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EFFECTS OF 670 NM AND 830 NM LIGHT ON THE IMMUNE

RESPONSE TO *BORRELIA BURGDORFERI*

by

Felice Chen

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ABSTRACT
EFFECTS OF 670 NM AND 830 NM LIGHT ON THE IMMUNE
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by

Felice Chen

The University of Wisconsin-Milwaukee, 2015
Under the Supervision of Dean T. Nardelli, Ph.D. and Janis T. Eells, Ph.D.

Lyme arthritis is a debilitating joint disorder that arises from Lyme disease, which is a result of infection by the spirochete *Borrelia burgdorferi.* Antibiotics are the traditional treatment for Lyme disease, but chronic arthritis may persist in some patients despite antibiotic treatment. Therefore, other forms of treatment for Lyme arthritis are needed. Photobiomodulation (PBM) using red or near-infrared light, with wavelengths between 630 nm and 900 nm, has been found to be beneficial in alleviating inflammatory symptoms of many disorders. The purpose of this thesis was to determine whether photobiomodulation using either 670 nm or 830 nm light would help alleviate inflammation in a murine Lyme arthritis model. The central hypothesis for this study was that PBM, using 670 nm or 830 nm light, will decrease inflammation in Lyme arthritis. Two specific aims were pursued to test this hypothesis. The first aim was to determine the effect that 670 nm and 830 nm light had on paw swelling and pro-inflammatory cytokine levels in *B. burgdorferi*-infected

mice. The working hypothesis for this specific aim was that 670 nm and 830 nm light would ameliorate swelling and reduce interleukin-17 (IL-17) and interferon-gamma (IFN-γ) production in *B. burgdorferi*-infected mice. The second aim was to determine the effect of 670 nm or 830 nm light had on production of borreliacidal antibodies in infected mice. The working hypothesis for this specific aim was that neither 670 nm nor 830 nm light would affect borreliacidal antibody production in *B. burgdorferi*-infected mice.

Wild-type or interleukin-10-deficient C57BL/6 mice were infected with *B. burgdorferi* and treated with either 670 nm light or 830 nm light for six or seven consecutive days. Infected paws were then measured for changes in paw swelling, and IL-17 and IFN-γ production by spleen cells were determined. Sera were used to determine borreliacidal antibody titers. Our hypothesis was partially supported, in that PBM using 670 nm light was capable of significantly decreasing IFN-γ production. Surprisingly, 670 nm light also increased the borreliacidal antibody titer in a model of dysregulated disease. However, it was unable to decrease the paw swelling exhibited in murine Lyme arthritis. Our hypothesis was not supported by the use of 830 nm light. Treatment with 830 nm light did not significantly affect paw swelling, and it significantly increased IL-17 production and reduced borreliacidal antibody titers in the model of

dysregulated Lyme arthritis. Our results indicate that different wavelengths may possess varying effects on inflammation in Lyme arthritis.

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LIST OF ABBREVIATIONS

APC: Antigen-presenting cell

ATP: Adenosine triphosphate

BSK: Barbour-Stoenner-Kelley

CcO: Cytochrome c oxidase

CDC: Centers for Disease Control and Prevention

COX-2: Cyclooxygenase-2

DbpA: Decorin-binding protein A

DC: Dendritic cell

EM: Erythema migrans

FR: Far-red

GM-CSF: Granulocyte macrophage colony-stimulating factor

HLA: Human leukocyte antigen

IL-6: Interleukin-6

IL-10: Interkeukin-10

IL-17: Interleukin-17

IFN-γ: Interferon-gamma

iNOS: Inducible nitric oxide synthase

KO: Knock-out

LED: Light-emitting diode

NapA: Neutrophil-activating protein A

NF-κB: Nuclear factor-kappa B

NIR: Near-Infrared

NK: Natural killer T cell

x

NO: Nitric oxide

Osp: Outer surface protein

PBM: Photobiomodulation

ROS: Reactive oxygen species

TGF-β: Transforming growth factor-beta

Th1: T helper type 1 cell

Th17: T helper type 17 cell

TLR: Toll-like receptor

Treg: Regulatory T cell

VlsE: Variable surface antigen

WT: Wild-type

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CHAPTER 1: INTRODUCTION

I. Lyme Disease

1. Introduction

Lyme disease is a tick-borne disorder caused by infection with the spirochete *Borrelia burgdorferi* after bites from infected ticks of the *Ixodes* genus (Bhate and Schwartz, 2010)*.* The disease is endemic to the Northeast, upper Midwest, and far West regions of the United States, as well as to parts of Europe (Hu, 2005; Nardelli et al., 2008a). The deer tick *I. scapularis* serves as a primary vector for *B. burgdorferi* (Steere et al., 2004), and reservoirs for *Ixodes* ticks vary from region to region. A common reservoir for *I. scapularis* is the white-footed mouse (Bhate and Schwartz, 2010), although other small mammals and the white-tailed deer also serve as reservoirs for the species (Magnarelli et al., 1991). Transmission is highest during the late spring to early summer months due to the high feeding rate exhibited by ticks at the nymph stage of development (Steere et al., 2004). A recent report by the Centers for Disease Control and Prevention indicated that there are about 300,000 cases of Lyme disease per year in the U.S., which is ten times higher than has been reported in the past (Centers for Disease Control and Prevention, 2013a).

B. burgdorferi was first isolated from *Ixodes* ticks in 1982 (Burgdorfer et al., 1982). It is a Gram-negative, microaerophilic, spiral-shaped organism

that averages about 4 to 30 µm in length. *B. burgdorferi* possesses various lipoproteins that aid in its transmission by the tick vector and promote infection in the human host. Outer surface protein (Osp) A binds to a tick receptor for Osp A, TROPSA, in the tick midgut (Pal et al., 2004), facilitating its anchorage within the tick between blood meals. During feeding, expression of OspA is downregulated, allowing for movement of *B. burgdorferi* into the host. At the same time, OspC is upregulated. This protein, which is required for infection, binds to tick salivary protein Salp15, which increases infectivity in the host (Ramamoorthi et al., 2005). Expression of other proteins, such as variable surface antigen (VlsE) and decorin-binding protein (Dbp) A (Guo et al., 1998; Indest et al., 2001; Bhate and Schwartz, 2010), facilitate spirochete transmission and dissemination within the host. VlsE was found to be involved in promoting antigenic variation for the spirochete through random recombination at the *vls* locus (McDowell et al., 2002), while DbpA binds to collagen-rich sites in the host and aid spirochete attachment (Guo et al., 1998). While in the host, *B. burgdorferi* induces inflammatory responses that account for the various symptoms of Lyme disease.

The interaction of OspC with Salp15 illustrates the contribution of tick salivary products in aiding infection with *B. burgdorferi*. Salp15-bound *B. burgdorferi* were found to be resistant to antibody-mediated killing, indicating a protective function of tick saliva for the spirochete (Hovius et

al., 2008). Tick saliva is known to contain other proteins that facilitate spirochete transmission to humans. Immunosuppressive proteins such as B-cell inhibitory protein and sialostatin L have been found in tick saliva, as well (Hannier et al., 2004; Kotsyfakis et al., 2006; Horka et al., 2012), and appear to help reduce host immunological barriers during transmission.

2. Clinical Manifestations of Lyme Disease

Clinical manifestations of Lyme disease typically consist of three stages. In the first stage, erythema migrans (EM), a rash that is occasionally characterized by concentric circles surrounding a central clearing, can develop from the original site of the tick bite (Centers for Disease Control and Prevention, 2013b). EM is exhibited by about 50% of adults and 90% of children infected with *B. burgdorferi*, and it is considered a hallmark sign of Lyme disease in endemic areas (Bhate and Schwartz, 2010). In addition to EM rashes, typical symptoms during the first stage include pain in the joints, muscles, and bones, fever, malaise, and enlarged lymph nodes (Biesiada et al., 2012; Bhate and Schwartz, 2010).

Over the days and weeks following untreated infection, the spirochetes disseminate to various regions of the body, and may cause the development of multiple EM rashes, neck stiffness, headaches, asymmetrical joint inflammation, and, in rare cases, atrioventricular blockage and carditis (Hu, 2005; Bhate and Schwartz, 2010). An

asymptomatic period may occur after the second stage, followed by the third stage, which may occur months to years after initial infection. In the final stage of untreated infection, EM rashes are rare, and manifestations may include memory impairment and cardiac disorders. In addition, persistent joint inflammation, known as Lyme arthritis, may develop (Bhate and Schwartz, 2010).

3. Lyme Arthritis

Lyme arthritis is a chronic, debilitating complication of later-stage *B. burgdorferi* infection that results from persistent joint inflammation. Lyme arthritis is characterized by infiltration of the synovium by inflammatory cells and, in its most severe forms, pannus formation, gradual degradation of the cartilage, and bone erosion. These immune events cause recurrent pain and inflammation of the joints and may hinder normal movement. Lyme arthritis manifests in about 50% of untreated Lyme disease patients (Centers for Disease Control and Prevention, 2013b). Antibiotics, including ceftriaxone and doxycycline, are commonly used to cure infection and alleviate symptoms of Lyme arthritis, and have proven to be effective for most patients (Dattwyler et al., 1988; Steere et al., 1994). However, instances of chronic arthritic symptoms may exist even after antibiotic treatment in some genetically predisposed patients. This condition, commonly called antibiotic-refractory Lyme arthritis, occurs in about 10% of Lyme arthritis patients (Steere and Angelis, 2006; Wormser et al.,

2012). These symptoms can remain even after *B. burgdorferi* levels become undetectable (Carlson et al., 1999). In addition, Li et al. (2011) demonstrated that synovial tissues of patients experiencing relapses in arthritic symptoms, after an antibiotic course, exhibited negative polymerase chain reaction results for presence of *B. burgdorferi*. These studies indicate that infection with viable *B. burgdorferi* may not be the sole cause of arthritic inflammation in some patients.

4. Theories on Antibiotic-Refractory Lyme Arthritis

Currently, it is unclear why some patients develop antibiotic-refractory Lyme arthritis. Several explanations may account for this occurrence. One possible explanation is that antibiotic-refractory patients may be genetically predisposed to experiencing prolonged inflammatory symptoms after infection (Steere and Glickstein, 2004). A study conducted by Steere et al. (1990) demonstrated that patients who experienced antibiotic-refractory Lyme arthritis possessed a significantly greater frequency of either human leukocyte antigen (HLA)-DR4 or HLA-DR2 major histocompatibility complex alleles. DR4+ mice lacking CD28 and MHC II were also shown to exhibit a higher percentage of arthritis development and possess persistent paw inflammation even after antibiotic administration, as opposed to mice only deficient in CD28 (Iliopoulou et al., 2008). Lymph node cells of DR4 transgenic mice also produced significantly greater interferon-γ (IFN-γ) levels than those of

DR11 transgenic mice, which are resistant to antibiotic-refractory arthritis (Iliopoulou et al., 2009). Collectively, these studies support the hypothesis that presence of HLA-DR4 alleles may contribute to the genetic predisposition to antibiotic-refractory Lyme arthritis in some patients.

Another possible explanation for antibiotic-refractory Lyme arthritis involves possible dysregulation of the immune system (Steere and Glickstein, 2004). In this case, it is hypothesized that the host immune response is unable to control the pro-inflammatory responses induced by initial infection. Therefore, pro-inflammatory mediators are persistently elicited and may result in chronic joint inflammation (Steere and Glickstein, 2004).

Another hypothesis is that the occurrence of persistent inflammation in Lyme arthritis may be due to a theory known as the "amber hypothesis". This theory suggests that persistent, antibiotic-resistant arthritis may be the result of the presence of non-viable *B. burgdorferi* or its fragments retained in, or dislodged from, tissue within the joints (Wormser et al., 2012). The remnants of spirochetal antigens may trigger inflammatory responses by the host and result in the persistent bouts of inflammation in Lyme arthritis. Indeed, administration of borrelial antigens into the joint spaces of rats induced mild arthritis and resulted in the antigens being embedded in the synovial membrane of joints (Gondolf et al., 1994). In

addition, Petzke et al. (2009) showed that both viable and dead spirochetes induced similar inflammatory transcriptional profiles in the peripheral blood mononuclear cells (PBMCs) of humans. These findings suggest that viable, whole spirochetes may not be required to induce an immune response.

5. Mouse Models for Lyme Arthritis

Animal models, including rhesus monkeys, rabbits, dogs, and various rodents, have been used to study Lyme arthritis progression (Barthold et al., 2010). Mice are the most commonly used models for this disease. C3H mice are particularly susceptible to developing severe arthritis once infected with *B. burgdorferi* and are useful for analyzing the immune events following infection (Ma et al., 1998). BALB/c mice are typically arthritis-resistant, but may develop mild to severe arthritis when administered high doses of spirochete (Ma et al., 1998). C57BL/6 mice are arthritis-resistant, exhibiting mild inflammation after infection (Barthold et al., 1990). However, interleukin-10 (IL-10)-deficient mice in a C57BL/6 background have been shown to develop severe arthritis upon infection (Brown et al., 1999). The use of these, and other, animal models of Lyme arthritis are key to understanding the wide variety of immune responses that occur in humans.

6. Innate Response to B. burgdorferi-*Infection*

Toll-like receptors (TLRs) play a crucial role in the initial recognition of, and subsequent responses to, the presence of a pathogen. Several TLRs, such as TLR2, 5, 7, and 9, have been implicated in the initial responses to *B. burgdorferi*. In particular, expression of TLR2 was found to be associated with the response to OspA, which triggers pro-inflammatory responses via the NF-κB pathway (Hirschfeld et al., 1999). Hirschfeld et al. (1999) discovered that transfection of TLR2 into TLR2-negative U373 cell lines, which do not respond to OspA, led to responsiveness to the lipoprotein, and that presence of CD14, a monocyte differentiation marker, was important for this responsiveness. This finding supports evidence of a vital role for TLR2 in the initial recognition of OspA. In addition, the joints of *Borrelia*-infected TLR2-deficient mice have been shown to possess a greater spirochete load and exhibit more swelling than their wild-type counterparts (Wooten et al., 2002), suggesting that activation of TLR2 contributes significantly to pathogen clearance.

Myeloid differentiation antigen 88 (MyD88), an adaptor molecule for TLR signaling and activation of NF-κB, has been found to be important in the innate response to *B. burgdorferi*. Spirochete clearance in *B. burgdorferi*-infected, MyD88-deficient mice was less efficient than in their wild-type counterparts (Liu et al., 2004). Macrophages of MyD88-deficient mice also degraded *B. burgdorferi* at a slower rate and in an incomplete fashion

compared to those of wild type mice (Liu et al., 2004). These results demonstrate the role of both TLR2 and MyD88 in effective spirochete clearance. However, as disease due to *B. burgdorferi* infection may still occur in TLR2- or MyD88-deficient mice, other inflammatory pathways are triggered in response to infection.

TLR5, TLR7, and TLR9 were also demonstrated to be involved in the initial recognition of *B. burgdorferi* antigens. The silencing of TLR5 mRNA resulted in decreased gene expression of pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) in *B. burgdorferi*-stimulated macrophages (Shin et al., 2008), signifying that TLR5 may have a role in inflammation upon stimulation by *B. burgdorferi*. In addition, inhibition of TLR7 and TLR9 signaling led to a decrease in interferon-α production in human peripheral blood mononuclear cells (Petzke et al., 2009).

Various innate cells of the human host, such as macrophages, dendritic cells, natural killer (NK) cells, and neutrophils, are elicited upon infection with *B. burgdorferi*. Dendritic cells (DCs) and macrophages play a role in the activation of the adaptive immune response in Lyme arthritis. It was demonstrated, in an adoptive transfer study, that DCs exposed to *B. burgdorferi* are capable of generating a protective response in naïve BALB/c and C3H mice by stimulating the production of antibodies against

OspA (Mbow et al., 1997). Phagocytosis of *B. burgdorferi* by macrophages is also crucial in eliminating the presence of the spirochete during infection (Montgomery and Malawista, 1996). It was found by Ma et al. (1994) that OspA is capable of inducing the release of a variety of pro-inflammatory mediators, including interleukins-1 and- 12, as well as interferon-β, from bone marrow-derived macrophages of C3H mice. Macrophage stimulation by OspA also subsequently induced production of IFN-γ by NK cells and T cells in C3H mice (Ma et al., 1994). In addition, both OspA and OspB were found to elicit the production of nitric oxide (NO) from the macrophages of C3H mice (Ma et al., 1994), which is a crucial component in pathogen elimination by macrophages (Green et al., 1990). Both macrophages and DCs are important in eliciting T cell responses in the presence of *B. burgdorferi*, with macrophages capable of inducing both naïve and mature T cell proliferation after *B. burgdorferi* infection (Altenschmidt et al., 1996).

Brown and Reiner (1998) demonstrated that NK cells produced more IFN-γ in the lymph nodes of *B. burgdorferi*-infected, arthritis-susceptible, C3H/HeJ mice than those of arthritis-resistant DBA mice. This suggests that NK cell functions may contribute to the severity of inflammation in Lyme arthritis. In addition, the presence of neutrophils is also important in the control of *B. burgdorferi*. For example, a chemokine-induced presence of neutrophils led to a more effective control in the number of *B. burgdorferi* organisms present in the heart, skin, and joints of severe

combined immunodeficient (SCID) mice of a BALB/c background (Xu et al., 2007). In addition, it was found by Guo et al. (2009) that tick salivary proteins decreased neutrophil function by inhibition of integrins and reduction of superoxide production, which may explain the disappearance of neutrophil aggregates in EM rashes (Xu et al., 2007). Finally, Codolo et al. (2013) showed that neutrophils are recruited to the site of infection by the *B. burgdorferi* antigen neutrophil-activating protein A (NapA) and that these cells release T cell attractants to induce T cell-mediated immune responses. These studies demonstrate that neutrophils act as a crucial initial defense against the spirochete, while supporting additional inflammatory cells during infection.

7. Involvement of IFN-γ and IL-17 in Lyme Arthritis

Studies have demonstrated that T helper type 1 (Th1) cells and the cytokines they secrete, particularly IFN-γ, are involved throughout the course of inflammation in Lyme arthritis. Cloned T cells obtained from synovial fluid and peripheral blood mononuclear cells of patients with chronic Lyme arthritis displayed a Th1 cytokine pattern when stimulated with *B. burgdorferi* antigens *in vitro* (Yssel et al., 1991). Specifically, OspA was shown to stimulate an IFN-γ response (Ma et al., 1994). It was also found that cells of synovial fluid from Lyme arthritis patients produced a high amount of IFN-γ when stimulated by *B. burgdorferi* (Yin et al., 1997). In further support of the role of IFN-γ in Lyme arthritis, it was found that

lymph nodes of the arthritis-susceptible C3H mouse produced a significantly greater amount of the cytokine upon stimulation with *B. burgdorferi* antigens than those of arthritis-resistant BALB/c mice (Keane Myers and Nickell, 1995). Another study demonstrated that a greater severity of Lyme arthritis in patients is directly correlated with a greater ratio of Th1 to Th2 cells, and that these Th1 cells are specific for OspA antigens and localized in the inflamed joints (Gross et al., 1998). In addition, C3H mice, which are prone to arthritis after infection with *B. burgdorferi*, display a Th1 profile during infection, whereas BALB/c mice, which experience milder arthritis, exhibit a Th2 profile during infection (Matyniak and Reiner, 1995). Moreover, patients unresponsive to antibiotics produced higher levels IFN-γ than responsive patients (Shin et al., 2007). Finally, it was demonstrated by Codolo et al. (2013) that the T cells in synovial fluid of Lyme arthritis patients produced IFN-γ when stimulated by NapA. These studies collectively suggest that IFN-γ strongly contributes to ongoing inflammation of the joints in Lyme arthritis.

However, there are some indications that IFN-γ is not solely responsible for the induction of Lyme arthritis. Brown and Reiner (1999) demonstrated that C3H mice deficient in IFN-γ exhibited a greater severity of arthritis compared to wild-type C3H mice, indicating that the presence of IFN-γ may not be required for arthritis development. In a different model of disease, Christopherson et al. (2003) demonstrated that persistent arthritis

was still exhibited by IFN-γ-deficient mice. Glickstein et al. (2001) further showed that IFN-γ-receptor-deficient mice controlled infection in a manner comparable to wild type control mice. These studies provide evidence that Lyme arthritis may not be mediated solely by IFN-γ activity, and that other cytokines may also contribute to inflammation.

Type 17 helper T (Th17) cells are another inflammatory subset of T helper cells. Transforming growth factor-β (TGF-β) and IL-6 are both key players in the differentiation of Th17 cells, and both are required for the differentiation of naïve T cells into IL-17-producing T cells (Veldhoen et al., 2006). Th17 cells secrete the pro-inflammatory cytokine interleukin-17 (IL-17), along with other pro-inflammatory mediators, such as TNF-α and granulocyte macrophage colony-stimulating factor (GM-CSF). Recombinant human IL-17 was found to stimulate the release of other pro-inflammatory cytokines, such as IL-1β and TNF-α, in macrophages (Jovanovic et al., 1998). IL-17 also induces a variety of chemoattractants, such as monocyte chemoattractant protein 1 (Shahrara et al., 2010). IL-17 has also been shown to induce the expression of intracellular adhesion molecule-1 in human fibroblasts (Yao et al., 1995).

The involvement of IL-17 has been documented in various arthropathies. For example, IL-17 is involved in cartilage and joint destruction in a collagen-induced arthritis model (Lubberts et al., 2001). In addition, high

levels of IL-17 were found in the synovial fluid of patients with rheumatoid arthritis (Ziolkowska et al., 2000), and in the sera of patients with early inflammatory arthritis (Gullick et al., 2013). Fluid in the joints of juvenile idiopathic arthritis patients was also shown to be dominated by Th17 cells (Nistala et al., 2008). IL-17 also was shown to be associated with increased NO levels in human cartilage (Attur et al., 1997), which is a possible mode of cartilage destruction. Collectively, these findings suggest a role for IL-17 in enhancing inflammation and exacerbating arthritis.

Indeed, there are a growing number of studies which demonstrate the contribution of Th17 cells and IL-17 to joint inflammation in Lyme arthritis. It was first shown by Infante-Duarte et al. (2000) that *B. burgdorferi* antigens are capable of stimulating IL-17 production by T cells of synovial fluid in humans and by spleen cells of mice. It was also found, using a model of *Borrelia*-induced arthritis in IFN-γ-deficient C57BL/6 mice, that neutralization of IL-17 or its receptor reduced paw swelling and histopathologic changes of the joint (Burchill et al., 2003). In addition, it was found that inhibition of IL-17 was associated with an increased presence of local T cells with immunoregulatory function (Nardelli et al., 2004). This study suggested that a possible factor for the pro-inflammatory conditions elicited during Lyme arthritis is insufficient regulatory T cell activity. Furthermore, NapA has also been found to stimulate the production of IL-17, IL-1β, IL-6, interleukin-23 (IL-23), and TGF-β (Codolo

et al., 2008). NapA also acts as a chemoattractant for IFN-γ- and IL-17-producing T cells in the synovial fluid of Lyme arthritis patients (Codolo et al., 2013). In addition, spleen cells of infected, arthritis-resistant C57BL/6 mice produced less IL-17 compared to an IL-10-deficient strain of the same genotype, which is known to develop arthritis (Hansen et al., 2013). This finding further demonstrates the inflammatory role of IL-17 in Lyme arthritis and provides evidence of a possible regulator of IL-17 after infection with *B. burgdorferi*.

Finally, other cytokines involved in the production of IL-17 have been shown to be involved in the development of Lyme arthritis. The presence of IL-23, a promoter of Th17-derived IL-17 (Langrish et al., 2005), was found to be required for arthritis development in vaccinated C57BL/6 mice infected with *B. burgdorferi* (Kotloski et al., 2008). In addition, Codolo et al. (2008) found that IL-23 production by monocytes and neutrophils was stimulated by NapA. Moreover, increased serum IL-23 levels were found in patients who experienced antibiotic-refractory Lyme disease symptoms (Strle et al., 2013). Taken together, this information suggests that IL-23 may contribute to the pathogenesis of Lyme arthritis inflammation. In addition, TGF-β and IL-6 are involved in the development of Th17 cells. The presence of both IL-6 and TGF-β are required for arthritis development in *B. burgdorferi*-vaccinated and -challenged C57BL/6 mice (Nardelli et al., 2008b). Since the pro-inflammatory inducers of IL-17 are

required for increasing the severity of arthritis, these findings suggest that IL-17 plays a significant role in promoting inflammation in Lyme arthritis.

Identifying inflammatory mediators of Lyme arthritis allows for better understanding of targets for treatments for the disease. The use of antibiotics to treat Lyme disease and arthritis, while effective for most patients, is not effective for some. In addition, prolonged use of antibiotics may cause various harmful effects. Therefore, alternative therapies may need to be developed to alleviate the symptoms of Lyme arthritis. Phototherapy may be one such alternative, since it is non-invasive and has been shown to reduce inflammation in other disease models.

II. Photobiomodulation

1. Background

Exposure to light from the far-red to near-infrared (NIR) regions (630—900nm) of the electromagnetic spectrum has been shown to modulate various cellular and molecular functions (Karu, 2003). This phenomenon has been termed "photobiomodulation" (PBM). PBM has been applied clinically in the treatment of soft tissue injuries and acceleration of wound healing for more than 40 years (Chung et al., 2012). Recent studies have demonstrated that FR/NIR photons penetrate diseased tissues including muscle, spinal cord and brain. PBM is an increasingly studied form of therapy due to its documented benefits in other disorders, including retinal

degeneration and neural inflammation (Eells et al., 2003; Desmet et al., 2006; Muili et al., 2012). Two types of light sources are employed in PBM: low-power lasers and light-emitting diode (LED) arrays. Low-power lasers were the first type of light source used in PBM. However, certain limitations, including potential damage induced by heat production and inability to treat large wound areas, increased the use of LED arrays (Whelan et al., 2001). LEDs can be produced to emit different wavelengths of light and can be manufactured in the form of arrays, allowing larger surface areas to be treated. There is also little risk of tissue damage from FR/NIR LED units. Moreover, the FDA has approved FR/NIR LED arrays for applications in soft-tissue injury and inflammation in humans (Whelan et al., 2001).

One reason PBM continues to possess a low profile in medicine is because there is an incomplete understanding of its mechanism(s) of action. PBM obeys the fundamental laws of photochemistry: 1) FR or NIR light must be absorbed by a photoacceptor molecule or molecules for PBM to occur, 2) only one molecule is activated per photon, 3) effectiveness depends on the degree of absorbance associated with various wavelengths, as demonstrated by the absorption spectrum of the photoacceptor molecule, and finally, 4) effectiveness also depends on the degree to which a particular wavelength induces biochemical changes, as defined by the action spectrum, a plot which illustrates the ability of the various wavelengths of light to induce biochemical or physiological

changes. The comparison between the action spectrum and absorption spectrum indicate which wavelengths are the most effective in stimulating cellular activity (Karu, 1989). Studies have also shown that PBM is more effective at inducing biochemical changes in cells or tissues which have sustained some form of injury or are dysfunctional (Karu, 1989). Moreover, it has been found that the absorption spectrum of cytochrome c oxidase and the action spectrum of FR/NIR light correspond with each other (Wong-Riley et al., 2005). Light at wavelengths between 600 nm—700 nm are generally used to treat more superficial wounds, while wavelengths between 780—950 nm have been shown to be more effective in treating deeper wounds (Chung et al., 2012). Commonly employed wavelengths for PBM studies include 670 nm in the far-red region of the spectrum and 830 nm in the NIR region of the spectrum (Fitzgerald et al., 2013).

Other key factors limiting the use of PBM in medicine are the selection of wavelengths, the most efficacious dose of light, and the dosing schedule. Many different wavelengths and dosage regimens have been applied with varying success in the treatment of wound and soft tissue injuries (Fitzgerald et al., 2013). Although the action spectrum and absorption spectrum have established certain wavelengths that are associated with greater biochemical changes, factors such as the fluence, intensity of the light, the duration of treatment, and area of the wound being treated are also crucial for the success of PBM (Chung et al., 2012). The dosage, or

fluence (J/cm^2), is defined as the number of photons applied per square centimeter of the area treated. It is a product of the light intensity (mW/cm^2) and duration of treatment (seconds). Fluences employed in PBM have been reported to be as low as 0.5 J/cm^2 or as high as 268 J/cm^2. Similarly, the intensity of light used in studies ranges from 2 mW/cm^2 to 2230 mW/cm^2 (Fitzgerald et al., 2013). In addition, variability in other treatment parameters, including total number of treatments and number of treatments per day, have been reported. For acceptance into mainstream medicine, reproducible dosage regimens need to be established for different disease states.

2. Mechanism(s) of Action of Photobiomodulation

Considerable experimental evidence indicates that cytochrome c oxidase is a primary photoacceptor molecule in photobiomodulation mediated by FR/NIR light (Wong-Riley et al., 2005). Cytochrome c oxidase (CcO) is the terminal electron acceptor in the mitochondrial electron transport chain. Importantly, CcO plays a key role in mitochondrial function and in retrograde signaling from the mitochondrion to the nucleus. PBM using FR/NIR light can induce a variety of biochemical signaling pathways after light absorption (Karu et al., 2003). Cellular events including up-regulation of the synthesis of anti-inflammatory cytokines may occur, along with simultaneous downregulation of pro-inflammatory cytokines.

One proposal for the mechanism of action of PBM is the displacement of NO from CcO, resulting in both increased ATP synthesis and activation of transcription factors (Karu, 2010). When NO is bound to CcO, it prevents oxygen from binding to the photoacceptor, thereby inhibiting cellular respiration and ATP production. The interaction of FR/NIR light with CcO causes a conformational change in CcO, resulting in the release of NO (Prindeze et al., 2012). This release allows oxygen to take the place of NO, and triggers cellular respiration and ATP production. The increase in ATP production ultimately enhances the potential for cellular repair (Karu, 2014). The replacement of NO with oxygen binding to CcO leads to a switch in redox states for the photoacceptor, and a subsequent increase in proton transport, resulting in an increase in mitochondrial membrane potential and, thus, increased ATP synthesis (Prindeze et al., 2012).

The NO displaced has been postulated to diffuse into the cytosol and activate transcription factors resulting in enhanced production of cytoprotective proteins (Karu et al., 2014), as light treatment has been found to change chromatin structure in human lymphocytes, which was indicative of a possible increase in cellular proliferation (Fedoseyeva et al., 1988). Additionally, studies have shown an increased release of antioxidants, such as manganese superoxide dismutase (MnSOD) and glutathione, after light treatment (Hodgson et al., 2011; Kirk et al., 2013), which indicates a possible cytoprotective mechanism induced by PBM.

Since NO possesses a variety of immunoregulatory functions (Bogdan,
2001), the NO displaced by cytochrome c oxidase may exert an anti-
inflammatory effect in cells. NO is produced by a variety of immune cells,
including macrophages and neutrophils, as well as endothelial cells,
epithelial cells, fibroblasts, keratinocytes, and Schwann cells (Bogdan,
2001). It is responsible for regulation of a wide variety of immunological
activities, including T and B cell inhibition, regulation and production of
various cytokines and chemokines, and regulation of transcription factors
such as NF-κB, which is responsible for inducing genes involved in pro-
inflammatory responses (Connelly et al., 2001; Prindeze et al., 2012).
Therefore, the release of NO from CcO after FR/NIR light absorption may
result in increased NO signaling to regulate immune events.

3. Photobiomodulation Effects on Various Arthropathies

Despite the uncertainty about the mechanisms of PBM and the
parameters that make it successful, studies have documented the
increased cellular regeneration and anti-inflammatory effects of PBM in
various types of arthritis. Whelan et al. (2001) demonstrated that LED
treatment of various cell lines using 670 nm and 880 nm, at a dosage of 4
J/cm^2 or 8 J/cm^2, promotes osteoblast and fibroblast proliferation, as well
as increased collagen synthesis by epithelial cells. In addition, 810 nm
light treatment with fluences of 5 J/cm^2 and 25 J/cm^2 was an effective

regimen for decreasing inflammatory cytokine levels in human rheumatoid arthritis synoviocytes (Yamaura et al., 2009). Treatment with 780 nm light was also effective in decreasing levels of inflammatory mononuclear cells and exudate in the inflamed area, while increasing the presence of chondrocytes and lubrication of the synovial joints of Wistar rats with collagen-induced arthritis (Alves et al., 2013). Da Rosa et al. (2012) compared the effects of 660 nm and 808 nm light in a rat model of osteoarthritis, using a fluence of 142 J/cm^2, and it was shown that light therapy using 808 nm light was more successful than 660 nm light in stimulating angiogenesis and reducing fibrosis and exudate. Lastly, Castano et al. (2007) showed that treatment with 810 nm light reduced inflammation in zymosan-induced arthritis in rats. These studies indicate the therapeutic potential of PBM in joint-related inflammation.

4. Effect of Photobiomodulation on Pro-Inflammatory Cytokines

As the mechanisms for PBM are still not established, it is unclear how FR/NIR light absorption leads to cytokine regulation and reduction in inflammation. It has been found that NO production is associated with regulation of a variety of both pro-inflammatory and anti-inflammatory cytokines, including IL-1, IL-6, IL-8, IL-10, IL-12, interleukin-18, IFN-γ, and TNF-α (Prindeze et al., 2012). Umansky et al. (1998) demonstrated that NO concentration dictates whether the presence of NO will exert a pro-inflammatory or anti-inflammatory effect in cells. It was found that when a

low concentration of the NO-generating compound glycerol trinitrate was added to endothelial cells, NF-κB activation was increased. In contrast, Muili et al. (2013) showed that low NO production was associated with anti-inflammatory effects of light treatment. When treating myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalitis (EAE) with 670 nm light in C57BL/6 mice, a reduction in NO production was observed (Muili et al., 2013). Treatment with 670 nm light was efficient in ameliorating inflammation in EAE in the presence of inducible nitric oxide synthase (iNOS), but had no effect in iNOS-deficient mice (Muili et al., 2013). Collectively, these studies suggest that the concentration of NO dictates whether its presence exerts a pro-inflammatory of anti-inflammatory effect.

Indeed, various studies have demonstrated the anti-inflammatory effects of PBM by reduction of pro-inflammatory mediators in various types of disorders. Pires et al. (2011) found that PBM via 780 nm light decreased the mRNA expression of IL-6, TGF-β, and the pro-inflammatory mediator cyclooxygenase-2 but not IL-1β, in both acute and chronic phases of tendinopathy in rats (Pires et al., 2011). Albertini et al.'s (2008) study supported the suppressive effect PBM exerts on TNF-α and IL-6 by demonstrating a reduction in these cytokines' mRNA expression. They also showed that IL-1β mRNA expression in carrageenan-induced inflammation in rat paws was downregulated after treatment with 660 nm

and 684 nm light (Albertini et al., 2008). Finally, Muili et al. (2012) showed that treatment with 670 nm LED light led to a downregulation of IFN-γ and TNF-α in mice with autoimmune encephalomyelitis. These studies demonstrate the ability of NIR light to reduce pro-inflammatory cytokine levels.

III. Hypothesis and Specific Aims

Since it has been found that some Lyme arthritis patients experience persistent inflammatory symptoms even after a course of antibiotics, an additional form of therapy would be beneficial to alleviate their symptoms. The production of IL-17 and IFN-γ during the course of Lyme arthritis may contribute to ongoing joint inflammation. Therapy which decreases the levels of these cytokines may reduce inflammation and ease symptoms in Lyme arthritis patients. Since FR/NIR light therapy was found to have a therapeutic effect in other inflammatory disorders, including various types of arthritis, it is hypothesized that FR/NIR light therapy will also ameliorate joint inflammation in Lyme arthritis.

The objective of this thesis is to determine the effects of 670 nm and 830 nm red or NIR light on pro-inflammatory cytokine levels in Lyme arthritis. The hypothesis for this thesis is that PBM using 670 nm or 830 nm light will decrease inflammation in Lyme arthritis. This hypothesis was tested using two specific aims:

1. **Determine the effect that 670 nm light and 830 nm light has on paw swelling and pro-inflammatory cytokine levels in *B. burgdorferi*-infected mice.** The working hypothesis for this aim is that 670 nm and 830 nm light will ameliorate swelling and reduce IL-17 and INF-γ production in *B. burgdorferi*-infected mice.

2. **Determine the effect of 670 nm and 830 nm light on production of borreliacidal antibodies in infected mice.** The working hypothesis for this aim is neither 670 nm nor 830 nm light will affect borreliacidal antibody production in *B. burgdorferi*-infected mice.

CHAPTER 2: MATERIALS AND METHODS

I. Mice

Six-to-12-week old, male and female C57BL/6 wild-type and IL-10-knock-out (KO) mice, weighing 20-30 g, were used in this study. Mice were housed at the University of Wisconsin-Milwaukee animal facility in a humidity-controlled environment, with light and dark cycles of 12 hours each, at a room temperature of 21°C and were provided food and water *ad libitum*. Laboratory protocols were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee.

II. Infection of Mice with *B. burgdorferi*

B. burgdorferi strain 297 organisms were obtained from S. M. Callister (Gundersen Medical Center, La Crosse, WI) and stored at -80°C until use. Spirochetes were grown in Barbour-Stoenner-Kelly (BSK) medium at 34°C until a concentration of 2×10^7 organisms/mL was reached. Viability was confirmed by darkfield microscopy.

Wild-type and IL-10 KO mice (four to six per group) were injected subcutaneously in the hind paws with 10^6 viable *B. burgdorferi* strain 297 organisms in 0.05 mL Barbour-Stoenner-Kelly (BSK) medium. Uninfected control mice were not used, due to a limited availability of experimental

animals and because the focus of this work is assessing efficacy of light therapy following infection.

III. Light Treatment

Groups of *B. burgdorferi*-infected wild-type and IL-10 KO mice received no light treatment or light treatment. 670 nm light treatment was administered with a Gallium/Aluminum/Arsenide (GaAlA) LED and consisted of illumination for 180 seconds at an intensity of 25 mW/cm^2, resulting in a fluence/dose of 4.5 J/cm^2. For 670 nm light treatment, mice were placed in a plexiglass box, which was positioned above the 670 nm array. Light treatment with 830 nm was administered with a hand-held WARP-10 LED device, and consisted of illumination for 90 seconds at an intensity of 50 mW/cm^2, resulting in a fluence/dose of 4.5 J/cm^2. For the 830nm light treatment, the WARP-10 device was placed in a manner in which the entire area of the paw was covered by the light. Untreated mice were handled in the same manner as light-treated mice, with the exception that they were not exposed to light.

In this infection model, resolution of mouse paw swelling typically occurs approximately 20 days after infection, with a peak of swelling exhibited approximately 8 days after infection. In one experiment, groups of four to six infected mice were untreated or received one course of daily 670 nm light treatment between days 8 and 14 after infection. These mice were euthanized by isoflurane inhalation followed by cervical dislocation on day

20 after infection. In another experiment, groups of four mice received one course of 670 nm or 830 nm light treatment daily between days 2 and 7 after infection. These mice were euthanized by isoflurane inhalation followed by cervical dislocation on day 8 after infection.

IV. Measurement of Hind Paw Swelling

The width and thickness of swelling in the ankle joints of mice were measured with a digital caliper with a sensitivity of 0.01 mm. Baseline measurements of hind paws of all mice were obtained before infection with *B. burgdorferi.* Measurements were obtained every other day following the initiation of light treatment and were obtained immediately prior to the treatment course. The data are presented as the average paw size for the respective groups +/- standard error of the mean.

V. Cell Culture and ELISA

Spleens were harvested on day 8 after infection. Single-cell, whole spleen suspensions were obtained by passing cells through a nylon mesh screen into cold RPMI 1640 medium supplemented with L-glutamine and 25 mM HEPES. 1×10^6 whole spleen cells were incubated with 1×10^5 *B. burgdorferi* strain 297 organisms at 37°C in 5% CO_2 for 24, 48, and 72 hours. Control cells were incubated without *B. burgdorferi* organisms. IL-17 and IFN-γ levels in the supernatants were measured using eBioscience mouse IL-17A and IFN-gamma ELISA Ready-Set-Go! Kits, respectively.

Plates were read at 450 nm and values were expressed as optical

densities. Standard curves were obtained and used to calculate pg/mL for

both cytokines.

VI. Borreliacidal Assay

Sera were used to determine titers of borreliacidal antibodies. Blood was

collected from anesthetized mice by intracardiac puncture, and sera were

obtained by centrifugation of blood samples at 1000 rpm for 10 minutes.

Equal volumes of serum were pooled from each mouse within a group.

Sera samples were serially diluted with BSK and then heated at 56°C for

30 minutes to inactivate complement components. Twenty microliters of

guinea pig complement and $1x10^4$ viable *B. burgdorferi* strain 297

organisms were added to the heat-inactivated sera. Samples were

incubated for 16 hours at 34°C. Control samples included complement

and *B. burgdorferi* without serum, serum and *B. burgdorferi* without

complement, and serum and complement without *B. burgdorferi*. After

incubation, presence of motile spirochetes was visualized (20 fields) using

dark-field microscopy. The reciprocal of the greatest dilution with no viable

B. burgdorferi was considered the borreliacidal antibody titer.

VII. Data Analysis

Swelling data between untreated and light-treated groups were analyzed

using Student's T-test. Cytokine levels in the supernatants of cells

obtained from untreated and treated mice were analyzed using the Mann-Whitney U Test. Alpha levels were set at 0.05 prior to the beginning of the experiments, and P values less than or equal to 0.05 considered significant.

CHAPTER 3: RESULTS

I. Specific Aim 1: Determine the effect that 670 nm light and 830 nm light has on paw swelling and pro-inflammatory cytokine levels in *B. burgdorferi*-infected mice

1. Effects of Phototherapy on Hind Paw Swelling

Wild-type and IL-10 KO mice were infected with *B. burgdorferi* strain 297 and then treated or untreated with 670 nm light daily between days 8 and 14 after infection. Wild-type mice exhibited mild paw swelling after infection (Figure 1A). Treatment of infected wild-type mice with 670 nm light caused a slight, but insignificant, increase in hind paw swelling throughout the duration of treatment (Figure 1A). This difference approached statistical significance (P=0.06) at day 14, the final day of treatment. However, paw swelling of treated wild-type mice began to decline on the fifth day of treatment and continued to decline after treatment. By contrast, the paw swelling of untreated mice increased during this time, peaking at day 18 after infection. At this time point, the paw swelling of previously-treated mice was lower than that of untreated mice. However, this difference was not significant (P=0.09).

Infected IL-10 KO mice exhibited much greater hind paw swelling than wild-type mice (Figure 1B). Swelling peaked at day 6 after infection and began to decline just before 670 nm light treatment began. 670 nm light-treated mice exhibited less paw swelling than untreated mice during and

after treatment (Figure 1B). However, this reduction in swelling was not statistically significant.

Figure 1: Average change in paw swelling after *B. burgdorferi* strain 297 infection of wild-type C57BL/6 mice (A; n=4 per group) and IL-10 KO mice (B; n=6 per group) treated with 670 nm light between days 8 and 14 after infection. Untreated mice are denoted by empty squares and treated mice are represented by filled squares. Bars show standard error of the mean.

Because the results for the first study were not statistically significant, the effect of light was tested earlier during inflammation. Wild-type and IL-10 KO mice were infected with *B. burgdorferi*, and then either untreated or treated with 670 nm or 830 nm light daily between days 2 and 7 after infection. As expected, infected IL-10 KO mice (Figures 2B and 3B) exhibited greater paw swelling than their wild-type counterparts (Figures 2A and 3A).

B. burgdorferi-infected wild-type mice exhibited mild hind paw swelling, and no changes in paw swelling were observed due to treatment of these mice with 670 nm light (Figure 2A). In addition, there were no significant differences between the paw swelling of 670 nm light-treated and untreated IL-10 KO mice infected with *B. burgdorferi* (Figure 2B). Both groups of IL-10 KO mice experienced a comparable decrease in paw swelling after the peak of inflammation.
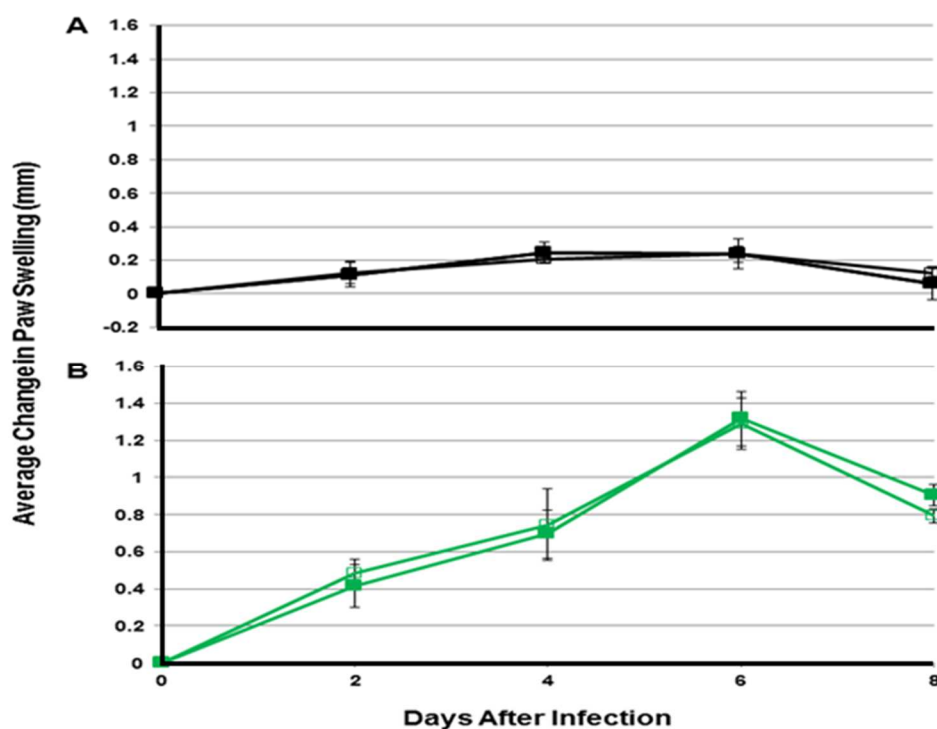
Figure 2: Average change in paw swelling after *B. burgdorferi* strain 297 infection of wild-type mice (A) or IL-10 KO mice (B) treated with 670 nm light between days 2 and 7 after infection. Untreated mice are denoted by empty squares and treated mice are represented by filled squares. n=4 in each group. Bars show standard error of the mean.

Similarly, in a different study, wild-type mice did not exhibit hind paw swelling after infection with *B. burgdorferi*, and swelling was not affected by treatment with 830 nm light (Figure 3A). In addition, treatment with 830

nm light did not cause significant changes in paw swelling of *B. burgdorferi*-infected IL-10 KO mice (Figure 3B). However, 830 nm light-treated mice showed an overall increase in paw swelling by day 8 after infection, whereas untreated mice exhibited a decrease in paw swelling at that time (Figure 3B). However, this difference was not statistically significant.

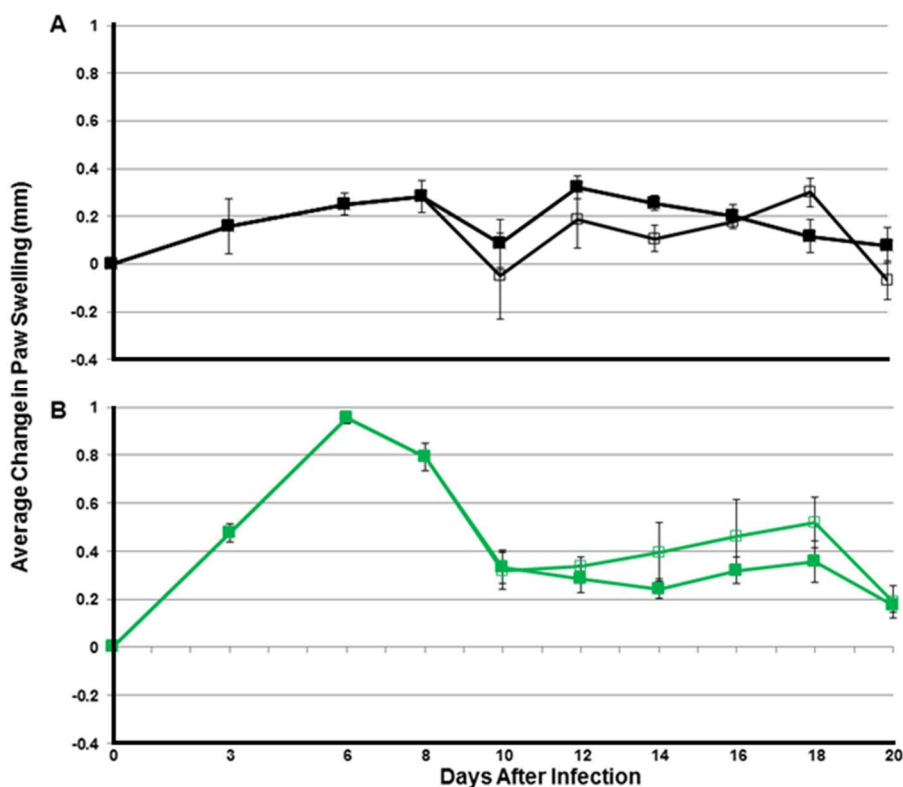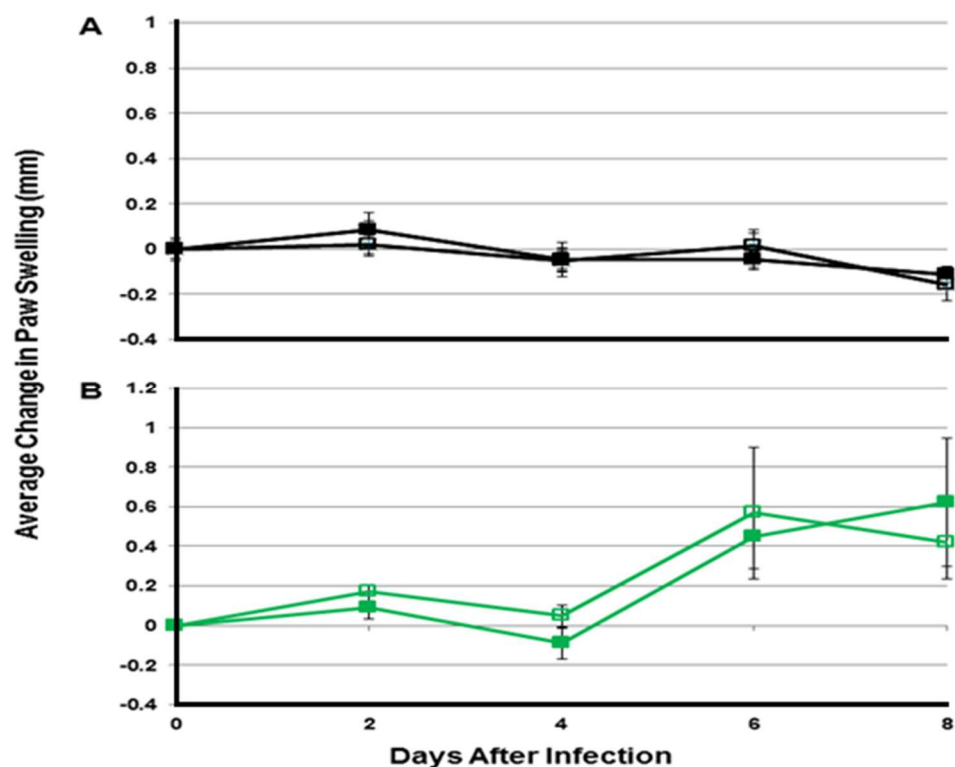**Figure 3: Average change in paw swelling after *B. burgdorferi* strain 297 infection of wild-type mice (A) or IL-10 KO mice (B) treated with 830 nm light between days 2 and 7 after infection. Untreated mice are denoted by empty squares and treated mice are represented by filled squares. n=4 in each group. Bars show standard error of the mean.**

2. Effects of Phototherapy on Inflammatory Cytokine Production

On day 8 after infection, spleens were harvested from *B. burgdorferi-*
infected wild-type and IL-10 KO mice that were untreated or treated with
670 nm or 830 nm light between days 2 and 7 after infection. 10^6 spleen
cells were incubated with or without 10^5 *B. burgdorferi* for 24, 48, or 72
hours. Supernatants were analyzed by ELISA for production of IL-17 and
IFN-γ.

i. Effects of 670 nm Light on Production of IL-17

Treatment with 670 nm light did not affect the production of IL-17 from
spleen cells of infected wild-type mice, regardless of whether the cells
were unstimulated (Figure 4A) or stimulated with *B. burgdorferi* (Figure
4B). However, in each case, the greatest amount of IL-17 production was
observed among cells from treated mice after 48 hours of culture. In
addition, IL-17 production by these cells was generally low (less than 60
pg/ml supernatant), regardless of whether the cells were stimulated.

Figure 4: IL-17 production by unstimulated (A) or stimulated (B) spleen cells of *B. burgdorferi*-infected wild-type mice that were untreated or treated with 670 nm light. n=4 in each group. In some cases, samples with equal values are shown as overlapping symbols.

Similarly, treatment with 670 nm light did not significantly affect the

production of IL-17 from cells of infected IL-10 KO mice, regardless of

whether the cells were unstimulated (Figure 5A) or stimulated with *B.*

burgdorferi (Figure 5B). A reduction in IL-17 production was observed

after 24 hours of culture among stimulated cells of 670 nm light-treated IL-

10 KO mice (Figure 5B); however, this reduction was not statistically

significant. In general, cells from unstimulated mice tended to produce

more IL-17 than cells from stimulated mice.

Figure 5: IL-17 production by unstimulated (A) or stimulated (B) cells of *B. burgdorferi*-infected IL-10 KO mice that were untreated (n=4) or treated with 670 nm light (n=3). In some cases, samples with equal values are shown as overlapping symbols.

ii. Effects of 670 nm Light on Production of IFN-γ

Treatment with 670 nm light did not have a significant effect on IFN-γ

production by unstimulated or stimulated cells of *B. burgdorferi*-infected

wild-type mice (Figure 6A and Figure 6B). However, in general, cells from

untreated mice exhibited more IFN-γ production than cells from treated

mice after 24 hours of culture. No IFN-γ production was detected from cultures of unstimulated or stimulated cells at 72 hours of incubation.

Figure 6: IFN-γ production by unstimulated (A) or stimulated (B) cells of *B. burgdorferi*-infected wild-type mice that were untreated or treated with 670 nm light. n=4 in each group. In some cases, samples with equal values are shown as overlapping symbols.

However, we observed a significant reduction in IFN-γ production of cells from *B. burgdorferi*-infected, 670 nm light-treated, IL-10 KO mice. This significant reduction was observed after 24 hours of culture of both unstimulated (Figure 7A) and stimulated (Figure 7B) cells. Treatment of mice with 670 nm light also caused an overall reduction in IFN-γ

production by unstimulated cells after 72 hours (Figure 7A). However, this reduction was not statistically significant. Overall, splenocytes of infected IL-10 KO mice (Figure 7) exhibited greater production of IFN-γ than infected wild-type mice (Figure 6), regardless of whether the mice were treated with 670 nm light.

Figure 7: IFN-γ production by unstimulated (A) or stimulated (B) cells of *B. burgdorferi*-infected IL-10 KO mice that were untreated (n=4) or treated with 670 nm light (n=3). * denotes significant reduction compared to untreated mice (*U* = 0, p<0.05). Data points with a "glow" effect denote samples that possessed IFN-γ levels that exceeded the sensitivity of the assay. In some cases, samples with equal values are shown as overlapping symbols.

iii. Effects of 830 nm Light on Production of IL-17

Treatment with 830 nm light did not significantly affect the production of IL-17 by cells of *B. burgdorferi*-infected wild-type mice (Figure 8). An increase in IL-17 production was observed among unstimulated cells of 830 nm light-treated mice after 72 hours of incubation (Figure 8A); however, these increases were not statistically significant. In addition, a similar increase was observed among stimulated cells of treated wild-type mice after 48 hours of incubation (Figure 8B). However, this increase also was not statistically significant.
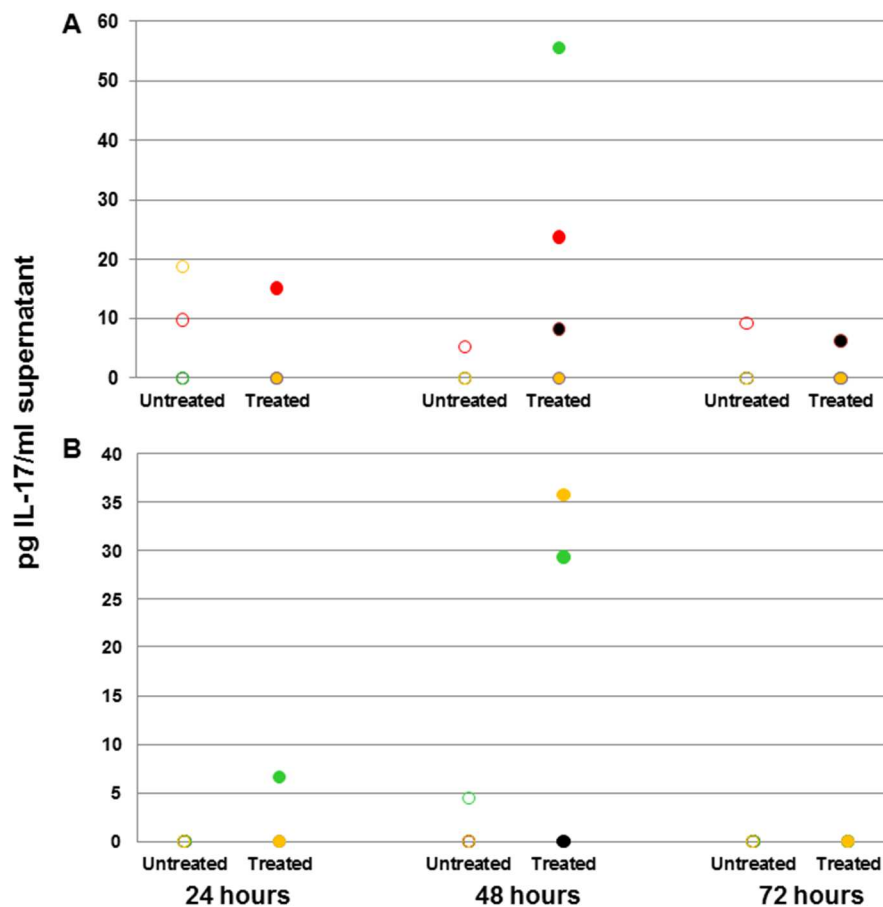
Figure 8: IL-17 production by unstimulated (A) or stimulated (B) cells of *B. burgdorferi*-infected wild-type mice that were untreated or treated with 830 nm light. n=4 in each group. Data points with a "glow" effect denote samples that possessed IL-17 levels that exceeded the sensitivity of the assay. In some cases, samples with equal values are shown as overlapping symbols.

Treatment of infected IL-10 KO mice with 830 nm light caused a significant increase in IL-17 production by unstimulated cells after 24 hours of incubation (Figure 9A). High levels of IL-17 production were observed among cells of both untreated and treated mice after longer incubation times. However, as multiple levels were higher than the sensitivity of the assay, no comparisons between these groups could be made. In addition,

high levels of IL-17 were produced by stimulated cells of untreated and treated mice at all time points (Figure 9B).
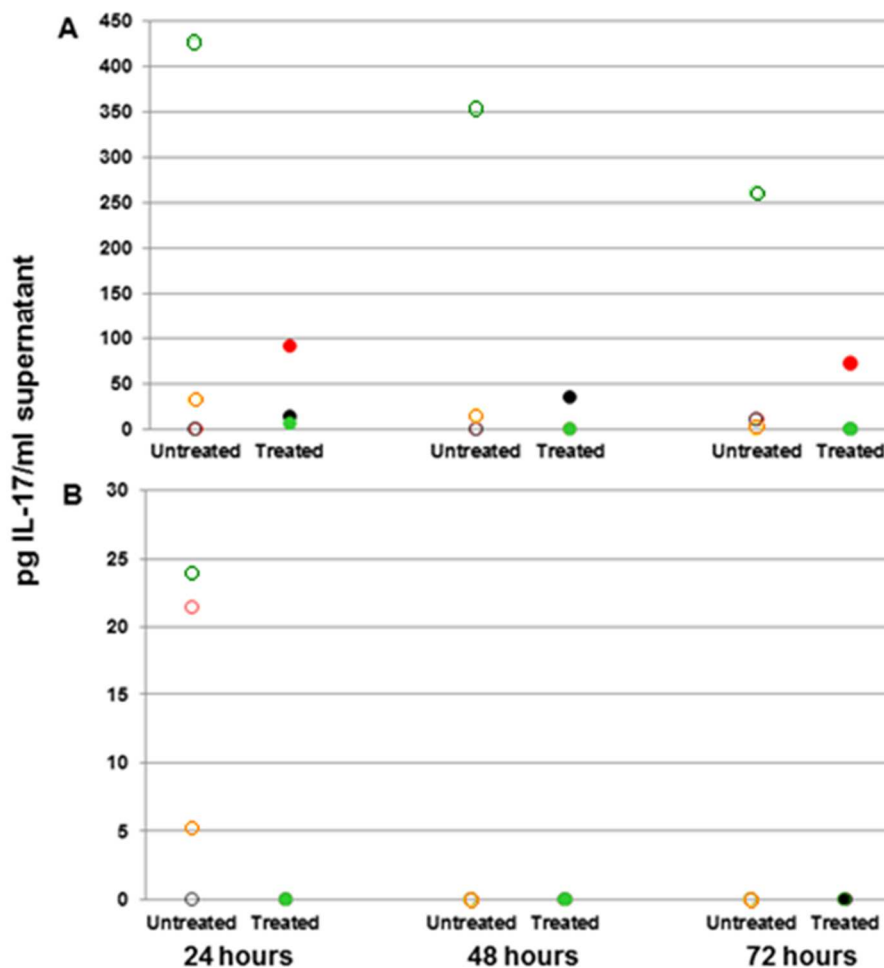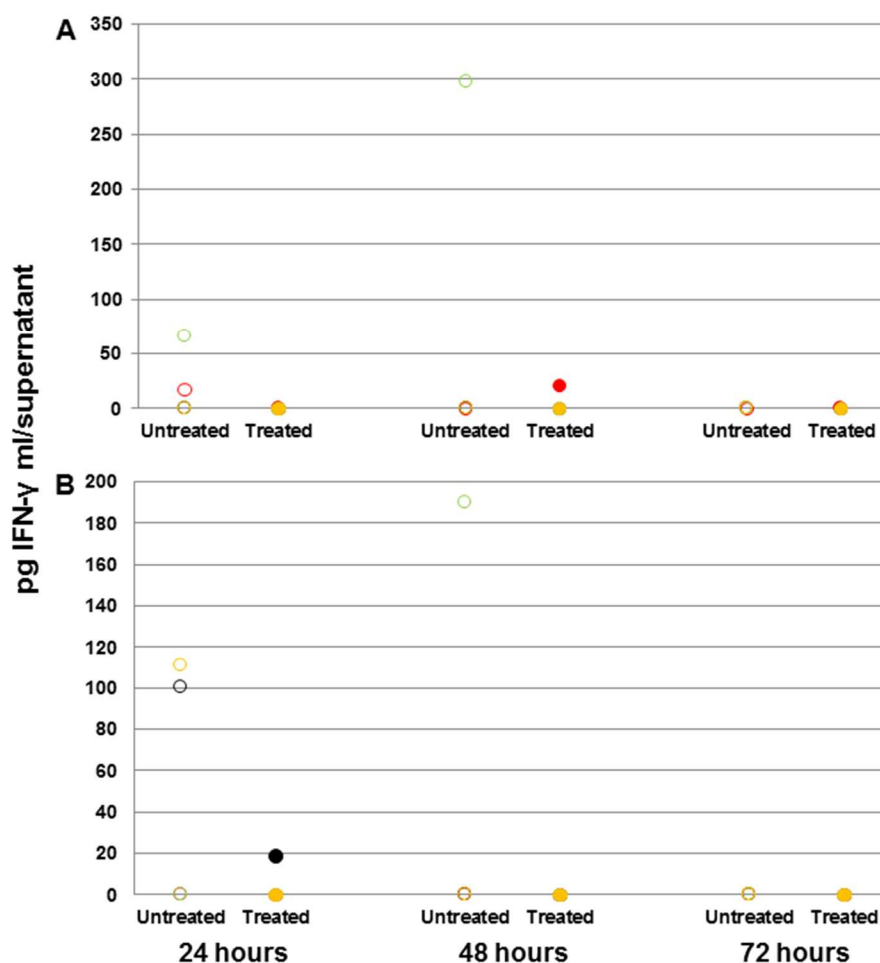

Figure 9: IL-17 production by unstimulated (A) or stimulated (B) cells of *B. burgdorferi*-infected IL-10 KO mice that were untreated (n=3) or treated with 830 nm light (n=4). * denotes significant increase compared to untreated mice (*U* = 0, p<0.05). Data points with a "glow" effect denote samples that possessed IL-17 levels that exceeded the sensitivity of the assay. In some cases, samples with equal values are shown as overlapping symbols.

iv. Effects of 830 nm Light on Production of IFN-γ

Treatment of infected wild-type mice with 830 nm light did not cause a significant effect on IFN-γ production (Figure 10). While an increase in IL-17 production was observed among unstimulated cells of untreated mice

after 72 hours, it was not statistically significant (Figure 10A). In general, higher levels of IFN-γ were produced by stimulated cells than unstimulated cells, from both untreated and treated mice (Figure 10B). However, as multiple readings of 24- and 72-hour cultures of stimulated cells were higher than the sensitivity of the assay, no comparisons between these groups could be made. There were no differences in IFN-γ production after 48 hours of incubation of stimulated cells from untreated and treated mice.
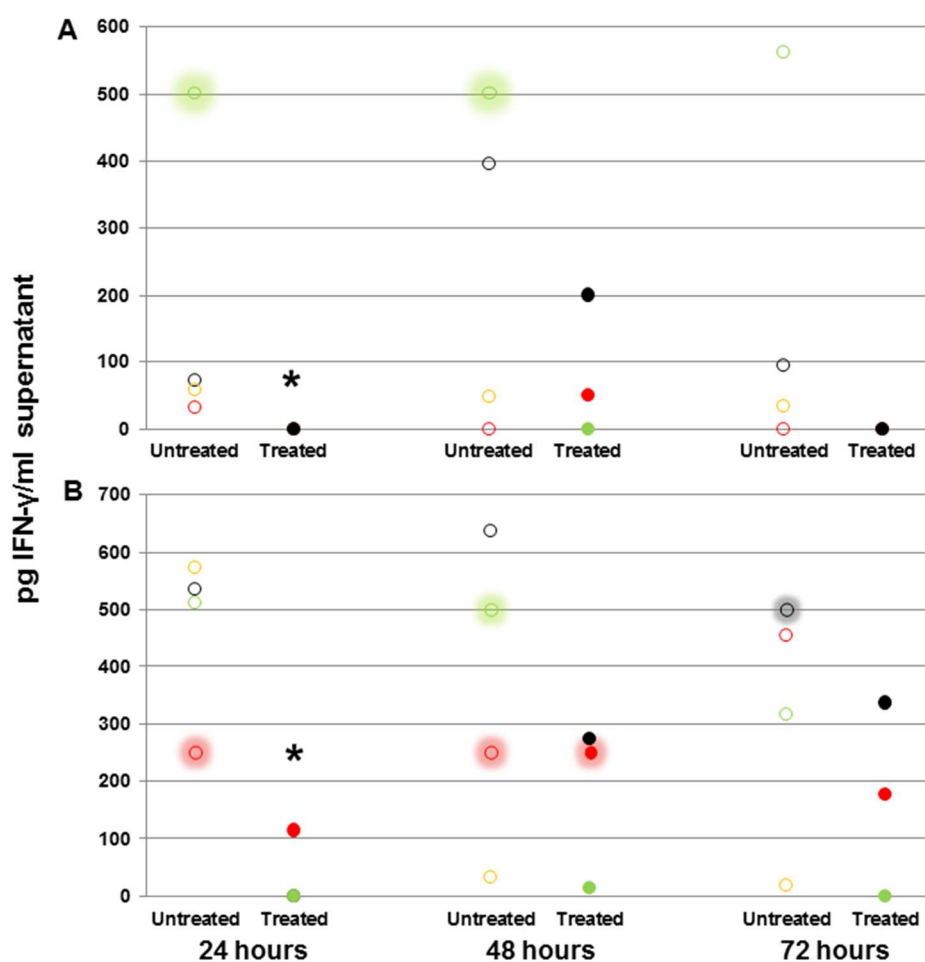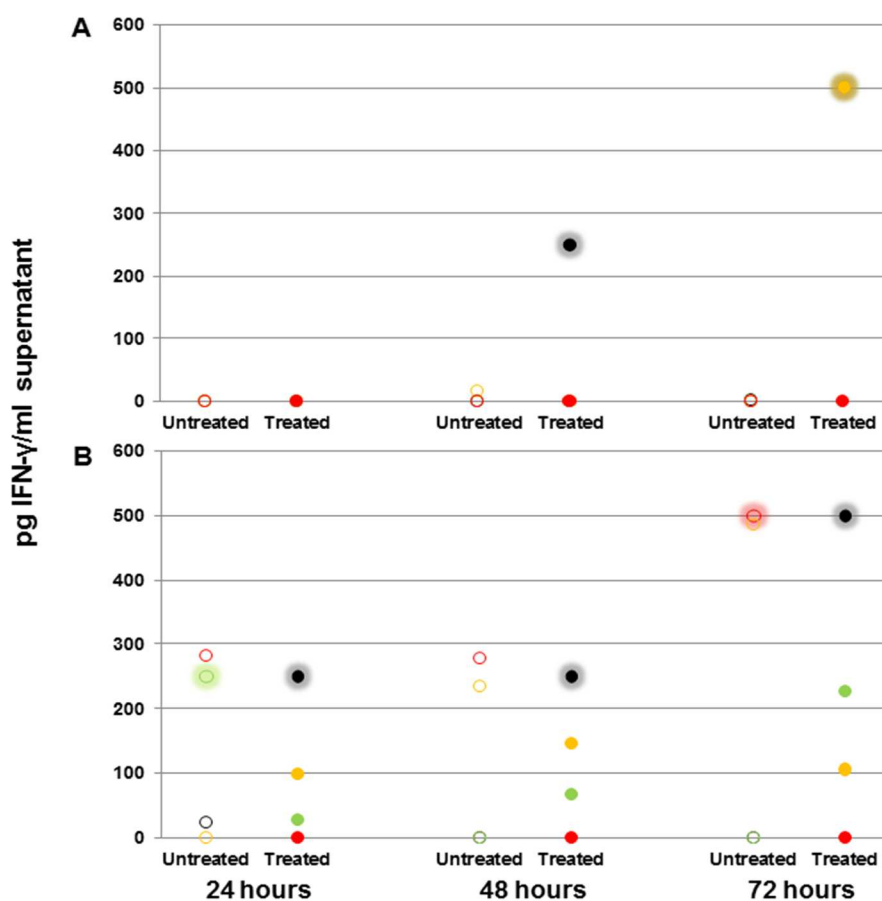

Figure 10: IFN-γ production by unstimulated (A) or stimulated (B) cells of *B. burgdorferi*-infected wild-type mice that were untreated or treated with 830 nm light. n=4 in each group. Data points with a "glow" effect denote samples that possessed IFN-γ levels that exceeded the sensitivity of the assay. In some cases, samples with equal values are shown as overlapping symbols.

We were able to make few claims about the effect of 830 nm light treatment on IFN-γ production of infected, IL-10 KO mice. While there was no difference in IFN-γ production by unstimulated cells after 24 hours of incubation (Figure 11A), multiple readings that exceeded the sensitivity of the assay for 48- and 72-hour cultures of unstimulated cells prevented a comparison. This was also the case for all cultures of stimulated cells obtained from 830 nm-light treated, IFN-γ mice (Figure 11B).
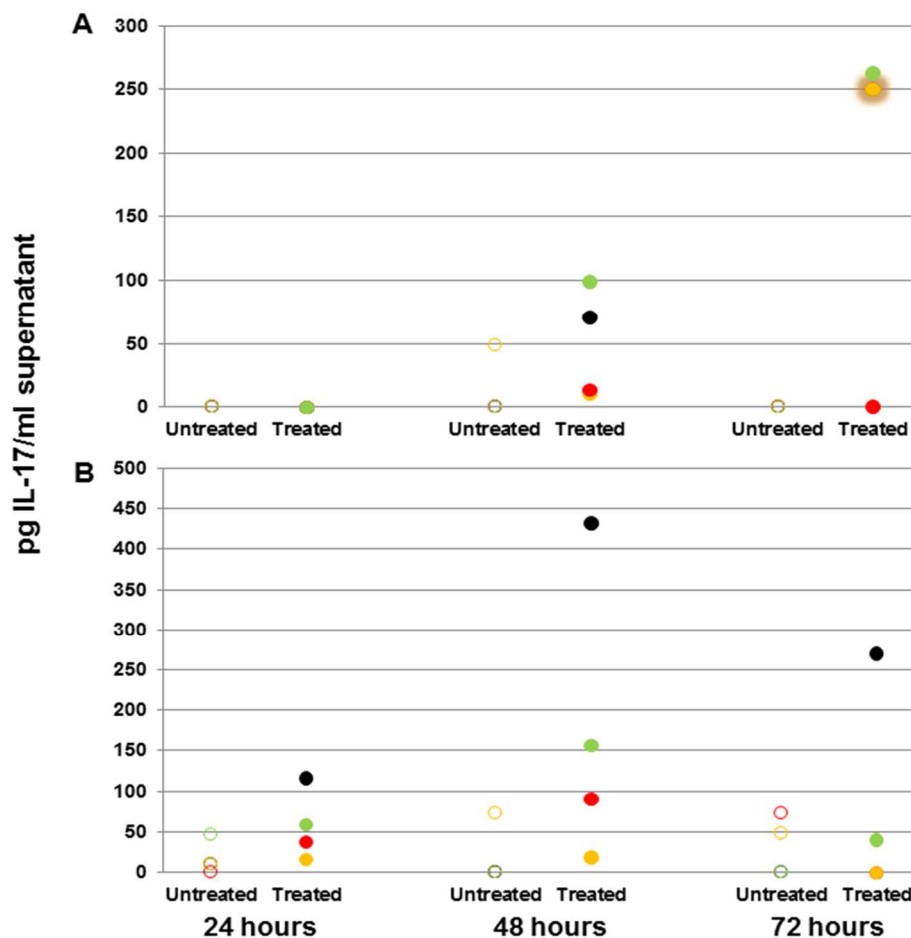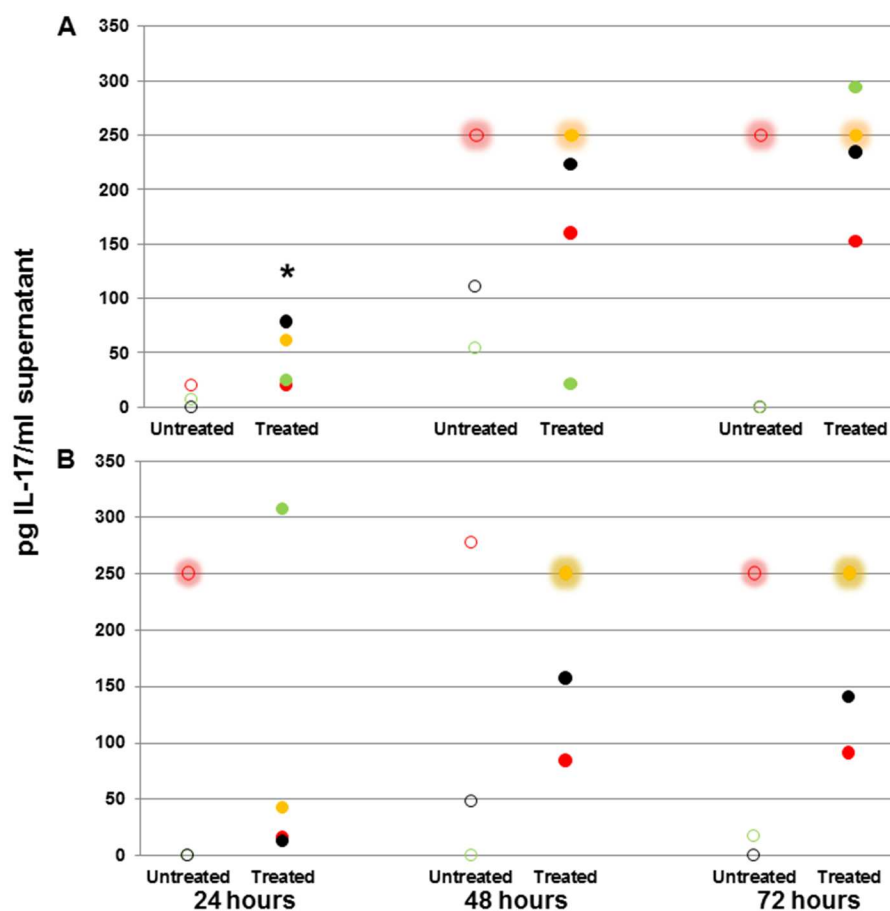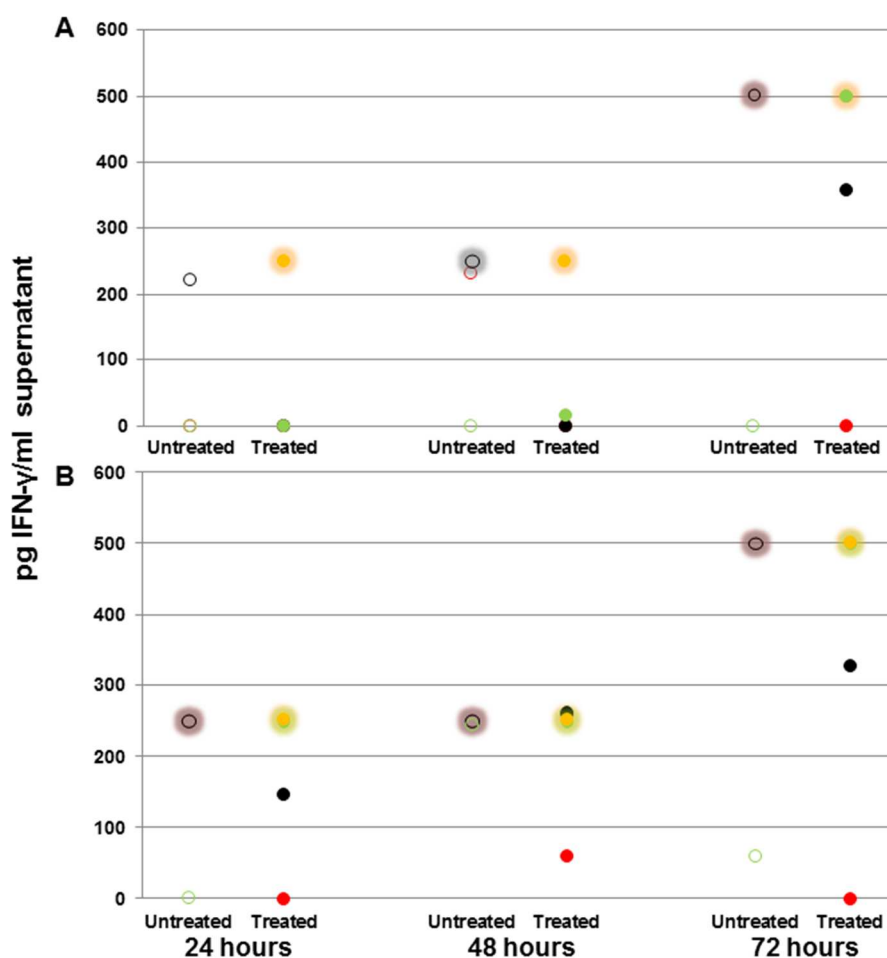

Figure 11: IFN-γ production by unstimulated (A) or stimulated (B) cells of *B. burgdorferi*-infected IL-10 KO mice that were untreated (n=3) or treated with 830 nm light (n=4). Data points with a "glow" effect denote samples that possessed IFN-γ levels that exceeded the sensitivity of the assay. In some cases, samples with equal values are shown as overlapping symbols.

II. Specific Aim 2: Determine the effect of 670 nm and 830 nm light on production of borreliacidal antibodies in infected mice

1. Effects of Phototherapy on Borreliacidal Antibody Titers

The effect of 670 nm and 830 nm light on borreliacidal antibody titers was determined. Sera were obtained on day 8 after infection of wild-type and IL-10 KO mice with *B. burgdorferi*. Mice were untreated or treated daily between days 2 and 7 after infection with either 670nm or 830nm light.

It could not be determined whether treatment of *B. burgdorferi*-infected wild-type mice with 670 nm light affected borreliacidal antibody titers (Figure 12A). Both titers were high (≥40,960) and exceeded the level of detection of the assay. By contrast, borreliacidal antibody titers of infected IL-10 KO mice were notably lower. Untreated, infected IL-10 KO mice exhibited a borreliacidal antibody titer of 160. Treatment of infected IL-10 KO mice with 670 nm light increased borreliacidal antibody titers to 20,480 (Figure 12A).

In another experiment, untreated *B. burgdorferi*-infected wild-type mice exhibited a borreliacidal antibody titer of 5120. By contrast, treatment of *B. burgdorferi*-infected wild-type mice with 830 nm light resulted in a higher borreliacidal antibody titer (≥40,960) (Figure 12B). In addition, untreated *B. burgdorferi*-infected, IL-10 KO mice exhibited a borreliacidal antibody titer

of 10,240. Treatment of infected IL-10 KO mice with 830 nm light reduced

borreliacidal antibody titers to 2,560 (Figure 12B).
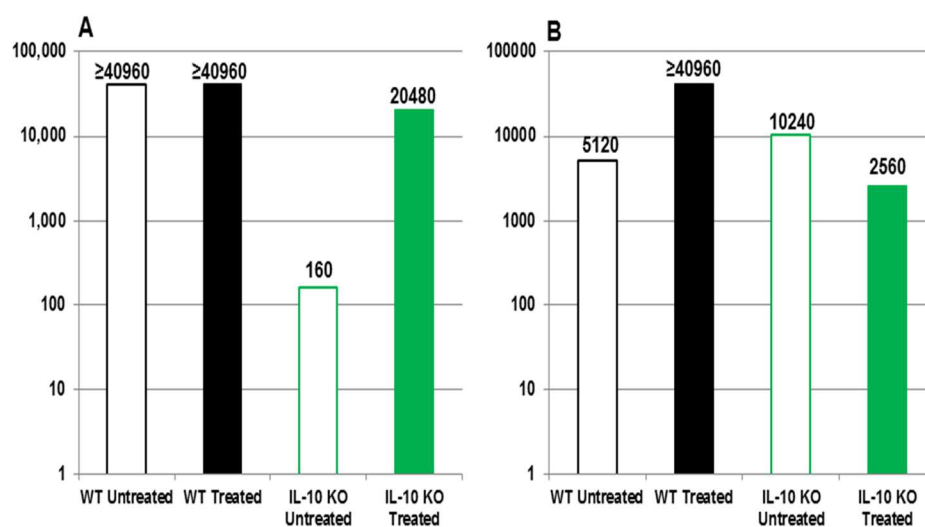
Figure 12: Serum borreliacidal antibody titers of *B. burgdorferi*-infected wild-type (black) and IL-10 KO (green) mice that were untreated (open bars) or treated (filled bars) with 670 nm light (A) or 830 nm light (B). Sera were collected on day 8 after infection. WT, wild-type mice.

CHAPTER 4: DISCUSSION

I. Introduction

Lyme arthritis is a complication resulting from infection with the spirochete *Borrelia burgdorferi*. The incidence of Lyme disease has recently been shown to be ten times more common than previously thought (Centers for Disease Control and Prevention, 2013a). Although antibiotics are typically administered for treatment of the disease, 10% of treated Lyme arthritis patients still experience arthritis. Therefore, it would be beneficial to identify other forms of therapy for Lyme arthritis in the event that treatment with antibiotics is ineffective.

PBM with light from far-red to near-infrared regions (630-900 nm) has been used to treat a wide variety of disorders, such as traumatic brain injury, retinal degeneration, and delayed wound healing (Whelan et al., 2001; Eells et al., 2003; Muili et al., 2013), although the mechanism of action of PBM remains to be completely elucidated (Fitzgerald et al., 2013). It is established that a photoacceptor must absorb photons to stimulate the biological processes of PBM (Karu, 1989). PBM has been found to be effective in reducing a number of pro-inflammatory mediators in various arthropathies (Castano et al., 2007; Yamaura et al., 2009; Hsieh et al., 2014). Since Lyme arthritis is known to be propagated by pro-inflammatory cytokines, including IFN-γ and IL-17 (Yin et al., 1997;

Burchill et al., 2003), PBM may have potential therapeutic effects on the inflammation associated with disease.

The purpose of this thesis was to determine the effects that photobiomodulation using 670 nm and 830 nm light had on the inflammatory state in Lyme arthritis, as well as on the borreliacidal antibody titers of infected mice. The hypothesis of this thesis was that _PBM using 670 nm or 830 nm light will decrease inflammation in Lyme arthritis._ This hypothesis was tested using two specific aims.

II. Specific Aim 1: Determine the effect that 670 nm light and 830 nm light has on paw swelling and pro-inflammatory cytokine levels in _B. burgdorferi_-infected mice

The working hypothesis of this specific aim was that 670 nm and 830 nm light will ameliorate swelling and reduce IL-17 and INF-γ production in _B. burgdorferi_-infected mice. To determine the effectiveness of 670 nm and 830 nm light on alleviating inflammation, the paws of _B. burgdorferi_-infected mice were measured, and IL-17 and IFN-γ levels were obtained from cultured spleen cells. This hypothesis was partially supported. While treatment with either wavelength of light did not significantly affect hind paw swelling of infected mice (Figures 1-3), IFN-γ production by spleen cells of infected IL-10 KO mice was significantly lower in 670 nm light-treated mice than untreated controls (Figure 7). However, treatment with

830 nm light tended to increase IL-17 production by spleen cells of infected IL-10 KO mice (Figure 9).

An initial experiment testing the effect of 670 nm light on murine Lyme arthritis was conducted by administering treatment starting near the peak of inflammation (day 8 after infection) for seven consecutive days. Although only one observation approached statistical significance (Figure 1A), some patterns did emerge. Treatment of wild-type mice using 670 nm light did cause a slight ($P=0.06$) increase in paw swelling at day 14 after infection, the day treatment ended (Figure 1A). The swelling of treated mice gradually subsided, while the swelling of untreated mice exhibited a second increase. By day 18 after infection, the swelling of treated mice was slightly ($P=0.09$) less than that of untreated mice. In addition, treatment of infected IL-10 KO mice lowered swelling, although not significantly (Figure 1B). It is possible that 670 nm light treatment had minimal effects on paw swelling because wild-type C57BL/6 mice develop mild swelling and minimal arthritis after infection with *B. burgdorferi* in the first place. In addition, the lack of significant effects in infected IL-10 KO mice could be due to treatment beginning too late. These mice exhibited a dramatic increase in paw swelling by day 6 after infection, with both treated and untreated groups showing a similar decline by day 10 after infection. It is possible that by this point, the inflammatory mediators may have exerted effects that light treatment could not alleviate. By day 8 after

infection with *B. burgdorferi*, IL-10 KO mice develop moderate inflammation of the perisynovial tissues of the ankle joint and the knee (Hansen et al., 2013). However, the degree of paw swelling in our IL-10 KO mice was nearly double than that which our laboratory has previously observed (Hansen et al., 2013). The inflammation in these mice may have been too great to be affected by light therapy. It has been suggested by Karu (1989) that the rate of cellular proliferation and tissue regeneration must occur at a maximal rate for PBM to be effective. Thus, it is possible that treatment efficacy would be enhanced when maximal levels of regenerative factors are not reached and are not overwhelmed by the production of inflammatory mediators.

To determine whether PBM administration would be more effective in reducing inflammation at an earlier course of disease, we assessed the effects of 670 nm light treatment starting on day 2 after infection. We also wanted to know whether 830 nm light could be efficient in reducing inflammation, since 830 nm light has a greater ability to penetrate tissue than 630 nm light (Giacci et al., 2014) and it demonstrates therapeutic effects in some systems (Freitas et al., 2001; Stadler et al., 2001; Mendez et al., 2004). Our results showed that early treatment with 670 nm light did not significantly affect the paw swelling that resulted from *B. burgdorferi* infection (Figures 2A and 2B). As expected, untreated, infected wild-type mice did not exhibit significant increases in paw swelling after infection

(Figure 2A). We also found that there were no significant differences in the changes in paw size between infected, treated IL-10 KO mice and their untreated counterparts. Both the treated and untreated groups saw resolution in paw swelling beginning after day 6 post-infection (Figure 2B). We observed that by day 8 after infection, 670 nm light-treated IL-10 KO mice exhibited slightly greater paw swelling than untreated mice. However, this change was not statistically significant.

In another experiment, we treated infected mice with 830 nm light at this same, early time. Wild type mice again did not show any significant changes in paw sizes, whether they were untreated or treated (Figure 3A). There were also no statistically significant differences observed when *B. burgdorferi*-infected IL-10 KO mice were treated with 830 nm light. However, the untreated group exhibited a reduction in paw swelling beginning after day 6 after infection (Figure 3B), while the 830 nm light-treated group exhibited a continued increase in paw swelling at the same time. While the differences in swelling between untreated and treated mice at day 8 were not significant, it is possible that 830 nm light treatment of infected IL-10 KO mice may have either induced even greater paw swelling or delayed its onset.

Some factors may account for why 670 nm light had little effect on paw swelling, while 830 nm light appeared to cause potentially more swelling.

The more penetrative nature of 830 nm light, compared to the 670 nm wavelength (Giacci et al., 2014), may be a reason why the 830 nm light treatment may have resulted in these trends. It is possible that this higher degree of penetration led to a greater production of inflammatory molecules, resulting in greater edema and paw swelling. For example, 830 nm light treatment has been shown to increase NO production by fibroblast cells in a wound-healing study (Houreld et al., 2010). Since NO has been found to modulate NF-κB activity (Connelly et al., 2001), NO displacement by cytochrome c oxidase after light absorption may lead to NF-κB-induced transcription of pro-inflammatory mediators. These events could result in increased paw swelling after several days of 830 nm light treatment. Another potential reason for the different effects of 670 nm and 830 nm light treatment is that longer wavelengths may induce different effects on cells than shorter wavelengths. For example, treatment with 675 nm light induced maximum fibroblast proliferation, whereas 810 nm light treatment inhibited it (Moore et al., 2005). 830 nm light could potentially inhibit cellular proliferation, and could be a reason why 670 nm light had little to no effect on paw swelling, while mice treated with 830 nm light exhibited trends toward slightly increased swelling.

The sacrifice of the mice at day 8 after infection prevented a more complete examination of paw swelling, as well as cytokine levels and borreliacidal antibody titers. This was due to an unavailability of a

sufficient number of animals. When 670 nm light was administered from day 8 to day 14 after infection, paw swelling was examined for an additional six days after treatment ended, and some differences in paw swelling between the light-treated and untreated wild type mice were observed (Figure 1A). Therefore, in a future study, mice will be observed for longer periods after this early light treatment regimen.

To investigate the effects of PBM on pro-inflammatory cytokines in *B. burgdorferi*-infected mice, IL-17 and IFN-γ levels were measured. We found that 670 nm light had no significant effect on the production of IL-17 from unstimulated (Figure 4A) or stimulated (Figure 4B) splenocytes of infected wild-type C57BL/6 mice. This was expected, as wild-type C57BL/6 mice only exhibit mild arthritis after infection. This is due, in part, to their ability to produce lower levels of pro-inflammatory cytokines and higher levels of anti-inflammatory cytokines, such as IL-10, than arthritis-susceptible mice (Brown et al., 1999). In general, unstimulated cells from treated, infected wild-type mice appeared to produce more IL-17 than those from untreated mice after 48 hours of stimulation. However, these changes were not significant. It is possible that 670 nm light treatment was able to change the levels of IL-10, or other anti-inflammatory cytokines, such that there was an increasing pro-inflammatory microenvironment *in vitro*. However, this is not supported by our paw swelling data (Figure 2A).

We will additionally measure the levels of anti-inflammatory mediators in a future study.

IL-17 production by unstimulated spleen cells of infected IL-10 KO mice (Figure 5A) was, overall, greater than that of their wild-type counterparts (Figure 4A). This was expected, as C57BL/6 mice deficient in IL-10 develop greater inflammation than wild type mice (Brown et al., 1999). These results also support recent findings that a lack of IL-10 in these infected mice is associated with a higher production of IL-17 (Hansen et al., 2013). Stimulated spleen cells of 670 nm light-treated, infected IL-10 KO mice showed a reduction (albeit insignificant) of IL-17 after 24 hours of incubation (Figure 5B). Although these results did not reach statistical significance, they suggest that 670 nm light treatment may disrupt IL-17 production, despite conditions in which inflammation is generally favored. However, IL-17 production was notably less in stimulated cultures than in unstimulated cultures (Figure 5). Our laboratory has recently shown that stimulation of cells with *B. burgdorferi* can cause an immediate decrease in IL-17 production, and that this decrease can occur independently of IL-10 (Hansen et al., 2013). This suggests that anti-inflammatory mediators other than IL-10 may be affecting this reduction in IL-17, and that these factors may be upregulated by 670 nm light.

We also found that 670 nm light treatment did not significantly affect IFN-γ production by the spleen cells of infected wild-type mice, regardless of whether the cells were unstimulated or stimulated (Figure 6A and Figure 6B). However, we did observe that cells of treated mice produced less IFN-γ overall after 24 hours, although these results were not statistically significant. In addition, we found that infected IL-10 KO mice produced greater levels of IFN-γ (Figure 7) than their wild-type counterparts (Figure 6). This would also be attributed to the absence of IL-10 in these mice, which have a reduced ability to inhibit inflammatory mediators such as IFN-γ (Gazzinelli et al., 1996). Importantly, we found that cells of 670 nm-treated IL-10 KO mice produced significantly less IFN-γ than cells from untreated mice, regardless of whether the cells were unstimulated or stimulated, after 24 hours of incubation (Figure 7A and Figure 7B). We observed a similar, although insignificant, trend among unstimulated cells after 72 hours of incubation. Collectively, this suggests that 670 nm light treatment could reduce inflammatory cytokine production in this system. This was more clearly seen among cells of IL-10 KO mice, which exhibit clear inflammation and develop arthritis after *B. burgdorferi* infection (Hansen et al., 2013).

The effects of 670 nm light on IL-17 and IFN-γ levels were not consistent with our results of paw swelling. While 670 nm light treatment did not reduce paw swelling in infected IL-10 KO mice, it did significantly reduce

IFN-γ production. It is possible that inflammation may still occur even with relatively lower production of inflammatory cytokines, especially in the mice that lack IL-10. Histopathologic examination of the paws would be required to determine whether the differences in cytokine production are consistent with structural changes. Blocking IL-17 (Hansen et al., 2013) or IFN-γ (Sonderegger et al., 2012) reduced the severity of arthritis in *B. burgdorferi*-infected, IL-10 KO mice. Therefore, it is possible that the reduction in inflammatory cytokine production we observed among 670 nm light-treated mice may be sufficient to reduce pathology, if not edema

Treatment of infected wild-type mice with 830 nm light did not induce significant changes in the production of IL-17 by either unstimulated or stimulated cells (Figure 8A and Figure 8B). However, in general, cells from these treated wild-type mice produced greater levels of IL-17 levels than their untreated counterparts, regardless of whether the cells were unstimulated or stimulated. These increases were observed among unstimulated cells after 48 and 72 hours of incubation (Figure 8A) and among stimulated cells after 24 and 48 hours of incubation (Figure 8B). Importantly, a significant increase in IL-17 production was observed among unstimulated cells of treated IL-10 KO mice after 24 hours of incubation (Figure 9A). These results suggest that treatment with 830 nm light stimulates the IL-17 response, rather than alleviating it, in *B. burgdorferi*-infected mice. Based on the general increase of IL-17

production observed among cells of treated wild-type mice (Figure 8), it is
not surprising that IL-10 KO mice would exhibit even higher IL-17 levels
(Figure 9) and, in one case, a significant increase in its production (Figure
9A). It was surprising that 830 nm light treatment resulted in these
increases, even if most of these increases were not statistically significant.
However, the fact that most of these changes in IL-17 production were not
significant is consistent with the lack of significant changes we observed in
paw swelling (Figure 3). However, there was a trend towards increased
swelling in infected IL-10 KO mice following 830 nm light treatment, which
may be partially explained by the significant increases in IL-17 production
(Figure 9A).

Similarly, 830 nm light treatment did not significantly affect IFN-γ
production by cells of either wild type or IL-10 KO mice (Figure 10).
However, unstimulated cells of 830 nm light-treated, infected wild-type
mice exhibited a trend toward increased IFN-γ production after 72 hours of
incubation (Figure 10A). We are unable to determine the effect of 830 nm
light treatment on IFN-γ production among cells of infected IL-10 KO mice,
as many of these values exceeded the sensitivity of the assay (Figures
11A and 11B).

Collectively, these results suggest that 830 nm light treatment may
actually induce pro-inflammatory cytokine production and possibly cause

increased inflammation, especially in mice lacking IL-10. *B. burgdorferi-*
infected IL-10 KO mice have been considered to be a model of
dysregulated Lyme arthritis (Sonderegger et al. 2012), which may reflect
the mechanism of disease in many people. Thus, 830 nm light treatment
may exacerbate these effects, should this treatment regimen be used on
Lyme arthritis patients. As with 670 nm light treatment, whether 830 nm
light treatment has any clinical effect would require histopathological
examination of the paws. In addition, a transfer study using the spleen cell
supernatants of the 830 nm light-treated IL-10 KO mice could further
determine whether the increase in IL-17 production contributes to a pro-
inflammatory environment in paw inflammation.

Varying the parameters of light treatment may increase effectiveness in
future studies. It is possible that the 830 nm light treatment induced
increases in paw swelling (albeit insignificantly), increased IL-17
production, and failed to reduce IFN-γ levels due to a higher intensity or
shorter treatment time than what could have been effective for this Lyme
arthritis mouse model. While the use of 670 nm and 830 nm lights both
utilized a total fluence of 4.5 J/cm^2, the treatment times and intensity used
for each were different. 670 nm light was applied for 180 seconds, with an
intensity of 25 mW, while 830 nm light was applied for 90 seconds with an
intensity of 50 mW. Since duration of treatment is inversely related to
intensity, changing one of these parameters would inevitably change the

other. Irradiation time was shown to be an important factor in the effectiveness of PBM, regardless of fluence or intensity (Castano et al., 2007). Therefore, treatment time with 830 nm light could be increased in future studies.

A major question is how PBM can have such varying effects, as seen in this, and other, studies. A possible explanation for this is based on the phenomenon of hormesis, in which molecules that are typically considered harmful in high amounts could induce a helpful response at lower dosages (Mattson, 2008). In the context of PBM, light effectiveness is based on the biphasic dose response, which reflects hormesis. In cases which PBM was effective or did not cause damage, factors that are typically associated with an increase in inflammation, such as NO, reactive oxygen species (ROS), and NF-κB induction, may be immunoprotective, depending on the their concentrations. The 830 nm light may have triggered a higher production of NO, ROS, and other pro-inflammatory mediators than the 670 nm light, resulting in anti-therapeutic results. On the other hand, 670 nm light may have triggered a more "optimal" level of NO than the 830 nm treatment, which, in turn, causes cells to produce more cytoprotective factors that increase cellular protection. In this sense, it is possible that the 670 nm light triggers an amount of NO that is too small to cause damage, so that negligible damage is seen.

III. Specific Aim 2: Determine the effect of 670 nm and 830 nm light on *B. burgdorferi* retention in infected mice

The working hypothesis for this specific aim was that neither 670 nm nor 830 nm light would affect borreliacidal antibody production in *B. burgdorferi*-infected mice. To test this hypothesis, borreliacidal antibody titers were determined for both untreated, infected mice and infected mice treated with either 670 nm or 830 nm light. Our hypothesis was not supported. We saw that light treatment with either wavelength induced changes in borreliacidal antibody titers in both infected wild-type and IL-10 KO mice. This was surprising, since we did not expect systemic anti-pathogen changes to occur with this therapy. However, the effects of 670 nm and 830 nm light treatment support our observations of cytokine production, particularly in IL-10 KO mice.

We could not determine whether treatment with 670 nm light induced changes in borreliacidal titers in infected wild-type mice, as both groups exhibited high titers (≥40,960) that exceeded the detection level of the assay (Figure 12A). However, significant differences were observed in the borreliacidal antibody titers between untreated and 670 nm light-treated, infected IL-10 KO mice (Figure 12A). Untreated, infected IL-10 KO mice possessed a low borreliacidal antibody titer (160), indicating a reduced ability to eradicate the spirochete. In contrast, 670 nm light-treated, infected IL-10 KO mice possessed a considerably higher borreliacidal

antibody titer of 20,480. These results suggest that treatment with 670 nm light may have increased the ability of infected IL-10 KO mice to eliminate the spirochete. This is consistent with the reduced levels of inflammatory cytokine production, especially that of IFN-γ (Figures 6 and 7), observed with treatment at this wavelength.

In another experiment, untreated, infected wild-type mice exhibited a borreliacidal antibody titer of 5120. This was significantly lower than that of our study using 670 nm light. Treatment of infected wild-type mice with 830 nm light resulted in a higher borreliacidal antibody titer, with levels exceeding that which were detectable by the assay (≥40,960) (Figure 12B). By contrast, untreated, infected IL-10 KO mice possessed a borreliacidal antibody titer of 10,240. This also was significantly higher than that of our study using 670 nm light. However, treatment of infected IL-10 KO mice with 830 nm light reduced the borreliacidal antibody titer to 2560, suggesting that 830 nm light treatment reduced the ability of infected IL-10 KO mice to eliminate *B. burgdorferi*. This is consistent with the trends toward increased inflammatory cytokine production, especially that of IL-17 (Figures 8 and 9), that we observed in 830 nm light-treated mice.

IL-10 is a known regulator of B cell differentiation and antibody production (Rousset et al., 1992), so the low borreliacidal antibody titer exhibited by

untreated IL-10 KO mice was not surprising. However, it is interesting that 670 nm light treatment was capable of increasing the borreliacidal antibody titer of infected IL-10 KO mice. These results suggest that 670 nm light treatment was capable of inducing B cell antibody production via other mediators. IL-21 has been shown to play a role in the differentiation of B cells (Bryant et al., 2007). Therefore, 670 nm light may have increased the levels of IL-21, as well. However, IL-21 is a known Th17 cytokine, and the Th17 (IL-17) response was not increased in these mice. Alternatively, the increase in borreliacidal capability of the 670 nm treated IL-10 KO mice may have been due to the decrease in IFN-γ production of these mice. Recombinant IFN-γ has been demonstrated to decrease the borreliacidal antibody production by lymph node cells, while also decreasing the number of B cells of *B. burgdorferi*-infected C3H mice (Munson et al., 2002).

By contrast, 830 nm light treatment reduced borreliacidal antibody titers in IL-10 KO mice, indicating that B cell differentiation and/or antibody production may have been reduced after treatment. However, it is possible that the increase in borreliacidal antibody production in 830 nm light-treated wild type mice may be due to an increase in IL-21, as these mice exhibited trends toward increased IL-17 production (Figure 8). It is likely that light treatment affected additional B cell-associated cytokines, as well.

Chapter 5: Conclusions and Future Directions

The purpose of this thesis was to determine whether photobiomodulation using either 670 nm light or 830 nm light would help alleviate inflammation in murine Lyme arthritis. The central hypothesis for this study was that *PBM using 670 nm or 830 nm light will decrease inflammation in Lyme arthritis*. Two specific aims were pursued to test this hypothesis. The first aim was to determine the effect that 670 nm light and 830 nm light has on paw swelling and pro-inflammatory cytokine levels in *B. burgdorferi*-infected mice. The working hypothesis for this specific aim was that 670 nm and 830 nm light will ameliorate swelling and reduce IL-17 and IFN-γ production in *B. burgdorferi*-infected mice. The second aim was to determine the effect of 670 nm and 830 nm light on production of borreliacidal antibodies in infected mice. The working hypothesis for this specific aim was that neither 670 nm nor 830 nm light will affect borreliacidal antibody production in *B. burgdorferi*-infected mice.

Our hypothesis was partially supported, in that PBM using 670 nm light was capable of significantly decreasing IFN-γ production. However, it was unable to decrease the paw swelling exhibited in murine Lyme arthritis, and, surprisingly, it increased the borreliacidal antibody titer in a model of dysregulated disease. In addition, our hypothesis was not supported by the use of 830 nm light. Treatment with 830 nm light did not significantly

affect paw swelling, it significantly increased IL-17 production, it and reduced borreliacidal antibody titers in the model of dysregulated Lyme arthritis. Our results indicate that different wavelengths may possess varying effects on inflammation in Lyme arthritis. Histopathologic analysis of these paws is required to determine the most relevant effects of these treatments.

The timing of treatment administration is critical for the success of PBM. A potential reason for the lack of significant effects of PBM in this study is that the "optimal" degree of inflammation has not been inflicted in the mice. While an underlying principle of how photobiomodulation works is that tissue must be damaged in order for it to be repaired by red or NIR light, it is unknown to what degree the tissue must be damaged. It would be reasonable to hypothesize that cells cannot be too damaged, to the point in which the light cannot induce protective factors to repair the damage. It is possible that too early of a treatment may not be effective, as there may not be enough regenerative mediators involved in tissue repair to promote adequate regeneration at the time of LED application. In addition, the severe inflammation in our infected IL-10 KO mice may have prevented any noticeable change in swelling to occur. Also, our study used extremely small sample sizes. Naturally, wide variations among the data could be expected in this system. In cases in which significant cytokine changes were observed, all of the values of one group were

higher than all of the values of the other group. In some cases, all of the values of one group were higher than all but one of those in the other group, which resulted in statistically insignificant results. A larger sample size is needed in future studies.

Following this experiment, one of the next steps would be to investigate the histopathologic changes in the paws after light treatment to determine the severity of arthritis. This would also provide a clearer indication of the tissue or cellular changes by light treatment. It would also be beneficial to determine the time point at which peak levels of particular growth factors, collagen, osteoblasts, and synoviocytes are present, and start treatment then to determine whether PBM has any therapeutic effects. Treatment could also begin on days of peak growth factor and cellular generation rates. Doing so could enhance the effects of these mediators and potentially significantly decrease inflammation and increase regeneration of cells in the joints.

It is also possible that the particular time of day treatment was administered was ineffective for the treatment of disease. Effects of light vary depending on the circadian rhythm of experimental animals. For example, treatment of neural inflammation in an EAE mouse model of multiple sclerosis showed that 670 nm light loses clinical effect when administered in the afternoon (J. Lyons, personal communication). In this

thesis, light treatment was given in the afternoon. As mice are nocturnal animals, 670 nm light treatment may be more effective if the light was applied in the evening, at a time when metabolic rates of mice were higher. Similarly, 830 nm light treatment may exert an anti-inflammatory effect in IL-10 KO mice if treatment had been given during the evening. Future studies should test this hypothesis by administrating 670 nm or 830 nm at a later time of day in *B. burgdorferi*-infected mice.

In addition, the effect 670 nm light may have on inducers and products of IL-17 activity should be investigated. IL-17 has been found to trigger the generation of IL-6 in the synoviocytes of rheumatoid arthritis patients (Chabaud et al., 1999), indicating a possible positive feedback loop between IL-17 and IL-6. Nardelli et al. (2008b) demonstrated that *Borrelia*-vaccinated and-infected mice administered both anti-IL-6 and anti-TGF-β antibodies exhibited a significant decrease in the severity of joint pathology, showing that both cytokines play a role in joint inflammation. Since Th17 cell differentiation is induced by IL-6 and TGF-β (Veldhoen et al., 2006), the levels of these two cytokines could also be affected by PBM. Investigating whether PBM has any effect on these cytokines, and whether these levels are consistent with that of IL-17, would lend greater information about its effect on IL-17.

Our results showed that infected IL-10 KO mice treated with 670 nm light produced less IL-17 and IFN-γ and higher borreliacidal antibody titers than untreated mice. Since IL-10 is required for the attenuation of IL-17-induced inflammation in murine Lyme arthritis (Hansen et al., 2013), as well as IFN-γ-mediated inflammation in general (Fiorentino et al., 1989), understanding the effects PBM may have on IL-10, specifically, would be necessary. This is particularly important, as we observed increases in inflammatory cytokines following treatment of infected IL-10 KO mice with 830 nm light.

In conclusion, we showed that treatment with 670 nm light resulted in general reductions of inflammation in the IL-10 KO mouse model of dysregulated Lyme arthritis. By contrast, treatment with 830 nm light resulted in general increases in inflammation in these mice. Additional studies are required to further define the effects of photobiomodulation on the symptoms of Lyme arthritis.

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