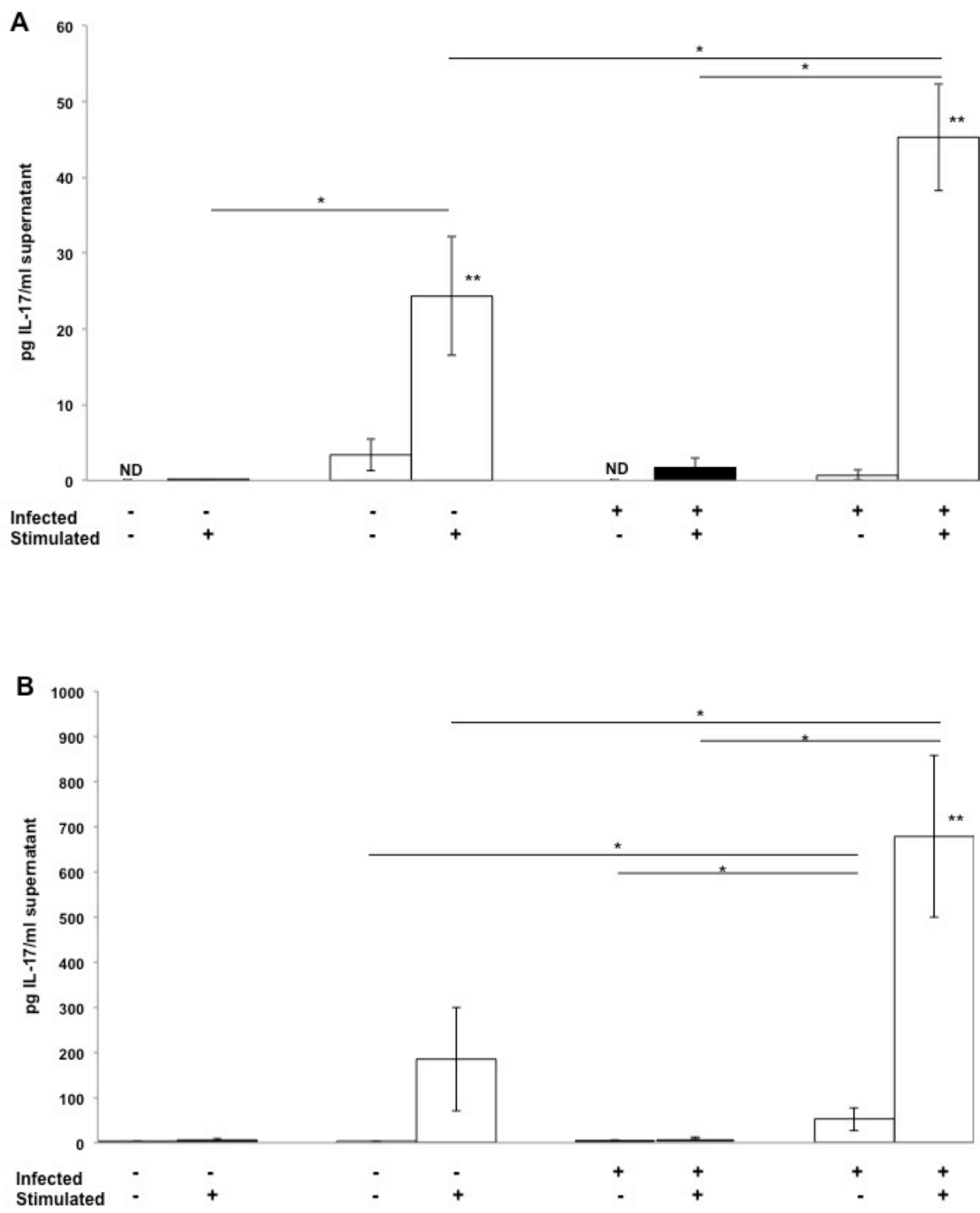


Figure 1. IL-17 production is increased in the absence of IL-10 eight days after infection with *B. burgdorferi*. Splenic cells obtained from wild-type (black bars) and IL-10-deficient (white bars) mice eight days after injection of *B. burgdorferi* or BSK medium were cultured in the presence or absence of the spirochete for 6 (A) and 24 (B) hours. (A) After 6 hours of incubation, cells from infected or uninfected wild-type mice produced minimal IL-17, regardless of *in vitro* stimulation. By contrast, stimulated cells from uninfected and *B. burgdorferi*-infected IL-10-deficient mice produced significant amounts of IL-17. The IL-17 production by these stimulated IL-10-deficient cells was significantly greater than that of stimulated wild-type counterparts, as well as that of unstimulated IL-10-deficient counterparts. Additionally, stimulated cells from infected IL-10-deficient mice produced significantly more IL-17 than stimulated cells from uninfected IL-10-deficient mice. (B) Production of IL-17 by wild-type cells remained low after 24 hours of incubation with or without the spirochete. By contrast, stimulated cells from *B. burgdorferi*-infected IL-10-deficient mice produced a large amount of IL-17. The production of IL-17 by these cells was significantly greater than that of stimulated cells from infected wild-type mice, stimulated cells from uninfected IL-10-deficient mice, and unstimulated cells from infected IL-10-deficient mice. Additionally, unstimulated cells from infected IL-10-deficient mice produced significantly more IL-17 than did unstimulated cells from infected wild-type mice and uninfected IL-10-deficient mice. Data are mean levels of IL-17/ml culture supernatant \pm SEM. * $P \leq 0.05$. ND, not detected.

Figure 2.

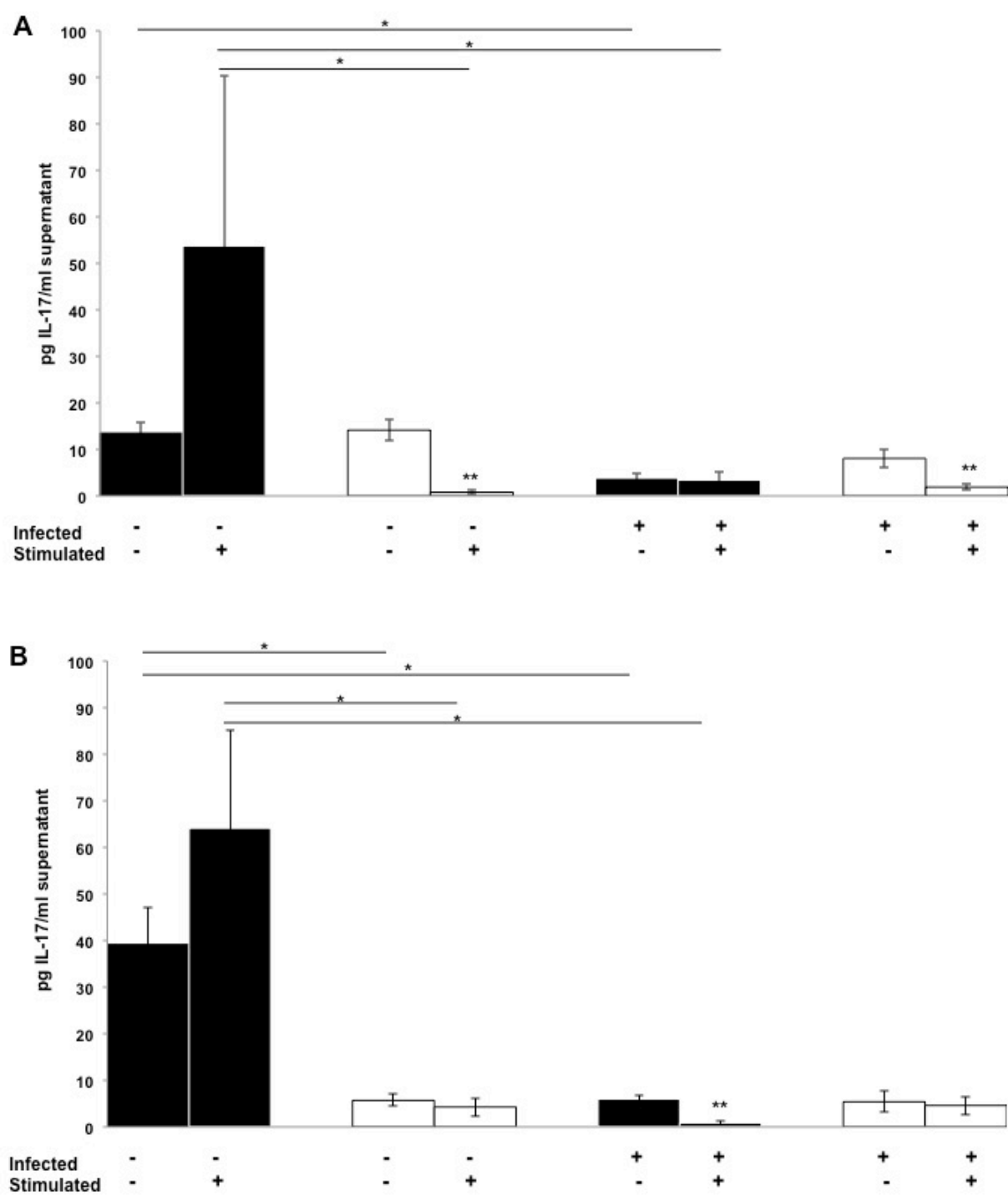


Figure 2. Effects of early *B. burgdorferi* exposure on production of IL-17. Splenic cells obtained from wild-type (black bars) and IL-10-deficient (white bars) mice one day after injection of *B. burgdorferi* or BSK medium were cultured in the presence or absence of the spirochete for 6 (A) and 24 (B) hours. (A) After 6 hours of incubation, cells from uninfected wild-type mice produced significantly more IL-17 than did cells from infected wild-type mice, regardless of stimulation *in vitro*. Stimulated cells from uninfected wild-type mice also produced significantly more IL-17 than stimulated cells from uninfected IL-10-deficient mice. In addition, *in vitro* stimulation with *B. burgdorferi* for 6 hours caused a significant decrease in IL-17 production by cells from IL-10-deficient, but not wild-type, mice, regardless of whether the mice were previously infected. (B) After 24 hours of incubation, cells from uninfected wild-type mice produced significantly more IL-17 than did cells from infected wild-type mice, regardless of stimulation *in vitro*. Similarly, these cells produced significantly more IL-17 than those of uninfected IL-10-deficient counterparts. In addition, *in vitro* stimulation with *B. burgdorferi* for 24 hours caused a significant decrease in IL-17 production by cells from infected wild-type mice, but not by those of uninfected wild-type mice, uninfected interleukin-10-deficient mice, or *B. burgdorferi*-infected interleukin-10-deficient mice. Data are mean levels of IL-17/ml culture supernatant \pm SEM. *P \leq 0.05.

Figure 3.

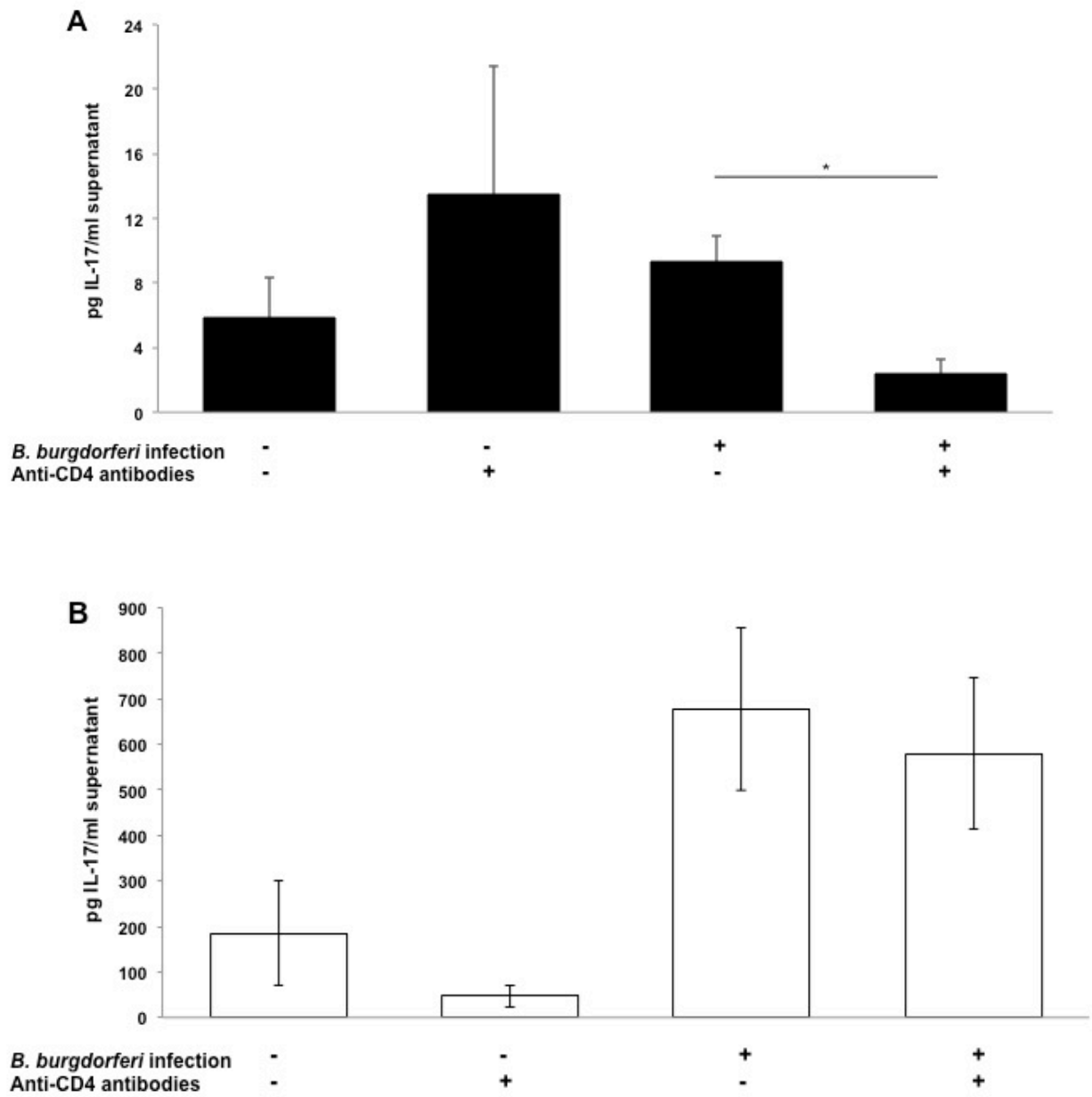


Figure 3. Partial inhibition of IL-17 production by blocking CD4 *in vitro*. Wild-type (A) and IL-10-deficient (B) splenic cells obtained from uninfected mice and mice infected with *B. burgdorferi* for 8 days were stimulated with the spirochete in the presence or absence of 5 µg/ml anti-CD4 antibody for 24 hours. (A) Addition of anti-CD4 antibody to cells of uninfected, wild-type mice did not significantly affect IL-17 production. In contrast, anti-CD4 antibody treatment of cells from *B. burgdorferi*-infected wild-type mice caused a significant reduction in IL-17 production. (B) Anti-CD4 antibody treatment of cells from uninfected or *B. burgdorferi*-infected IL-10-deficient mice reduced the production of IL-17. However, these reductions were not statistically significant. Data are mean levels of IL-17/ml culture supernatant ± SEM. * $P \leq 0.05$.

Figure 4.

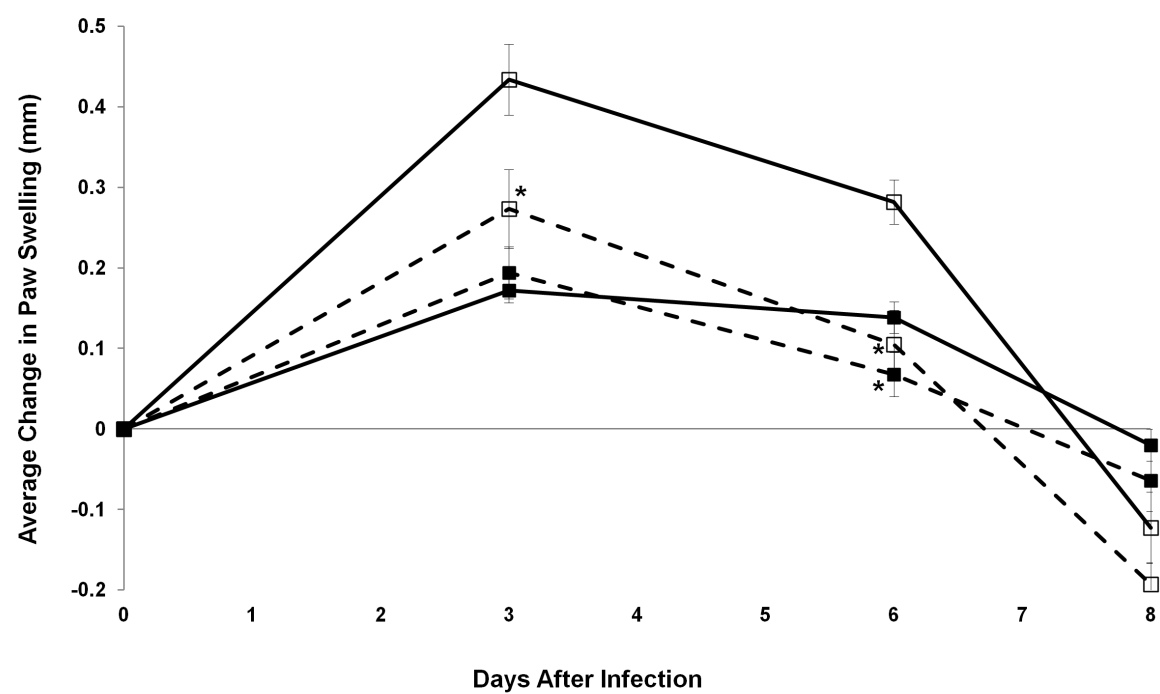


Figure 4. Treatment of *B. burgdorferi*-infected IL-10-deficient mice with anti-IL-17 antibody significantly reduces hind paw swelling.

Wild-type (black squares) and IL-10-deficient (white squares) mice were infected with *B. burgdorferi* and administered anti-IL-17 antibody (dashed lines) or isotype control antibody (solid lines) within 3 hours of infection and daily thereafter for 7 days. Hind paw swelling of infected, but untreated, IL-10-deficient mice was greater than that of infected, but untreated, wild-type mice.

Anti-IL-17 antibody treatment of infected wild-type mice caused a significant reduction in hind paw swelling that was evident on day 6 after infection. Treatment of *B. burgdorferi*-infected IL-10-deficient mice with anti-IL-17 antibody caused a substantial reduction in hind paw swelling that was evident on day 3 after infection and which lasted through day 6 after infection. Data are mean changes in paw swelling relative to baseline \pm SEM. * $P \leq 0.05$.

Table 1.

	Synovium (Tibiotarsal)	Perisynovium (Tibiotarsal)	Tibiotarsal Bone Erosion	Knee
Wild-type + isotype antibody	0.50	0.00	0.00	0.50
Wild-type + anti-IL- 17 antibody	0.50	0.00	0.00	0.25
IL-10-deficient + isotype antibody	1.75	1.75*	0.75	2.50*
IL-10-deficient + anti-IL-17 antibody	1.50	1.00*	0.75	1.00**

Table 1. Treatment of *B. burgdorferi*-infected IL-10-deficient mice with anti-IL-17 antibody reduces the severity of histopathology. *B. burgdorferi*-infected wild-type mice that were administered anti-IL-17 antibody or an isotype control antibody exhibited minimal histopathological changes of the ankle joint 8 days after infection. *B. burgdorferi*-infected IL-10-deficient mice exhibited arthritic changes of the hind paws, including bone erosion of the ankle joint and cellular infiltration and hyperplasia of the perisynovial tissues and knee, 8 days after infection. Treatment of these mice with anti-IL-17 antibody caused a significant reduction in the severity of pathology observed in the perisynovium of the tibiotarsal joint and knee. *Significantly greater ($P \leq 0.05$) than wild-type counterpart. **Significantly ($P \leq 0.05$) less than isotype antibody-treated counterpart.

Figure 5.

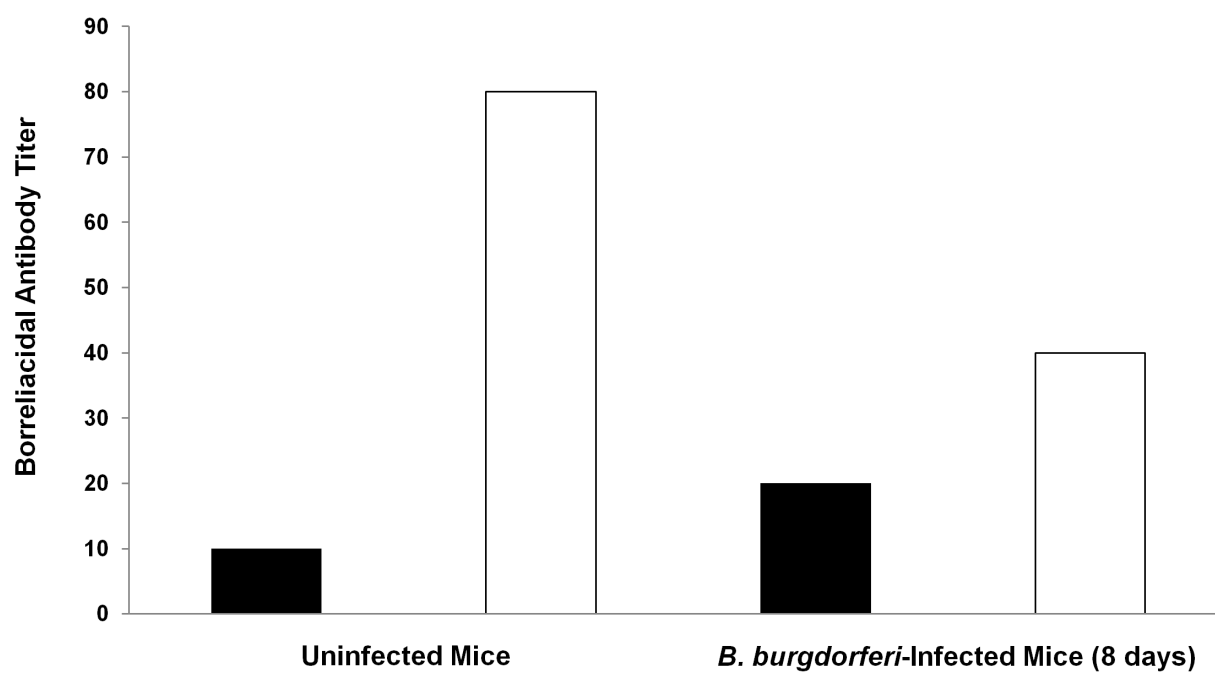


Figure 5. IL-10 deficiency does not affect borreliacidal antibody titers eight days after infection. Sera from uninfected and *B. burgdorferi*-infected wild-type (black bars) and IL-10-deficient (white bars) mice were collected on day 8 after infection. Borreliacidal antibody titers of all groups were low (≤ 80). No significant differences in titers between groups were detected.

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CHAPTER 3: SPECIFIC AIM 2

CD4+ Cell-Derived Interleukin-17 in a Model of Dysregulated, *Borrelia*-Induced Arthritis

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Abstract

Lyme borreliosis, which is caused in the United States by the spirochete *Borrelia burgdorferi*, may manifest as different arrays of signs, symptoms, and severities between infected individuals. Recent studies have indicated that particularly severe forms of Lyme borreliosis in humans are associated with an increased Th17 response. Here, we hypothesized that a murine model combining the dysregulated immune response of an environment lacking interleukin-10 (IL-10) with a robust T cell-driven inflammatory response would reflect arthritis associated with the production of interleukin-17 (IL-17) by CD4+ cells. We demonstrate that IL-10 regulates the production of IL-17 by *Borrelia*-primed CD4+ cells early after interaction with Lyme spirochetes *in vitro* and that infection of *Borrelia*-primed mice with *B. burgdorferi* leads

to significant production of IL-17 that contributes to the development of severe arthritis. These results extend our previous findings by demonstrating that a dysregulated adaptive immune response to Lyme spirochetes can contribute to severe, Th17-associated arthritis. These findings may lead to therapeutic measures for individuals with particularly severe symptoms of Lyme borreliosis.

Introduction

The causative agent of Lyme borreliosis in the United States, *Borrelia burgdorferi*, may induce a potent immune response, which can result in multi-system pathology in untreated individuals. The signs and symptoms of Lyme borreliosis are determined, in part, by the degree to which inflammatory mediators of innate and adaptive immunity are activated and regulated throughout infection. That Lyme borreliosis within a population is manifested as different arrays of signs and symptoms, each with varying degrees of severity, indicates that inflammatory responses to the spirochete differ in robustness and mechanism. This assertion is supported by recent findings that different immune pathways exist for the development of Lyme arthritis in experimental animals (Sonderegger *et al.* 2012, Hansen *et al.* 2013) and humans (Crandall *et al.* 2006, Strle *et al.* 2014). As a result, no one animal model of *B. burgdorferi* infection is sufficient to reflect the complex immune responses and varying clinical outcomes exhibited by humans.

The differences in pathology between genotypes of experimental animals have been partially linked to the anti-inflammatory cytokine interleukin-10 (IL-10). IL-10 is produced

following exposure to borrelial antigens (Giambartolomei *et al.* 1998; Brown J *et al.* 1999; Lazarus *et al.* 2008), possibly aiding infection by suppressing various effectors of the innate immune response (Yin *et al.* 1997; Brown J *et al.* 1999; Lazarus *et al.* 2006; Gautam *et al.* 2011). However, the early production of IL-10 following *B. burgdorferi* infection also affects the adaptive immune response. For example, IL-10 produced by macrophages plays a significant role in inhibiting an interferon-gamma (IFN- γ)-driven arthropathy in mice (Sonderegger *et al.* 2012). Also, *B. burgdorferi*-induced IL-10 inhibits events involved in the activation of adaptive immunity, including phagocytosis and expression of MHC class II and various co-stimulatory molecules (Chung *et al.* 2013). Additionally, we showed that the anti-inflammatory effects of IL-10 in the days following infection with *B. burgdorferi* extend to preventing an interleukin-17 (IL-17)-dependent arthropathy (Hansen *et al.* 2013).

We first identified a role for an arthritic IL-17 response to *B. burgdorferi* using a model exhibiting a particularly robust T cell response to Lyme spirochetes (Lim *et al.* 1995a, 1995b). Specifically, we showed that wild-type and IFN- γ -deficient C57BL/6 mice that are primed and subsequently infected with heterologous Lyme spirochetes develop arthritis that is dependent, in part, on various Th17-associated cytokines (Burchill *et al.* 2003; Nardelli *et al.* 2004; Nardelli *et al.* 2005; Amlong *et al.* 2006; Kotloski *et al.* 2008; Nardelli *et al.* 2008b; Kuo *et al.* 2011). Importantly, the presence of Th17 cells or their associated cytokines in humans with Lyme arthritis has been demonstrated in several studies. For example, IL-17-producing T cells have been isolated from the joints of humans with Lyme arthritis (Infante-Duarte *et al.* 2000; Codolo *et al.* 2008; Codolo *et al.*

2013). In addition, while Th1 cells have long been observed in patients with Lyme arthritis (Yssel *et al.* 1991; Gross *et al.* 1998; Shen *et al.* 2010; Strle *et al.* 2012), a substantial proportion of Th17 cells has been shown to be present among individuals with the disease (Shen *et al.* 2010). Also, the Th17 cell-supporting cytokine IL-23, which, like IL-17, is induced by *B. burgdorferi* (Knauer *et al.* 2007), has been shown to be elevated in certain patients with severe, persisting symptoms after treatment for Lyme borreliosis (Strle *et al.* 2014).

It has been hypothesized that the severe symptoms of Lyme arthritis may be due partially to a dysregulated immune response (Nardelli *et al.* 2004; Steere and Glickstein 2004; Nardelli *et al.* 2008a). In this report, we used a model combining the dysregulation of an environment devoid of IL-10 (Sondergger *et al.* 2012) with the potency of a robust T cell-driven inflammatory response (Lim *et al.* 1995a, 1995b) to characterize factors that may influence the severe disease, which occurs in some patients. We show that *Borrelia*-primed CD4⁺ cells produce IL-17 in an IL-10-regulated manner soon after interaction with Lyme spirochetes *in vitro*. In addition, infection of *Borrelia*-primed, IL-10-deficient mice induces a substantial increase in IL-17 production that may contribute to the development of severe arthritis. These results extend our previous findings by demonstrating that a dysregulated adaptive immune response to Lyme spirochetes can lead to Th17-associated inflammation. These findings may lead to therapeutic measures for individuals with particularly severe symptoms of Lyme borreliosis that are associated with an increased Th17 response.

Materials and Methods

Mice

Male and female, six- to twelve-week old, IL-10-deficient and wild-type mice on the C57BL/6 background, originally obtained from Jackson Laboratories, were provided by J.-A. Lyons (University of Wisconsin-Milwaukee). Mice were housed at the University of Wisconsin-Milwaukee animal facility at an ambient temperature in a humidity-controlled environment under a 12-hour light/dark schedule. Food and water were provided *ad libitum*. All experiments were reviewed and approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee.

Priming of mice

It has been shown that injection of experimental animals with inactivated, whole Lyme spirochetes in adjuvant primes the animals for a robust, arthritic T cell response (Lim *et al.* 1995a, 1995b) that is activated following infection with heterologous spirochetes. A homologous infection strain also was used to induce arthritis after priming of hamsters. This depended on infection occurring prior to the development of protective antibodies produced in response to priming (Lim *et al.* 1994); however, this has yielded inconsistent results in mice. We used this heterologous priming/infection model in mice to identify a role for IL-17 in the development of *Borrelia*-induced arthritis (Burchill *et al.* 2003; Nardelli *et al.* 2008b), which has been reported in some humans (Codolo *et al.* 2008, 2013) but which is not evident upon infection of disease-susceptible C3H mice (Nardelli *et al.* 2010; Lasky *et al.* 2015).

Borrelia bissetii organisms (provided by S. M. Callister, Gundersen Health System, La Crosse, WI) were maintained as 1.0 mL frozen stocks at -80°C. Aliquots were thawed and inactivated by incubating in a 56°C water bath for 30 minutes. Inactivation of the organisms was determined by lack of motility when viewed under dark-field microscopy and lack of growth after culture in Barbour-Stoenner-Kelly (BSK) medium for seven days. Heat-inactivated bacteria were washed four times by centrifuging (10,000 x g) with phosphate buffered saline (PBS) for 10 minutes, resuspended in PBS, and enumerated using a Petroff-Hausser counting chamber. After washing, inactivated bacteria were mixed with the adjuvant (1% aluminum hydroxide; General Chemical Performance Products, Berkeley Heights, NJ) to obtain a final concentration of 4×10^6 organisms mL⁻¹ in the preparation. Mice were injected with 0.25 mL of the antigen suspension subcutaneously in each inguinal region while under anesthesia with isoflurane in a nose-and-mouth cup. Control groups consisted of mice injected with aluminum hydroxide without *B. bissetii*.

Infection of mice

Borrelia burgdorferi 297 organisms (provided by S. M. Callister) were maintained as 1.0 mL frozen stocks at -80°C. Aliquots were grown at 34°C for three to six days in BSK medium prior to infection. Twenty-one days after priming, groups of four to six mice were injected with 10^6 viable *B. burgdorferi* 297 organisms in 50 µl BSK medium subcutaneously in each hind paw while anesthetized with isoflurane in a nose-and-mouth cup. Control groups consisted of primed mice injected with BSK medium alone.

Administration of anti-IL-17 antibody

Monoclonal antibodies specific for mouse IL-17A (clone eBioMM17F3; IgG1) and mouse IgG1 isotype control antibodies were purchased from eBioscience (San Diego, CA). Antibodies were reconstituted with filter-sterilized PBS to create a final concentration of $50 \mu\text{g mL}^{-1}$. Three hours after infection, mice were injected with 50 μL (2.5 μg) of anti-IL-17 antibodies subcutaneously in each hind paw while anesthetized with isoflurane in a nose-and-mouth cup. Antibody treatment was conducted daily for seven days. Control mice were injected in the same manner with IgG isotype control antibody (eBioscience).

Assessment of swelling

The width and thickness of the tibiotarsal joints were measured using a digital caliper (Marathon; Richmond Hill, Ontario, Canada) with a sensitivity of 0.01 mm.

Measurements were conducted immediately prior to, and throughout, infection with *B. burgdorferi*. The paws of antibody-injected mice were measured before injection.

Spleen cell culture

Spleen cells were harvested from mice one or eight days after infection (or 22 or 29 days after priming, in uninfected animals). Single-cell suspensions of whole spleen cells were created by passing the tissue gently through a nylon mesh screen (BD Falcon; BD Biosciences, San Jose, CA) into cold RPMI 1640 medium (Cellgro; Corning, Manassas, VA) supplemented with L-glutamine and 25 mM HEPES. 1×10^6 spleen cells were incubated with or without 1×10^5 viable *B. burgdorferi* strain 297 organisms and with or

without 5 µg anti-mouse CD4 antibodies (clone G1.5; eBioscience) or isotype control antibodies (eBioscience). Cell culture supernatants were collected after six or 24 hours of incubation at 37°C in an atmosphere of 5% CO₂.

ELISA

IL-17 levels in cell culture supernatants were determined using IL-17A ELISA Ready-Set-Go! Kits (eBioscience; San Diego, CA) according to the manufacturer's instructions. The plates were read at an absorbance of 450 nm to generate optical density values. Standard curves were then created to calculate the amount of IL-17 in each sample as pg mL⁻¹.

Histological examination

Histological examination was conducted by a board-certified pathologist (Thomas F. Warner, University of Wisconsin-Madison) as described previously (Hansen *et al.* 2013). Briefly, paws were dissected eight days after infection, fixed in formalin, decalcified, sectioned, and stained with hematoxylin & eosin. The histopathological changes were scored according to the following scale: 0, no change; 1, mild leukocytic infiltration of subsynovial tissue; 2, moderate infiltration of subsynovial tissue, synovium, and synovial space; 3, profuse leukocytic infiltration of subsynovial tissue, synovium and synovial space; and 4, profuse leukocytic infiltration of tissues with destruction of bone or cartilage present.

Statistics

Data were analyzed using ANOVA followed by the Tukey-Kramer *post hoc* test when a significant *F* value indicated reliable mean differences. Paired data were analyzed using two-tailed Student's *t* test. Data were analyzed using Prism and SigmaPlot. The alpha level was set at 0.05 prior to initiation of experiments. Data are expressed as mean \pm standard error of the mean (SEM) and reflect the results of two experiments, unless otherwise stated.

Results

Production of IL-17 eight days after infection of primed mice

Spleen cells from *B. bissettii*-primed, but uninfected, wild-type C57BL/6 mice produced low amounts of IL-17 (5 pg mL⁻¹ supernatant) after six hours of incubation in media (Figure 1A). When *B. bissettii*-primed wild-type cells were stimulated *in vitro* for six hours with a viable heterologous spirochete, *B. burgdorferi* strain 297, no IL-17 was detected. In addition, spleen cells from *B. bissettii*-primed, IL-10-deficient mice produced amounts of IL-17 that were below the level of detection of the assay following incubation in media for six hours (Figure 1A). However, incubation of *B. bissettii*-primed, IL-10-deficient cells with a heterologous *Borrelia* strain significantly increased the production of IL-17 (19 pg mL⁻¹; $P \leq 0.05$). The production of IL-17 by these primed, stimulated IL-10-deficient cells also was significantly greater ($P \leq 0.05$) than that of their wild-type counterparts. After 24 hours of stimulation with *B. burgdorferi* strain 297, cells from *Borrelia*-primed, but uninfected, wild-type mice produced more IL-17 than their unstimulated counterparts (Figure 1C). However, this increase was not statistically

significant. By contrast, primed cells from IL-10-deficient mice produced a large amount of IL-17 (approximately 500 pg mL⁻¹) following exposure *in vitro* to the heterologous strain, which was significantly greater ($P \leq 0.05$) than that of primed, but unstimulated, IL-10-deficient cells (74 pg mL⁻¹) and primed, stimulated wild-type cells.

Similar findings were observed among cells that were obtained from *B. bissettii*-primed mice that were subsequently infected with *B. burgdorferi* strain 297 for eight days. Spleen cells from primed, infected wild-type mice exhibited no differences in IL-17 production when incubated for six hours with the infection strain (Figure 1B). By contrast, incubation of cells from IL-10-deficient, primed and infected mice for six hours with the challenge strain significantly increased the production of IL-17, from 9 pg mL⁻¹ supernatant to 43 pg mL⁻¹ supernatant ($P \leq 0.05$; Figure 1B). In addition, *in vitro* stimulation of spleen cells from *Borrelia*-primed and -infected mice resulted in a significant increase in IL-17 production in the absence of IL-10 compared to wild-type cells. Moreover, spleen cells from primed, infected wild-type mice exhibited increased, but not statistically significant, IL-17 production when incubated for 24 hours with the infection strain (Figure 1D). Spleen cells from IL-10-deficient, *Borrelia*-primed and -infected mice that were stimulated with the infection strain *in vitro* produced significantly more ($P \leq 0.05$) IL-17 (186 pg mL⁻¹) than unstimulated IL-10-deficient cells (5 pg mL⁻¹) and stimulated wild-type cells.

Overall, these findings show that in the absence of IL-10, stimulation of primed splenocytes induced production of IL-17, which increased dramatically with duration of

exposure to the spirochete. In addition, the magnitude of increased IL-17 production by primed, stimulated IL-10-deficient cells was much greater when the primed mice were additionally infected.

Effects of CD4 blockage among cells of primed mice infected for eight days

Spleen cells obtained from *B. bissettii*-primed mice that were uninfected or infected with *B. burgdorferi* strain 297 for eight days, described above, also were stimulated *in vitro* with the challenge isolate in the presence of anti-CD4 antibodies. The presence of anti-CD4 antibodies for six or 24 hours did not affect the production of IL-17 from stimulated cells of *Borrelia*-primed, uninfected wild-type mice (Figures 2A and 2C). While we did observe that anti-CD4 antibodies caused a 31% reduction in IL-17 production among primed wild-type cells that were stimulated for 24 hours, this decrease was not statistically significant. Blockage of CD4 also had no effect on IL-17 production by primed and stimulated IL-10-deficient cells (Figures 2A and 2C). The production of IL-17 by these cells was still significantly greater than that of wild-type counterparts, even with the addition of anti-CD4 antibodies.

Similarly, addition of anti-CD4 antibodies had no statistically significant effects on IL-17 production by stimulated cells that were obtained from mice that were primed and then infected for eight days with *B. burgdorferi* strain 297 (Figures 2B and 2D). However, some trends were evident. Stimulated cells obtained from primed and infected wild-type mice, when incubated in the presence of anti-CD4 antibodies, exhibited 30% and 60% reductions in IL-17 production after six (Figure 2B) and 24 (Figure 2D) hours of

incubation, respectively. Additionally, stimulated cells obtained from primed and infected IL-10-deficient mice, when incubated with anti-CD4 antibodies, exhibited a 43% reduction in IL-17 production after six hours of incubation (Figure 2B). However, these decreases were not statistically significant.

Collectively, these findings demonstrate that CD4⁺ cells obtained from the spleen eight days after infection of primed mice may not be a statistically significant source of IL-17, regardless of whether IL-10 is present. However, trends indicating a possible contribution of CD4⁺ cells to the production of IL-17 in this model led us to investigate these immune events at an earlier time point following infection.

Production of IL-17 one day after infection of primed mice

We investigated the role of IL-10 on IL-17 production on the day following infection of *Borrelia*-primed mice. Spleen cells obtained from *B. bissettii*-primed wild-type mice that were infected for one day with *B. burgdorferi* strain 297 produced approximately 32 pg mL⁻¹ IL-17 after incubation in media for six hours. Stimulation of these cells *in vitro* with the infection strain caused a significant decrease in IL-17 production (24 pg mL⁻¹; $P \leq 0.05$, Figure 3A). Similarly, stimulated cells from primed IL-10-deficient mice that were infected for one day produced significantly less IL-17 than did unstimulated cells (16.0 pg mL⁻¹ vs. 23.23 pg mL⁻¹; $P \leq 0.05$). Additionally, unstimulated and stimulated cells from these wild-type mice each produced significantly more IL-17 than did their IL-10-deficient counterparts after six hours of incubation.

After 24 hours of incubation in media, cells from primed wild-type mice that were infected with *B. burgdorferi* strain 297 for one day produced minimal amounts of IL-17 (5 pg mL⁻¹), which were not affected by *in vitro* stimulation (Figure 3B). By contrast, stimulation of cells from primed, IL-10-deficient mice that were infected for one day increased IL-17 production. This increase was notable but did not achieve statistical significance ($P = 0.07$). Collectively, these findings show that splenocytes obtained following early infection of primed mice initially down-regulated their production of IL-17 for a short time upon stimulation with viable spirochetes.

Effects of CD4 blockage among cells of primed mice infected for one day

Spleen cells obtained from *B. bissettii*-primed mice that were infected with *B. burgdorferi* strain 297 for one day, described above, were stimulated *in vitro* with the challenge isolate in the presence of anti-CD4 antibodies. Incubation of stimulated wild-type cells with anti-CD4 antibodies for six hours did not affect the production of IL-17 (Figure 4A). By contrast, incubation of stimulated, IL-10-deficient cells with anti-CD4 antibodies for six hours resulted in a greater than 50% reduction in the production of IL-17 compared to stimulated cells incubated with isotype control antibodies, a decrease which was statistically significant ($P \leq 0.05$; Figure 4A). The amount of IL-17 produced by these stimulated IL-10-deficient cells following incubation with anti-CD4 antibodies was also significantly less than that of stimulated wild-type cells incubated with the antibodies. By contrast, after 24 hours of incubation, no effects on IL-17 production due to the presence of anti-CD4 antibodies were observed (Figure 4B). Collectively, these findings

suggest that in the absence of IL-10, production of IL-17 by primed CD4⁺ cells occurs early upon exposure to viable spirochetes.

Effects of IL-10 on hind paw swelling and arthritis

We measured the average changes in hind paw swelling, compared to baseline levels, following infection of *B. bissettii*-primed mice with *B. burgdorferi* strain 297. Primed wild-type mice exhibited no swelling of the hind paws in the eight days following injection of BSK medium (Figure 5). By contrast, infection of primed wild-type mice caused an increase in hind paw swelling that was significantly greater ($P \leq 0.05$) three and six days after infection. By day eight after infection, the paw swelling observed among primed, infected wild-type mice was still greater than that of uninfected controls. However, this increase was not statistically significant ($P = 0.06$). Similarly, *Borrelia*-primed, IL-10-deficient mice did not display hind paw swelling after injection of BSK medium.

However, infection of primed IL-10-deficient mice led to severe hind paw swelling that was significantly greater than that of uninfected counterparts throughout the study ($P \leq 0.05$). Moreover, *Borrelia*-primed, infected IL-10-deficient mice exhibited significantly greater hind paw swelling throughout the study compared to primed, infected wild-type mice ($P \leq 0.05$).

Mild cellular infiltration into the synovium of the tibiotarsal joint and into the knee was observed when *Borrelia*-primed wild-type mice were infected with *B. burgdorferi* strain 297 for eight days. Minimal infiltration into the perisynovial tissues of the tibiotarsal joint also was observed in these mice. By contrast, primed IL-10-deficient mice exhibited

moderate infiltration of cells, mostly lymphocytes, into these tissues eight days after infection. In particular, the lymphocytic infiltration observed in the perisynovial tissues of the tibiotarsal joint in primed, infected IL-10-deficient mice was significantly greater than that of wild-type counterparts ($P \leq 0.05$). In addition, *Borrelia*-primed mice that were not infected exhibited no histopathological changes of the tibiotarsal or knee joints (Table 1). Collectively, these results demonstrate that the swelling and arthritis observed in this disease model is significantly regulated by IL-10.

Effect of IL-10-regulated IL-17 on hind paw swelling and arthritis

In a separate trial, *B. bissettii*-primed mice were infected with *B. burgdorferi* strain 297 and then administered anti-IL-17 antibodies or isotype-control antibodies. Primed wild-type mice exhibited moderate hind paw swelling following infection with *B. burgdorferi* strain 297, returning to baseline levels by day eight of infection (Figure 6). As we have shown previously, administration of anti-IL-17 antibodies to these mice inhibited the development of hind paw swelling and, here, also reduced the average paw size below baseline measurements. This reduction was statistically significant ($P \leq 0.05$) throughout the experiment. In addition, *B. bissettii*-primed, IL-10-deficient mice infected with *B. burgdorferi* strain 297 exhibited pronounced hind paw swelling that was apparent by day three after infection and which also returned to baseline levels eight days after infection. These mice exhibited significantly greater paw swelling than wild-type counterparts ($P \leq 0.05$) at days three and six after infection. Administration of anti-IL-17 antibodies to primed and infected IL-10-deficient mice delayed the onset of paw swelling, significantly reducing it at day three after infection ($P \leq 0.05$; Figure 6).

Additionally, even after treatment with anti-IL-17 antibodies, primed and infected IL-10-deficient mice displayed greater paw swelling than anti-IL-17 antibody-treated, primed and infected wild-type mice. This difference was not statistically significant three days after infection ($P = 0.057$) but was significant thereafter ($P \leq 0.05$).

B. bissettii-primed IL-10-deficient mice that were infected with *B. burgdorferi* strain 297 for eight days exhibited severe and moderate infiltration of lymphocytes into the synovial and perisynovial tissues, respectively, of the tibiotarsal joints (Table 2). Treatment of primed, infected IL-10-deficient mice with anti-IL-17 antibodies reduced the degree of pathology in these tissues; however, these changes were not statistically significant. Additionally, no histopathological changes to the knee joints were observed. Collectively, these results demonstrate that IL-17 significantly contributes to the edema and may potentially contribute to the pathology in this dysregulated disease model.

Discussion

It is well documented that adaptive immunity is not absolutely required for the development of Lyme arthritis in C3H mice (Barthold *et al.* 1993; Brown & Reiner 1999). However, CD4⁺ T cells have been shown to contribute to disease in other animal models (Lim *et al.* 1995a, 1995b; McKisic *et al.* 2000; Wang *et al.* 2008). This is an important consideration, as inflammatory T cell responses are observed in individuals with Lyme arthritis, including antibiotic-refractory disease (Strle *et al.* 2012). Th1 (Sonderegger *et al.* 2012) and IL-17-associated (Hansen *et al.* 2013) responses also have been reported in *B. burgdorferi*-infected IL-10-deficient C57BL/6 mice. These mice

allow for examination of a dysregulated immune response (Sonderegger *et al.* 2012) involving inflammatory T cells, which contribute to the symptoms of severe Lyme arthritis.

The variable incidence and severity of symptoms that may result among individuals infected with *B. burgdorferi* suggest that different immune mechanisms, and various intensities thereof, may be elicited in response to the spirochete. Conflicting findings of the involvement of IL-17 in experimental animals highlight the likelihood that a Th17 response to the spirochete varies by genetic makeup and model of disease induction. For example, various groups have shown that murine cells produce Th17-associated cytokines in response to *B. burgdorferi* (Infante-Duarte *et al.* 2000; Knauer *et al.* 2007; Oosting *et al.* 2011). Additionally, we showed that priming of otherwise arthritis-resistant C57BL/6 mice prior to heterologous infection induces arthritis dependent on these Th17-associated cytokines (Burchill *et al.* 2003; Nardelli *et al.* 2004; Nardelli *et al.* 2005; Amlong *et al.* 2006; Kotloski *et al.* 2008; Nardelli *et al.* 2008b; Kuo *et al.* 2011), particularly in the absence of IFN- γ (Burchill *et al.* 2003; Nardelli *et al.* 2004; Amlong *et al.* 2006). Also, we recently showed that infected C57BL/6 mice lacking IL-10 developed destructive pathology that was prevented by administration of anti-IL-17 antibodies (Hansen *et al.* 2013). However, we also observed that infected C3H mice, with or without prior priming, produced little serum IL-17, and that administration of anti-IL-17 antibodies to these mice actually increased some aspects of disease (Nardelli *et al.* 2010). In addition, Lasky *et al.* (2015) provided convincing evidence that mice deficient in the IL-17A receptor displayed similar degrees of paw swelling and histopathology as

did infected wild-type controls. Therefore, the contribution of IL-17 to experimental Lyme arthritis likely depends on a variety of factors, including genetic makeup, method of disease induction, and the presence of functional immunoregulatory mechanisms.

Studies using human samples also have demonstrated conflicting findings pertaining to the role of IL-17 in Lyme arthritis. Synovial fluid T cells from Lyme arthritis patients produce IL-17 in response to borrelial lipoproteins (Infante-Duarte *et al.* 2000), including neutrophil activating protein A (NapA) (Codolo *et al.* 2008, 2013). Additionally, NapA induces the production of IL-23 from various innate cells (Codolo *et al.* 2008). By contrast, Bachmann *et al.* (2010) showed that stimulation of human peripheral blood mononuclear cells with various Lyme spirochetes failed to induce production of IL-17. Additionally, while Oosting, et al. (2011) showed that *B. burgdorferi* stimulated IL-17 and IL-23 from human peripheral blood mononuclear cells, and that a single nucleotide polymorphism in the IL-23 receptor gene resulted in less IL-17 production, this polymorphism did not affect the incidence of disease. Collectively, these conflicting findings in humans demonstrate that the robustness of the Th17 response to *B. burgdorferi* may be as varied as the individuals from which the samples were obtained.

However, several reports describe an interesting association between Th17 responses and particularly severe or prolonged disease following infection with *B. burgdorferi*, including cases of antibiotic-refractory disease. Codolo *et al.* (2008) showed that patients with antibodies to the IL-17-inducing protein NapA tended to have a prolonged resolution of arthritis following treatment, and they more frequently exhibited antibiotic-

refractory disease, than Lyme arthritis patients who did not have detectable levels of the antibodies. In addition, Shen *et al.* (2010) showed that among patients with responsive or refractory Lyme arthritis, the percentage of Th17 cells among total CD4+ T cells was, overall, relatively low (about 5%). However, Th17 cells comprised over 10% of the synovial fluid CD4+ T cells in two of six patients with antibiotic-responsive disease. Among 12 patients with antibiotic-refractory disease, this percentage approached 10% in multiple patients, including one in whom approximately 25% of the synovial CD4+ T cells were Th17 cells (Shen *et al.* 2010). Furthermore, the results of a study by Strle *et al.* (2014) demonstrated that higher levels of IL-23 were associated with more symptomatic infection prior to treatment. Also, IL-23 levels were higher in a group that eventually exhibited post-Lyme symptoms than a group in whom disease resolved. In addition, nearly two-thirds of patients with detectable IL-23 had symptoms after treatment, and all patients with exceedingly high levels of IL-23 had these symptoms (Strle *et al.* 2014). Collectively, these findings support our observations of IL-17 responses in the dysregulated, *Borrelia*-induced, T cell-associated arthropathy that we describe here. However, the compelling findings of Lasky *et al.* (2015) and Oosting *et al.* (2011) that an IL-17 response is not involved in the development of Lyme arthritis are also supported by the fact that not all patients with Lyme arthritis produce detectable IL-17. It is possible that those with severe, prolonged disease are predisposed to exhibiting particularly dysregulated inflammation indicative of a Th17 cell response. This hypothesis is supported by our current findings and by our previous work showing that depletion of putative regulatory T cells leads to severe, IL-17-dependent arthritis using the priming/infection model (Nardelli *et al.* 2004, 2005, 2006). Therefore, it is possible

that an inability to properly regulate the adaptive immune response may result in severe or persistent Lyme arthritis, and that a Th17 response may be involved.

Here, we extend our previous findings by showing that IL-10 controls an inflammatory, CD4⁺ cell-mediated IL-17 response in an immune-dysregulated system characterized by repeated exposure to *Borrelia* antigens. Splenocytes from *Borrelia*-primed mice infected with *B. burgdorferi* for one day responded to *in vitro* stimulation with an initial decrease in IL-17 production (Figure 3A), supporting our previous findings in a different disease model (Hansen *et al.* 2013). However, in the absence of IL-10, a significant source of this early IL-17 was CD4⁺ cells (Figure 4A). As infection progressed in these primed, IL-10-deficient mice, their splenocytes responded to *in vitro* stimulation with a vigorous IL-17 response (Figure 1D). These primed, IL-10-deficient mice infected with *B. burgdorferi* eventually developed massive hind paw swelling that was significantly reduced by administration of IL-17 antibodies (Figure 6). Our findings support our previous report of an additional regulatory role for IL-10 in Lyme arthritis (Hansen *et al.* 2013) and extend it by providing evidence that *Borrelia*-primed, IL-17-producing CD4⁺ cells may contribute to the histopathologic effects of a dysregulated response to the spirochete. It is necessary to state that while administration of anti-IL-17 antibodies to *Borrelia*-primed, infected IL-10-deficient mice significantly reduced hind paw swelling, the corresponding decrease in histopathological score was not statistically significant. This was surprising, as blockage of IL-17 has significantly reduced pathology consistently in *Borrelia*-primed and -infected C57BL/6 mice, regardless of whether the mice were wild-type (Nardelli *et al.* 2008b) or IFN- γ -deficient (Burchill *et al.* 2003;

Nardelli *et al.* 2004). This may have been due to a single mouse in which pathology was notable despite receiving anti-IL-17 antibodies; all others displayed no histopathological changes. However, we previously showed that administration of anti-IL-17 antibodies did not completely prevent the pathology of the tibiotarsal joint following infection of unprimed, IL-10-deficient mice (Hansen *et al.* 2013); therefore, it perhaps is not surprising that blocking IL-17 failed to completely abrogate pathology in this dysregulated system. It would be interesting to determine the mechanisms by which this dysregulated immunity contributed to cellular effects in the spleen early after infection of the footpad. As later-stage Lyme arthritis in humans, including antibiotic-refractory disease, is associated with heightened T cell responses, it is important to consider the contribution that Th17 cells may play to the development of pathology.

While our findings support our previous report (Hansen *et al.* 2013), the use of *Borrelia*-primed mice here yielded unique information. For example, the significant increase in IL-17 production we observed among primed cells of uninfected, IL-10-deficient mice after six hours of stimulation (Figure 1A) was maintained after 24 hours (Figure 1B). By contrast, we previously showed that stimulation of cells from non-primed, uninfected IL-10-deficient mice caused a significant increase in IL-17 production that, while still elevated by 24 hours, was not statistically different from unstimulated cells at this later time point (Hansen *et al.* 2013). Therefore, previous exposure to borrelial antigens in the absence of IL-10 primed mice for a significantly sustained IL-17 response. When these primed, stimulated IL-10-deficient cells were additionally incubated with anti-CD4 antibodies, we observed no decrease in IL-17 production (Figures 2A and 2C). This

result was unexpected. It is possible that a reduction would have been apparent if we had used a higher concentration of anti-CD4 antibodies. Alternatively, it is possible that primed mice may require active infection to generate IL-17-producing CD4⁺ cells more efficiently, or, possibly, that CD4⁺ cells are not a major source of IL-17 in these primed, uninfected mice. Nonetheless, these results show that priming alone, without subsequent infection, is sufficient to induce an IL-17 response upon encountering viable spirochetes *in vitro*, and that IL-10 plays a significant role in preventing a robust IL-17 response.

In addition, the use of this priming/infection model shows that in the context of dysregulated immunity, repeated exposure to viable *Borrelia* organisms leads to the potential for a massive IL-17 response. While stimulation of cells from primed, uninfected IL-10-deficient mice caused a 7-fold increase in IL-17 production (Figure 1C), stimulation of cells from primed IL-10-deficient mice infected for eight days caused a 33-fold increase in IL-17 production (Figure 1D). Blocking CD4 among these cells for six hours reduced IL-17 production by 43% (Figure 2B). Although this decrease was not statistically significant, it was greater than the decrease we observed among wild-type counterparts. Nonetheless, the lack of a statistically significant decrease also was surprising. Perhaps a higher concentration of CD4-neutralizing antibodies would have exerted greater effects on this significant production of IL-17. Alternatively, it is possible that by eight days after infection in this system, the capacity of CD4⁺ cells to mount an IL-17 response has waned. In support of this hypothesis, CD4⁺ cells were a significant source of IL-17 as soon as one day after infection of primed IL-10-deficient mice, which

eventually developed moderate-to-severe arthritis. It is possible that in a system of dysregulated immunity, Th17-associated arthritic responses are established early following activation of lymphocytes by *B. burgdorferi*. This may account for the occurrence of potent Th17 responses in subsets of patients with particularly severe or prolonged Lyme arthritis.

In conclusion, we support and extend our previous findings by showing that in the context of dysregulated immunity, *Borrelia*-experienced CD4⁺ cells subsequently exposed to *B. burgdorferi* have the potential to contribute to IL-17-mediated edema and pathology. This response occurs soon after restimulation and may increase substantially with the duration of infection. Importantly, our previous and current assertions that Th17-related pathology contributes to Lyme arthritis have been supported by recent reports linking these responses to persistent Lyme arthritis and post-Lyme disease sequelae in humans. Consideration of Th17-mediated pathology may be important for those symptomatic patients with prolonged, untreated infection with *B. burgdorferi*. This consideration may also be important for patients who exhibit symptoms following antimicrobial treatment for confirmed infection, particularly in a potential scenario in which inflammation might be prolonged due to repeated responses to borrelial antigens released from host tissues (Wormser *et al.*, 2012). Further characterization of a dysregulated IL-17 response in the development of Lyme borreliosis could lead to novel therapeutic and preventative measures against the disease, particularly in subsets of patients with especially severe or prolonged symptoms.

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Figure 1.

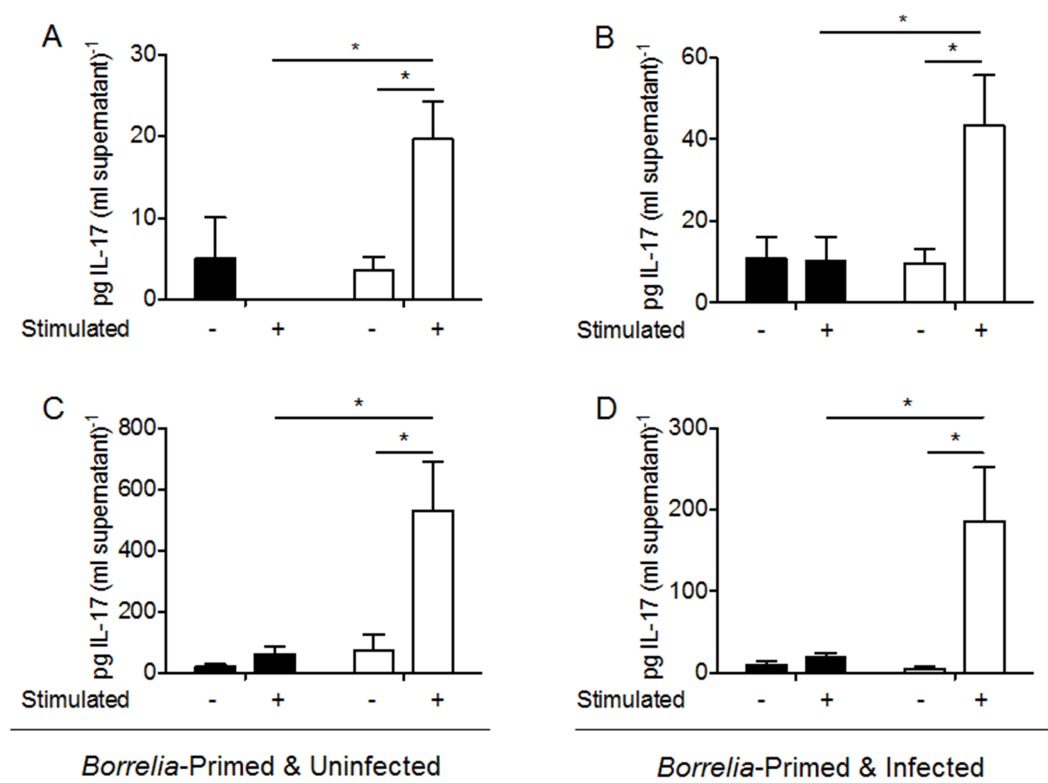


Figure 1. Levels of IL-17 in supernatants of spleen cells that were stimulated or not stimulated with *B. burgdorferi* strain 297 for 6 (A and B) or 24 (C and D) hours. Spleen cells were obtained from *Borrelia*-primed wild-type (black bars) or IL-10-deficient (white bars) mice that were not infected (A & C) or infected (B & D) with *B. burgdorferi* strain 297 for 8 days. *, $P \leq 0.05$. Bars indicate \pm SEM.

Figure 2.

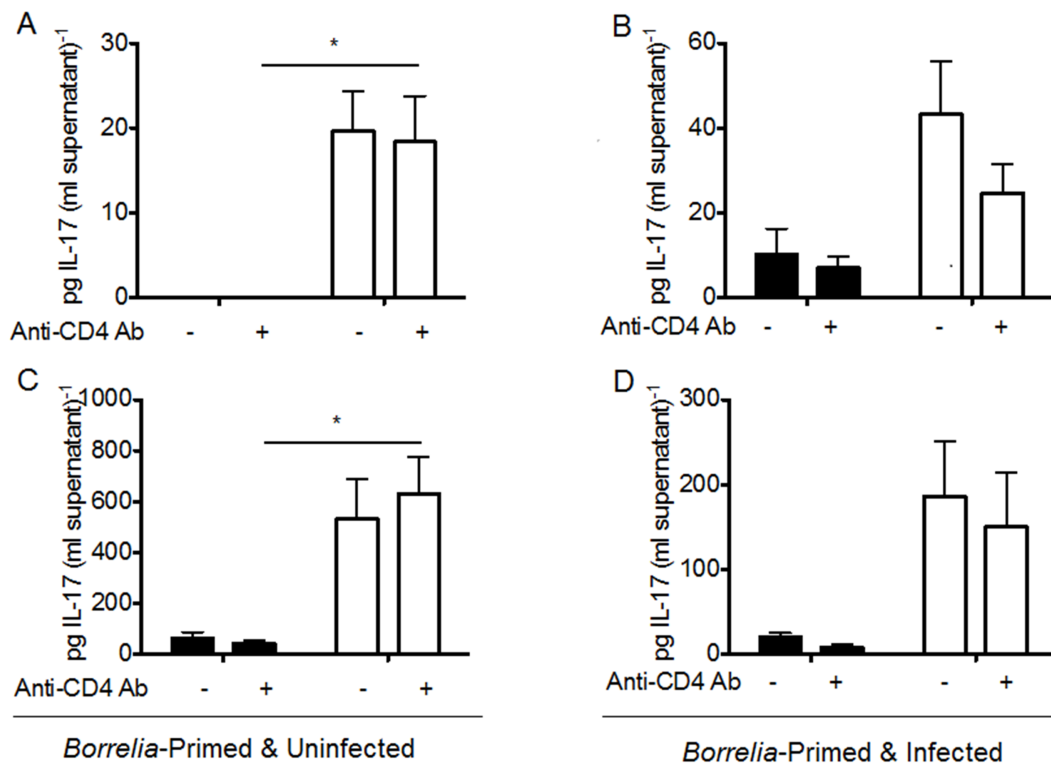


Figure 2. Levels of IL-17 in supernatants of spleen cells that were stimulated with *B. burgdorferi* strain 297 and treated with either anti-CD4 antibodies or isotype control antibodies for 6 (A and B) or 24 (C and D) hours. Spleen cells were obtained from *Borrelia*-primed wild-type (black bars) or IL-10-deficient (white bars) mice that were not infected (A & C) or infected (B & D) with *B. burgdorferi* strain 297 for 8 days. *, $P \leq 0.05$. Bars indicate \pm SEM.

Figure 3.

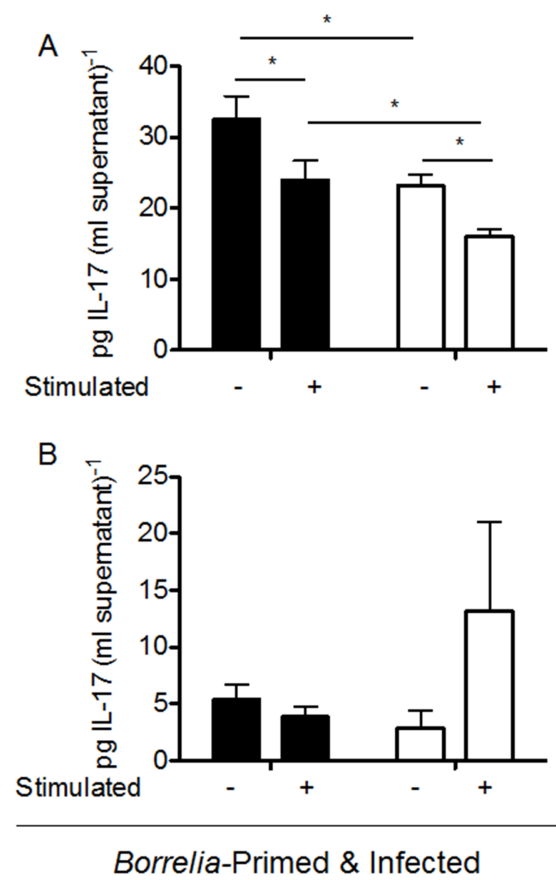


Figure 3. Levels of IL-17 in supernatants of spleen cells that were stimulated or not stimulated with *B. burgdorferi* strain 297 for 6 (A) or 24 (B) hours. Spleen cells were obtained from *Borrelia*-primed wild-type (black bars) or IL-10-deficient (white bars) mice that were infected with *B. burgdorferi* strain 297 for 1 day. *, $P \leq 0.05$. Bars indicate \pm SEM.

Figure 4.

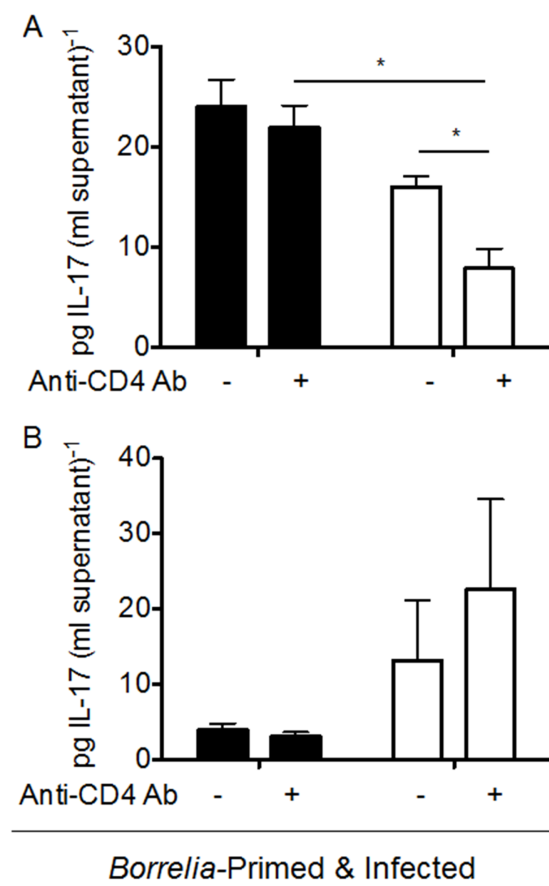


Figure 4. Levels of IL-17 in supernatants of spleen cells that were stimulated with *B. burgdorferi* strain 297 and treated with either anti-CD4 antibodies or isotype control antibodies for 6 (A) or 24 (B) hours. Spleen cells were obtained from *Borrelia*-primed wild-type (black bars) or IL-10-deficient (white bars) mice that were infected with *B. burgdorferi* strain 297 for 1 day. *, $P \leq 0.05$. Bars indicate \pm SEM.

Figure 5.

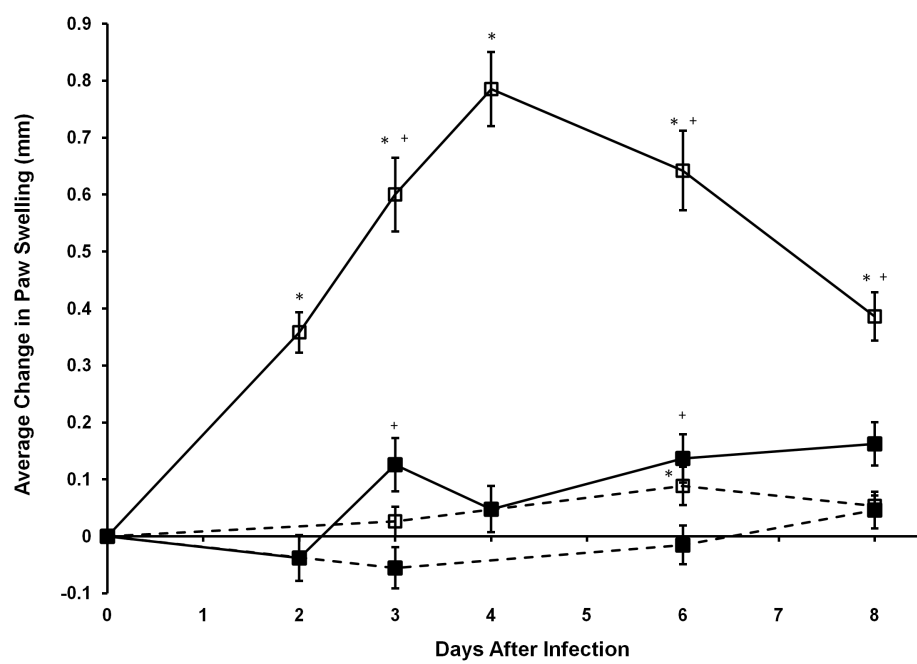


Figure 5. Average change in hind paw swelling among *Borrelia*-primed wild-type (filled squares) and IL-10-deficient (open squares) mice that were subsequently infected with *B. burgdorferi* strain 297 (solid lines) or injected with BSK medium (dashed lines). *, significant ($P \leq 0.05$) difference in degree of hind paw swelling due to genotype. +, significant ($P \leq 0.05$) difference in degree of hind paw swelling due to infection. Bars indicate \pm SEM.

Figure 6.

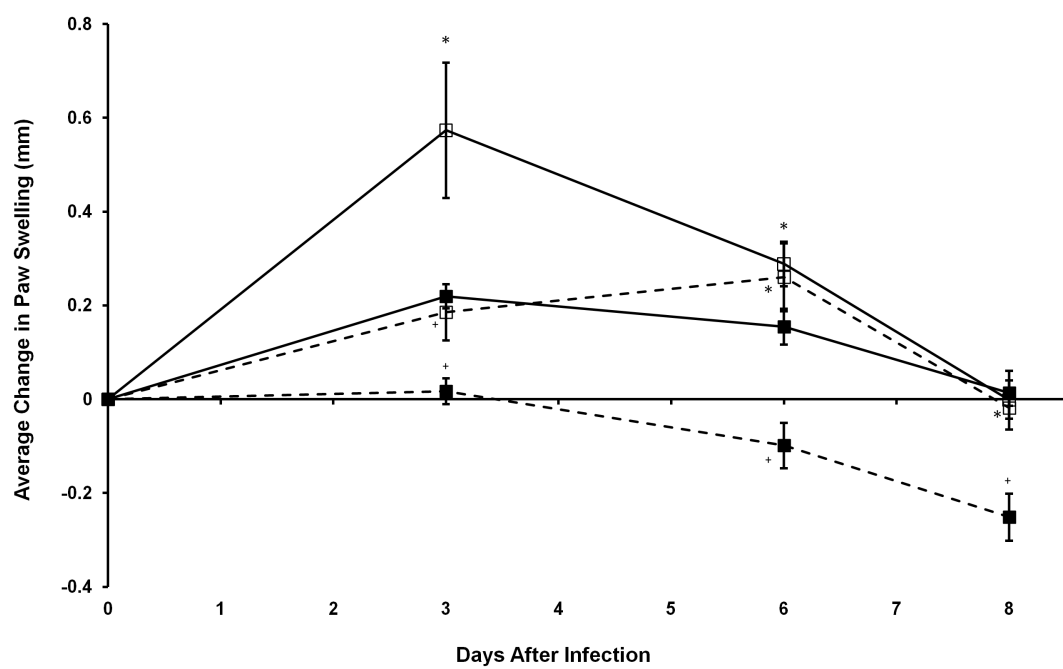


Figure 6. Average change in hind paw swelling among *Borrelia*-primed wild-type (filled squares) and IL-10-deficient (open squares) mice that were subsequently infected with *B. burgdorferi* strain 297 and injected with anti-IL-17 antibodies (dashed lines) or isotype control antibodies (solid lines). *, significant ($P \leq 0.05$) difference in degree of hind paw swelling due to genotype. **+**, significant ($P \leq 0.05$) difference in degree of hind paw swelling due to antibody treatment. Bars indicate \pm SEM.

Table 1.

	Synovium (Tibiotarsal)	Perisynovium (Tibiotarsal)	Knee
Primed wild-type mice	0	0	0
Primed, infected wild-type mice	0.88 ± 0.64	0.38 ± 0.26	0.94 ± 0.49
Primed IL-10-deficient mice	0	0	0
Primed, infected IL-10-deficient mice	2.17 ± 0.48	2.0 ± 0.37*	2.0 ± 0.52

Table 1. Effects of IL-10 deficiency on development of histopathology following infection of *Borrelia*-primed mice with *B. burgdorferi* strain 297. Primed wild-type mice exhibited mild infiltration of inflammatory cells into the synovium of the tibiotarsal joint and into the knee eight days after infection with viable spirochetes. These mice also exhibited minimal infiltrates into the perisynovial tissues of the tibiotarsal joints. By contrast, primed IL-10-deficient mice exhibited moderate cellular infiltration into all of these tissues eight days after infection, with a significant ($P \leq 0.05$) increase manifested in the perisynovial tissues of the tibiotarsal joint. No histopathological changes were observed in primed, uninfected mice. Histopathology was graded on a scale of 0-4, as described in Materials and Methods. Data \pm SEM; n = 4-6 mice per group.

Table 2.

	Synovium (Tibiotarsal)	Perisynovium (Tibiotarsal)
Primed, infected IL-10 deficient-mice + isotype control antibody	3.0 ± 1.78	1.75 ± 0.75
Primed, infected IL-10- deficient mice + anti-IL-17 Ab	1.5 ± 1.5	0.5 ± 0.5

Table 2. Effects of anti-IL-17 antibody treatment on development of histopathology following infection of *Borrelia*-primed, IL-10-deficient mice with *B. burgdorferi* strain 297. Primed, IL-10-deficient mice that received isotype control antibodies exhibited severe and moderate cellular infiltration into the synovial and perisynovial tissues, respectively, of the tibiotarsal joint eight days after infection. Treatment of primed, infected IL-10-deficient mice with anti-IL-17 antibodies reduced the degree of pathology in these tissues; however, these changes were not statistically significant. No changes to the knee were noted. Histopathology was graded on a scale of 0-4, as described in Materials and Methods. Data \pm SEM; n = 4 mice per group.

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CHAPTER 4: SPECIFIC AIM 3

The Role of Existing Regulatory T Cells on Induction of *Borrelia burgdorferi*-Induced Arthritis

Abstract

The anti-inflammatory mechanisms that regulate the pathogenic T cell response to *Borrelia burgdorferi* are poorly understood. Previous studies have suggested a role for interleukin-10 (IL-10) and regulatory immune cells in the modulation of Lyme arthritis. Here, we utilized the “depletion of regulatory T cell” (DEREG) mouse model, which allows for the selective depletion of CD4⁺CD25⁺Foxp3⁺ regulatory T (Treg) cells, to directly examine the effects of Treg cells on the development of arthritis following *B. burgdorferi* infection. We demonstrate that Treg cell depletion prior to infection results in greater hind paw swelling and the development of arthritis, along with an increased *B. burgdorferi*-specific antibody response. However, we observed limited effects on the production of certain inflammatory cytokines. Additionally, we found that Treg cells have no effect on the levels of *Borrelia* DNA in the joint tissue. These findings add to accumulating knowledge regarding the immunomodulatory function of Treg cells in *B. burgdorferi*-induced arthritis, as well as provide additional considerations for the impact of Treg cells on the inhibition of the antibody response during infection with *B. burgdorferi*.

Introduction

Infection with *Borrelia burgdorferi* results in a variety of symptoms, including inflammation of the joint tissue, which may progress to a more severe condition known as Lyme arthritis. CD4⁺CD25⁺Foxp3⁺ regulatory T (Treg) cells are important regulators of the inflammatory response, in part, through production of interleukin-10 (IL-10) (Rubtsov *et al.* 2008). *B. burgdorferi* induces production of IL-10 early after stimulation of host cells (Giambartolomei *et al.* 1998). We recently showed that IL-10 regulates the production of interleukin-17 (IL-17) from CD4⁺ T cells and moderates the development of pathology in two different murine models of *B. burgdorferi*-induced arthritis (Hansen *et al.* 2013, Hansen *et al.* 2016). Although the source of IL-10 was not determined in these studies, accumulating evidence suggests that Treg cells, a notable source of IL-10, may inhibit the inflammatory response induced by *B. burgdorferi* infection.

Specifically, evidence supports a role for Treg cells in reducing inflammation in Lyme arthritis. Inhibition of IL-17 in an interferon- γ (IFN- γ)-deficient mouse model of Lyme arthritis results in an increased number of CD4⁺CD25⁺ T cells and prevents the development of arthritis (Nardelli *et al.* 2004). Depletion of CD25-expressing cells in these mice resulted in destructive arthritis (Nardelli *et al.* 2004). In addition, CD4⁺CD25⁺ T cells isolated from these infected mice treated with anti-IL-17 antibodies inhibited arthritis when adoptively transferred into infected mice (Nardelli *et al.* 2005). By contrast, depletion of CD25⁺ cells in *Borrelia*-infected mice in which IL-17 was not neutralized did not affect the development of arthritis (Nardelli *et al.* 2006). These findings suggest that in the absence of IL-17, a specific population of CD4⁺CD25⁺ T

cells developed in this system. Notably, Th17 and CD4+CD25+Foxp3+ Treg cells develop from a common precursor, which suggests that the CD4+CD25+ cell population induced in the absence of IL-17 were Foxp3+ Treg cells (Bettelli *et al.* 2006).

Additionally, patients with antibiotic-refractory Lyme arthritis who experience a longer duration of joint inflammation have fewer numbers of Treg cells in the synovial fluid than patients whose arthritis resolved following antibiotic therapy (Shen *et al.* 2010). Also, Treg cells isolated from the synovial fluid of patients with antibiotic-refractory Lyme arthritis had a decreased ability to suppress effector T cell proliferation, resulting in greater inflammatory cytokine production (Vudattu *et al.* 2013). Therefore, Treg cells may inhibit *B. burgdorferi*-induced joint inflammation; however, the direct role of Treg cells in Lyme arthritis has not been investigated.

In order to characterize the effects of Treg cells on the early inflammatory events of Lyme arthritis, we utilized “depletion of regulatory T cell” (DEREG) mice. DEREG mice express a diphtheria toxin (DTx) receptor linked to the Treg cell-specific Foxp3 promoter, which allows for the selective elimination of Foxp3+ Treg cells following administration of DTx at the desired time point (Lahl & Sparwasser 2011). We utilized DEREG mice on the C57BL/6 background, a strain that is naturally resistant to the development of Lyme arthritis, in order to determine the effect of Treg cells on the inflammatory response to *B. burgdorferi*. We hypothesized that Treg cells prevent the onset of arthritis following infection with *B. burgdorferi*. We found that depletion of Treg cells prior to infection with *B. burgdorferi* results in decreased IL-10 production in early infection. Additionally, depletion of Treg cells leads to increased paw swelling and

pathology associated with arthritis, but does not affect spirochete load in the joint tissue. Moreover, depletion of Treg cells leads to increased *Borrelia*-specific antibody production. These results demonstrate that Treg cells are important mediators of the immune response to *B. burgdorferi*.

Materials and Methods

Mice

Six-to-12 week old, male and female C57BL/6 DREG mice (The Jackson Laboratory) were housed at the University of Wisconsin-Milwaukee animal research facility in a humidity-controlled environment at 21°C under a 12-hour light-and-dark cycle. Mice had access to food and acidified water *ad libitum*. The Institutional Animal Care and Use Committee at the University of Wisconsin-Milwaukee approved all experimental procedures.

Depletion of Treg cells

Diphtheria toxin (Calbiochem, San Diego, CA) was reconstituted in filter-sterilized PBS. Mice were injected with 1 µg diphtheria toxin in 100 µl of PBS intraperitoneally (i.p.) on the two consecutive days prior to infection with *B. burgdorferi*. Control groups included DREG mice injected with PBS prior to infection and DREG mice administered diphtheria toxin injected that were not infected. Groups consisted of 3-4 mice each.

Preparation of bacteria

B. burgdorferi strain B31-A3 organisms were provided by J. Coburn (Medical College of Wisconsin, Milwaukee, WI). Low passage organisms were grown at 32°C in modified Barbour-Stoenner-Kelly (BSK) medium until they reached a concentration of 10^6 microbes/ml before being aliquoted into 1.5-ml screw-cap tubes (Sarstedt, Newton, NC) and stored at -80°C. On the day of infection, 1 mL frozen suspensions of spirochetes were thawed, washed, and resuspended in PBS supplemented with 0.2% non-immune serum, as described by Ristow *et al.* (2012). Dark-field microscopy was used to determine motility and enumerate microbes using a Petroff-Hausser counting chamber.

Infection of mice

Naïve mice were anaesthetized with isoflurane in a nose-and-mouth cup and infected by subcutaneous injection between the scapulae with 2×10^4 viable *B. burgdorferi* strain B31-A3 organisms in a volume of 0.05 mL of PBS supplemented with 0.2% non-immune mouse serum. Control groups consisted of naïve DEREK mice injected with PBS supplemented with non-immune serum.

Assessment of swelling

Swelling of each hind paw was assessed every other day in order to determine changes in edema of the tibiotarsal joint following *B. burgdorferi* infection using a digital caliper (Marathon, Richmond Hill, Ontario) with a sensitivity of 0.01 mm. The width and thickness of each tibiotarsal joint were measured and averaged to provide the mean caliper value. Baseline swelling levels of each group were measured prior to infection

on day 0, and the changes in swelling from these baseline levels were determined every other day after infection and at the time of euthanasia.

Determination of Treg cell frequency

At day 7, 14, or 32 after infection, mice were anesthetized with isoflurane in a nose-and-mouth cup, and blood was collected via terminal cardiac puncture. Peripheral blood mononuclear cells (PBMCs) and plasma were isolated from whole blood using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO). 1×10^6 PBMCs were incubated with Fc block (anti-CD16/CD32) before staining with fluorescein isothiocyanate (FITC)-labeled anti-mouse CD4 (clone RM4-5) and phycoerythrin (PE)-labeled anti-mouse CD25 (PC61.5). Surface-labeled cells were fixed with 2% paraformaldehyde, permeabilized with 0.5% saponin, and stained with intracellular antibody allophycocyanin (APC)-labeled anti-mouse Foxp3 (clone FJK-16s). Appropriate fluorescently labeled isotype control antibodies were used to control for non-specific labeling of surface molecules. Treg cells were categorized as CD4⁺CD25⁺Foxp3⁺ and CD4⁺CD25⁺FoxP3⁺. Quantification was conducted using a BD FACSCaliber flow cytometer with FlowJo software. All reagents were obtained from eBioscience (eBioscience, San Diego, CA).

Histological examination

Following euthanasia on day 7, 14, or 32, one hind paw from each mouse was amputated above the knee joint. Paws were placed in cryptically coded tissue-embedding cassettes and fixed in 10% buffered zinc formalin before being embedded in

paraffin. The paraffin-embedded tissues were cut in 4 μm sections, placed on glass slides, and stained with hematoxylin and eosin. Unbiased histological examination of the tissues was conducted by a board-certified pathologist (Dr. Michael Lawlor, Children's Hospital of Wisconsin, Milwaukee, WI). Histopathological changes were scored according to the following scale: 0, no pathology; 1, mild lymphocyte infiltration restricted to joint surfaces; 2, mild lymphocyte infiltration restricted to subcutaneous soft tissue; and 3, mild lymphocyte infiltration of muscle and soft tissue (Figure 1).

Assessment of cytokine production

Spleens were harvested on day 7, 14, or 32 after infection, and single-cell suspensions of splenocytes were created by passing the tissue through a nylon mesh screen (BD Falcon, BD Biosciences, San Jose, CA) into cold Dulbecco's Modified Eagle Medium (DMEM). 1×10^6 cells were incubated at 37 °C in 5% CO₂ in the absence or presence of 1×10^5 viable *B. burgdorferi* strain B31-A3 organisms. Cell culture supernatants were collected after 24, 48, and 72 hours of incubation. Levels of IL-17, IFN- γ , and IL-10 in the cell culture supernatants were determined using IL-17A, IFN- γ , or IL-10 Ready-Set-Go! ELISA kits (eBiosciences, San Diego, CA), respectively, according to the manufacturer's instructions. Plates were read at an absorbance of 450 nm. Standard curves were used to calculate the cytokine amount in each sample as pg/ml.

Quantification of spirochetes by PCR

Bacterial load was determined as described by Ristow *et al.* (2012). One hind paw was collected at the time of euthanasia, frozen in liquid nitrogen, and stored at -80°C until

the time of DNA isolation. DNA was isolated using the QIAamp genomic DNA and RNA kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Both *B. burgdorferi* genome copies and mouse genome copies were enumerated. PCR amplification was performed in 20 µl reaction mixtures containing 5 µl DNA, 10 µl of PowerUp SYBR Green Master Mix (Life Technologies, Carlsbad, CA), 2 µl magnesium chloride, 1 µl water, and 1 µl of both forward and reverse primer for either *B. burgdorferi* *recA* (forward: 5'- GCAGCTATCCCACCTTCTTT-3', reverse: 5'- ATGAGGCTCT-CGGCATTG-3') or *Mus musculus* β-actin (forward: '5-TCACCCACACTGTGC-CCATCTACGA-3', reverse: 5'-GGATGCCACAGGATTCCATACCCA-3'). The thermal cycling profile consisted of one two-minute cycle at 50°C and one two-minute cycle at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A melting curve was acquired by heating the product to 95°C for 15 seconds, cooling to 60°C for 1 minute, and heating to 95°C for 15 seconds. *B. burgdorferi* *recA* gene copies were normalized to mouse β-actin after being calculated from standard curves using StepOnePlus Real-Time PCR System.

Quantification of antibody production

Pooled sera from each group were analyzed in duplicate for *B. burgdorferi*-specific IgG and IgM antibody content by a protocol adapted from Yang *et al.* (2011). Briefly, 96-well plates were coated with 5 µg/ml sonicated *B. burgdorferi* B31-A3 and incubated overnight at 4°C. Control wells were coated with goat anti-mouse IgM or IgG isotype control antibodies. Plates were washed three times with PBS containing 0.05% Tween 20 before blocking for 1 hour at room temperature with PBS containing 10% fetal bovine

serum and 0.2% Tween 20. After blocking, plates were washed once. 1:2 serial dilutions of serum ranging from undiluted to 1:65,536 were added to wells and incubated for 2 hours at room temperature. Murine IgM or IgG isotype control antibodies were added to wells coated with goat anti-mouse IgM or IgG as positive controls. Plates were washed 3 times before addition of goat anti-mouse IgM or IgG antibodies conjugated with horseradish peroxidase. Wells were developed with 3,3',5,5'-tetramethyl-benzidine (eBioscience). After 5 minutes, the reaction was stopped with 2 N sulfuric acid. Optical density was measured at 450 nm. Reciprocal endpoint dilutions were determined from average titration curves and defined as twofold above the buffer background (Coligan 1991). All antibodies were obtained from Southern Biotech (Birmingham, AL).

Statistics

Data were analyzed using two-way ANOVA followed by the Bonferroni correction for *post hoc* analysis when a significant *F* value indicated reliable mean differences. Paired data were analyzed using two-tailed Student's *t* test. Data were analyzed using GraphPad Prism. The alpha level was set at 0.05 prior to initiation of experiments. Data are expressed as mean \pm standard error of the mean (SEM) unless otherwise stated.

Results

Effect of diphtheria toxin administration on Treg cell frequency

First, we verified that injection of DTx leads to depletion of Foxp3⁺ Treg cells *in vivo*. DERE mice were injected with 1 μ g DTx per mouse for two consecutive days and

blood was collected 3 days later. Analysis of pooled PBMCs based on light scattering revealed that 3.12% of lymphocytes were Foxp3+ in DERE mice injected with PBS (Figure 2). By contrast, 0.75% of lymphocytes were Foxp3+ in DERE mice administered DTx (Figure 2). DTx treatment resulted in a 75% reduction in the frequency of Foxp3+ T cells.

Administration of DTx has been shown to cause a reduction in the body weight of mice beginning the second day of DTx administration (zu Hörste *et al.* 2010). Therefore, we measured the weight of mice throughout the study to ensure that the toxin did not adversely cause weight loss in the mice. Mice injected with PBS displayed a steady weight gain throughout the course of the experiment (Figure 3). By contrast, mice administered DTx maintained, but did not gain, weight during the first two weeks of the experiment (Figure 3). At day 16 after infection, mice administered DTx two weeks prior experienced a rapid increase in weight, with overall weight increases being equivalent to those in mice injected with PBS (Figure 3). Both groups then gained weight at the same rate through day 32 after infection (Figure 3). Uninfected mice administered DTx displayed the same pattern of weight gain as mice that received DTx prior to infection (data not shown).

Effect of DTx administration on hind paw swelling and arthritis

Baseline levels of hind paw size were determined by measuring the width and thickness of tibiotarsal joints prior to infection with *B. burgdorferi*. Paw size was measured every other day, and baseline measurements were subtracted to determine the average

change in paw size. Mice injected with PBS prior to infection experienced minimal paw swelling throughout the course of infection (Figure 4). By contrast, mice depleted of Treg cells prior to infection with *B. burgdorferi* displayed significantly greater paw swelling ($P \leq 0.05$) than PBS-injected mice beginning 10 days after infection (Figure 4). The increased change in hind paw size observed in Treg cell-depleted mice persisted through day 22 of infection and was significantly greater than *B. burgdorferi*-infected mice injected with PBS on days 10, 12, 14, and 22 after infection (Figure 4). No paw swelling was observed in mice administered DTx that were not infected (data not shown).

Histopathology of hind paws was assessed. Minimal histopathological changes limited strictly to joint surfaces were observed seven days after infection in mice injected with PBS (Figure 5A). Similarly, minimal histopathological changes were observed 7 days after infection in mice depleted of Treg cells (Figure 5A). By day 14 after infection, mice previously injected with PBS continued to display minimal histopathological changes (Figure 5B), with only mild infiltration of the joint surface in one mouse (Figure 5D). Mice depleted of Treg cells prior to infection, however, developed inflammation in the muscle and dermal tissues and inflammation of the ligaments of the joint (Figure 5D). No histopathological changes were observed in either group 32 days after infection (Figure 5C). In addition, no pathology was observed in diphtheria toxin-injected, uninfected mice at any time point (data not shown).

Effect of Treg cell depletion on cytokine production

We investigated the role of Treg cell ablation on the production of cytokines at various time points after infection with *B. burgdorferi*. Spleen cells harvested seven days after *B. burgdorferi* infection from mice previously injected with PBS produced undetectable levels of IL-17 after incubation in media for 24 hours (Figure 6A, left). Similarly, cells from mice depleted of Treg cells prior to infection did not produce any detectable amounts of IL-17. *In vitro* stimulation with live *B. burgdorferi* for 24 hours did not affect IL-17 production by cells from PBS-injected mice. By contrast, stimulated cells from mice depleted of Treg cells prior to infection produced a greater amount of IL-17 than unstimulated cells. However, this increased IL-17 production was not statistically significant. After 48 hours of incubation, no significant differences were observed in IL-17 production between cells from PBS-injected or Treg cell-depleted mice in both unstimulated and stimulated cultures (Figure 6A, middle). After 72 hours of incubation, stimulated cells from PBS-injected mice produced significantly more IL-17 than stimulated cells from Treg cell-depleted, *B. burgdorferi*-infected mice ($p < 0.01$; Figure 6A, right). Additionally, stimulation of cells isolated from PBS-injected, *B. burgdorferi*-infected mice 72 hours of incubation led to significantly greater production of IL-17 compared to unstimulated cells ($p < 0.05$). By contrast, stimulation had no effect on IL-17 production by cells from Treg cell-depleted mice.

Additionally, IFN- γ was measured in the cell culture supernatants of the same cells, which were harvested after 7 days of infection. No significant differences in IFN- γ production were observed in supernatants of cells from PBS-injected or Treg cell-

depleted mice after 24 hours (Figure 6B, left). Additionally, no differences were observed due to Treg cell depletion after 48 (Figure 6B, middle) or 72 (Figure 6B, right) hours. However, *in vitro* stimulation resulted in significantly increased IFN- γ production by cells from both PBS-injected and Treg cell-depleted mice infected with *B. burgdorferi* at all incubation times.

Production of IL-10 was also assessed from the same cell culture supernatants. Spleen cells harvested from mice injected with PBS prior to *B. burgdorferi* infection produced large amounts of IL-10 after 24 hours of incubation in media (Figure 6C, left). Cells from mice that were depleted of Treg cells prior to infection produced lower levels of IL-10 compared to cells from PBS-injected mice. However, this decrease in IL-10 production was not statically significant. Stimulation of these cells for 24 hours resulted in increased IL-10 production by cells from both PBS-injected and Treg cell-depleted mice. Cells from mice depleted of Treg cells 7 days prior produced significantly less IL-10 than cells from PBS-injected mice after 24 hours of stimulation ($p < 0.05$). After 48 hours of incubation, cells from mice injected with PBS prior to *B. burgdorferi* infection and mice depleted of Treg cells prior to infection produced low levels of IL-10 (Figure 6C, middle). Stimulation with viable *B. burgdorferi* led to a significant increase in IL-10 production in both groups; however, there was no difference in IL-10 production by cells isolated from PBS-injected and Treg-depleted mice. After 72 hours of incubation, unstimulated cells from mice injected with PBS or DTx before infection produced moderate amounts of IL-10 after 72 hours of incubation (Figure 6C, right). Stimulation with *B. burgdorferi* resulted in an increase in IL-10 production by cells from PBS-injected

mice, but the increase was not statistically significant. However, stimulation induced a statistically significant increase in IL-10 production from cells of Treg cell-depleted mice ($p < 0.05$).

Spleen cells harvested 14 days after infection from PBS-injected mice produced low amounts of IL-17 after 24 hours of incubation (Figure 7A, left). Cells from Treg cell-depleted mice produced similar amounts of IL-17. Stimulation of these cells with *B. burgdorferi* for 24 hours had no effect on IL-17 production. After 48 and 72 hours of incubation, production of IL-17 remained low (Figure 7A, middle and right). No differences in IL-17 production were observed between PBS-injected and Treg cell-depleted mice, regardless of stimulation.

Production of IFN- γ was not detected from supernatants of cells harvested from PBS-injected and Treg cell-depleted mice 14 days after infection following 24, 48, and 72 hours of incubation in media (Figure 7B). IFN- γ production was increased following *in vitro* stimulation at all incubation times; however, the increases were not statistically significant. No differences in IFN- γ production from cells of PBS-injected and Treg cell-depleted mice were observed. Additionally, no significant differences in IL-10 production between cells from PBS-injected and Treg cell-depleted mice were observed at any incubation time (Figure 7C).

Spleen cells harvested after 32 days of infection produced low amounts of IL-17 at all time points, with no significant differences occurring due to stimulation or Treg cell

depletion (Figure 8A). Additionally, IFN- γ was undetectable at most incubation times (Figure 8B). Moreover, no significant differences were observed in IL-10 production due to Treg cell depletion (Figure 8C).

Effect of Treg cell ablation on antibody production

B. burgdorferi B31-A3-specific total IgM antibody levels were determined by ELISA using pooled sera harvested from mice at 7, 14, or 32 days after infection. Sera collected 7 days after infection from PBS-injected mice had an average IgM endpoint dilution of 1:1024 (Figure 9A). By contrast, sera from Treg cell-depleted mice had a greater IgM average endpoint dilution of 1:2048. After 14 days of infection, PBS-injected mice had an average IgM endpoint dilution of 1:256 (Figure 9B). IgM serum levels of Treg cell-depleted mice were again greater, with an average endpoint dilution of 1:512. After 32 days of infection, PBS-injected mice had an average IgM endpoint dilution of 1:32. Once again, sera from Treg cell-depleted mice had greater IgM levels, with an average endpoint dilution of 1:256. Overall, Treg cell depletion prior to infection increased IgM antibody production.

IgG levels were also determined in serum samples. Seven days after infection, sera from PBS-injected mice had an average IgG endpoint dilution of 1:16 (Figure 10A). Sera from mice depleted of Treg cells had a lower IgG endpoint dilution of 1:8. Fourteen days after infection, sera from PBS-injected mice had an average endpoint dilution of 1:8 (Figure 10B). By contrast, sera from Treg cell-depleted mice had greater IgG antibody production, with an average endpoint dilution of 1:16. Thirty-two days after

infection, sera from PBS-injected mice had an average endpoint dilution of 1:16 (Figure 10C). Sera from mice depleted of Treg cells had a greater endpoint dilution of 1:32. Overall, sera from Treg cell-depleted mice had increased IgG antibody levels.

Effect of Treg ablation on bacterial load

We determined the amount of bacteria within the tibiotarsal joints of mice injected with PBS or depleted of Treg cells before infection to assess the impact of Treg cells on dissemination of the spirochete to these tissues. After 7 days of infection, no difference in the number of *B. burgdorferi* genomes per 10^4 mouse genomes was observed in the joints of mice previously injected with PBS or depleted of Treg cells (Figure 11A). Additionally, 14 days after infection, no differences were observed in joints of PBS-injected mice and the joints of Treg cell-depleted mice (Figure 11B). However, more *B. burgdorferi* DNA was detected on day 14 than on day 7. Also, no significant difference in the colonization of tibiotarsal joints was observed between PBS-injected and Treg cell-depleted mice on day 32 after infection (Figure 11C).

Discussion

In this report, we examined the effects of early Treg cell ablation on the development of *B. burgdorferi*-induced arthritis. We found that depletion of Treg cells prior to infection with *B. burgdorferi* led to increased joint swelling and pathological changes indicative of arthritis. These findings support our hypothesis that Treg cells prevent the onset of arthritis following *B. burgdorferi* infection. Additionally, Treg cell depletion was associated with an increase in IgM antibody production and an early decrease in IgG

production followed by an increased IgG response. Collectively, these findings provide evidence that Treg cells prevent arthritis and affect the immune response to *B.*

burgdorferi in a model of natural resistance to disease.

Previous studies have suggested that regulatory immune cells affect *B. burgdorferi*-induced arthritis. Neutralization of IL-17 in IFN- γ -deficient mice reduced the severity of disease in a model of *B. burgdorferi*-induced, T cell-driven arthritis (Burchill *et al.* 2003). The severity of arthritis correlated with the number of CD4+CD25+ putative Treg cells in the draining lymph nodes (Nardelli *et al.* 2004). Additionally, depletion of CD25+ cells in infected mice in the absence of IL-17 led to severe arthritis (Nardelli *et al.* 2004).

Adoptive transfer of CD4+CD25+ cells that developed in infected mice in which IL-17 was neutralized was shown to prevent the development of arthritis following adoptive transfer into mice infected with *B. burgdorferi* (Nardelli *et al.* 2004). However, depletion of CD25+ cells in *Borrelia*-infected mice in which IL-17 was not neutralized did not affect the development of arthritis (Nardelli *et al.* 2006). These findings suggest that different populations of CD4+CD25+ T cells developed in the presence and absence of IL-17.

Although Foxp3 expression was not recognized as the definitive Treg cell marker at the time of these studies, it is likely that they were Treg cells. Th17 and Treg cells are derived from a common precursor, and differentiation is reciprocal and dependent on the cytokine environment (Bettelli *et al.* 2006). It is likely that neutralization of IL-17 in these experiments may have promoted the development of Treg cells. These findings suggest that Treg cells likely reduce the inflammatory response to *B. burgdorferi*, ameliorating the severity of Lyme arthritis.

Other studies support a role for Treg cells in antibiotic-refractory Lyme arthritis. Patients in whom arthritis failed to resolve despite antibiotic treatment were found to have fewer numbers of Treg cells in the synovial fluid than those in whom arthritis responded to treatment (Vudattu *et al.* 2013). Additionally, the Treg cells in these patients do not effectively inhibit the pro-inflammatory response (Vudattu *et al.* 2013). Moreover, in patients with antibiotic-refractory arthritis, a higher percentage of Treg cells was associated with a faster resolution of arthritis (Shen *et al.* 2010). Overall, these findings suggest that Treg cells are important for the eventual resolution of antibiotic-refractory Lyme arthritis.

C57BL/6 mice are naturally resistant to *B. burgdorferi*-induced arthritis (Schaible *et al.* 1990). This is attributed to decreased levels of IFN- γ and increased levels of IL-10 (Brown *et al.* 1999, Ganapamo *et al.* 2000). Also, IL-10, the major cytokine produced by Treg cells, is known to attenuate *B. burgdorferi*-induced arthritis through suppression of inflammatory cytokine production by macrophages (Brown *et al.* 1999). While reduced levels of IL-10 during initial infection may be beneficial to induce a robust immune response against the bacterial infection, the prolonged inflammation can result in induced pathology. Previous studies have not addressed if Treg cells are responsible for resistance to arthritis in C57BL/6 mice.

We examined the role of Treg cells in Lyme arthritis by depleting Treg cells prior to infection with *B. burgdorferi*. Using the DREG mouse model, which allows for the selective depletion of Foxp3⁺ Treg cells, we demonstrate that Treg cell depletion prior

to infection results in greater hind paw swelling and the development of arthritis. Differences in hind paw swelling, however, did not become evident until 10 days after infection. Our findings are consistent with swelling patterns in arthritis-susceptible C3H mice, which develop significant paw swelling 14 days after infection (Ma *et al.* 1998). By day 14 after infection, not only were differences in hind paw swelling observed, but, also, histopathological changes indicative of arthritis were evident in mice depleted of Treg cells. Only one of four *B. burgdorferi*-infected mice administered PBS displayed even minimal histopathological changes 14 days after infection. By contrast, all four mice depleted of Treg cells prior to infection had lymphocyte infiltration into the joint tissue. Our findings are supported by the findings of others, as Treg cells have been shown to limit severity of disease by inhibiting pulmonary pathology associated with lymphocyte infiltration in respiratory syncytial virus infection (Fulton *et al.* 2010). The exact mechanisms by which Treg cells inhibit inflammation during *B. burgdorferi* infection require further examination. It is possible that the initial depletion of Treg cells resulted in a persistent or gradually excessive immune response that caused pathology. Our findings that IL-10 production by spleen cells harvested 7 days after infection was significantly decreased in mice depleted of Treg cells supports this idea (Figure 6C). However, regulation of the inflammatory response eventually led to resolution of pathology, as Treg cell depletion in our model is likely only temporary. Our findings provide strong support that Treg cells modulate the severity of Lyme arthritis.

Others have suggested that the induction of Treg cells following *B. burgdorferi* infection leads to immune suppression that is responsible for chronic symptoms. Specifically,

patients with chronic neuroborreliosis were found to have an increased Treg cell response to *B. burgdorferi* protein NapA that led to greater production of IL-10 (Amedei *et al.* 2013). Additionally, patients with chronic neuroborreliosis were found to have increased Foxp3 mRNA in the blood compared to healthy controls (Jarefors *et al.* 2007). These findings suggest that Treg cells may also function by inhibiting the immune response to *B. burgdorferi*, thereby leading to chronic symptoms. Our findings contrast with these previous studies by providing evidence that the presence of Treg cells correlated with disease resolution. However, our model does not allow for long-term Treg cell depletion.

We expected Treg cell depletion to result in greater inflammatory cytokine production, as Treg cells can limit pathology through inhibition of inflammatory T cells. After 7 days of infection, we found that depletion of Treg cells prior to *B. burgdorferi* infection resulted in significantly lower IL-17 production by spleen cells after 72 hours of incubation. While surprising, our results may be explained by the temporary depletion of Treg cells. A newly emerging Treg population on day 7 may inhibit IL-17 production in the *in vitro* environment. In support of this, we found that cells of Treg cell-depleted mice harvested on day 7 produced increasing levels of IL-10 throughout incubation (Figure 6C). After 72 hours of incubation, IL-10 production by cells of Treg cell-depleted mice was significantly greater than that of PBS-injected mice (Figure 6C). At the same time, we found that cells from Treg cell-depleted mice produced significantly less IL-17 than cells from PBS-injected mice (Figure 6A). These findings are consistent with our previous work, in which we demonstrated that IL-10 inhibits IL-17 production during B.

burgdorferi infection (Hansen *et al.* 2013). Alternatively, decreased IL-17 production following Treg cell depletion may occur due to the effects of IL-17+Foxp3+ Treg cells, known as Tr17 cells (Beriou *et al.* 2009). Tr17 cells produce IL-17 and display suppressive functions (Beriou *et al.* 2009). In fact, Tr17 cells have a stronger suppressive capacity on effector T cells than IL-17- Treg cells (Li *et al.* 2012). Administration of DTx would have also depleted the Tr17 population, and could account for both the decreased levels of IL-17 observed in our cultures and the increase in pathology. Notably, increased percentages of Tr17 cells have been described in a mouse model of collagen-induced arthritis and in humans with rheumatoid arthritis (Komatsu *et al.* 2014). It is also possible that IL-17 is not a significant contributor to Lyme arthritis, as mice deficient in IL-17 or its receptor still develop arthritis (Kuo *et al.* 2016, Lasky *et al.* 2015). Additionally, reduced levels of IL-17 in humans were not found to correlate with protection from induction of Lyme arthritis (Oosting *et al.* 2011). In the event that IL-17 may not be a contributing factor in arthritis, severe or persistent disease may reflect an inability of Treg cells to properly regulate the effects of other cytokines.

Later-stage Lyme arthritis is caused, in part, by the inflammatory effects of type 1 T helper (Th1) cells through the production IFN- γ . We found that depletion of Treg cells prior to infection with *B. burgdorferi* had no effect on IFN- γ production. Limited effects of Treg cell depletion on IFN- γ production have been described in other bacterial infections. For example, Treg cell depletion had no significant effect on IFN- γ production by lymph node cells from mice infected with *Helicobacter pylori* (Kaparakis *et al.* 2006).

Additionally, spleen cells harvested from *Mycobacterium bovis*-infected mice produced similar amounts of IFN- γ , regardless of Treg cell depletion (Berod *et al.* 2014). Notably, *B. burgdorferi*-induced arthritis occurs even in the absence of IFN- γ (Brown & Reiner 1999, Christopherson *et al.* 2003). Our findings further demonstrate that IFN- γ is not the sole cytokine responsible for the development of pathology. Further, our findings indicate that Treg cells may have limited suppressive effects on IFN- γ during *B. burgdorferi* infection.

One mechanism by which Treg cells may limit paw swelling and pathology is through IL-10 production. Notably, IL-10-deficient C57BL/6 mice develop greater paw swelling and histopathological changes than wild-type C57BL/6 mice (Brown *et al.* 1999). Our findings are consistent with these, as we observed a significant decrease in IL-10 production after 24 hours of incubation from spleen cells harvested seven days after infection from Treg cell-depleted mice (Figure 6C). Notably, it was at this time at which paw swelling began (Figure 4). However, no differences in IL-10 production were observed at any other times. The increase in IL-10 production by cells from Treg cell-depleted mice seen after 48 and 72 hours may have been due to the presence of other IL-10-secreting cell located within the spleen (Yanaba *et al.* 2009, Ostroukhova *et al.* 2004, Saraiva & O'Garra 2010). Alternatively, remaining IL-10 producing cells, or, possibly, reemerging Treg cells, may have proliferated during the incubation time, resulting in more IL-10 production at later time points. Overall, we found that Treg cell depletion resulted in a significantly decrease in their main cytokine, IL-10.

Interestingly, no differences in cytokine levels due to Treg cell depletion were observed after 14 days of infection, when pathology was observed. It is possible that differences in cytokine production are transient in the DREG mouse and occur much earlier than 1 week after infection, as Treg cell populations recover to normal levels by day 6 after depletion (Christiaansen *et al.* 2014). The lack of a Treg response may have resulted in increased inflammation in early infection that eventually resulted in increased lymphocyte infiltration of the tissue seen after 14 days. Additionally, our measurement of cytokine production by spleen cells may not necessarily reflect the immune response within the joint. Notably, significantly larger numbers of Th1 and Treg cells are present in the synovial fluid than the peripheral blood in patients with Lyme arthritis (Shen *et al.* 2010). By measuring cytokine production by spleen cells, we investigated systemic differences due to Treg cell depletion. Further investigation may reveal a more robust effect of Treg cell depletion on cytokine levels within the joint tissue.

In addition to inhibiting inflammation, Treg cells directly suppress the immunoglobulin response (Lim *et al.* 2005). Therefore, we investigated the effect of Treg cells on *B. burgdorferi*-specific antibody production. We found that Treg cell depletion results in an increased IgM and IgG response. Our findings are consistent with the findings of others, as Treg cell depletion using antibodies to CD25 selectively increased IgG1 antibody titers (Kaparakis *et al.* 2006). While the use of anti-CD25 antibodies does not selectively deplete Foxp3-expressing cells, it is possible that the increased antibody response following Treg cell depletion is a result of an enhanced effector T cell response. For example, C57BL/6 mice depleted of CD4⁺ T cells had reduced IgM and

IgG production 10 days following *B. burgdorferi* infection (Hastey *et al.* 2012). We surmise that enhanced effector function in the absence of Treg cells increases the antibody response. It is known that Treg cells can suppress antibody production through suppression of germinal center reactions (Chung *et al.* 2011) and inhibition of CD4⁺ T-follicular helper cells (Wing *et al.* 2014). T-follicular helper cells are important for the conversion of B cells to antibody-secreting plasma cells (Vinuesa *et al.* 2005). Notably, depletion of Treg cells using the DREG mouse model resulted in increased numbers of T-follicular helper cells and increased IgG antibody production (Wing *et al.* 2014). We speculate that Treg cells may suppress T-follicular helper cells within the germinal centers of lymph nodes following *B. burgdorferi*-infection, thereby decreasing antibody production. Our hypothesis would explain why both the IgM and IgG antibody responses were increased following Treg cell depletion. In general, our findings suggest that Treg cells existing prior to infection may inhibit the antibody response to *B. burgdorferi*.

Moreover, we found that Treg cell depletion did not have an effect on bacterial burden in the joint tissue at any of the time points following infection that we investigated. Others have also shown that depletion of Treg cells does not affect bacterial burden. For example, Treg cell depletion did not alter bacterial burden in murine infection with *M. bovis* (Berod *et al.* 2014) or *Staphylococcus aureus* (Tebartz *et al.* 2015). Additionally, Treg cell depletion had no effect on bacterial dissemination or bacterial burden in a murine model of sepsis (Kuhlhorn *et al.* 2013). Together, these studies support our findings that Treg cells do not impact bacterial burden. Perhaps the suppressive

mechanisms utilized by Treg cells do not affect bacterial clearance. It is known that C57BL/6 mice are resistant to the development of arthritis following *B. burgdorferi* infection, in part, due to increased production of IL-10 (Brown *et al.* 1999). However, C57BL/6 mice and C3H, arthritis-susceptible, mice display a similar bacterial burden in the joints (Brown *et al.* 1999), which suggests that Treg cell production of IL-10 may not affect spirochete load. Additionally, IL-10-deficient mice develop severe swelling of the hind paws and arthritis, yet have a lower bacterial burden in the joint (Brown *et al.* 1999). Together, these findings suggest that Treg cell modulation of arthritis may be unrelated to spirochete numbers in the joint tissue. Our findings further suggest that Treg cells present prior to infection do not affect *B. burgdorferi* burden in the joint tissue. However, we are currently assessing the effect of Treg cell depletion on bacterial burden of other tissues. It may be that while Treg cells do not affect bacterial burden, they may inhibit dissemination of the bacteria. We found that heart tissue isolated from infected mice injected with PBS became culture positive after 3 weeks (unpublished data). By contrast, mice administered DTx had culture positive heart tissue after only 2 weeks. These findings suggest that Treg cells may impede bacterial dissemination to some tissue sites. Additional studies are needed to define the effects of Treg cells on dissemination of spirochetes within the host.

We did not find any correlation between the effects of Treg cell depletion on antibody production and bacterial load. Our findings are not surprising, since total antibodies against *B. burgdorferi* differ from borreliacidal antibodies. Borreliacidal antibodies are important for clearance of the spirochete via induction of complement-mediated killing

(Schmitz *et al.* 1991). Borreliacidal antibody titers correlate with the severity of arthritis (Schmitz *et al.* 1991) and wane rapidly after clearance of infection (Creson *et al.* 1996). By contrast, the total antibody response remains elevated for months after infection (Padilla *et al.* 1996). Although depletion of Treg cells resulted in increased antibody production, we hypothesize that borreliacidal antibody production would not be affected in the absence of Treg cells. Our hypothesis is based on our previous reports, which found that IL-10 does not affect borreliacidal production (Hansen *et al.* 2013). It is possible, however, that Treg cells may affect borreliacidal antibody production via other suppressive mechanisms.

In conclusion, we provide evidence that depletion of Treg cells existing prior to infection with *B. burgdorferi* results in significantly greater paw swelling and development of arthritis along with an increased *B. burgdorferi*-specific antibody response. However, we observed limited effects on the production of certain inflammatory cytokines.

Additionally, we found that Treg cells have no effect on *Borrelia* DNA in the joint tissue.

These findings add to accumulating knowledge regarding the immunomodulatory function of Treg cells in *B. burgdorferi*-induced arthritis, as well as provide additional considerations for the impact of Treg cells on the inhibition of the antibody response.

More importantly, our findings suggest that individuals with decreased numbers of Treg cells may be more likely to develop arthritis after infection with *B. burgdorferi*. Further studies examining the role of Treg cells in *B. burgdorferi* infection is vital for understanding how a dysregulated immune response leads to joint pathology, which could lead to new therapies for the prevention of Lyme arthritis.

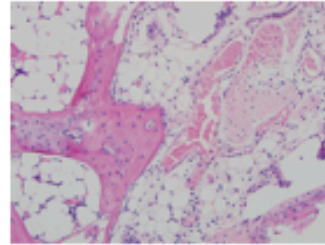
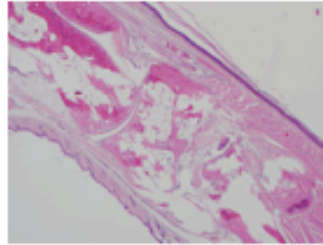
Figure 1.

Histology Score

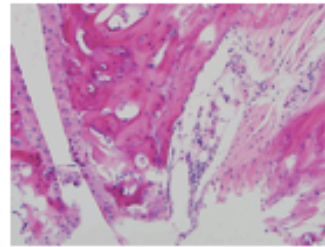
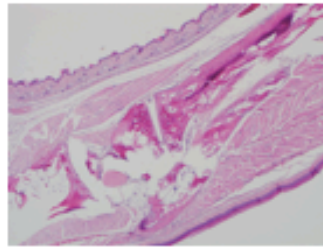
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200X H&E

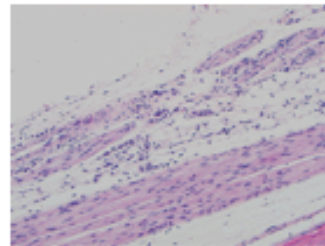
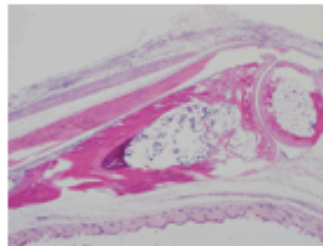
0



1



2



3

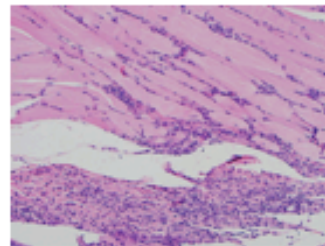
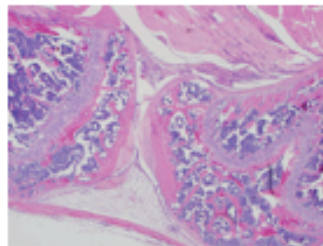


Figure 1. Representative images of the severity of pathology in the tibiotarsal joint of mice infected with *B. burgdorferi* rated on a 4-point scale. 0 = no detectable pathology across the entire section, 1 = mild chronic inflammation restricted to the joint surface, 2 = mild chronic inflammation restricted to subcutaneous soft tissue, 3 = mild chronic inflammation of the muscle and soft tissue.

Figure 2.

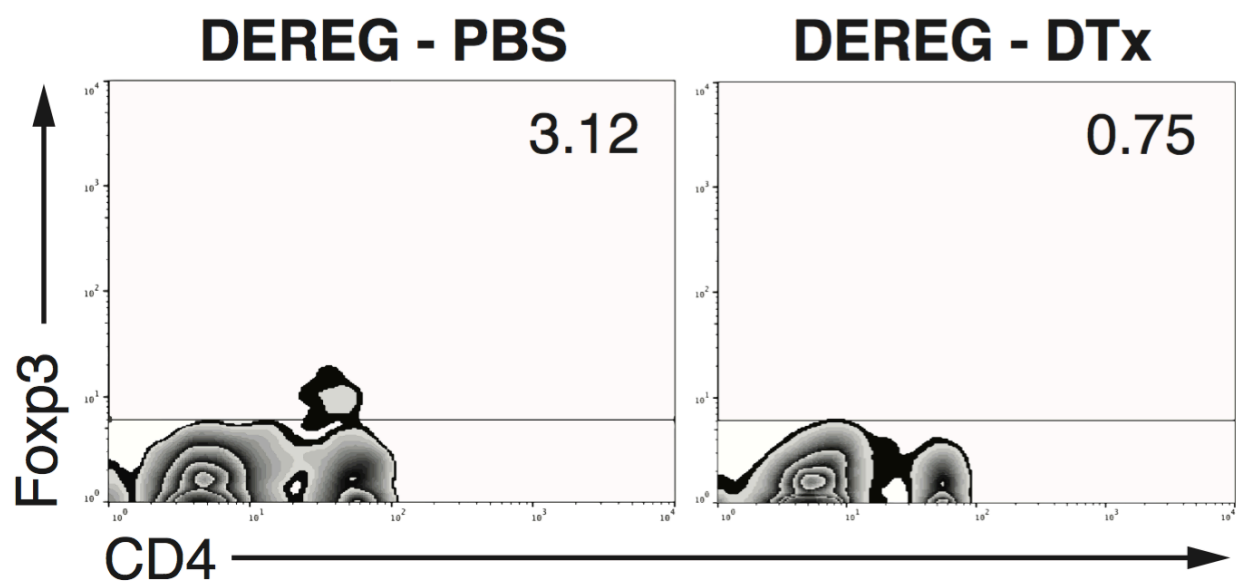


Figure 2. Efficiency of Foxp3+ cell depletion in DERE mice. DERE mice were treated i.p. with PBS (left panel) or 1 µg of DTx (right panel) to deplete Treg cells on two consecutive days. Three days later, percentage of CD4+Foxp3+ cells in the blood was determined by flow cytometry. Numbers indicate percentage of Foxp3+ cells. 75% of Treg cells were depleted in DERE mice 3 days after administration of DTx.

Figure 3.

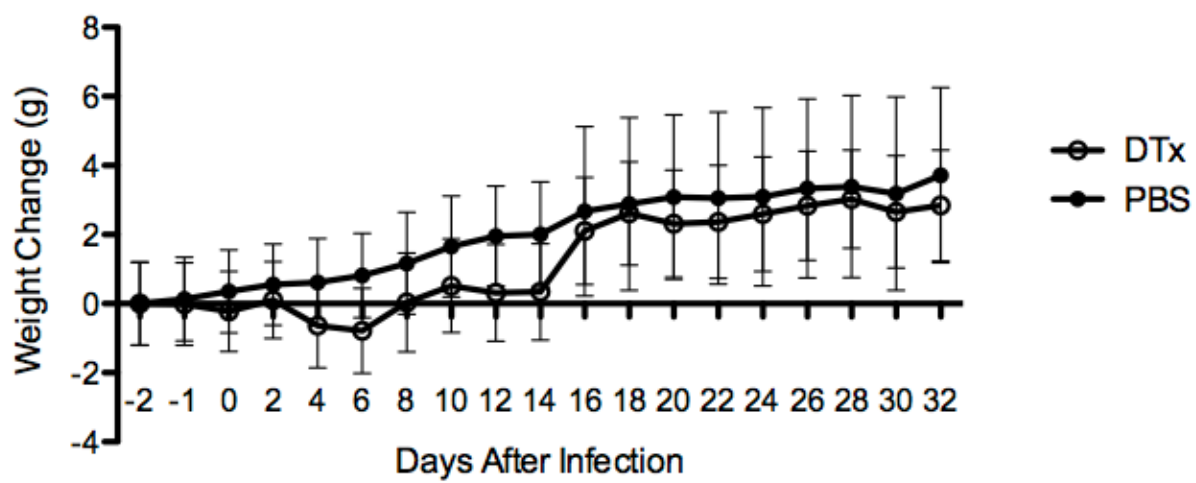


Figure 3. Diphtheria toxin (DTx) induces a transient delay in weight gain. Mice were injected on days -2 and -1 with PBS (closed circles) or 1 µg DTx (open circles). Additionally, mice were infected with 2×10^4 viable *B. burgdorferi* strain B31-A3 on day 0. Weight gain was delayed in mice administered DTx, but eventually recovered to that occurring in mice injected with PBS 16 days later. Data are mean weights \pm SEM

Figure 4.

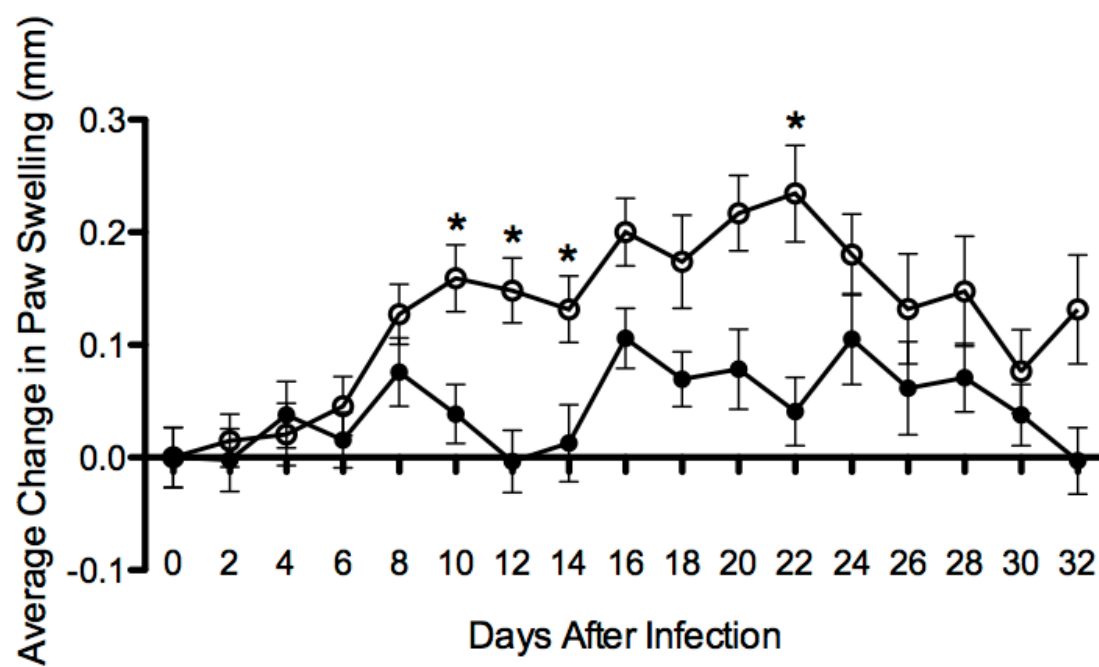


Figure 4. Administration of diphtheria toxin significantly increased hind paw swelling in *B. burgdorferi*-infected DERE mice. DERE mice were administered PBS (black circles) or DTx (white circles) two consecutive days prior to infection with *B. burgdorferi*. DTx administration caused a significant increase in hind paw swelling that was evident on day 10 after infection. No changes in hind paw measurements in DERE mice administered DTx that were not infected (not shown). Data are mean changes in paw swelling relative to baseline \pm SEM. * $P < 0.05$.

Figure 5.

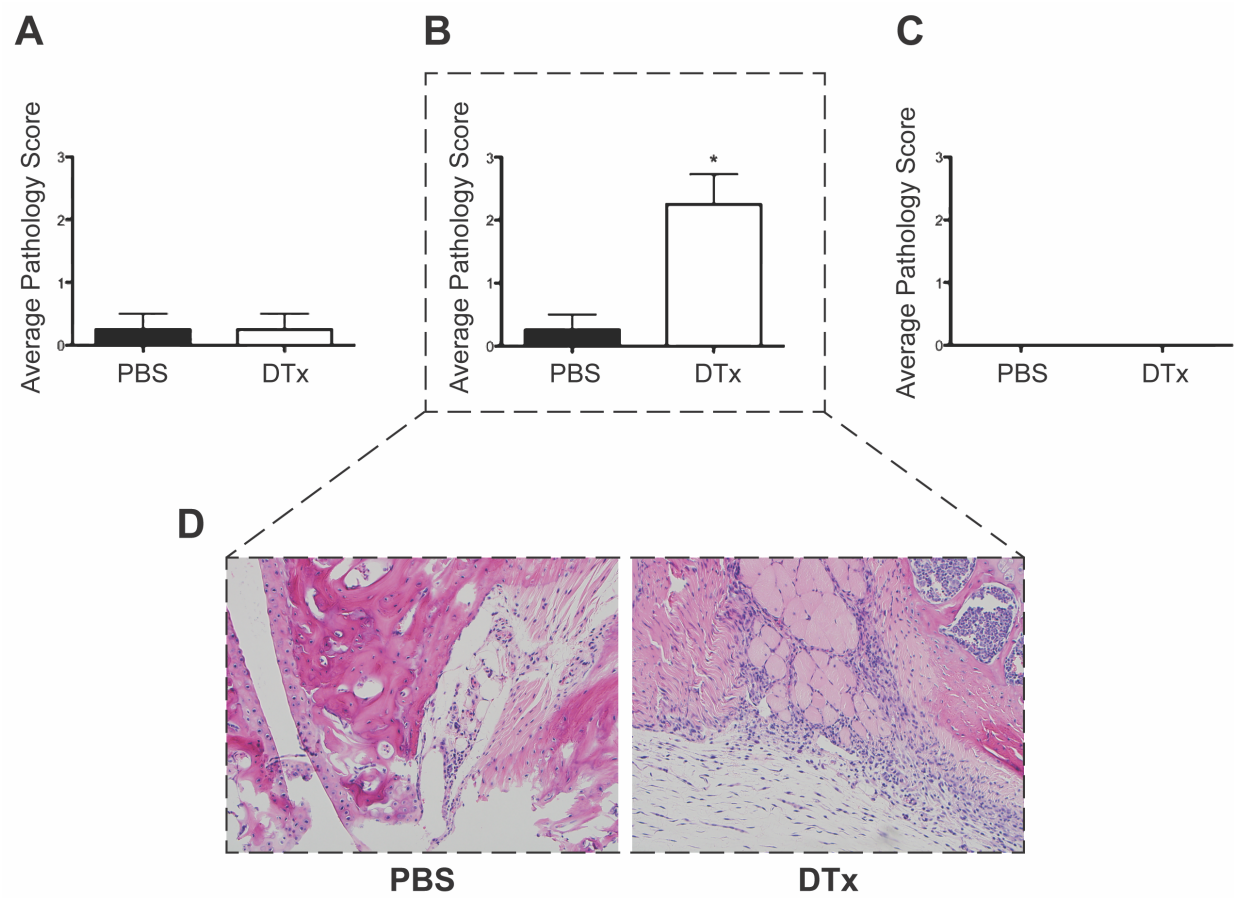


Figure 5. Administration of diphtheria toxin increases joint pathology in *B. burgdorferi*-infected DERE mice. Histopathology in H&E-stained longitudinal sections of the tibiotarsal joints of *B. burgdorferi*-infected DERE mice injected with PBS or administered DTx on two consecutive days prior to infection were assessed as described in *Materials and Methods*. A: Mean pathology scores 7 days after infection. B: Mean pathology scores 14 days after infection. C: Mean pathology scores 32 days after infection. D: Representative images of pathology observed 14 days after infection. Error bars indicate SEM. * = $P < 0.05$

Figure 6.

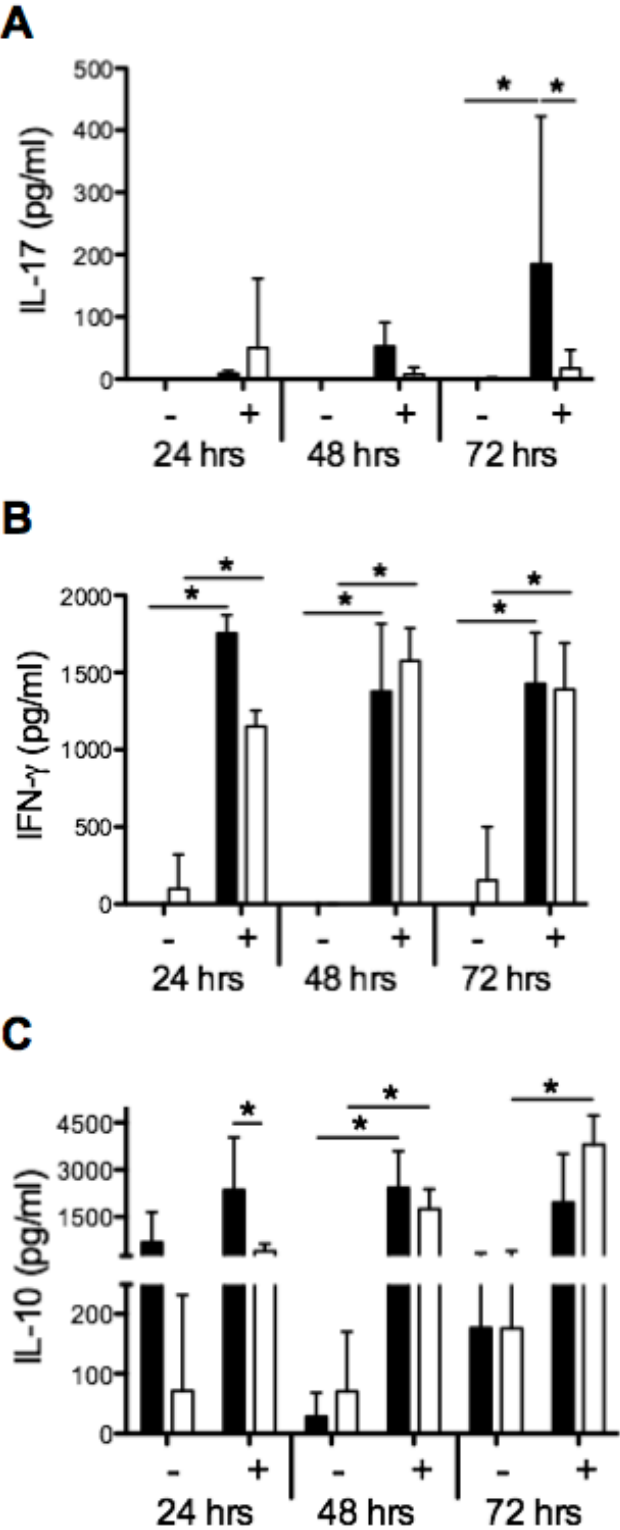
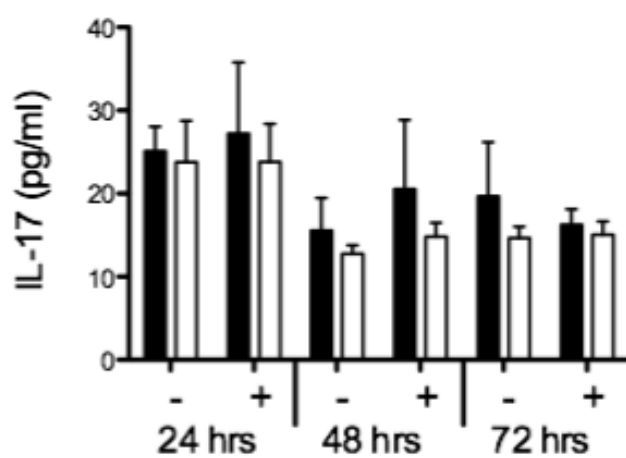


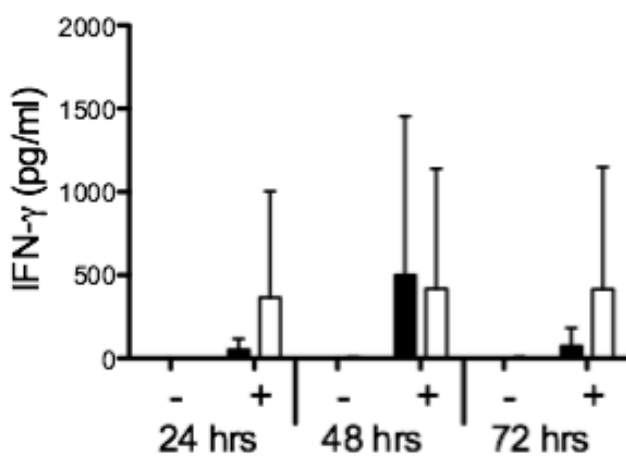
Figure 6. Effects of Treg cell depletion on production of cytokines after 7 days of *B. burgdorferi*-infected mice. Spleen cells obtained from DERE mice administered PBS (black bars) and DTx (white bars) seven days after infection with *B. burgdorferi* cultured in the presence (+) or absence (-) of the spirochete. Levels of IL-17 (A), IFN- γ (B), and IL-10 (C) were measured after 24, 48, and 72 hours of incubation. Data are mean levels of the cytokines/ml culture supernatant \pm SEM. *P < 0.05.

Figure 7.

A



B



C

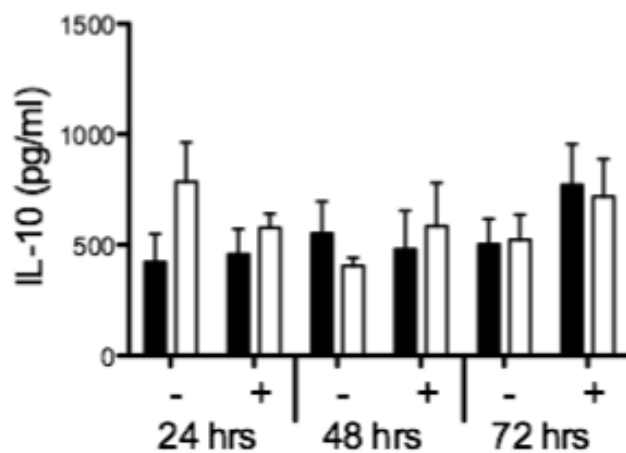
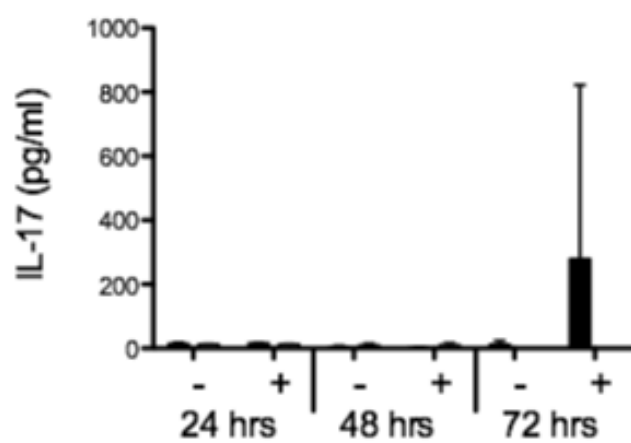


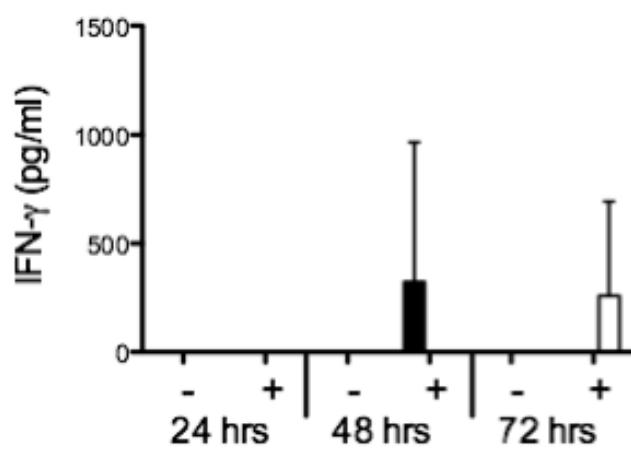
Figure 7. Effects of Treg cell depletion on production of cytokines after 14 days of *B. burgdorferi*-infected mice. Spleen cells obtained from DERE mice administered PBS (black bars) and DTx (white bars) 14 days after infection of *B. burgdorferi* cultured in the presence (+) or absence (-) of the spirochete. Levels of IL-17 (A), IFN- γ (B), and IL-10 (C) were measured after 24, 48, and 72 hours of incubation. Data are mean levels of the cytokines/ml culture supernatant \pm SEM. No significant differences in cytokine production were detected.

Figure 8.

A



B



C

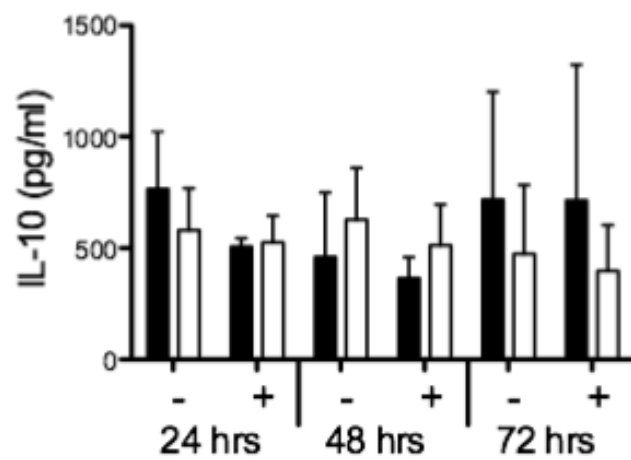
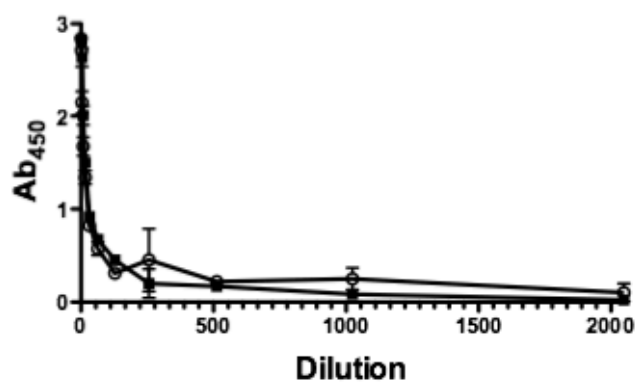


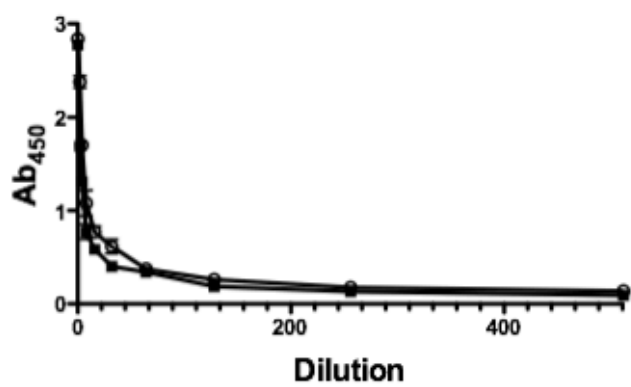
Figure 8. Effects of Treg cell depletion on production of cytokines after 32 days of *B. burgdorferi*-infected mice. Spleen cells obtained from DERE mice administered PBS (black bars) and DTx (white bars) 32 days after infection of *B. burgdorferi* cultured in the presence (+) or absence (-) of the spirochete. Levels of IL-17 (A), IFN- γ (B), and IL-10 (C) were measured after 24, 48, and 72 hours of incubation. Data are mean levels of the cytokines/ml culture supernatant \pm SEM. No significant differences in cytokine production were detected.

Figure 9.

A



B



C

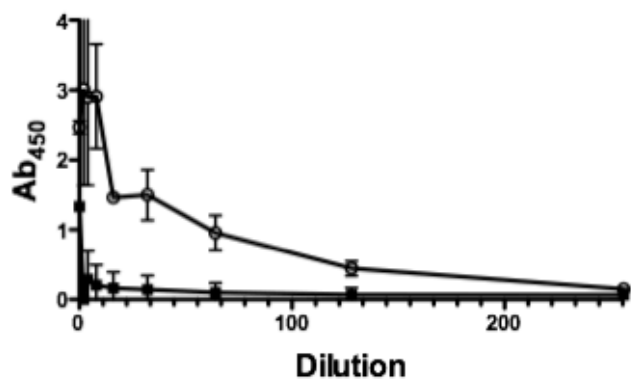
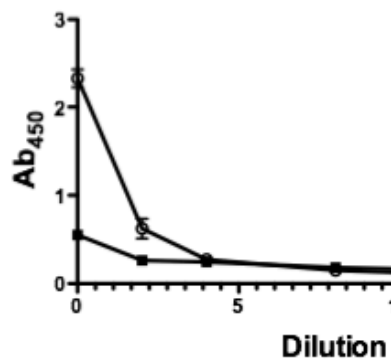


Figure 9. Effect of Treg cell depletion on IgM antibody production in *B.*

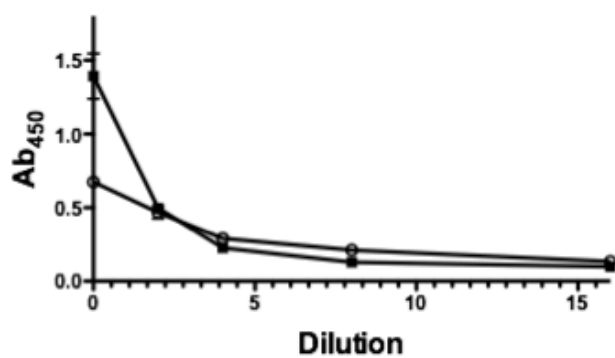
***burgdorferi*-infected mice.** Pooled sera collected from DERE mice administered PBS (closed circles) or DTx (open circles) on 2 consecutive days prior to *B. burgdorferi* infection were assessed for IgM antibody production after 7 (A), 14 (B), and 32 (C) days after infection. Values are expressed as the absorbance at 450 nm (Ab_{450}). Error bars indicate SEM.

Figure 10.

A



B



C

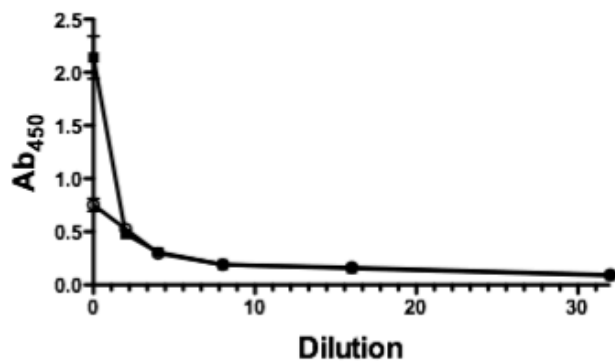


Figure 10. Effect of Treg cell depletion on IgG antibody production in *B.*

***burgdorferi*-infected mice.** Pooled sera collected from DERE mice administered PBS (closed circles) or DTx (open circles) on 2 consecutive days prior to *B. burgdorferi* infection were assessed for IgM antibody production after 7 (A), 14 (B), and 32 (C) days after infection. Values are expressed as the absorbance at 450 nm (Ab_{450}). Error bars indicate SEM.

Figure 11.

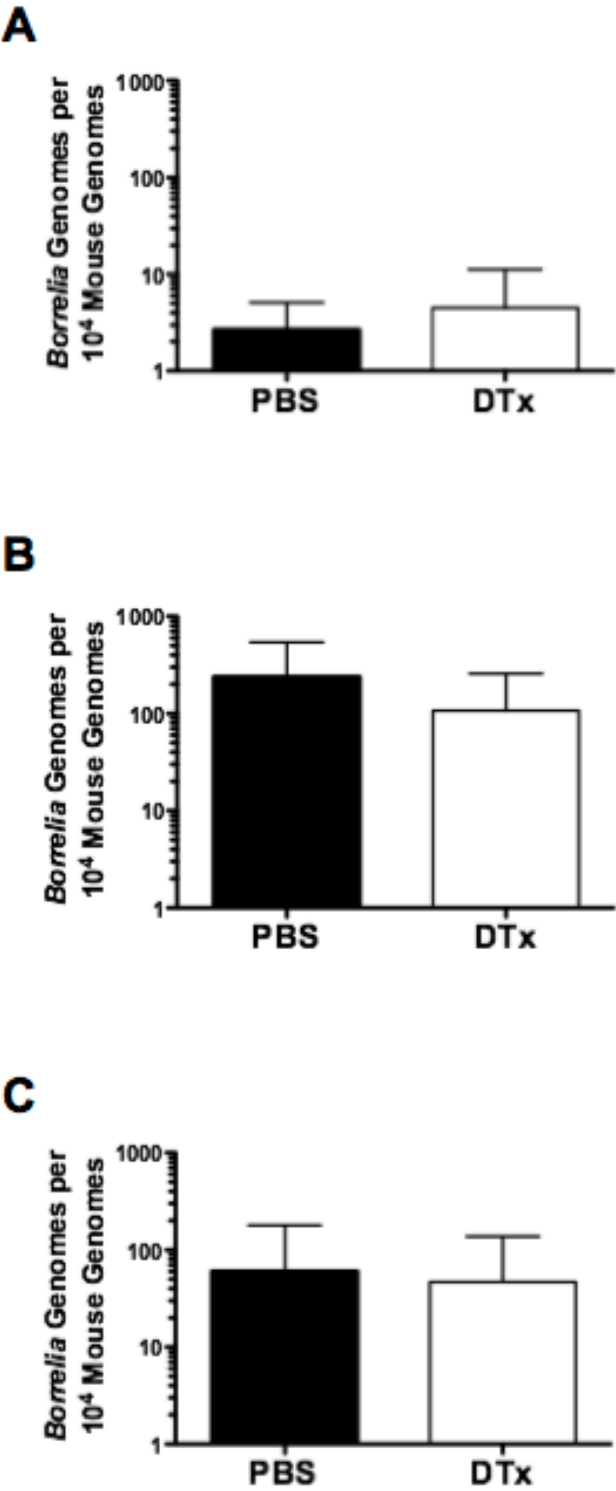


Figure 11. Effect of Treg cell depletion on bacterial burden in the tibiotarsal joint in *B. burgdorferi*-infected mice. The number of bacterial genomes was determined in the tibiotarsal joints of DERE mice administered PBS (black bars) or DTx (white bars) for 2 consecutive days prior to infection with *B. burgdorferi* after 7 (A), 14 (B), or 32 (C) days of infection. Data are mean number of *B. burgdorferi* genomes per 10^4 mouse genomes \pm SEM. No significant differences in bacterial burden between groups were detected.

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CHAPTER 5: SPECIFIC AIM 4

The Role of Regulatory T Cells During *Borrelia burgdorferi* Infection

Abstract

We previously showed that depletion of CD4⁺CD25⁺Foxp3⁺ regulatory T (Treg) cells prior to infection with *B. burgdorferi* results in increased paw swelling and arthritis in C57BL/6 mice. Here, we demonstrate that depletion of Treg cells 7 days after infection with *B. burgdorferi* caused greater IL-17 production by spleen cells 14 days after infection. Additionally, depletion of Treg cells 21 days after infection resulted in decreased IL-10 production by spleen cells 28 days after infection. Treg cell depletion at all time points resulted in significantly increased paw swelling in the week following depletion. Despite changes in paw swelling, Treg cell depletion did not have an effect on the development of pathology at the time points we examined. We also found that Treg cells inhibit, or promote, *B. burgdorferi*-specific antibody production depending on the timing of Treg cell depletion. Our findings indicate that Treg cells may inhibit the inflammatory response following *B. burgdorferi* infection that causes joint swelling. These findings support the need for further research into the role of Treg cells in Lyme arthritis.

Introduction

Regulatory T (Treg) cells are important mediators of the immune response by inhibiting inflammation and preventing tissue destruction. We have shown that depletion of regulatory T (Treg) cells in arthritis-resistant C57BL/6 mice prior to infection with

Borrelia burgdorferi resulted in significantly greater paw swelling beginning 10 days after infection, which continued through day 22 after infection. Additionally, Treg cell depletion followed by infection with *B. burgdorferi* also resulted in the development of arthritis 14 days after infection. However, Treg cell depletion did not affect pathology 7 days after infection or 32 days after infection. These findings suggest that Treg cell depletion prior to infection affects the inflammatory response that leads to the development of pathology. In addition, depletion of Treg cells led to an increase in *B. burgdorferi*-specific antibody production. These results suggest that Treg cells are central immunomodulatory cells that can affect the immune response to *B. burgdorferi* infection.

Accumulating evidence supports our assertion that Treg cells modulate the immune response to prevent the development of *B. burgdorferi*-induced arthritis. Neutralization of interleukin-17 (IL-17) in an interferon- γ (IFN- γ)-deficient mouse model of Lyme arthritis led to increased numbers of CD4+CD25+ putative Treg cells (Nardelli *et al.* 2004). Depletion of CD25-expressing cells that developed when IL-17 was neutralized resulted in destructive arthritis in infected mice (Nardelli *et al.* 2004). By contrast, depletion of CD25+ cells when IL-17 was not depleted had no effect on the development of arthritis (Nardelli *et al.* 2006). Adoptive transfer of CD4+CD25+ T cells induced by neutralization of IL-17 into *B. burgdorferi*-infected mice prevented the development of arthritis (Nardelli *et al.* 2005). Since Th17 and Treg cells differentiate from a common precursor (Bettelli *et al.* 2006), the putative CD4+CD25+ cells that developed in the absence of IL-17 were likely true Treg cells. In addition, patients with

antibiotic-refractory Lyme arthritis have fewer numbers of Treg cells than patients who respond to treatment (Vudattu *et al.* 2013). Overall, patients with antibiotic-refractory Lyme arthritis were also found to have Treg cells that were less effective in controlling the inflammatory immune response (Vudattu *et al.* 2013). Additionally, patients with antibiotic-refractory disease who have fewer numbers of Treg cells in the synovial fluid resolve symptoms of arthritis slower than patients whose arthritis resolved after antibiotic treatment (Shen *et al.* 2010). Treg cells isolated from patients with antibiotic-refractory Lyme arthritis were functionally suppressive, yet were still unable to control inflammation to resolve arthritis (Shen *et al.* 2010). Taken together, these studies suggest that Treg cells may inhibit the development of *B. burgdorferi*-induced arthritis.

Here, we extend our previous findings by assessing the role of Treg cells during the course of *B. burgdorferi* infection. We hypothesized that Treg cells present following *B. burgdorferi* infection decrease the severity of arthritis. Using the DREG mouse model, we depleted Treg cells at different points following infection with *B. burgdorferi*. We found that Treg cell depletion led to an increased inflammatory response and paw swelling, but did not affect severity of arthritis, one week after depletion. Additionally, Treg cell depletion resulted in an increased IgM antibody response, while depletion increased or decreased the IgG response depending on the timing of depletion. These results indicate that Treg cell populations mediate the immune response during *B. burgdorferi* infection.

Materials and Methods

Mice

Six-to-12 week old, male and female C57BL/6 DREG mice (The Jackson Laboratory) were housed in a humidity-controlled environment at 21°C under a 12-hour light-and-dark cycle at the University of Wisconsin-Milwaukee animal research facility. Mice had access to food and acidified water *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Milwaukee.

Preparation of Bacteria

B. burgdorferi strain B31-A3 organisms were provided by J. Coburn (Medical College of Wisconsin, Milwaukee, WI). Low passage organisms were grown at 32°C in modified Barbour-Stoenner-Kelly (BSK) medium until they reached a concentration of 10^6 microbes/ml before being aliquoted into 1.5-ml screw-cap tubes (Sarstedt, Newton, NC) and stored at -80°C. On the day of infection, 1 mL frozen suspensions of spirochetes were thawed, washed, and resuspended in PBS supplemented with 0.2% non-immune serum as described by Ristow *et al.* (2012). Dark-field microscopy was used to determine motility and enumerate microbes using a Petroff-Hausser counting chamber.

Infection of mice

Naïve mice were anaesthetized with isoflourane in a nose-and-mouth cup and infected by subcutaneous injection between the scapulae with 2×10^4 viable *B. burgdorferi* strain B31-A3 organisms in a volume of 0.05 mL of PBS supplemented with 0.2% non-

immune serum. Control groups consisted of DERE mice injected with PBS supplemented with non-immune serum.

Depletion of Treg cells

Diphtheria toxin (Calbiochem, San Diego, CA) was reconstituted in filter-sterilized PBS. Mice were injected with 1 µg diphtheria toxin (Calbiochem, San Diego, CA) in 100 µl of PBS intraperitoneally (i.p.) on two consecutive days beginning at day 7, day 14, or day 21 after infection with *B. burgdorferi*. Control groups included infected DERE mice injected with PBS and non-infected DERE mice administered DTx at the same time points as experimental mice. Groups consisted of 3-4 mice each.

Assessment of swelling

Swelling of each hind paw was assessed every other day in order to determine changes in edema of the tibiotarsal joint following infection with *B. burgdorferi* using a digital caliper (Marathon, Richmond Hill, Ontario) with a sensitivity of 0.01 mm. The width and thickness of each tibiotarsal joint were measured and averaged to provide the mean caliper value. Baseline swelling levels of each group were measured prior to infection, and the changes in swelling from these baseline levels were determined every other day after infection and at the time of euthanasia.

Histological examination

Mice were euthanized one week after administration of DTx, on days 14, 21, or 28 after infection. One hind paw from each mouse was amputated above the knee joint. Paws

were placed in cryptically coded tissue-embedding cassettes and fixed in 10% buffered zinc formalin before being embedded in paraffin. The paraffin-embedded tissues were cut in 4µm sections, placed on glass slides, and stained with hematoxylin and eosin. Unbiased histological examination of the tissues was conducted by a board-certified pathologist (Dr. Michael Lawlor, Children's Hospital of Wisconsin, Milwaukee, WI). Histopathological changes were scored according to the following scale: 0, no pathology; 1, mild lymphocyte infiltration restricted to joint surfaces; 2, mild lymphocyte infiltration restricted to subcutaneous soft tissue; and 3, mild lymphocyte infiltration of muscle and soft tissue.

Assessment of cytokine production

Spleens were harvested on days 14, 21, or 28 after infection, and single-cell suspensions of splenocytes were created by passing the tissue through a nylon mesh screen (BD Falcon, BD Biosciences, San Jose, CA) into cold Dulbecco's Modified Eagle Medium (DMEM). 1×10^6 cells were incubated at 37 °C in 5% CO₂ in the absence or presence of 1×10^5 viable *B. burgdorferi* strain B31-A3 organisms. Cell culture supernatants were collected after 24, 48, and 72 hours of incubation. Levels of IL-17, IFN-γ, and IL-10 in the cell culture supernatants were determined using IL-17A, IFN-γ, or IL-10 Ready-Set-Go! ELISA kits (eBioscience, San Diego, CA), respectively, according to the manufacturer's instructions. Plates were read at an absorbance of 450 nm. Standard curves were used to calculate the cytokine amount in each sample as pg/ml.

Quantification of spirochetes by PCR

Bacterial load was determined as described by Ristow *et al.* (2012). One hind paw was collected at the time of euthanasia, frozen in liquid nitrogen, and stored at -80°C until the time of DNA isolation. DNA was isolated using the QIAamp genomic DNA and RNA kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Both *B. burgdorferi* genome copies and mouse genome copies were enumerated. PCR amplification was performed in 20- μ l reaction mixtures containing 5 μ l DNA, 10 μ l of PowerUp SYBR Green Master Mix (Life Technologies, Carlsbad, CA), 2 μ l magnesium chloride, 1 μ l water, and 1 μ l of both forward and reverse primer for either *B. burgdorferi* *recA* (forward: 5'- GCAGCTATCCCACCTTCTTT-3', reverse: 5'- ATGAGGCTC-TCGGCATTG-3') or *Mus musculus* β -actin (forward: '5-TCACCCACACTGTGCCCATCTACGA-3', reverse: 5'-GGATGCCACAGGATTCCATACCCA-3'). The thermal cycling profile consisted of one 2-minute cycle at 50°C and one 2-minute cycle at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A melting curve was acquired by heating the product to 95°C for 15 seconds, cooling to 60°C for 1 minute, and heating to 95°C for 15 seconds. *B. burgdorferi* *recA* gene copies were normalized to mouse β -actin after being calculated from standard curves using StepOnePlus Real-Time PCR System.

Quantification of antibody production

Pooled sera from each group were analyzed in duplicate for *B. burgdorferi*-specific IgG and IgM antibody content by a protocol adapted from Yang *et al.* (2011). Briefly, 96-well plates were coated overnight at 4°C with 5 μ g/ml sonicated *B. burgdorferi* B31-A3.

Control wells were coated with goat anti-mouse IgM or IgG isotype control antibodies. Plates were washed three times with PBS containing 0.05% Tween 20 before blocking for 1 hour at room temperature with PBS containing 10% fetal bovine serum and 0.2% Tween 20. After blocking, plates were washed once. 1:2 serial dilutions of serum ranging from undiluted to 1:65,536 were added to wells and incubated for 2 hours at room temperature. Murine IgM or IgG isotype control antibodies were added to wells coated with goat anti-mouse IgM or IgG as positive controls. Plates were washed 3 times before addition of goat anti-mouse IgM or IgG antibodies conjugated with horseradish peroxidase. Wells were developed with 3,3',5,5'-tetramethyl-benzidine (eBioscience). After 5 minutes, the reaction was stopped with 2 N sulfuric acid. Optical density was measured at 450 nm. Reciprocal endpoint dilutions were determined from average titration curves and defined as twofold above the buffer background (Coligan 1991). All antibodies were obtained from Southern Biotech (Birmingham, AL).

Statistics

Data were analyzed using two-way ANOVA followed by the Bonferroni correction for *post hoc* analysis when a significant *F* value indicated reliable mean differences. Paired data were analyzed using two-tailed Student's *t* test. Data were analyzed using GraphPad Prism. The alpha level was set at 0.05 prior to initiation of experiments. Data are expressed as mean \pm standard error of the mean (SEM) unless otherwise stated.

Results

Effect of diphtheria toxin administration

We previously showed that administration of diphtheria toxin in DERE mice reduced Treg cell populations by 75% (Chapter 4). However, we previously showed that diphtheria toxin administration results in a maintenance in body weight shortly after injection, before eventually increasing to levels observed in untreated mice (Chapter 4). To ensure the health of the mice administered diphtheria toxin, we recorded body weight every other day following infection. Mice that were injected with PBS gained weight over the course of the experiment. By contrast, mice administered DTx on days 7 and 8 after infection exhibited a mild, statistically insignificant decrease in body weight beginning at day 10 until the end of the experiment on day 14 (Figure 1A). Similarly, infected mice administered DTx on days 14 and 15 after infection exhibited decreased body weight 6 days later (Figure 1B). In addition, infected mice administered DTx on days 21 and 22 displayed a decrease in body weight beginning at day 24 and continuing through day 28 (Figure 1C). Naïve mice administered DTx displayed the same pattern of weight loss as infected mice that received DTx (data not shown).

Effect of DTx administration on hind paw swelling and arthritis

Baseline levels of hind paw size were determined by measuring the width and thickness of tibiotarsal joints prior to infection with *B. burgdorferi*. Paw size was measured every other day, and baseline measurements were subtracted to determine average change in paw size. Infected mice injected with PBS displayed minimal paw swelling in all cohorts. By contrast, mice depleted of Treg cells beginning at day 7 after infection displayed significantly greater paw swelling than PBS-injected mice beginning at day 8

after infection (Figure 2A). Mice depleted of Treg cells on day 7 after infection continued to display increased paw swelling until the end of the experiment, at day 14 after infection (Figure 2A). Mice depleted of Treg cells beginning at day 14 after infection also exhibited significantly greater paw swelling than PBS-injected mice beginning at day 18 after infection, which remained through the end of the experiment at day 21 after infection (Figure 2B). Additionally, mice depleted of Treg cells beginning at day 21 after infection displayed greater paw swelling than PBS-injected mice at day 26 after infection (Figure 2C).

No histopathological changes were observed 14 days after infection in PBS-injected mice or mice depleted of Treg cells on day 7 after infection (Figure 3A). PBS-injected mice displayed minimal histopathological changes on day 21 that were not significantly greater than the minimal pathology observed in mice depleted of Treg cells on day 14 (Figure 3B). Both PBS-injected mice and mice depleted of Treg cells on day 21 displayed minimal histopathological changes on day 28 (Figure 3C). No significant difference in pathology was observed due to Treg cell depletion at any time point.

Effect of Treg cell depletion on cytokine production

We investigated the role of Treg cell ablation on the production of cytokines at varying time points after infection with *B. burgdorferi*. Spleen cells harvested 14 days after *B. burgdorferi* infection from mice injected with PBS on days 7 and 8 produced low levels of IL-17 after 24, 48, and 72 hours of incubation in media (Figure 4A). Unstimulated spleen cells harvested on day 14 after infection from mice that were depleted of Treg

cells on day 7 after infection produced a greater amount of IL-17 after 24 and 48 hours of incubation than PBS-injected mice. However, the increases were not statistically significant. Upon *in vitro* stimulation with *B. burgdorferi*, cells from PBS-injected mice produced slightly greater levels of IL-17 at all time points than unstimulated cultures. However, these increases were not statistically significant. Additionally, after 48 hours of incubation, stimulated spleen cells from Treg cell-depleted mice produced a significantly greater amount of IL-17 than unstimulated cells ($p < 0.05$). Depletion of Treg cells had no effect on the production of IL-17 after 72 hours of incubation, regardless of stimulation.

In addition, we measured IFN- γ production by the same spleen cells. No differences were observed due to Treg cell depletion at any duration of incubation. We found that IFN- γ production on day 14 after infection was similar between unstimulated cells from PBS-injected mice and Treg cell-depleted mice at all incubation times. IFN- γ production was increased following *in vitro* stimulation of cells from PBS-injected mice after 24, 48, and 72 hours of incubation. However, the increases were not statistically significant (Figure 4B). By contrast, stimulation led to significantly increased IFN- γ production by spleen cells isolated from mice depleted of Treg cells on day 7 after infection at all incubation times ($p < 0.05$). In addition, IL-10 production was measured in the same cell cultures. Cells from PBS-injected mice and cells from Treg cell-depleted mice produced negligible amounts of IL-10 at all incubation times, regardless of stimulation (Figure 4C).

Cytokine production by spleen cells harvested 21 days after infection was also assessed in PBS-injected mice and mice depleted of Treg cells on day 14 after infection. Spleen cells harvested 21 days after *B. burgdorferi* infection from mice injected with PBS produced low levels of IL-17 after 24, 48, and 72 hours of incubation in media (Figure 5A). In addition, production of IL-17 was below the level of detection at all time points by spleen cells from mice that were depleted of Treg cells on days 14 after infection. Upon stimulation, spleen cells from PBS-injected mice produced greater, but not significantly different, amounts of IL-17 than stimulated cells isolated from Treg cell-depleted mice. In general, stimulation resulted in increased IL-17 production by cells from both PBS-injected and Treg cell-depleted mice; however, the increases were not statistically significant.

Unstimulated spleen cells from PBS-injected mice produced levels of IFN- γ that were below the level of detection after 24, 48, and 72 hours of incubation (Figure 5B). By contrast, unstimulated cells from Treg cell-depleted mice produced detectable IFN- γ at all incubation times. Stimulation did not significantly increase IFN- γ production by cells from PBS-injected mice after 24 hours, as levels remained undetectable. Stimulation did increase IFN- γ production by these cells after 48 and 72 hours of incubation, although the increase was not statistically significant. Stimulation also resulted in increased, but not significantly greater, IFN- γ production by cells from Treg cell-depleted mice. Additionally, IL-10 production by the same cells was assessed. In addition, no differences in IL-10 production were observed due to Treg cell depletion (Figure 5C),

and cells from PBS-injected mice and cells from Treg cell-depleted mice produced negligible amounts of IL-10 at all incubation times, regardless of stimulation.

We further assessed cytokine production by spleen cells harvested 28 days after infection in PBS-injected mice and mice depleted of Treg cells on day 21 after infection. Unstimulated cells harvested 28 days after infection with *B. burgdorferi* from both PBS-injected and mice depleted of Treg cells on day 21 after infection produced similarly low levels of IL-17 at all incubation times (Figure 6A). While no significant differences were observed due to Treg cell depletion, stimulated cultures of cells from Treg cell-depleted mice produced less IL-17 than cell from PBS-injected mice at all incubation times. Stimulation of cells from both PBS-injected mice and mice depleted of Treg cells resulted in increased IL-17 production that was not statistically significant.

Twenty-eight days after infection, no statistically significant differences were observed due to Treg cell depletion in unstimulated or stimulated cultures after 24, 48, or 72 hours of incubation (Figure 6B). IFN- γ production by unstimulated cells isolated from PBS-injected mice was below the level of detection after 24 and 48 hours of incubation, with low, but detectable, levels produced after 72 hours. By contrast, unstimulated cells from mice depleted of Treg cells produced a greater, though not statistically significant, amount of IFN- γ at all incubation times than cells from PBS-injected mice. Following stimulation, cells from both PBS-injected and Treg cell-depleted mice produced similar amounts of IFN- γ at all incubation times. These stimulated cells produced significantly greater amounts of IFN- γ ($P < 0.05$) than their unstimulated counterparts.

Spleen cells isolated from PBS-injected mice produced large amounts of IL-10 after 24 hours of incubation (Figure 6C). IL-10 production by both stimulated and unstimulated cells from PBS-injected mice was significantly greater than that of cells isolated from Treg cell-depleted mice ($p<0.05$). After 48 hours of incubation, cells from PBS-injected mice produced negligible levels of IL-10, and unstimulated cells from Treg cell-depleted mice produced low, but measurable, amounts of IL-10. Stimulation had no effect on IL-10 production by cells from PBS-injected or Treg cell-depleted mice after 48 hours. No detectable IL-10 was produced by cells from PBS-injected or Treg cell-depleted mice after 72 hours of incubation.

Effect of Treg cell ablation on antibody production

B. burgdorferi B31-A3-specific total IgM antibody levels were determined by ELISA using pooled sera. Mice were injected with PBS or depleted of Treg cells on days 7, 14, or 21 after infection, and sera were harvested one week later, at 14, 21, and 28 days after infection, respectively. Sera collected 14 days after infection from PBS-injected mice had an average IgM endpoint dilution of 1:1024 (Figure 7A). By contrast, sera collected 14 days after infection from mice depleted of Treg cells on day 7 after infection had a greater IgM average endpoint dilution of 1:2048. Twenty-one days after infection, sera from PBS-injected mice had an average endpoint dilution of 1:1024 (Figure 7B). IgM serum levels of Treg cell-depleted mice were again greater, with an average endpoint dilution of 1:2048. Twenty-eight days after infection, sera from PBS-injected mice had an average IgM endpoint dilution of 1:2048 (Figure 7C). Sera from Treg cell-depleted mice had a lower IgM average endpoint dilution of 1:512. Overall, Treg cell

depletion on days 7 or 14 after infection increased IgM antibody production at 14 and 21 days, respectively. However, Treg cell depletion 21 days after infection resulted in decreased antibody production on day 28.

IgG levels in serum samples were also determined. On day 14 after infection, sera from PBS-injected mice had an average IgG endpoint dilution of 1:4 (Figure 8A). By contrast, sera from mice depleted of Treg cells on day 7 after infection had a greater average endpoint dilution of 1:8 on day 14 after infection. Twenty-one days after infection, sera from PBS-injected mice had an average IgG endpoint dilution of 1:8 (Figure 8B). Sera from mice depleted of Treg cells on day 14 after infection also had an average endpoint dilution of 1:8 21 days after infection. Twenty-eight days after infection, sera from PBS-injected mice had an average end point dilution of 1:32 (Figure 8C). By contrast, sera from Treg cell-depleted mice had a lower IgG average endpoint dilution of 1:2. Overall, depletion of Treg cells 1 week after infection slightly increased IgG production, but depletion of Treg cells 3 weeks after infection substantially reduced IgG production.

Effect of Treg cell ablation on bacterial load

We determined the number of bacterial genomes within the tibiotarsal joints of *B. burgdorferi*-infected mice injected with PBS and infected mice depleted of Treg cells to assess the impact of Treg cells on dissemination of the spirochete to the joints. *B. burgdorferi* DNA was measured 7 days after injection with PBS or Treg cell depletion on days 7, 14, or 21 after infection. No differences in borrelial load were observed in the

joints of PBS-injected mice and the joints of Treg cell-depleted mice after 14 days of infection (Figure 9A), 21 days of infection (Figure 9B), or 28 days of infection (Figure 9C).

Discussion

In this report, we examined the effects of Treg cells on the immune response to during *B. burgdorferi* in an arthritis-resistant mouse model by depleting them at different time points following infection. We found that depletion of Treg cells after infection with *B. burgdorferi* resulted in increased swelling of the hind paws, but did not increase arthritis 7 days after depletion. Also, depletion of Treg cells after 7 days of infection led to an increased capacity to produce IL-17 and IFN- γ , and depletion of Treg cells after 21 days of infection led to a decrease in IL-10 production. Additionally, Treg cell depletion 7 days after infection increased *B. burgdorferi*-specific antibody production 14 days after infection. However, Treg cell depletion 21 days after infection decreased antibody production 28 days after infection. These findings partially support our hypothesis by demonstrating that Treg cells inhibit swelling and inflammatory cytokine production following infection with *B. burgdorferi*.

Previous reports have suggested a role for Treg cells in modulating arthritis in *B. burgdorferi* infection. Neutralization of IL-17 in IFN- γ -deficient mice delayed the onset of swelling and prevented histopathological changes in a model of severe Lyme arthritis (Burchill *et al.* 2003). Reduced arthritis in these mice was associated with an increase in the number of CD4+CD25+ putative Treg cells in the draining lymph nodes beginning

2 days after infection (Nardelli *et al.* 2004). The addition of anti-CD25 antibody in anti-IL-17 antibody, IFN- γ -deficient mice led to the induction of pathology when administered prior to infection, though later administration of anti-CD25 had less pronounced effects (Nardelli *et al.* 2004). Additionally, adoptive transfer of CD4+CD25+ T cells from IFN- γ -deficient, anti-IL-17 antibody-treated mice prevented the development of arthritis in infected recipients, whereas pathology was observed in infected mice that received CD4+CD25- cells (Nardelli *et al.* 2005). In addition, administration of anti-CD25 antibody when IL-17 is not neutralized had no effect on pathology; however, CD4+CD25+ populations were reduced (Nardelli *et al.* 2006). Foxp3 expression was not determined in these studies, as it had not yet been identified as the Treg cell-specific transcription factor. However, the increased presence of CD4+CD25+ cells following IL-17 neutralization suggests these may be Treg cells, as Treg cells and Th17 cells have a reciprocal relationship and arise from a common lineage (Bettelli *et al.* 2006). Treg cells may not only influence the development of arthritis, but may also contribute to the resolution of disease. Notably, mice deficient in CD28 have decreased numbers of CD4+CD25+ T cells (Tang *et al.* 2003) along with increased production of IFN- γ (Shanafelt *et al.* 1998). Additionally, mice deficient in CD28 develop more severe arthritis (Iliopoulou *et al.* 2007). These findings suggest that Treg cells are important regulators of inflammation throughout the course of Lyme arthritis.

Reduced numbers of Treg cells may be a contributing factor as to why some patients with Lyme disease fail to resolve inflammation following antibiotic treatment. Analysis of T cell populations in patients who continued to experience symptoms of Lyme arthritis

following antibiotic therapy supports this notion. Patients with slow-resolving Lyme arthritis following antibiotic treatment were found to have lower numbers of Treg cells in the synovial fluid than patients whose symptoms resolved after antibiotic therapy (Shen *et al.* 2010). In addition, patients with antibiotic-refractory Lyme arthritis were also found to have low numbers of Treg cells, in addition to having Treg cells that were less effective in suppressing inflammatory cytokine production (Vudattu *et al.* 2013). Together, these studies indicate that patients with Lyme arthritis and who have fewer functional Treg cells develop an excessive inflammatory response that leads to the inability to resolve arthritis, even after clearance of the infection.

Treg cells are also important mediators of the immune response by inhibiting inflammation and preventing tissue destruction. In order to directly examine the effect of Treg cells during infection with *B. burgdorferi*, we utilized the DREG mouse model. The DREG mouse model allows for the selective depletion of Foxp3⁺ Treg cells following administration of diphtheria toxin. We previously showed that depletion of Treg cells prior to infection with *B. burgdorferi* results in increased paw swelling and arthritis in an arthritis-resistant mouse model. Here, we administered diphtheria toxin at different times after establishment of infection. We found that depletion of Treg cells on day 7, day 14, or day 21 after infection led to significant increases in hind paw swelling compared to mice in which Treg cells were not depleted. Our findings are consistent with our previous work, in which we found increased paw swelling following depletion of Treg cells prior to *B. burgdorferi*-infection. However, when Treg cells were depleted prior to infection with *B. burgdorferi*, increased paw swelling did not occur until 12 days

after infection. Here, we found that Treg cells depleted after infection results in a rapid increase in paw swelling, regardless of the time of depletion. Our findings suggest that paw swelling that occurs following *B. burgdorferi* infection is controlled by Treg cells throughout the course of infection.

Despite changes in paw swelling, Treg cell depletion did not have an effect on the development of pathology 7 days later. Previously, we found that depletion of Treg cells prior to infection with *B. burgdorferi* led to the development of pathology in arthritis-resistant mice. However, histopathological findings were not observed until 14 days after infection, and disease resolved by 32 days after infection. In arthritis-susceptible mice, arthritis generally peaks approximately 21 days after infection (Barthold 1996). Therefore, we assessed the effects of Treg cell depletion 7 days later, on days 14, 21, and 28 after infection, in order to capture these events. In our previous study, pathology was not observed until 2 weeks after depletion. Therefore, it is possible that the paw swelling seen in our current experiment due to Treg cell depletion indicates that pathology is developing, and a longer duration after depletion may be needed for pathological changes to be observed. Swelling likely occurred more rapidly when Treg cells were depleted after establishment of *B. burgdorferi* infection because an immune response to the spirochete had already been generated. Thus, the depletion of Treg cells prior to infection may not have affected swelling as quickly because of the time needed to generate a substantial immune response. Alternatively, depletion of Treg cells at these various times following infection may not affect pathology, as Treg cells present during the time of infection may have already suppressed inflammatory T cells and cytokine production that would have caused pathology. In other models of arthritis,

exacerbated pathology was observed when Treg cells were depleted during disease (Atkinson *et al.* 2016, Irmeler *et al.* 2014). However, our findings suggest that Treg cells present following infection control paw swelling, but reducing their numbers after *B. burgdorferi* infection may not increase the severity of arthritis if disease is already established. Despite this, the impact of Treg cells during *B. burgdorferi* infection may still be important. Approximately 18% of patients with Lyme disease may present with joint pain without evidence of inflammation (Steere *et al.* 1987). Treg cells may inhibit immune reactions involved in pain and swelling that are independent of hypercellularity of the joint tissue.

We also investigated the effect of Treg cell depletion on the production of certain T cell-associated cytokines by splenocytes. Treg cell depletion on day 7 after infection resulted in increased IL-17 production by stimulated cells harvested at 14 days after infection. It is possible that Th17 cells are the source of IL-17 in these cultures. Our hypothesis is supported by the findings of Bettelli *et al.*, which demonstrated that Th17 and Treg cells arise from a common precursor (2006). Additionally, the neutralization of IL-17 in mice infected with *B. burgdorferi* was found to increase the number of CD4⁺CD25⁺ T cells with suppressive functions (Nardelli *et al.* 2004). In addition, we previously showed that spleen cells from mice deficient in IL-10 produced increased levels of IL-17 following *B. burgdorferi* infection than wild-type mice (Hansen *et al.* 2013). Collectively, these findings suggest that depletion of Treg cells, which produce IL-10, may have increased the number of Th17 cells, thereby resulting in increased IL-17 production.

Additionally, we noticed a trend of decreased IL-17 production by cells from mice depleted of Treg cells on day 14 and day 21 after infection when harvested on day 21 and day 28 after infection, respectively. These trends in decreased IL-17 production occurred when trends in increased IFN- γ production were also observed. The source of IL-17 and IFN- γ in these cultures may have been CD4⁺ T cells, as Th1 and Th17 cells can have an antagonistic relationship (Damsker *et al.* 2010). This would explain why increased levels of IFN- γ were observed with decreased production of IL-17 by cells from Treg cell-depleted mice. Our assertions are supported by the fact that the Th1/IFN- γ response is a more prominent contributor to the development of Lyme arthritis compared to the Th17/IL-17 response. Therefore, our findings may also suggest an important role for Treg cells in the control of IFN- γ production during *B. burgdorferi* infection.

In addition to affecting IL-17 production, Treg cell depletion also impacted the level of IFN- γ production by spleen cells. We found that cells harvested on day 14 from mice depleted of Treg cells on day 7 produced significantly greater levels of IFN- γ following stimulation. While cells from PBS-injected mice did produce more IFN- γ following stimulation, the increase was not significant. At day 21 after infection, we also noticed a trend toward increased IFN- γ production by unstimulated spleen cells from mice depleted of Treg cells on day 14 after infection. Collectively, these findings suggest that depletion of Treg cells may increase the inflammatory capacity of T helper type 1 cells during *B. burgdorferi* infection. Others have shown that depletion of Treg cells results in increased IFN- γ production by spleen cells during bacterial infection (Raghavan *et al.*

2003); however, the effects of Treg cell depletion could only be observed after Treg cell depletion at day 3 and were no longer evident by day 7 (Prajeeth *et al.* 2014, Veiga-Parga *et al.* 2012). It has been shown that replenishment of Treg cells following depletion initiates 3 days after administration of DTx (Christiaansen *et al.* 2014). As a result, the effects of Treg cell depletion on cytokine production may not be evident at extended times after infection. Previously, we found that depletion of Treg cells prior to infection did not have a sustained effect on the production of inflammatory cytokines, despite resulting in increased paw swelling and arthritis. It is possible that Treg cells do not play a major role in suppressing effector T cells during *B. burgdorferi* infection; however, this seems unlikely. Overall, our findings suggest that Treg cells may not extensively inhibit T cell-associated inflammatory cytokine production throughout *B. burgdorferi* infection in our system, but, rather, may function in this capacity at an earlier time after infection.

Treg cells can modulate inflammation through the production of IL-10. It is known that Treg cells contribute to production of IL-10 in response to *B. burgdorferi* infection (Amedei *et al.* 2013). Therefore, we investigated the effects of Treg cell depletion on IL-10 production during *B. burgdorferi* infection. We found that spleen cells harvested at day 28 from mice depleted of Treg cells on days 21 after infection had significantly lower production of IL-10 compared to cells from mice injected with PBS. The effects of Treg cell depletion on IL-10 production occurring at day 28 are particularly interesting. During the course of *B. burgdorferi* infection in mice, arthritis typically peaks around day 28 and then subsides rapidly (Ma *et al.* 1998). Our findings suggest that IL-10 produced

by Treg cells may be important for the control of inflammation at later times. Despite displaying reduced levels of IL-10 and increased paw swelling, mice depleted of Treg cells did not develop increased pathology 7 days later. By contrast, we and others have shown that a lack of IL-10 results in increased pathology during *B. burgdorferi* infection. Specifically, IL-10-deficient mice develop more severe arthritis following *B. burgdorferi* infection than wild-type mice (Brown *et al.* 1999). In addition, IL-10 was found to attenuate arthritis following *B. burgdorferi* infection in a model of Lyme arthritis by decreasing the IL-17 response (Hansen *et al.* 2013). Perhaps the presence of other IL-10-producing cells mediated the development of arthritis in our experiments. Notably, macrophages are also a significant producer of IL-10 and help regulate the inflammatory response during infection with *B. burgdorferi* (Brown *et al.* 1999, Chung *et al.* 2013). Treg cells decrease the inflammatory effects of macrophages (Taams *et al.* 2005), and the ability of Treg cells to inhibit macrophages may explain why we saw arthritis when Treg cells were depleted prior to infection, but not when Treg cells were depleted at later times. Alternatively, Treg cells may have a more substantial impact in later infection, after initial regulation has already limited the extent of inflammation. In addition, we may observe disease if we examine pathology at later time points after Treg cell depletion. Collectively, our previous findings, along with our current report, suggest that Treg cell-derived IL-10 limits paw swelling in *B. burgdorferi* infection. However, additional work is needed to determine the effects of Treg cell depletion on pathology.

Treg cells have also been reported to suppress the antibody response (Lim *et al.* 2005). As such, we investigated the effect of Treg cell depletion on the production of *B. burgdorferi*-specific antibodies. We found that depletion of Treg cells on day 7 or day 14 led to increased IgM antibody production one week later, at 14 and 21 days after infection with *B. burgdorferi*, respectively. Treg cell depletion on day 7 also resulted in increased IgG antibody production at day 14 after infection, while Treg cell depletion on day 14 did not affect IgG production on day 21 after infection. Treg cell depletion 21 days after infection resulted in decreased IgM and IgG at 28 days after infection. We previously reported that depletion of Treg cells prior to infection caused increased production of IgM throughout the course of disease. We postulated that depletion of Treg cells enhanced the ability of effector T cells and/or T-follicular helper cells to enhance antibody production. Why, then, would delayed Treg cell depletion have different effects on antibody production depending on the time of depletion? While mainly described as suppressive, Treg cells can also enhance the antibody response (Vendetti *et al.* 2010). In fact, Treg cells can differentiate into T-follicular helper cells (Tsuji *et al.* 2009). We have already suggested that Treg cells may have a more profound effect in inhibiting inflammation during early infection. Perhaps, in late infection, when inflammation has already been limited, Treg cells become T-follicular helper cells to enhance antibody production. Or, after inflammation has been controlled, Treg cell numbers are reduced, thereby reversing the inhibition of antibody suppression. Overall, our findings suggest Treg cells inhibit antibody production in early *B. burgdorferi* infection, but may contribute to antibody production at later times.

We also investigated the ability of Treg cells to control clearance of *B. burgdorferi* within the joint tissue. We found that Treg cell depletion had no effect on bacterial burden in the tibiotarsal joint. Others have also shown that depletion of Treg cells in other infections had no effect on clearance of bacterial. For example, Treg cell depletion had no effect on clearance of *Mycobacterium bovis* (Berod *et al.* 2014) or *Staphylococcus aureus* infection (Tebartz *et al.* 2014). Additionally, we previously showed that depletion of Treg cells prior to infection with *B. burgdorferi* did not affect bacterial load in the tibiotarsal joints. Together, these findings indicate that Treg cells may not affect the clearance of *B. burgdorferi* from affected tissues. However, additional investigation of spirochete viability by other methods, such as culture of tissue, is required to affirm these findings.

In summary, we showed that the depletion of Treg cells after infection with *B. burgdorferi* results in significantly greater paw swelling, along with a greater capacity for IL-17 and IFN- γ production and decreased IL-10 production. Additionally, our findings indicate that cells from mice depleted of Treg cells display an increased potential for an inflammatory response following stimulation. We also found that Treg cells inhibit antibody production early after infection but increase antibody production at later times. However, we also found that Treg cell depletion had no effect on the *B. burgdorferi* levels in the joint tissue or the development of pathology 7 days later. These findings add to accumulating knowledge regarding the immunomodulatory function of Treg cells in *B. burgdorferi* infection. They also provide evidence of inhibitory and, possibly, effector function of Treg cells. More importantly, our findings suggest that Treg cells

display an anti-inflammatory effect and exert an initial control of inflammation during the development of Lyme arthritis. Additional research is required to determine the effects of Treg cells on the resolution of arthritis. Our findings provide additional support for the role of Treg cells in *B. burgdorferi*-induced arthritis, and provide a greater understanding of how a dysregulated immune response contributes to Lyme arthritis. Our findings suggest that Treg cells inhibit the early production of antibodies, which may affect vaccine development or the interpretation of serological diagnostic tests. Importantly, our findings may also provide new targets for the treatments of antibiotic-refractory Lyme arthritis, thereby reducing unnecessary antibiotic use.

Figure 1.

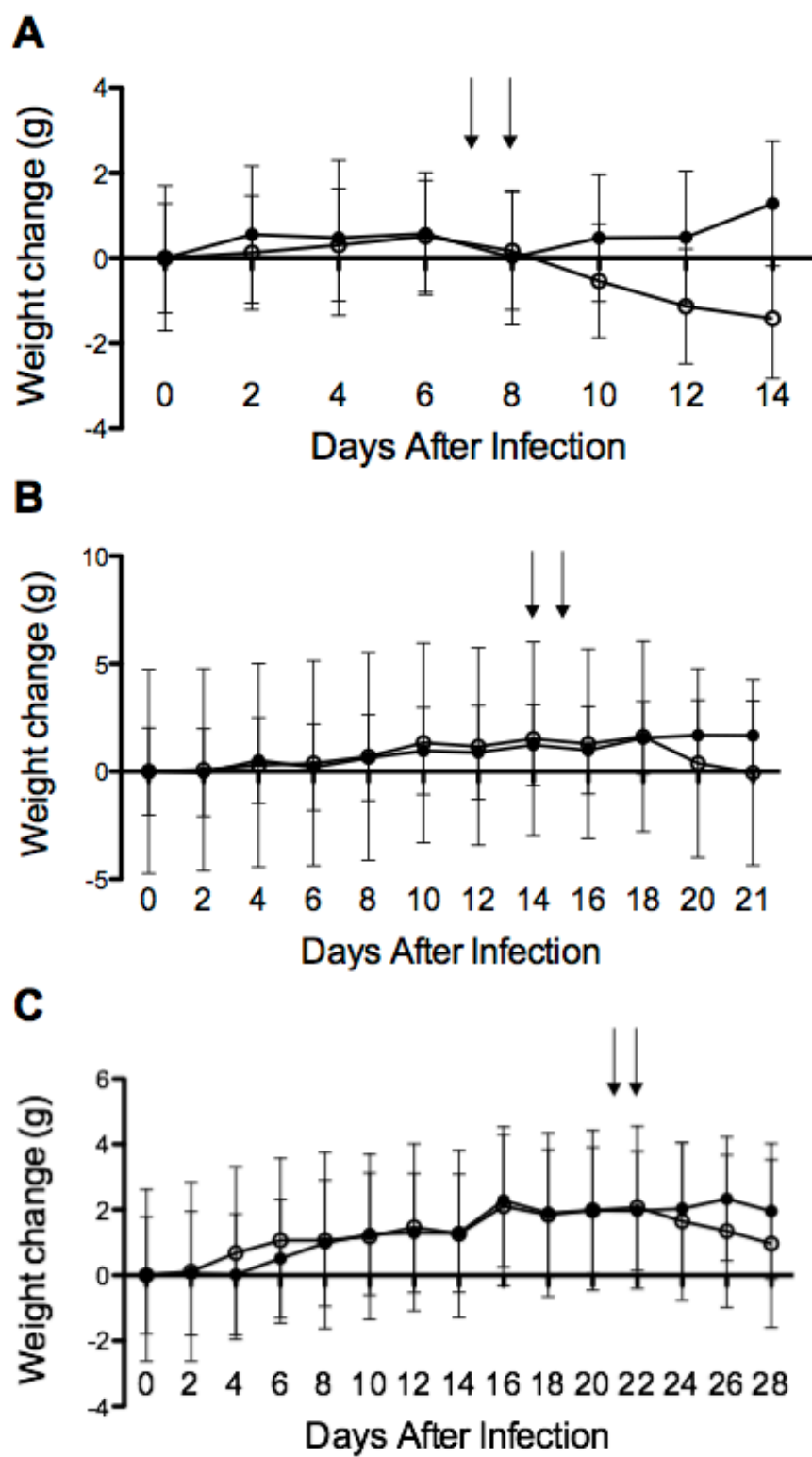


Figure 1. Diphtheria toxin (DTx) induces weight loss. Mice were infected with 2×10^4 viable *B. burgdorferi* strain B31-A3 on day 0. Additionally, mice were injected for 2 consecutive days beginning at day 7 (A), day 14 (B), or day 21 (C) with PBS (closed circles) or 1 μ g DTx (open circles). Weight loss occurred in mice administered DTx, but not in mice injected with PBS. Mice that were administered DTx, but were not infected, also displayed weight loss (data not shown). Arrows indicate days of DTx administration. Data are mean weights \pm SEM. * $P < 0.05$.

Figure 2.

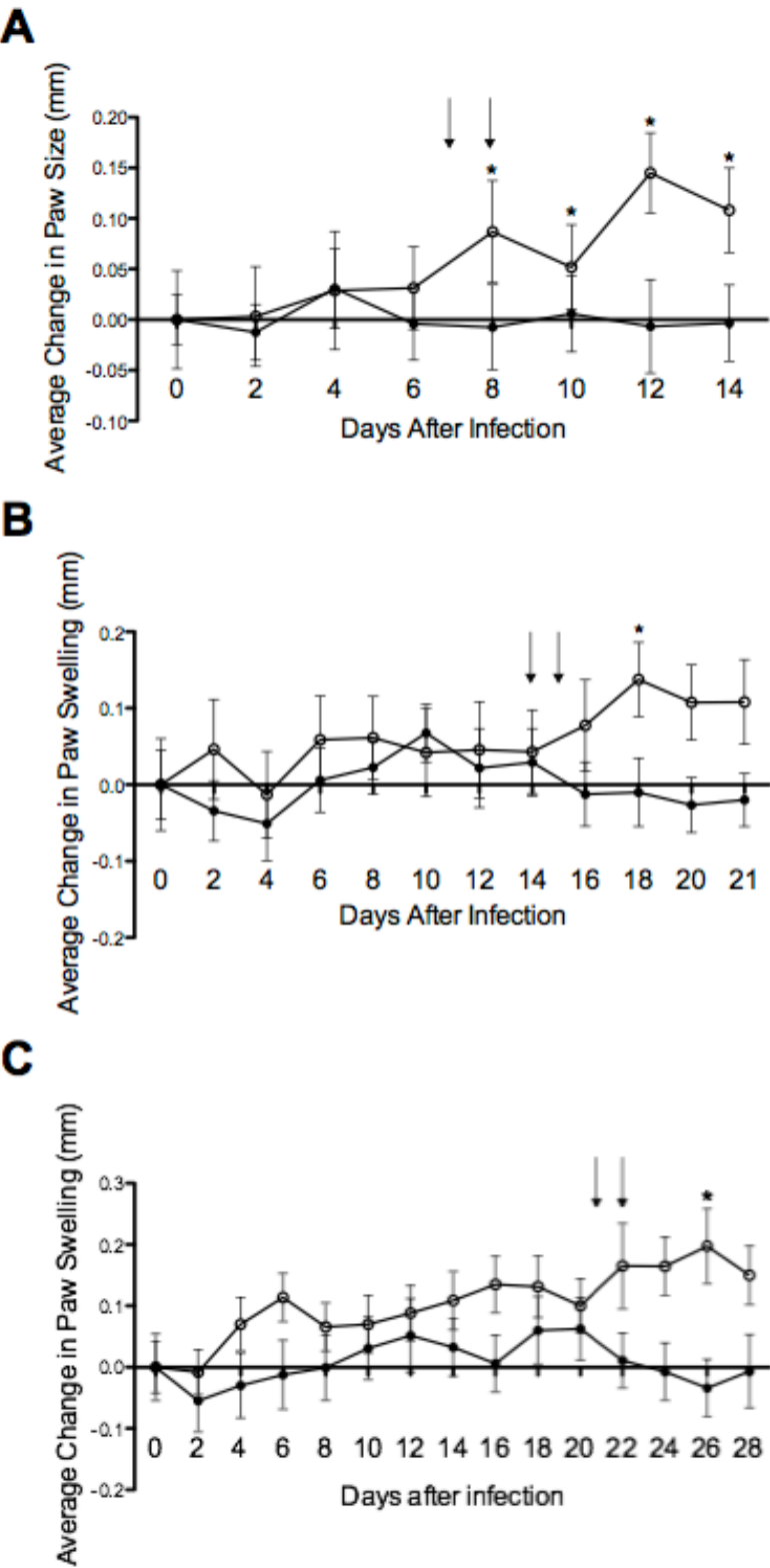


Figure 2. Administration of diphtheria toxin significantly increased hind paw swelling in *B. burgdorferi*-infected DERE mice. DERE mice were administered PBS (black circles) or DTx (white circles) two consecutive days beginning at day 7 (A), 14 (B), or 21 (C) after infection with *B. burgdorferi*. DTx administration caused a significant increase in hind paw swelling, regardless of time of administration. No swelling of hind paws occurred in uninfected DERE mice administered DTx (not shown). Arrows indicate days of DTx administration. Data are mean changes in paw swelling relative to baseline \pm SEM. * $P < 0.05$.

Figure 3.

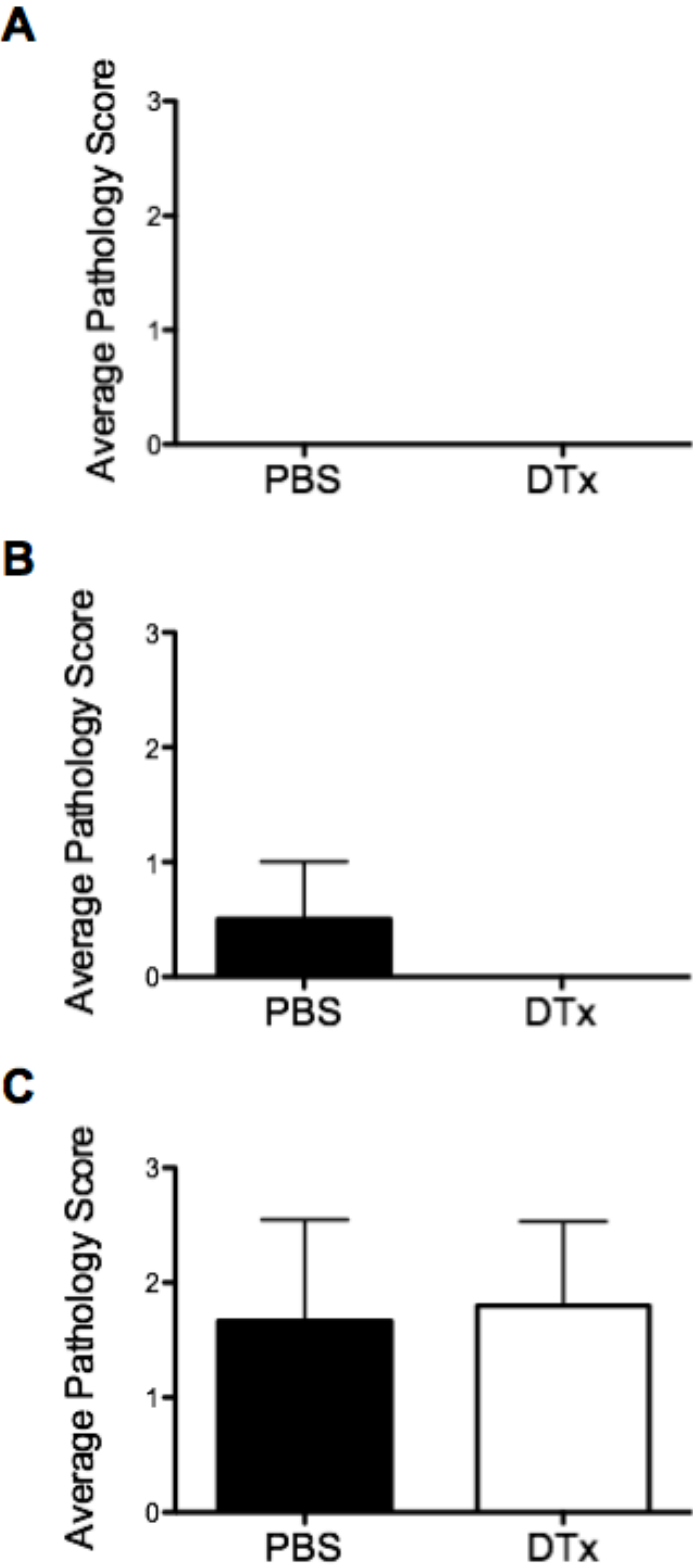


Figure 3. Administration of diphtheria toxin does not affect joint pathology in *B. burgdorferi*-infected DERE mice after 7 days. Histopathology in the tibiotarsal joints of *B. burgdorferi*-infected DERE mice injected with PBS or administered DTx on two consecutive days after infection beginning at day 7 (A), day 14 (B), or day 21 (C). Pathology was assessed as described in *Materials and Methods*. Error bars indicate SEM.

Figure 4.

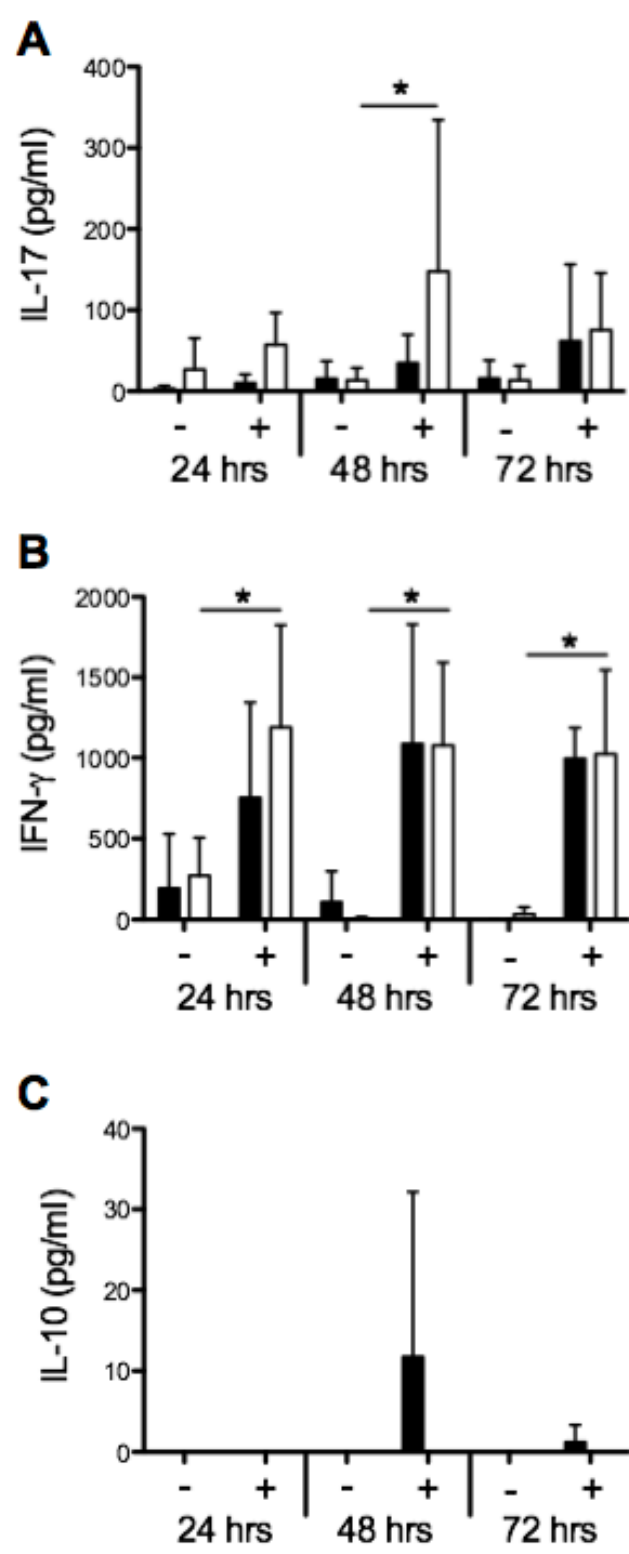


Figure 4. Effects of Treg cell depletion 7 days after *B. burgdorferi* infection on cytokine production 14 days after infection. Spleen cells obtained from DERE mice administered PBS (black bars) and DTx (white bars) 14 days after infection with *B. burgdorferi* were cultured in the presence (+) or absence (-) of the spirochete. Levels of IL-17 (A), IFN- γ (B), and IL-10 (C) were measured after 24, 48, and 72 hours of incubation. Data are mean levels of the cytokines/ml culture supernatant \pm SEM. * $P < 0.05$.

Figure 5.

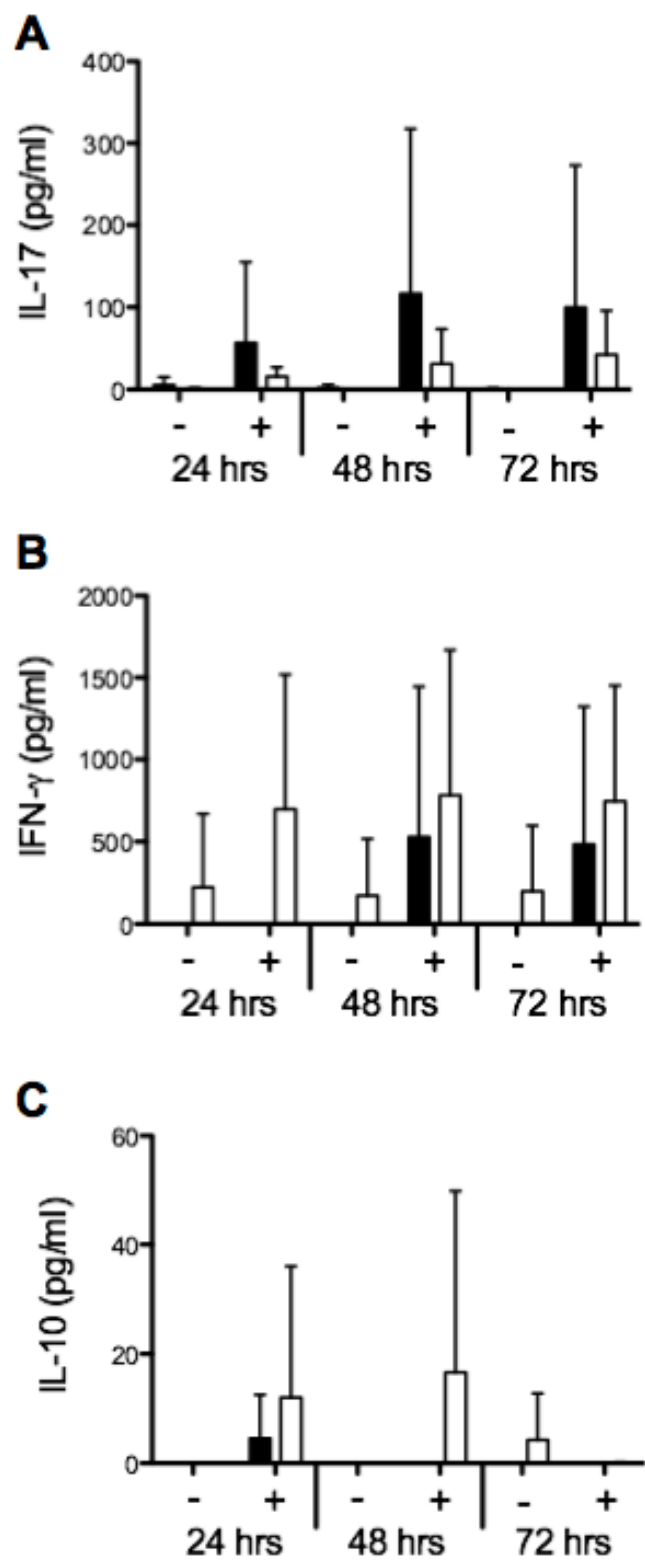


Figure 5. Effects of Treg cell depletion 14 days after *B. burgdorferi* infection on cytokine production 21 days after infection. Spleen cells obtained from DERE mice administered PBS (black bars) and DTx (white bars) 21 days after infection with *B. burgdorferi* were cultured in the presence (+) or absence (-) of the spirochete. Levels of IL-17 (A), IFN- γ (B), and IL-10 (C) were measured after 24, 48, and 72 hours of incubation. Data are mean levels of the cytokines/ml culture supernatant \pm SEM. No significant differences in cytokine production were detected.

Figure 6.

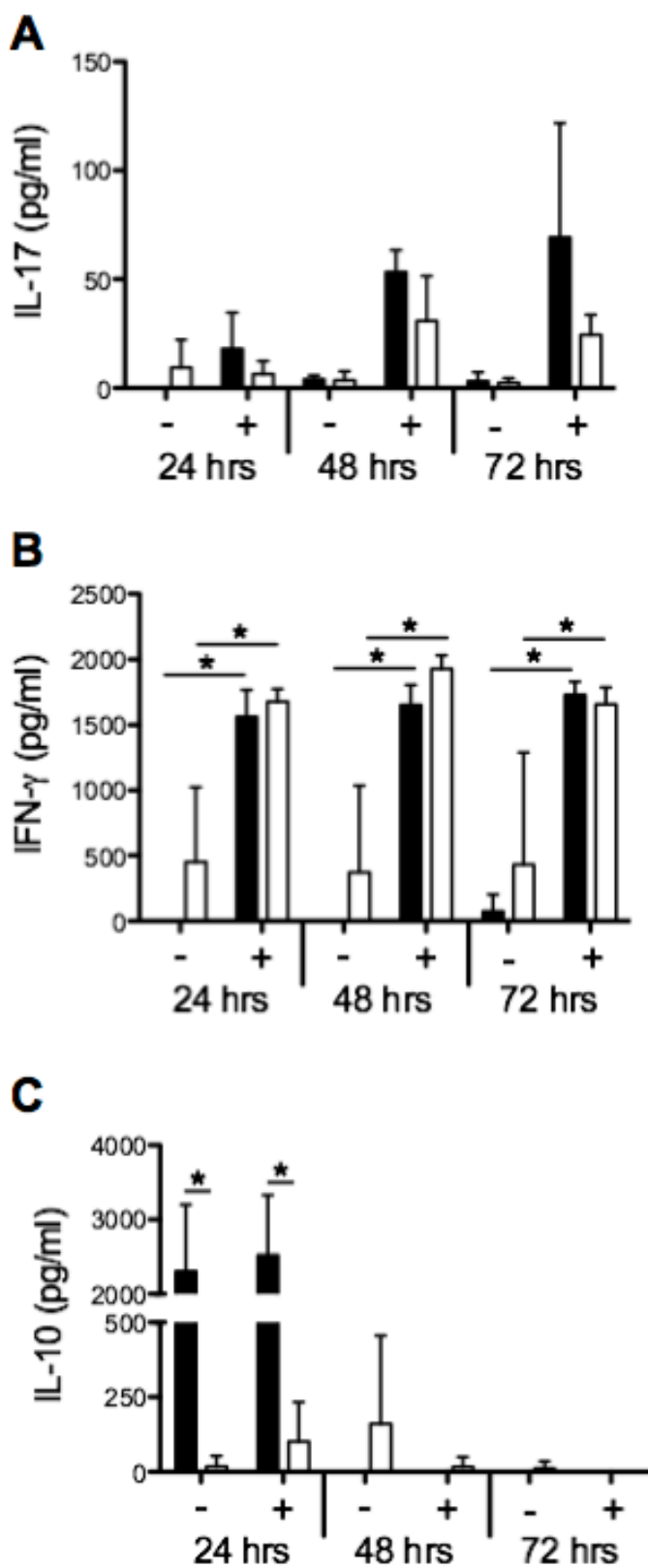
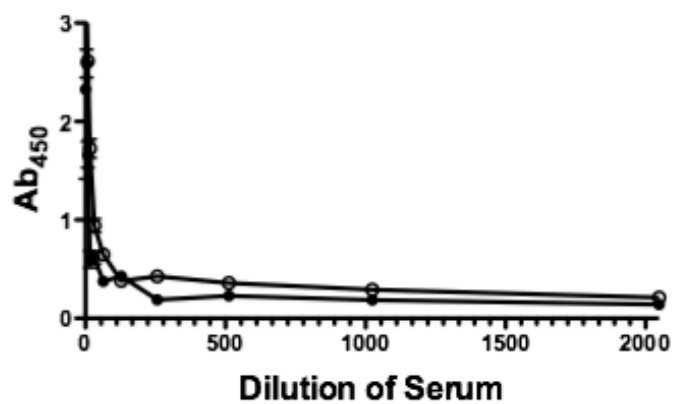


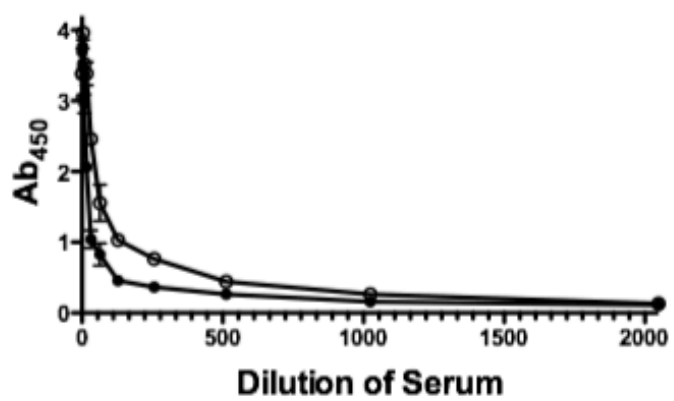
Figure 6. Effects of Treg cell depletion 21 days after *B. burgdorferi* infection on cytokine production 28 days after infection. Spleen cells obtained from DERE mice administered PBS (black bars) and DTx (white bars) 28 days after infection with *B. burgdorferi* were cultured in the presence (+) or absence (-) of the spirochete. Levels of IL-17 (A), IFN- γ (B), and IL-10 (C) were measured after 24, 48, and 72 hours of incubation. Data are mean levels of the cytokines/ml culture supernatant \pm SEM. * $P < 0.05$.

Figure 7.

A



B



C

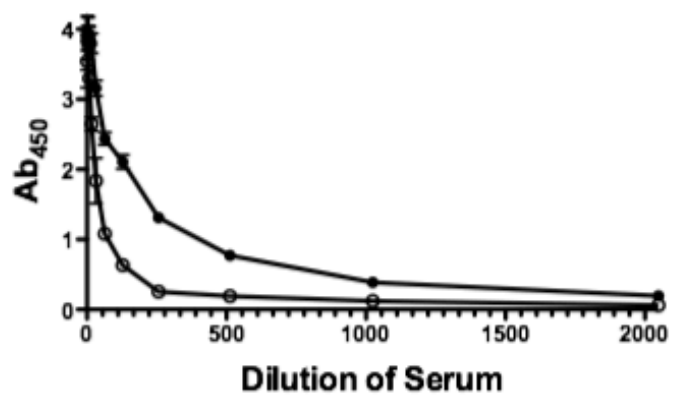
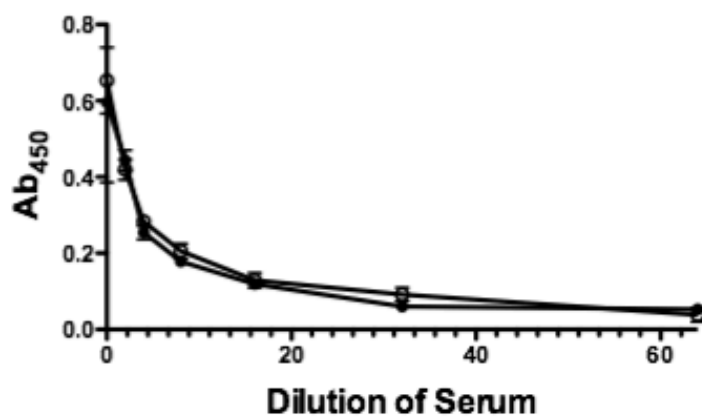


Figure 7. Effect of Treg cell depletion on IgM antibody production in *B.*

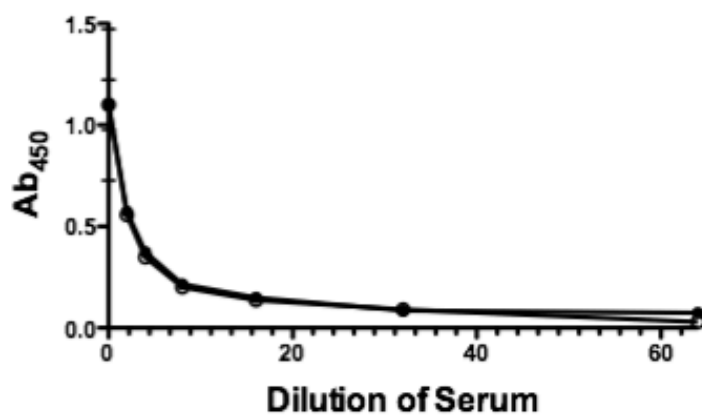
***burgdorferi*-infected mice.** Pooled sera collected from DEREg mice administered PBS (closed circles) or DTx (open circles) on 2 consecutive days prior to *B. burgdorferi* infection beginning at day 7 (A), 14 (B), or 21 (C) were assessed for IgM antibody production. Values are expressed as the absorbance at 450 nm (Ab_{450}). Error bars indicate SEM.

Figure 8.

A



B



C

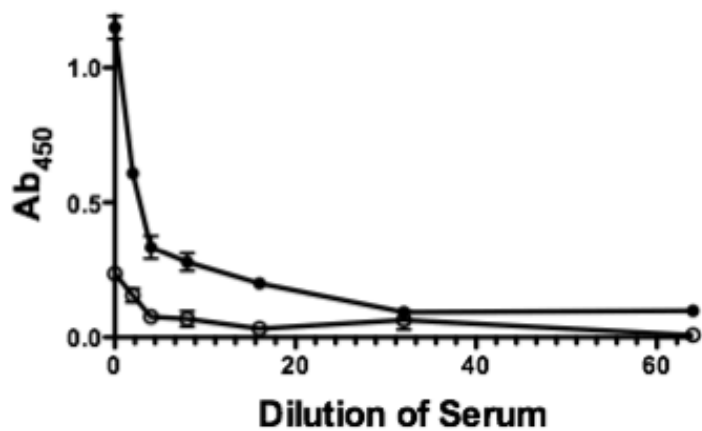
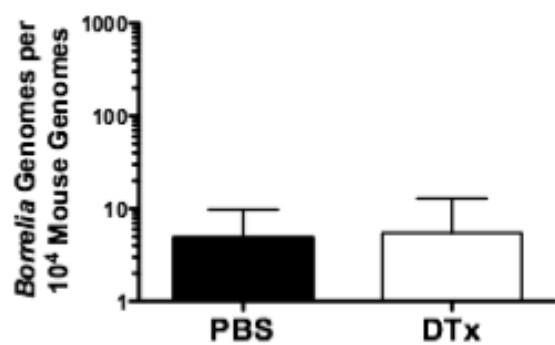


Figure 8. Effect of Treg cell depletion on IgG antibody production in *B.*

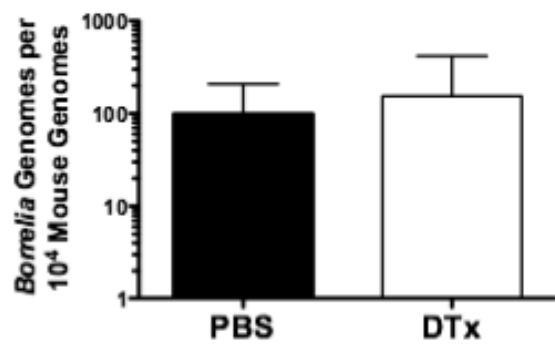
***burgdorferi*-infected mice.** Pooled sera collected from DREG mice administered PBS (closed circles) or DTx (open circles) on 2 consecutive days prior to *B. burgdorferi* infection beginning at day 7 (A), 14 (B), or 21 (C) were assessed for IgG antibody production. Values are expressed as the absorbance at 450 nm (Ab_{450}). Error bars indicate SEM.

Figure 9.

A



B



C

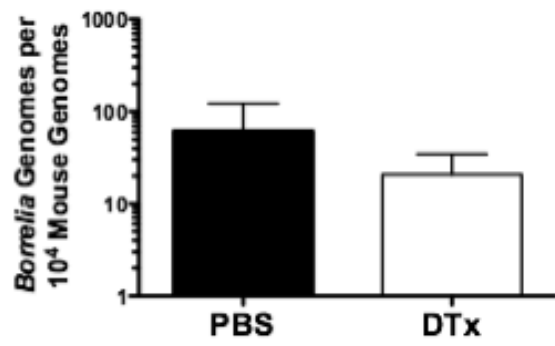


Figure 9. Effect of Treg cell depletion on bacterial burden in the tibiotarsal joint in *B. burgdorferi*-infected mice. The number of bacterial genomes was determined in the tibiotarsal joints of DREG mice administered PBS (black bars) or DTx (white bars) for 2 consecutive days beginning at day 7 (A), 14 (B), or 21 (C) after infection with *B. burgdorferi*. Data are mean number of *B. burgdorferi* genomes per 10^4 mouse genomes \pm SEM. No significant differences in bacterial burden between groups were detected.

References

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CHAPTER 6: SUMMARY AND CONCLUSIONS

The purpose of this research was to identify novel regulatory mechanisms of the inflammatory immune response upon infection with the causative agent of Lyme disease, *Borrelia burgdorferi*. Our findings may assist in the development of new therapeutic strategies for patients with Lyme arthritis. We hypothesized that a dysregulated immune response results in an excessive inflammatory response and the development of arthritis following *B. burgdorferi* infection. To this end, we tested the following hypotheses:

1. IL-10 modulates the inflammatory immune response in Lyme arthritis through inhibition of IL-17.
2. IL-10 reduces CD4⁺ cell-derived IL-17 production in Lyme arthritis.
3. Treg cells prevent the onset of arthritis following infection with *B. burgdorferi*.
4. Treg cells present following *B. burgdorferi* infection reduce arthritis severity.

C57BL/6 mice are genetically resistant to the development of arthritis following infection with *B. burgdorferi*, which is attributed, in part, to increased levels of IL-10 (Brown *et al.* 1999, Ganapamo *et al.* 2000). IL-10 is an anti-inflammatory cytokine with a variety of suppressive effects. Here, we examined a possible mechanism by which IL-10 modulates arthritis in *B. burgdorferi* infection. In accordance with previous studies, we observed that infection of IL-10-deficient mice results in moderate swelling of the hind paws and greater histopathological changes of the ankle joint. We also observed that cells from *B. burgdorferi*-infected wild-type mice produce less IL-17 early after infection

than cells from uninfected mice, which suggests that the bacteria induce anti-inflammatory mechanisms in initial infection. We further showed that spleen cells obtained from IL-10-deficient C57BL/6 mice produce more IL-17 at the time of arthritic manifestations, indicating that IL-10 inhibits IL-17 production during *B. burgdorferi* infection. We further showed that the significant hind paw swelling and histopathological changes observed in *B. burgdorferi*-infected, IL-10-deficient C57BL/6 mice were partially inhibited by administration of anti-IL-17 antibody. Our observations add to a growing body of data showing that IL-17 contributes to Lyme arthritis. Collectively, these findings support our first hypothesis, that IL-10 regulates inflammation in Lyme arthritis through inhibition of the IL-17 response.

We also examined the ability of IL-10 to regulate IL-17 production in a model of dysregulated *Borrelia*-induced arthritis. Lyme arthritis is thought to result, in part, from a dysregulated immune response (Nardelli *et al.* 2004, Steere & Glickstein 2004, Nardelli *et al.* 2008). Here, we combined the use of a model of dysregulation due to lack of IL-10 (Sondergger *et al.* 2012) and a model of T cell-driven arthritis that incorporates both innate and adaptive immune events that contribute to pathology (Burchill *et al.* 2003, Christopherson *et al.* 2003). We found that *Borrelia*-priming and infection resulted in decreased IL-17 production by spleen cells from mice infected for one day. Our findings are consistent with our previous findings that indicate *B. burgdorferi* induces downregulation of the immune response in early infection. After 8 days of infection, spleen cells from primed and infected mice deficient in IL-10 produced significantly greater levels of IL-17 than cells from primed and infected wild-type mice.

Moreover, we demonstrated that addition of anti-CD4-antibodies to spleen cell cultures significantly decreased IL-17 production, which indicates that CD4⁺ cells are a source of IL-17 in *B. burgdorferi*-induced arthritis. Additionally, mice deficient in IL-10 developed severe hind paw swelling, which was reduced by administration of IL-17 antibodies. Our findings support our second hypothesis, that IL-10 reduces CD4⁺ cell-derived IL-17 production in Lyme arthritis. These results suggest that *Borrelia*-primed, IL-17-producing CD4⁺ cells may contribute to the histopathologic effects of a dysregulated response to the spirochete. Coupled with our previous results, our findings provide further evidence that Th17-related pathology contributes to Lyme arthritis and that IL-10 regulates these events.

CD4⁺CD25⁺Foxp3⁺ regulatory T (Treg) cells are important mediators of the inflammatory response, in part, through production of IL-10 (Rubtsov *et al.* 2008). Therefore, we further investigated the effects of immune dysregulation by directly addressing the role of Foxp3⁺ Treg cells in the prevention of arthritis. Here, we utilized the DEREK mouse model, which allows for the selective depletion of Foxp3⁺ cells following administration of diphtheria toxin (Lahl & Sparwasser 2011). We found that depletion of Treg cells existing prior to infection results in significantly greater paw swelling and development of arthritis. We also demonstrated that Treg cell depletion resulted in an increased *B. burgdorferi*-specific antibody response, suggesting that Treg cells may inhibit the development of a protective immune response to *B. burgdorferi*. Our findings support our third hypothesis, that Treg cells prevent the onset of arthritis following infection with *B. burgdorferi*. More importantly, our findings suggest that

individuals with decreased numbers of Treg cells may be more likely to develop arthritis after infection with *B. burgdorferi*.

Finally, we investigated the role of Treg cells during the course of *B. burgdorferi* infection. We found that Treg cell depletion at 7, 14, or 21 days after infection caused an increase in paw swelling at days 14, 21, or 28 after infection, respectively. Treg cell depletion, however, did not cause pathology 7 days after depletion. The potential for the development of arthritis requires further investigation. We also found an increased potential to make IL-17 and IFN- γ , along with a significant decrease in IL-10 production. We also provided additional support that Treg cells inhibit antibody production in early disease; however, Treg cells may enhance antibody production at later times. These findings are important for understanding how regulation of the immune response during *B. burgdorferi* infection prevents joint swelling. Additionally, our findings suggest that Treg cells may also possess effector functions under certain conditions. These findings partially support our fourth hypothesis by demonstrating that Treg cells inhibit paw swelling following infection with *B. burgdorferi*. Together with our previous findings, this suggests that Treg cells possess an anti-inflammatory effect in initial *B. burgdorferi* infection to prevent arthritis, but may not contribute to the resolution of disease.

In summary, this work demonstrates that intact, functioning, immunoregulatory mechanisms are vital to control the immune response to *B. burgdorferi* and prevent the development of Lyme arthritis. We provided additional evidence for the role of IL-17 in Lyme arthritis and identified IL-10 as a regulator of its inflammatory effects. We also

identified CD4⁺ T cells as a source of IL-17 in Lyme arthritis, suggesting that Th17 cells contribute to disease. We then demonstrated that Treg cells prevent the onset of arthritis in *B. burgdorferi* infection. We further determined that Treg cells inhibit paw swelling and inflammatory cytokine production during *B. burgdorferi* infection.

In conclusion, our findings collectively suggest that a dysregulated immune response results in an excessive inflammatory response and the development of arthritis following *B. burgdorferi* infection (Figure 1). Additional research is needed to fully elucidate the regulatory mechanisms that inhibit the development and promote resolution of Lyme arthritis. Understanding the immune events that contribute to Lyme arthritis would reduce unnecessary antibiotic use and identify new strategies for treatment. The effects of Treg cells on the humoral response to *B. burgdorferi* should be considered in the context of new treatments for Lyme borreliosis, the development of new vaccines, and the use of serological diagnostic testing. Our findings suggest that individuals with decreased numbers of Treg cells may be more likely to develop arthritis after infection with *B. burgdorferi*. Finally, our findings are important for understanding the immune mechanisms involved in the immune response to *B. burgdorferi*, which has significant implications for the development of strategies to prevent or treat Lyme borreliosis.

Figure 1.

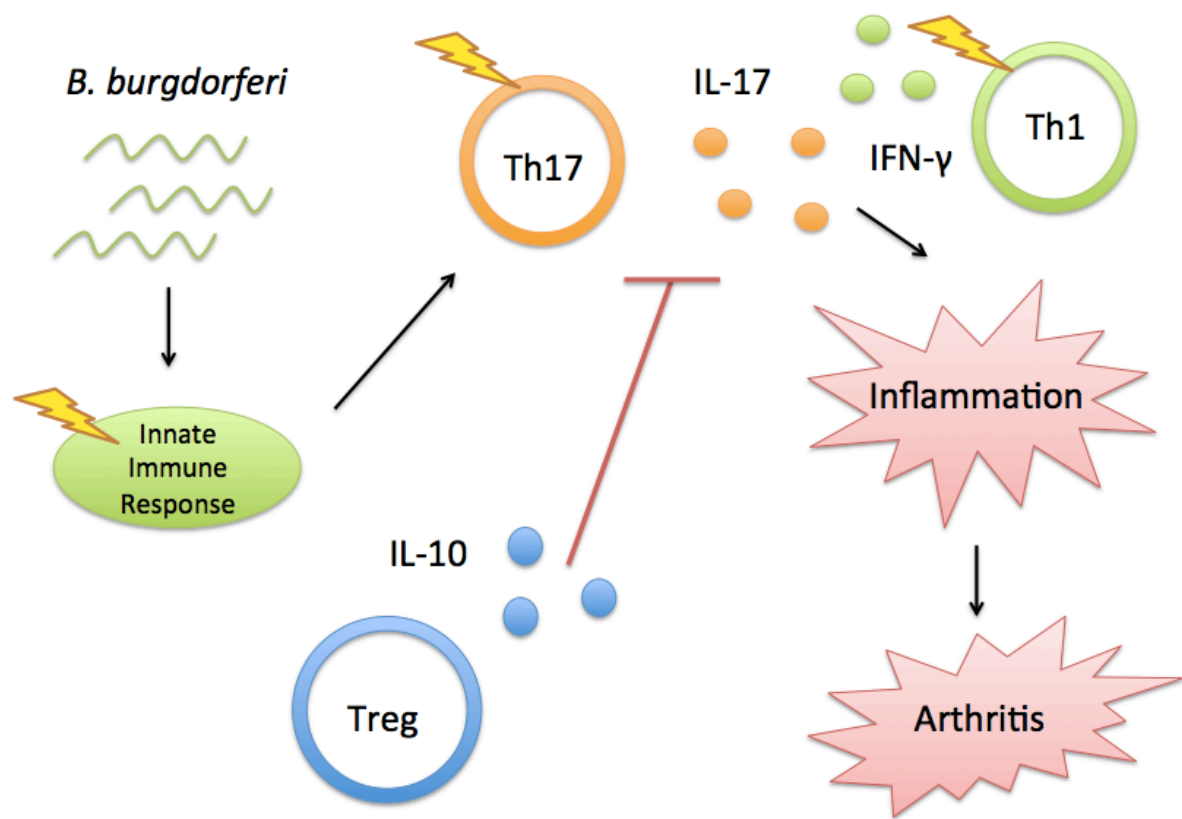


Figure 1. Proposed regulatory mechanisms in *B. burgdorferi*-induced arthritis.

Infection with *B. burgdorferi* results in activation of Th1 and Th17 cells, which contribute to inflammation and the development of Lyme arthritis. We propose that Treg cells, through production of IL-10, limit the inflammatory potential of Th1 and Th17 cells to control inflammation and prevent the development of Lyme arthritis.

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CURRICULUM VITAE

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EDUCATION

- 2012-2016 **PhD, Health Sciences**
University of Wisconsin – Milwaukee, Milwaukee, WI
Supervisor: Dean Nardelli, PhD
Dissertation: Regulatory Mechanisms in *B. burgdorferi*-Induced Arthritis
- 2009-2012 **MS, Biomedical Sciences**
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Thesis: Role of Interleukin-17 in the Adaptive Immune Response in Lyme Arthritis
- 2005-2009 **BS, Medical Microbiology and Immunology**
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RESEARCH EXPERIENCE

- 2011-Present **Research Technologist**
Medical College of Wisconsin, Milwaukee, WI
Supervisor: Cecilia Hillard, PhD
Manage multiple aspects of animal research including colony management and genotyping, database administration, rodent behavioral experiments, surgeries, and flow cytometry.
- 2014-2015 **Laboratory Assistant**
University of Wisconsin – Milwaukee, Milwaukee, WI
Supervisors: Elizabeth Liedhegner, PhD and Janis Eells, PhD
Manage animal research colony, assist with experiment planning, and conduct rodent experiments and tissue harvesting.
- 2009-Present **Graduate Student**
University of Wisconsin – Milwaukee, Milwaukee, WI
Supervisor: Dean Nardelli, PhD
Research conducted in Biomedical Sciences department related to the role of T helper 17 and regulatory T cells in *Borrelia burgdorferi*-induced arthritis.

2007-2009 **Undergraduate Research Assistant**
University of Wisconsin – Madison, Madison, WI
Supervisor: Ajit Verma, PhD
Maintained animal research colony, conducted rodent research experiments, and managed lab budget.

TEACHING EXPERIENCE

2009-2015 **Teaching Assistant**
University of Wisconsin – Milwaukee, Milwaukee, WI
Assisted with a variety of undergraduate courses, prepared materials for weekly labs, attended and assisted students in lab, monitored lecture attendance, proctored exams, and graded exams and lab reports.

2012-2015 **Instructor**
American Indian Science Scholar Program, Milwaukee, WI
Developed and taught laboratory exercises and participated in the planning and organizing of a 10 day summer science camp for American Indian high school students from across the country

2010 **Instructor**
High School Science Scholar Program, Milwaukee, WI
Developed and taught laboratory exercises during the weeklong summer program.

INTERNSHIPS

Fall 2008 **Student Intern**
Wisconsin Department of Health Services – Bureau of Communicable Diseases, Madison, WI
Supervisor: Lorna Will
Organized and monitored data for tuberculosis patients in Wisconsin. Completed paperwork for appropriate prescriptions, and conducted informational interviews.

STUDENT ORGANIZATIONS

2013- 2015 **Member**
Officer: Vice President 2013-2015
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2006-2009 **Member**
Officer: Social Events Coordinator 2009
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- | | |
|-----------|--|
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Hospice Helping Hands |
| 2005-2007 | Member
Students Improving Urban Healthcare |

PUBLICATIONS

Hansen ES, Johnson ME, Schell RF, Nardelli DT. 2016. CD4+ Cell-Derived Interleukin-17 in a Model of Dysregulated, *Borrelia*-Induced Arthritis. *Pathog Dis* 74(7):pii: ftw084.

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POSTERS AND PRESENTATIONS

- | | |
|---------------|---|
| January 2015 | Gordon Research Conference: The Biology of Spirochetes
<i>Ventura Beach Marriott, Ventura, CA</i>
Poster: Initial Characterization of DEREK Mice as a Potential Model of Lyme Borreliosis
Poster: Role of Endocannabinoid Receptor Type 2 (CB2R) in Lyme Arthritis |
| December 2015 | College of Health Sciences Research Symposium
<i>University of Wisconsin – Milwaukee, Milwaukee, WI</i>
Poster: Role of Endocannabinoid Receptor Type 2 (CB2R) in Lyme Arthritis
Presentation: Role of Endocannabinoid Receptor Type 2 (CB2R) in Lyme Arthritis (First Place Award) |
| January 2014 | Gordon Research Conference: The Biology of Spirochetes
<i>Ventura Beach Marriott, Ventura, CA</i> |

Poster: Regulation of Interleukin (IL)-17 Production and IL-17-Driven Inflammation by IL-10 in a Murine Model of *Borrelia*-Induced Arthritis

- December 2013 **College of Health Sciences Research Symposium**
University of Wisconsin – Milwaukee, Milwaukee, WI
Poster: IL-10 Inhibits IL-17 production and Lyme Arthritis Following *Borrelia burgdorferi* Infection
Presentation: IL-10 Inhibits IL-17 production and Lyme Arthritis Following *Borrelia burgdorferi* Infection
- September 2012 **Midwest Microbial Pathogenesis Conferences**
Medical College of Wisconsin, Milwaukee, WI
Poster: Interleukin-17 production by adaptive immune cells at the peak of *Borrelia burgdorferi*-induced inflammation
- May 2012 **College of Health Sciences Research Symposium**
University of Wisconsin – Milwaukee, Milwaukee, WI
Poster: Control of Interleukin-17-Associated Lyme Arthritis by Events of Adaptive Immunity
Presentation: Control of Interleukin-17-Associated Lyme Arthritis by Events of Adaptive Immunity

AWARDS

- Fall 2015 **Distinguished Dissertator Fellowship**
University of Wisconsin – Milwaukee, Milwaukee, WI
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Spring 2014
Fall 2013
Fall 2012 **Chancellor's Graduate Student Award**
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- Fall 2013 **Graduate Student Travel Award**
University of Wisconsin – Milwaukee, Milwaukee, WI
- Fall 2012 **Student Research Grant Award**
University of Wisconsin – Milwaukee, Milwaukee, WI

Summer 2011 **Chancellor's Graduate Student Award**
University of Wisconsin – Milwaukee, Milwaukee, WI
Clinical Laboratory Sciences

2005-2009 **Bright Futures Scholarship**
Kimberly-Clark, Irving, TX

UNIVERSITY SERVICE

2011- 2013 **Search and Screen Committee**
University of Wisconsin – Milwaukee, Milwaukee, WI
Student representative on the department committee responsible for reviewing, selecting and interview candidates for Lab Manager II position in department of Biomedical Sciences

COMMUNITY SERVICE

February 2015 **Nicolet High School Science and Engineering Fair Judge**
February 2014 *Nicolet High School, Glendale, WI*