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Identification of Immunomodulatory Cells Induced By 670 nm Light Therapy in an Animal Model of Multiple Sclerosis

Erin Christine Koester
University of Wisconsin-Milwaukee

IDENTIFICATION OF IMMUNOMODULATORY

CELLS INDUCED BY 670 NM LIGHT THERAPY IN

AN ANIMAL MODEL OF MULTIPLE SCLEROSIS

by

Erin C. Koester

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ABSTRACT

IDENTIFICATION OF IMMUNOMODULATORY CELLS INDUCED BY 670 NM LIGHT THERAPY IN AN ANIMAL MODEL OF MULTIPLE SCLEROSIS

by

Erin C. Koester

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Multiple sclerosis is an autoimmune, demyelinating disease characterized by neurodegeneration and inflammation of the central nervous system. It affects approximately 250,000 people in the United States alone, with women being affected two times more than men. Experimental Autoimmune Encephalomyelitis (EAE) is the primary animal model of MS, sharing clinical signs and histopathology with MS. The current paradigm supports MS/EAE induction by myelin reactive $CD4^+$ T cells that cross the blood brain barrier to induce an inflammatory response that leads to the destruction of the myelin sheath and eventual loss of axons. Recent data suggest that axonal loss and disease progression are due to accumulation of oxidative stress. Most of the current therapies for MS are only useful early in the disease process, slowing disease progression but not preventing it, probably because they do not affect oxidative stress. With previous studies showing relevance of mitochondrial dysfunction to neurodegeneration, new

therapies designed to maintain mitochondrial function could prove beneficial. Photobiomodulation is an alternative therapy proven effective in the treatment of chronic inflammation and neurodegeneration. Previous data demonstrated that 670nm near-infrared (NIR) light-emitting diode (LED) is effective in the amelioration of the disease in a mouse model of MS. Furthermore, experiments suggested an important role for anti-inflammatory cytokines, particularly Interleukin-10 (IL-10), and maintenance of mitochondrial function as important mechanisms mediating disease amelioration. The current studies sought to characterize the immune cell population induced by photobiomodulation which is responsible for the clinical effect noted. With a deeper understanding of the mechanism of protection against disease, the 670nm light would be a promising adjunct therapy for the treatment of MS.

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LIST OF ABBREVIATIONS

°C: Degree Celsius

AAALAC: Association for Assessment and Accreditation of Laboratory Care

Ag: Antigen

ANOVA: Analysis of variance

APC: Allophycocyanin

ATP: Adenosine triphosphate

AUC: Area under the curve

B6 mice: C57BL/6 mice

Breg: Regulatory B cell

CD: Cluster of differentiation

cDNA: Complementary Deoxyribonucleic acid

CFSE: Carboxyfluorescein succinimidyl ester

CNS: Central nervous system

CO_2: Carbon dioxide

CSF: Cerebrospinal fluid

Ct: Threshold cycle

DNA: Deoxyribonucleic acid

dpi: Day's post immunization

EAE: Experimental autoimmune encephalomyelitis

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme linked immunosorbent assay

FBS: Fetal bovine serum

FITC: Fluorescein isothiocyanate

FoxP3: Forkhead box P3

g: Grams

GFP: Green fluorescent protein

H_2SO_4: Sulphuric acid

HBSS: Hanks balanced salt solution

HCl: Hydrochloric acid

HRP: Horse radish peroxidase

IFA: Incomplete Freund's adjuvant

Ig: Immunoglobulin(s)

IgG: Immunoglobulin G

IL: Interleukin

IL10$^{-/-}$: Interleukin 10 deficient

IL10/GFP mice: Interleukin 10 Green fluorescent protein mice

IFN-γ: Interferon gamma

iTreg: Induced regulatory T cell

LED: Light-emitting diode

LLLT: Low-level light therapy

LSM: Lymphocyte separating medium

MBP: Myelin basic protein

MHC: Major histocompatibility complex

ml: Milliliter

mM: Millimolar

MOG: Myelin oligodendrocyte glycoprotein

mRNA: Messenger Ribonucleic acid

MS: Multiple Sclerosis

NFκ-B: Nuclear factor kappa-light-chain-enhancer of activated B cells

ng: Nangrams

NIH: National Institute of Health

NIR: Near-infrared

NO: Nitric oxide

nTreg: Natural regulatory T cell

OCBs: Oligoclonal bands

PBM: Photobiomodulation

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PE: Phycoerythrin

PerCP-Cy5.5: Peridinin chlorophyll 5.5

PLP: Proteolipid protein

x

PP-MS: Primary progressive multiple sclerosis

PT: Pertussis toxin

qPCR: Quantitative polymerase chain reaction

qRTPCR: Quantitative reverse Transcriptase Polymerase Chain reaction

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RPMI medium: Roswell Park Memorial Institute medium

RR-MS: Relapsing emitting multiple sclerosis

RT: Reverse transcription

SP-MS: Secondary progressive multiple sclerosis

TE buffer: Tris-EDTA buffer

TGF-β: Transforming growth factor beta

Th: T helper cell

TMB: Tetramethylbenzidine

TMEV: Theiler's murine encephalomyelitis virus

TNF-α: Tumor necrosis factor alpha

Treg: T regulatory cells

Tris-HCl: Tris Hydrochloric acid

WT: Wild type

β-actin: Beta actin

Δ: Delta

μl: Microliter

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CHAPTER I: INTRODUCTION

Multiple Sclerosis

Multiple sclerosis (MS) is a disease of unknown etiology characterized by inflammation, loss of myelin, axonal damage, and neurodegeneration of the central nervous system (CNS) [1]. MS is thought to be a CD4^{+} T cell-mediated autoimmune disorder [2]. It affects an estimated 250,000 people in the United States alone [3]. As seen with many autoimmune disorders, MS plagues more females than males and is typically diagnosed between 20 to 40 years of age [1]. While the general etiology of the disease is unknown, MS affects genetically susceptible individuals, with contribution from environmental factors [4].

The symptoms of MS, including sensory disturbances, memory loss, double vision, muscle weakness and paralysis, vary from individual to individual making it a difficult disease to diagnose [5].   Paralysis and loss of function are hallmark symptoms of MS seen in advanced stages of the disease. Studies suggest that a cascade of events beginning with mitochondrial dysfunction and immune infiltration lead to axonal loss that contributes to MS pathology and disease progression [6].   While current therapies exist to slow disease progression and to treat the symptoms of the disease, the overall goal of lasting disease amelioration has yet to be realized.

Three main clinical phenotypes of MS have been identified: relapsing-remitting MS (RR-MS), primary progressive MS (PP-MS), and secondary progressive MS (SP-MS) [1]. The majority of patients display RR-MS in the beginning stages of the disease. This phenotype is characterized by bouts of acute inflammation, resulting in neurological dysfunction, followed by periods of remission [3,7]. Typically, RR-MS patients enter

into the SP-MS within in 5-10 years of diagnosis, characterized by progressively worse disease without the relief of remission. Finally, approximately 15% of MS patients have PP-MS. From the onset of PP-MS, patients experience a progressively worse disease, which in severe cases can be quite rapid, again without any bouts of remission [3, 4]. It is not clear, however, the exact factors that trigger the disease into these distinct disease courses [4]. Figure 1 shows a representation of the typical disease course associated with each clinical phenotype [8].
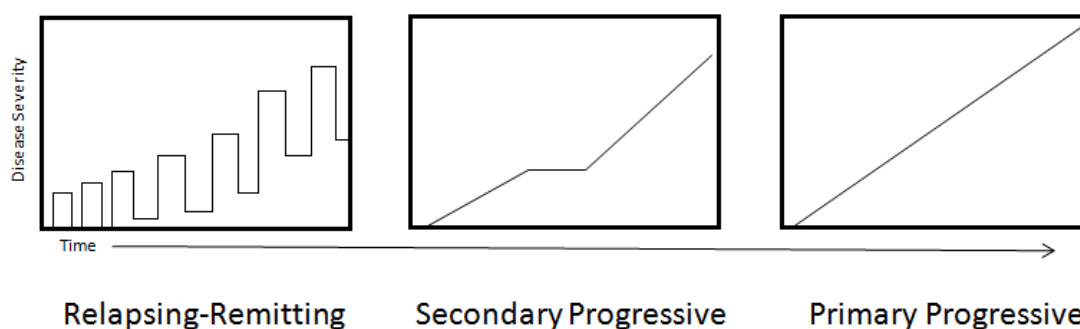
Figure 1. Graphical representation of the 3 main clinical phenotypes of MS.

Experimental Autoimmune Encephalomyelitis

Much has been learned about the pathogenesis of MS through experiments in the Experimental Autoimmune Encephalomyelitis (EAE) animal model. EAE is also routinely used to identify therapeutic targets and to investigate novel therapies [9]. EAE mimics the clinical signs, histopathology, and disease mechanisms associated with MS. The clinical course of EAE presents as bouts of partial to full hind-limb paralysis, progressing to forelimb involvement with severe disease. Like MS, EAE is a CD4$^+$ T cell-mediated demyelinating disease of the CNS. The disease is initiated through active

immunization using myelin basic protein (MBP), proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG) [10] emulsified in an adjuvant that will promote an inflammatory response to the myelin proteins [11]. PLP and MBP are major constituents of myelin, located internally, important to maintaining the structural integrity of the myelin sheath, whereas MOG, a minor contributor to myelin, is a type I transmembrane protein located on the outer surface of the myelin sheath and oligodendrocytes [12-14]. Due to its external location, MOG is considered a candidate antigen for the initiation of MS/EAE, whereas PLP and MBP are thought to be important to disease progression or relapse [14]. Several studies using anti-MOG antibodies have supported its role as an important auto-antigen in demyelinating CNS diseases.

Similar to MS, the EAE disease course varies depending on the species, strain and antigen used. Each of these different models previously mentioned is considered a model of the stage of MS that it mimics. For example, disease in Lewis rats is an acute, monophasic disease, while disease in SJL mice presents as a relapsing/remitting disease reminiscent of the early stage of MS in patients. EAE in C57BL/6 mice, initiated with MOG35-55, used in the proposed studies, presents as a chronic relapsing/progressive disease similar to the chronic phase noted in established MS patients. Figure 2 depicts disease course based on species, strain, and peptide used. Auto-antibodies reactive to MOG contribute to the immunopathological mechanisms of the disease, including demyelination and axonal loss with the CNS [12,13].
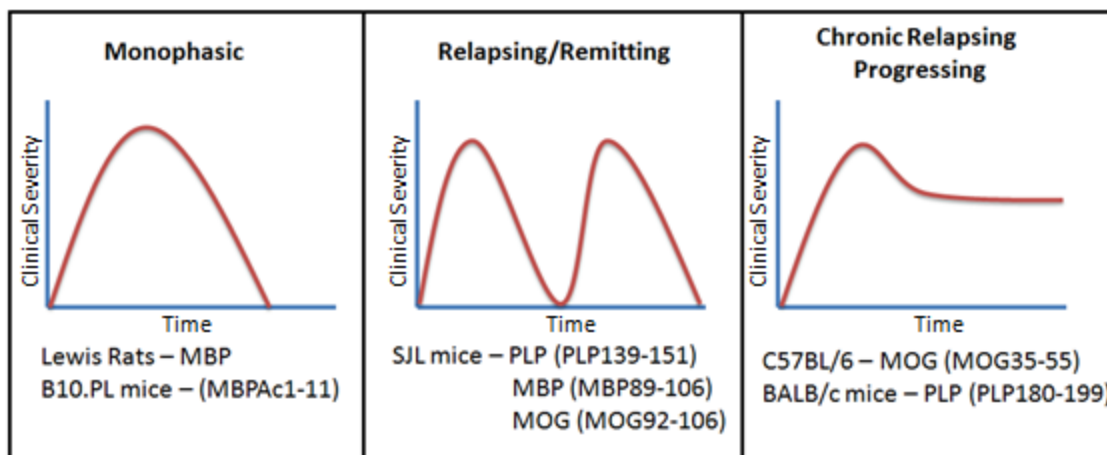
Figure 2. Disease course based on species, strain, and antigen used for onset of disease.

Immunopathogenesis of MS/EAE

Autoimmunity

In a healthy individual, CD4+ T cells regulate the immune system. In the case of autoimmunity, the body cannot distinguish between self and non-self, thus creating an immune response to its own cells and tissues. Typically, autoreactive T cells with a high affinity toward self-peptide are eliminated during maturation within the thymus, termed "central tolerance" [15]. When these autoreactive T cells evade deletion, autoimmunity may occur. In healthy individuals, these cells remain in a quiescent state, termed "peripheral tolerance". Multiple mechanisms of peripheral tolerance have been proposed [16]. Similarly, multiple mechanisms have been proposed by which tolerance is broken, leading to the onset of autoimmunity [16-19].

One hypothesis is put forth to explain the onset of autoimmunity is molecular mimicry. In this case, small pathogenic peptide fragments that share similar structures or sequences as self-antigens are delivered onto the major histocompatibility complex

(MHC) -class II molecules [15]. Similarly, this results in reactivity toward self-antigens and elicits a cascade of inflammation and neurodegeneration. Another hypothesis is that autoimmunity, including MS, is initiated through a viral infection. Theiler's murine encephalomyelitis virus (TMEV) is a CD4^{+} T cell-mediated demyelinating disease that mimics MS-like symptoms [17, 18]. In this model, low levels of the infectious virus can be detected months after inoculation within the CNS [18]. Demyelination is brought on by virus-specific Th1 cells and autoreactive T cells are stimulated containing virus-encoded superantigens [17]. The final hypothesis put forth is epitope spreading. Epitope spreading occurs when epitopes, distinct from the epitope inducing the disease, become reactive during chronic inflammation [19]. In this case, encephalitogenic myelin proteins are primed secondary to myelin destruction with CD4^{+} T cells specific for multiple epitopes [19]. These three hypotheses are represented in Figure 3. While all of these are plausible explanations of the evolution of MS, it is most likely the combination of the factors that play a role in disease initiation and progression [15].
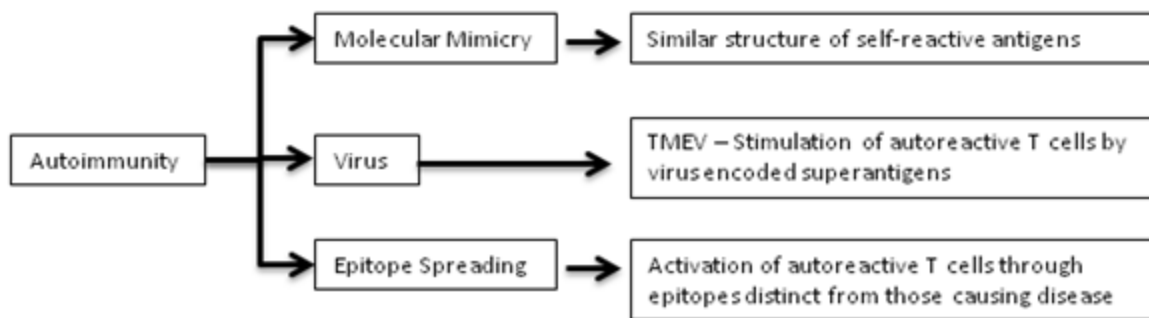
Figure 3. Plausible explanations for autoimmunity in MS/EAE

T cells

The role of T cells in the pathogenesis of MS/EAE was introduced over 30 years ago by Ben-Nun *et al.* through the adoptive transfer of T cells specific for CNS auto-antigens [20, 21]. Extensive research supports the understanding that MS/EAE are initiated by MHC class II restricted, myelin-reactive CD4+ T cells [4, 7, 22]. These cells are activated in the periphery and infiltrate the CNS, leading to disease pathology [23].

Effector T helper (Th) cells are responsible for secretion of cytokines, and are further broken down into subsets, including but not limited to, Th1, Th2, and Th17 cells. Th1 cells, generated in the presence of Interleukin (IL)-12 and IL-18, are characterized as pro-inflammatory cells. This population of cells is defined by secretion of pro-inflammatory cytokines, IL-2 and Interferon-gamma (IFN-γ) [20, 23]. These are cytokines that play a central role in initiation of encephalitogenic T cells, as wells as the progression of CNS inflammation in MS and animal models [23, 24]. Differentiation into the Th2 subset occurs in the presence of IL-4. These cells are considered anti-inflammatory and are known to be protective in EAE/MS [25]. This population is characterized by the secretion of the cytokines, IL-4, IL-5, IL-13, and IL-10. It has been recently hypothesized that Th17 cells also play a large role in MS pathology [26]. This population, induced in the presence of transforming growth factor beta (TGF-β) and IL-6, secrete the pro-inflammatory cytokine IL-17 [27]. Together, Th1/Th17 plays a role in MS induction and pathogenesis [27].

Th1 cytokines are responsible for the recruitment and activation of inflammatory cells. IFN-γ is thought to be central in mediating the pathogenesis of MS/EAE. IFN-γ facilitates the activation of macrophages. It is a pro-inflammatory cytokine that drives a

chronic inflammatory pathway leading to tissue injury [28]. However, the understanding of the role of IFNγ in the disease process is confounded by the observation that the deletion of IFN-γ does not eliminate the disease but increases the clinical disease and pathology [24]. This exacerbation is not only seen in EAE, but in other Th1 autoimmune conditions such as experimental autoimmune uveitis [24]. This finding indicates that IFN-γ is not only responsible for disease progression, but also aids in the regulatory functions of the immune system.

Tumor necrosis factor-α (TNF-α) is typically released in response to a bacterial infection and is associated with a Th1 cell response eliciting CNS inflammation. Like IFN-γ, however, the mice deficient of this cytokine go on to develop a worse disease than their wild-type (WT) counterparts [25]. In a study by Probert *et al. o*ver-expression of TNF-α has also been found to be cytotoxic to oligodendrocytes resulting in severe demyelination [29].

Elevated levels of IL-17, produced from Th17 cells, have also been established in MS patients [30]. A study by Komiyama *et al.* demonstrates the pathogenic role that IL-17 plays in MS/EAE.   Mice deficient in IL-17 display a less severe disease course than WT mice, indicating that IL-17 plays an important role in the development of EAE. Interestingly, mice devoid of IFN-γ had higher IL-17 production compared to the WT controls. This further blurs the role of IFN-γ as it displays both pathogenic and protective roles as a mediator of Th1 cells as well as a suppressor of IL-17 cytokines [31].

Recent data suggest that chronic inflammatory diseases such as MS, Th1 cells play a pathogenic role, whereas Th2 cells display protective qualities [32]. Th2 cells counter the roles of Th1 cells through the release of anti-inflammatory cytokines that aim

to down-regulate the production of pro-inflammatory cytokines. Th2 cells are protective in both MS and EAE through the release of IL-10 as well as another anti-inflammatory cytokine, IL-4. In addition to their anti-inflammatory processes, these cytokines also inhibit the demyelinating functions of macrophages [32]. In past studies, patients exhibiting elevated levels of Th2 cells due to parasitic infections have shown milder forms of MS [23]. Fleming and Fabry have proposed the use of parasites as a therapy for autoimmune diseases due to the increased production of Th2 they afford [33]. A representation of a naïve T cell into its effector and suppressive subsets is seen in Figure 4.
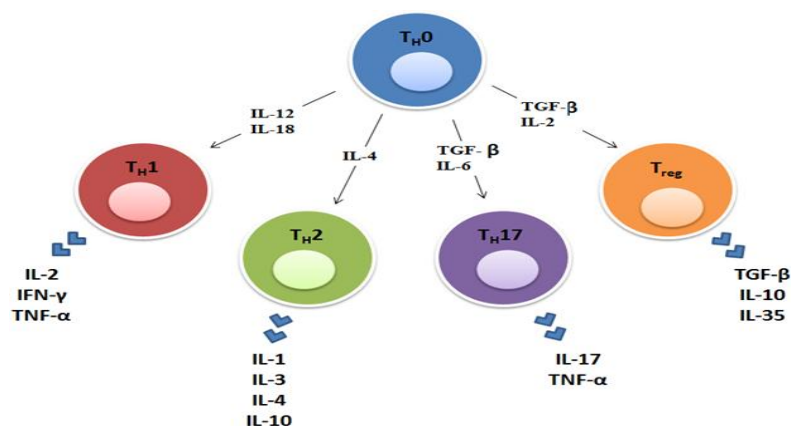
Figure 4. Cytokines involved and secreted in the differentiation of T cells.

Secretion of IL-10, through subsets of regulatory B cells (Bregs) and regulatory T cells (Tregs), aids in the regulation of the immune response. IL-10 mediates the response through the inhibition of pro-inflammatory cytokines as well as causing T cell hyporesponsiveness, or loss of T cell function. Its role as an anti-inflammatory cytokine is further confirmed in IL-10 deficient mice. Induction of EAE in these knockout mice is associated with a worse disease course compared to wild-type controls. Because these

mice are lacking IL-10, the balance is shifted to a pro-inflammatory state resulting in a worse disease course [34]. Furthermore, induction of IL-10 in current MS therapies proves to be an effective means to ameliorate the disease [35].

IL-4, like IL-10, is an anti-inflammatory cytokine as it amplifies the response of Th2 cells. Through the inhibition of Th1 cells, it decreases the production of TNF-α and IFN-γ [25]. Th1 and Th2 cells play antagonistic roles. Altering disease course from being Th1-dominated to being Th2-dominated typically result in positive clinical effects for the MS and EAE [36].

B cells

While the specific role of B cells in MS/EAE pathology remains unclear, they are thought to play a role in the disease process. B cells reactive to MOG have been found in the CNS of MS patients [37]. B cells, upon antigen challenge, differentiate into plasma cells and produce antibodies. Antibodies for myelin components are present in the cerebral spinal fluid (CSF) of MS patients [37]. Oligoclonal immunoglobulin G (IgG) bands (OCBs) are found within the CSF are present in 95-100% of MS patients and represent immunoglobulin synthesized within the CNS. The presence of OCBs is typically associated with a worse disease prognosis [37]. A study by Lucchinetti et al. indicates that high numbers of antibody deposits and complement activation are characteristic of demyelinated lesions found through histopatholigical studies of MS patients [38]. The activation of complement lends a hand to demyelination through pathogenic antibodies as well as antibody-mediated functions [39-42].

Regulatory Immune Cells

Regulatory T cells

The involvement of regulatory T cells (Tregs) in autoimmune disorders is still under debate [43]. While autoreactive T cells are found in all individuals, those with autoimmunity exhibited low levels or dysfunctional CD4$^+$ Tregs [43-45]. A study done by Viglietta et al. demonstrated reduced numbers of CD4$^+$ Tregs in MS patients compared to the healthy controls [44]. Contradictory studies have been publish which show no difference in the number of circulating Tregs, however, the suppressive functions of the Tregs seem to be decreased within the patient population in comparison to the healthy controls [43]. Whatever the case may be, these studies indicate the functional role of these cells in protection against an autoimmune response.

In contrast from effector Th cells, Tregs are responsible for the down-regulation of antigen-specific T-cell responses and are important in the prevention of autoimmunity. In the thymus, autoreactive T cell clones with high avidity are eliminated through negative selection mediated by programmed cell death [46]. The autoreactive cells that escape clonal deletion within the thymus and enter the peripheral circulation are those responsible for the onset of autoimmunity. While exact mechanisms are unknown, Tregs in EAE display protective qualities by increasing the Th2 phenotype toward an anti-inflammatory state [46].

Forkhead box P3 (FoxP3) is an X-linked transcription factor considered to be the most specific marker of Tregs [46]. The gene was originally found in scurfy mice in which the gene was mutated. These mice possess a mutated FoxP3 gene, leading to a fatal lymphoproliferative autoimmune disorder in these mice [43]. In transgenic mice

with a targeted disruption of the FoxP3 gene (and subsequently $CD4^+CD25^+$ cells), an almost identical condition was noted [43]. Thus, FoxP3 is considered to be a master regulatory molecule in Tregs [46].

The regulatory activity of CD4+ T cells is seemingly increased with high expressions of the surface marker CD25 [47] in natural Tregs (nTreg), or $CD4^+CD25^+$ T cells that develop and mature in the thymus for immune regulation [23]. These nTregs that express CTLA-4 (cytotoxic T cell lymphocyte antigen 4) and CCR4 (chemokine receptor) arise from previously activated cells [48] and acquire FoxP3 expression during maturation within the thymus [49].

Induced Tregs (iTreg) are $CD4^+CD25^-$ T cells that acquire CD25 and FoxP3 expression in the periphery instead of the thymus in response to inflammation or autoimmunity [48]. These Tregs constitute ~5-10% of circulating $CD4^+$ T cells [43]. iTregs are induced with IL-2 and TGF-β [48]. While they have a similar phenotype as nTregs, these cells arise from naïve $CD4^+$ T cells [48]. These cells are responsible for the secretion of the anti-inflammatory cytokine, IL-10, relevant to the decrease of MS severity [23].

While they function quite differently, there is a link between Th17 cells and Tregs. Wahl *et al.* was the first to show the importance of TGF-β in Treg development. TGF-β is required for the differentiation of both T cell lineages (Figure 5). However, IL-6 is also needed for Th17 production [49]. The dichotomy between Th17 and Treg cells is regulated at the transcriptional level. The transcription factor RORγt is required for Th17cells whereas Tregs require FoxP3 [49-51]. In findings from Zhou *et al.* FoxP3, from iTregs, has the ability to suppress RORγt, however, in the presence of IL-6, this

suppressive activity is abrogated [49, 51]. Additionally, iTregs are resistant to the effects

of IL-6 and display a noticeable decrease of IL-6 receptors on their surface compared to

nTregs [48]. From a study done using green fluorescence protein (GFP) cells from

FoxP3^{-/-} mice indicates that activation of nTregs with IL-6 results in a markedly

decreased FoxP3 expression [48]. After treatment with IL-6, these nTregs were unable to

increase the survival rate of mice with graft versus host disease [48]. In the presence of

IL-2, however, there is a down-regulation of IL-6 receptor expression and signaling [48].

These studies demonstrate the importance of the environment in which T cell activation

occurs.
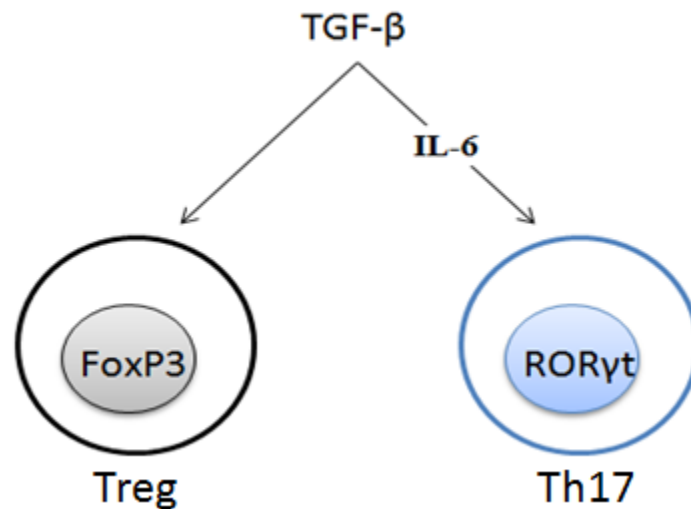
Figure 5. Differentiation between Tregs and TH17 with the induction of IL-6.

The role of CD8^{+} Tregs has been overlooked since the emergence of CD4+ Treg

involvement in autoimmunity. CD8^{+} Tregs are also important in the suppression of

MS/EAE. Through the secretion of IL-10, CD8^{+} Tregs directly control the production of

IFN-γ and the proliferation of CD8^{+} effector cells without the use of antigen presentation [52]. While the exact role of FoxP3 is not understood, recent studies have shown FoxP3 expression in CD8^{+} T cells occurs after antigen activation affording suppressive activity [53]. Because the role of CD8^{+} Tregs has not been extensively studied as CD^{+} Tregs, the distinctive cellular markers are not fully known [52]. Correale and Villa found that CD8^{+} Treg specific clones that recognize and lyse myelin-reactive CD4^{+} T cells responsible for the induction of MS, are down-regulated in MS patients, specifically within the CSF [52, 54]. Supporting this finding, lymph cells retrieved from immunized mice revealed high populations of CD8^{+} Tregs when analyzed using flow cytometry [54].

Regulatory B cells

Regulatory B cells (Bregs) also act as regulators of the immune response through the release of IL-10 [40, 41]. Bregs secrete cytokines that down-regulate the function of autoreactive T cells as well as inflammation [40]. A study by Matsushita et al. proposes that Bregs reduce disease severity during EAE initiation and Tregs inhibit the late stage effects of MS [40]. In this study, the depletion of Bregs before and during disease initiation led to increased disease severity, whereas depletion of Bregs after the immunization had no effect on the disease severity [40]. Conversely, Treg depletion before EAE onset delayed onset by 2 days and did not significantly disturb the severity, however, depletion of Tregs after onset resulted in a significantly worse disease course [40]. The exact interactions of B cells and Tregs in the activation and progression of the disease remain unknown [40, 41].

Role of Mitochondria

Evidence suggests that mitochondrial dysfunction actively contributes to axonal injury and neurodegeneration [55]. The mitochondria, or the "power house" of the cell, serve a variety of functions, including regulation of the electron transport chain, adenosine-triphosphate (ATP) synthesis, ion homeostasis, lipid metabolism and apoptosis [56, 57]. ATP synthesis mainly occurs within the inner membrane of the mitochondria consisting of the electron transport chain [58]. While low levels of reactive oxygen species (ROS) are generated by the mitochondria through oxidative phosphorylation, mitochondrial antioxidant enzymes are produced to effectively remove these by-products [58].

The mitochondrial membrane houses the electron transport chain. This chain is responsible for the production of ATP within the cell. The chain is made up of 4 transmembrane complexes known as Complex I, Complex II, Complex III, and Complex IV [59]. Throughout the transport chain, protons are translocated across the membrane to produce a proton gradient. The electron flow travels throughout the other complexes where it meets up with the final electron acceptor, oxygen. From here hydrogen and oxygen combine to form a water molecule. The proton gradient generated is then used for ATP production via oxidative phosphorylation [59]. Axonal injury and mitochondrial defects, due to MS, occur within each of these complexes. These defects are responsible for excessive ROS and loss of ion homeostasis [55]. When ion homeostasis is lost and energy demands exceed ATP production, the balance of ion levels is lost eventually resulting in axonal degeneration and progressive neurological disability [55].

The mitochondrial free radical theory of aging (MFRTA) involves the generation of potentially damaging ROS in the mitochondria [57]. This theory explains that the mitochondria generate ROS that have damaging effects within all types of molecules [57]. When the production of reactive oxygen species overwhelms the antioxidant mechanism of the cell, oxidative stress leading to damage of proteins, lipids, and nucleic acids occurs. This oxidative damage cannot be repaired [60, 61]. When oxidative damage is excessive, signaling mechanisms are in place, which lead to the death of the cell by apoptotic mechanisms [60]. Mitochondrial dysfunction is thought to play a central role in oxidative damage leading to apoptosis [27].

Recent data suggest that accumulation of oxidative stress within the CNS plays a critical role in the progression of MS/EAE [62, 63]. The current paradigm supports the notion that MS initiation and myelin destruction are autoimmune in nature, while axonal degeneration, mediated by accumulation of oxidative stress, is responsible for the progression of the disease and the onset of permanent disability [62]. It was initially thought that axonal degeneration coincided with bouts of inflammation. However, when inflammation subsided, axonal degeneration persisted, indicating other factors were at play [63]. A study done by Qi et al. showed that mitochondrial oxidative stress plays a role in neurodegeneration before the infiltration of the inflammatory cells. This study demonstrated that suppression of mitochondrial oxidative stress, which began before the infiltration of inflammatory cells, ameliorates MS/EAE neurodegeneration [62]. The increases of mitochondrial defenses against ROS suppressed the loss of the mitochondrial membrane potential and protect the axon [62].

With the loss of the myelin sheath surrounding the axon, sodium channels are redistributed along the axon, and axonal transport becomes inefficient. Therefore, the mitochondria are dispersed to the site of demyelination to maintain conduction [27,63]. With this localization, the mitochondria are exposed to large amounts of reactive oxygen and reactive nitrogen species, such as nitric oxide (NO), which inhibits the function of cytochrome c oxidase and cause mitochondrial DNA damage [63]. Initially, these mitochondria are able to function at full capacity. However, eventually their function is compromised [27], resulting in damage to the respiratory chain, leading to impairment of ATP synthesis [63]. This has been noted in a study by Dutta *et al.* comparing the motor cortex of six control patients in comparison to six MS patients. Their findings suggest that diminished products from the electron transport chain in the MS patients, indicating that mitochondria in these patients have limited capacities [64]. It is proposed that the compromised mitochondria contribute to the axonal degeneration as well as neurological disability within these MS patients [64].

FDA-approved therapies for MS exist to slow disease progression or manage symptoms but do not stop it. These include immunomodulatory and immunosuppressive therapies that work with RR-MS, however are less effective on later stages of the disease [22]. This is presumably because they fail to address the mechanism of oxidative stress and provide a neuroprotective component.

Photobiomodulation

Photobiomodulation (PBM), or low level light therapy (LLLT), uses light in the far-red (FR) to near-infrared (NIR) range of 630-1000nm to regulate many cellular

functions [65]. For over 40 years, light-emitting diodes (LED) and low-energy lasers have been used to heal soft tissue injuries at an accelerated rate and to relieve chronic inflammation [66]. Recently, it has also been proven beneficial in restoring or preserving mitochondrial dysfunction, increasing energy metabolism, and improving cell viability through the activation of cellular photoacceptors, such as cytochrome c oxidase, as well as activation of transcription factors [22, 65, 67].

Within the mitochondria, a balance between ATP production and the production of ROS must be met in order to prevent inflammation [61]. Several *in vitro* studies have demonstrated an increase in ATP production in the presence of the light therapy [61]. While mitochondria produce increased amounts of ATP due to NIR-LED treatment, it is also believed that PBM modulates ROS through a brief up-regulation, which induces transcription factors [61, 68]. The induction of transcription factors are directly linked to increased cell proliferation, modulation of cytokine levels, and inflammatory mediators [68].

PBM uses the absorption of specific wavelengths of light to cause biological changes within the cell through the activation of signaling pathways [69]. These effects for PBM are mediated through improving energy metabolism and increasing ATP production. In mammalian tissues, there are over 50 mammalian photoacceptors known to absorb light within this range [67, 69]. Three major photoacceptor molecules are hemoglobin, myoglobin, and cytochrome c oxidase [65]. Of these, cytochrome c oxidase of the electron transport chain, is the only tissue associated with energy functions [65, 67, 69]. Up to 50% of NIR light administered to cells is absorbed by mitochondrial chromophores, including cytochrome c oxidase [65]. Evidence of cytochrome c oxidase

as a photoacceptor is also supported by experiments in which cytochrome c oxidase is inhibited [66]. Action and absorption spectra have also been used as evidence for cytochrome c oxidase as a photacceptor [66]. The absorption spectrum indicates wavelengths of light in which light is removed from the spectrum, and the action spectrum indicates the biological responses [66]. Because the absorption spectrum for cytochrome c oxidase parallels that of the protective action spectrum of the FR/NIR wavelengths, it is believed that cytochrome c oxidase is the primary terminal electron acceptor of the mitochondrial electron transport chain [65, 67].

The exact mechanism by which the light influences cytochrome c oxidase has yet to be elucidated; however it is believed that a link between NO and cytochrome c oxidase is relevant [68]. Two pathways have been proposed as possible mechanisms for PBM [61, 68, 70]. NO decreases cell respiration by binding to cytochrome c oxidase and displacing oxygen [70]. PBM prevents binding of NO to cytochrome c oxidase through the dissociation of NO to cytochrome c oxidase [68, 70]. High levels of NO have been reported in cell cultures after PBM due to this dissociation [70]. The other plausible explanation is that cytochrome c oxidase acts as a nitrite reductase enzyme to dissociate the NO [68]. Both of these explanations result in increased ATP production [68].

The effect of PBM does not end with increased ATP production. It also affects the production of ROS [70]. When oxygen, the final electron acceptor of the electron transport chain, is metabolized, ROS are produced [68]. LLLT increases oxidation, resulting in an increased number of ROS [68, 70]. This results in the induction of many transcription factors, specifically nuclear factor kappa B (NF-κB) which transcribes protective gene products that are anti-apoptotic and promote cell survival [70]. Figure 6

is a representation of how PBM activates a cascade of events leading to transcriptional changes and prevents cellular toxicity [68, 70].
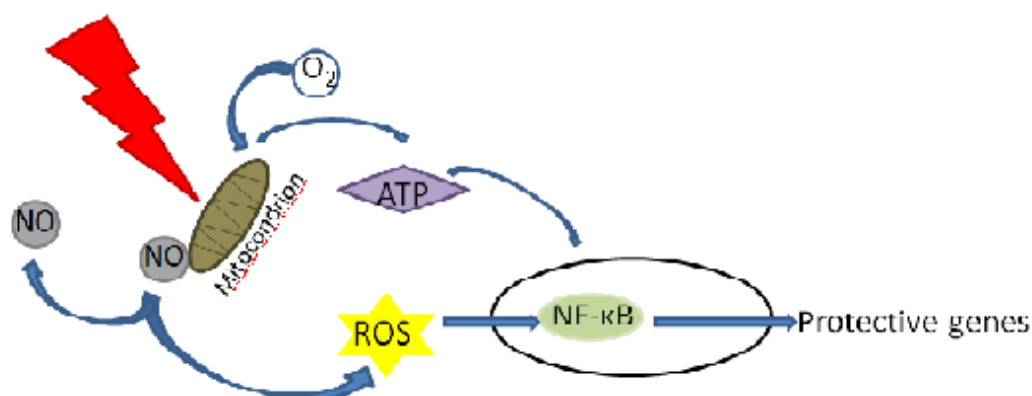
Figure 6. LLLT dissociates NO increasing ATP production and increases ROS which drives protection through the release of transcriptional factors, specifically NF-κB.

Because low doses of PBM are therapeutic, it has been argued that increasing the dosage will have an increased beneficial effect. While the topic is controversial, evidence as pointed to the fact that PBM is dose dependent, and overexposure to light can have detrimental effects [70]. At a low dose, PBM can enhance the proliferation of lymphocytes through the increased release of growth factors [70]. At elevated levels, PBM produces excessive ROS, excessive NO, and may activate a cytotoxic pathway all of which may cause cell apoptosis [70]. At low levels, PBM initiates signaling through cyclic AMP, but at increased doses mitochondrial permeability is compromised and apoptosis is induced [70].

Because MS/EAE pathogenesis is thought to be due, in part, to mitochondrial dysfunction, this lab previously hypothesized that the use of PBM would prove beneficial against disease progression [22]. Our published data indicate the effectiveness of once daily 670nm NIR-LED treatment ameliorated EAE severity in female C57BL/6 mice. Upon disease initiation, the mice were placed into two groups: 670nm light treated or sham control, in which they were placed in the treatment chamber with no light administration. In a blinded study, the mice were then graded using a clinical scale to judge disease severity. A double treatment protocol was used in which mice received 7 days of treatment, or stress only restraint, followed by a 7-day rest period (in which no light was administered), with a subsequent 7-day treatment and rest period. Figure 7 shows the influence the light therapy has by comparing the disease severity between treated and control groups. The WT mice treated with the 670nm light show an improved clinical score in comparison to the sham, or restraint only, counterparts.
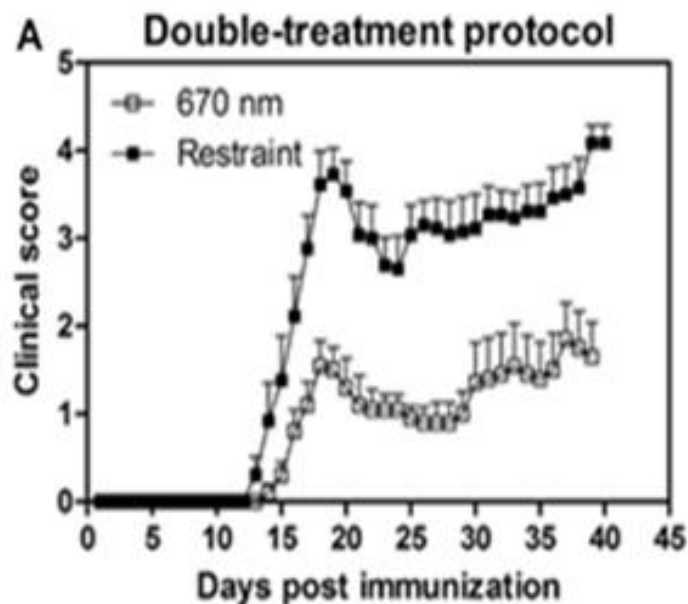
Figure 7. 670nm light reduces disease severity in MOG-induced mice. Treated mice immunized withMOG35-55 following the double treatment protocol display decreased disease severity in comparison to restraint only stress. (A)P<0.0001 by 2-way ANOVA
(Muili *et al., 2012)*

Again, using the double-treatment protocol, spinal cords were removed from MOG$_{35-55}$ immunized mice at 3 time points: 2 days post onset, peak, and 2 days after the start of the second treatment. Interested in cytokine involvement in EAE, QPCR analysis was done on IL-10 and IFN- γ. Figure 2 shows an up-regulation of IL-10 (Figure 8A) during the chronic disease phase with a non-significant down-regulation of IFN-γ (Figure 8B) [22], suggesting a role for IL-10 in the protection from clinical EAE by 670nm-mediated photobiomodulation.
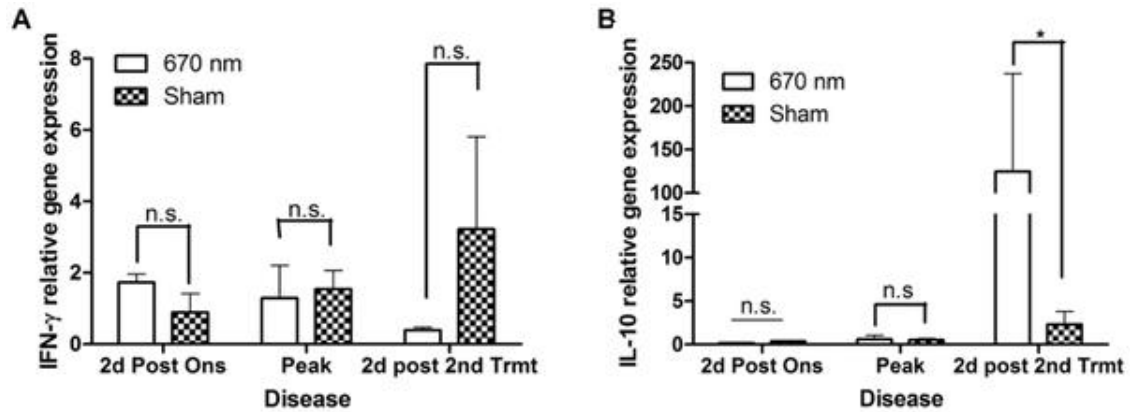
Figure 8: Cytokine modulation using the double-treatment protocol over the course of EAE. Spinal cords were isolated from mice immunized with MOG$_{35-55}$ receiving the double-treatment protocol and QPCR was performed to look at cytokine expression. **(A)** IFN-Y, **(B)** IL-10.
(Muili *et al., 2012)*

Through use of the suppression protocol, in which mice were immunized with MOG$_{35-55}$ and treated for 10 consecutive days post immunization, the disease course of WT mice were compared to IL-10$^{-/-}$ mice. In the findings from Muili *et al,* the WT mice subjected to 670nm light treatment developed less severe EAE in comparison to the WT mice that received restraint only stress, in which no light was administered (Figure 9A). This is indicative of a possible neuroprotective role afforded through light therapy. In contrast, IL-10$^{-/-}$ mice show no improvement in both 670nm treated and sham groups (Figure 9B) [22].
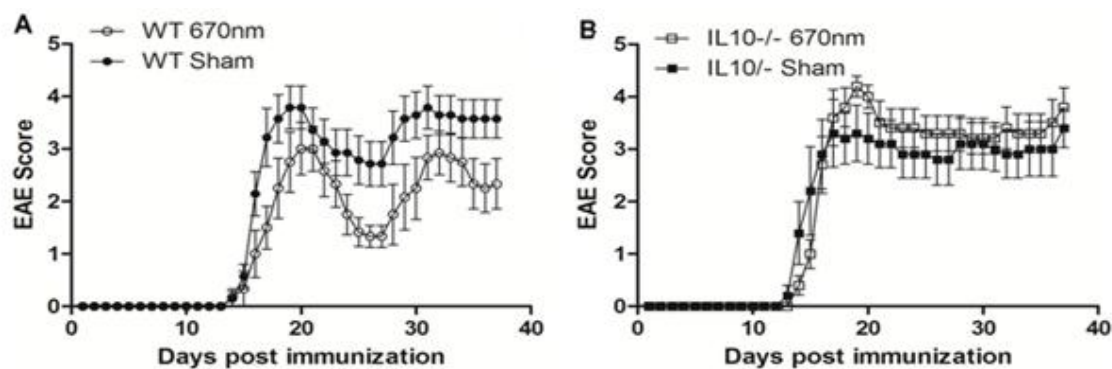
Figure 9. **670nm light decreases EAE severity in WT mice but not IL10-deficient mice.** Previous data demonstrates the protective nature of the light therapy in WT mice expressing IL-10 in comparison to the increased clinical scores of the IL-10$^{-/-}$ mice. **These data implicate IL10 as important in protection from EAE mediated by 670nm.** (Muili *et al., 2012)*

Hypothesis

Multiple sclerosis is a disease plaguing approximately 250,000 people in the US alone [1]. Previous data demonstrated the therapeutic potential of 670nm light therapy in the EAE model, in part through an immune mediated mechanism involving production of IL10 and down-regulation of the pro-inflammatory response. The goal of this new experiment is to identify the cellular source of IL10 implicated in the mechanism of disease protection. This data will provide a deeper understanding of disease protection by 670nm light, which will be necessary as this is developed as an adjunct therapy for the treatment of MS. The *central hypothesis* is that up-regulation of the anti-inflammatory cytokine, IL-10, by 670nm-mediated photobiomodulation decreases disease severity in the EAE model.

Specific Aims

I. Investigate the role of immune modulation in the amelioration of EAE by photobiomodulation.

II. Characterize the source anti-inflammatory cytokines in response to photobiomodulation by visualizing what cells are releasing IL-10 through the use of GFP reporter mice.

CHAPTER II: MATERIALS AND METHODS

Mice

Pathogen-free female WT, IL10 deficient (IL10$^{-/-}$), and mice genetically modified to express GFP following the IL-10 promoter on a C57BL/6 (B6) background were bred in-house from breeding pairs purchased from Jackson Laboratories (Bar Harbor, MN). The mice were housed in an Association for Assessment and Accreditation of Laboratory Care (AAALAC) accredited facility on the University of Wisconsin-Milwaukee campus following National Institutes of Health (NIH) and University guidelines. Food and water was provided ad libitum and a 12-hour light/dark schedule was used in a temperature and humidity-controlled environment. Protocols were certified by the Institutional Animal Care and Use Committee. All mice used were 6-8 weeks of age.

GFP, originally found in the jellyfish Aequorea Victoria, has been widely used in biochemical and cell biology studies to visualize proteins [3]. Through fusion with the desired protein, GFP allows visualization of the protein while it maintains its normal

functional properties. GFP fluoresces a vivid green color when exposed to blue light. When conditions prompt the expression of IL-10 in the mice expressing GFP under the control of the IL10 gene promoter, GFP will be expressed and the cells will glow allowing the location of IL-10 to be visualized in genetically altered heterozygous GFP/IL-10 mice.

Antigens

MOG$_{35-55}$ peptides (MEVGWYRSPFSRVVHLYRNGK) synthesized and purified by HPLC (GenScript, Piscataway, NJ) were used for the emulsion as well as in the resuspension for the cell culture.

EAE induction

EAE was induced by active immunization with an emulsion made with MOG$_{35-55}$ and *Mycobacterium tuberculosis*. The emulsion of 50 µg or 100 µg MOG$_{35-55}$ peptide emulsified (1:1) in incomplete freund's adjuvant (IFA, MP Biochemicals, Solon, OH) with 300 µg *M. tuberculosis*, strain H37RA (Difco Laboratories, Detroit, MI) was injected into mature mice (6-8 weeks old). Emulsions were prepared using an Omni Mixer Homogenizer (Omni International, Kennesaw, GA). Each mouse received 0.05 mL of emulsion subcutaneously at four sites. When immunized for clinical disease course, mice also received 300 ng pertussis toxin (PT, List Biological Labs Inc., Campbel, CA) prepared in with phosphate buffered saline (PBS) to keep the disease onset consistent throughout the mice. The PT/PBS mixture was injected intraperitoneally at 0 and 72 hours post immunization, per standard laboratory protocol.

Before the mice were injected with the emulsion, they were anesthetized using a ketamine (100mg/ml), xylazine (300 mg/ml) cocktail (1 ml ketamine; 0.15 ml xylazine; 4.6 ml water) which was injected intraperitoneally.

EAE clinical grading

Mice observed for clinical EAE beginning 7-10 days post immunization (dpi) and continuing daily for the duration of the experiment. Mice were graded by a blinded observer using a clinical scale of 0-5 [0: healthy, no sign of EAE; 1: loss of tail tone; 2: hind limb weakness; 3: paresis or paralysis of one hind limb; 4: paralysis of both hind limbs; 5: moribund or dead]. Mice receiving a clinical score of >4 were sacrificed.

Treatment

Once mice were deemed sick, (clinical score of 1) they were designated into either the treatment group or sham group, receiving no light treatment. Treatment of 670 nm wavelength light was administered using a Gallium/Aluminum/Arsenide (GaAlA) light-emitting diode (LED) array ($75cm^2$) (Quantum Devices, Barneveld, WI) at 67% intensity for 90 seconds at a power intensity of $28mW/cm^2$. During these treatments, unanesthetized mice were placed in a polypropylene restraint (12.7 x9 x7.6 cm) with the light placed directly above the dorsal surface of the mouse for the defined treatment time. For sham treatment, the mouse was placed in the treatment chamber for the same length of time as the groups receiving light treatment but without light exposure. This was to account for the effect the stress of the restraining the mice may have. Treatment was applied once daily as follows: 7 days treatment, followed by 7 days rest, with 7 days

treatment, and an additional rest period of 7 days. The mice received an energy density of $5J/cm^2$ (375 J daily total).

For *in vitro* treatment experiments, Lymph node and spleen cells that had been extracted and set up in a cell culture (see *cell culture*) were also designated to either the treatment or sham group. The treated cells received treated with 670 nm wavelength light at 100% intensity for 180 seconds using a hand held WARP 75 Light Delivery System (Quantum Devices, Barneveld, WI). Treatment began 2 hours after the cells were resuspended then every 24 hours for 3 days. During these treatments, the light was placed 2 cm above the flask for the given treatment time. For sham treatment, the flask was still placed in the treatment chamber for the same length of time as the groups receiving light treatment but without light exposure.

Cell Culture

For in vitro characterization of the response to NIR treatment, lymph nodes and spleen cells were isolated from mice immunized as above but with without PT 10-14 dpi or over the course of disease, as indicated. Samples isolated over the course of disease defined as follows: pre-clinical phase (~10 dpi) before clinical symptoms of EAE appear; 2 days post onset when clinical disease begins; during the peak of the disease (where disease course is typically the worst); 2 days into the second rest period; and 2 days post second treatment. Mice were sacrificed by anesthetizing them with the ketamine cocktail and cervical dislocation. Using a dissection tray and sterile technique including reagent grade ethanol, spleens and lymph nodes were removed and suspended separately in Hank's Balanced Salts (HBSS). Six draining peripheral lymph nodes (2 popliteal, 2

axilliary and 2 brachial) were removed from each immunized mouse. Single cells suspensions were then generated through homogenization of the removed nodes and spleens with autoclaved glass homogenizers. These cells were then centrifuged at 1250xg for 10 minutes to separate the cells from fat and debris. Trypan blue was used to visualize the cells (90 µl of 0.4% trypan blue; 10 µl cells) and determine cell viability using a hemacytometer. The cell count was determined using the following formula:

(No. of cells counted) (Dilution factor) (10^4)/ No. of squares counted = cells/ml

A total of 2×10^6 cells/ml were then cultured in RPMI 1640 complete [penicillin (100 U/mL)/streptomycin (100 µg/mL), L-glutamate (2 mM), sodium pyruvate (0.1 mM), 2-mecarptoethanol (50 mM)], 10% Fetal Bovine Serum (FBS), and MOG_{35-55} (10 µg/ml). The suspension was then distributed into T75 tissue culture flasks (TPP tissue culture; MidSci, St. Louis, MO); 670 nm light at 180 seconds and sham (e.g. no light) group and antigen vs no antigen, and stored in an incubator at 35-37 degrees Celsius at 10% CO_2.

RNA isolation

The cells harvested from spleens and lymph nodes (as described in *cell culture*) were centrifuged at either 24, 48, 72, or 96 hours after incubation. The cells were then homogenized in 1 ml of Trizol reagent (Invitrogen, Grand Island, NY) and frozen at -80 °C until further use. Total RNA was isolated by the Trizol method, according to manufacturer's instructions. Briefly, the frozen cells were allowed to thaw at room temperature and centrifuged at 12,000xg for 10 minutes at 2-8 °C. Chloroform (200 µl) was added for every 1 ml of Trizol and the cells were vigorously shaken for 15 seconds, incubated at room temperature for 2-3 minutes, then centrifuged at 12,000xg at 2-8°C for

15 minutes. The upper aqueous layer containing the RNA was carefully removed and placed in a clean tube. 0.5 ml isopropanol per 1 ml Trizol added to the original cell solution and mixed gently, followed by a10 minute incubation at room temperature. The RNA was recovered through centrifugation again at 12,000xg at 2-8°C for 10 minutes. The supernatant was removed and the RNA was washed with 1 ml of 70% ethanol (EtOH) for every 1 ml Trizol. The samples were again centrifuged at 75,000xg for 5 minutes at 2-8 °C. The supernatant was carefully removed and the RNA pellet was allowed to air-dry and resuspended in 50 µl molecular grade water. The concentrations and purities of the samples were assessed using a UV-2501 PC UV-VIS recording spectrophotometer (Shimadzu Scientific Instruments Inc.) using absorbances at 260 nm and 280 nm. The samples were diluted 1:50 in 1X TE buffer (10mM Tris-HCl, 1 mM EDTA, pH 7.5). When necessary, RNA was concentrated using the Qiagen RNEasy Min-Elute colums, according to manufacturer's instructions (Qiagen, Valencia, CA). Total RNA concentration was adjusted by dilution to assure reverse transcription of similar amounts of RNA for QPCR analysis.

Reverse Transcription

The isolated RNA samples were reverse transcribed (RT) to cDNA using the RT^2 HT First Strand Kit (SA Biosciences, Valencia, CA) according to manufacturer's instructions. Six microliters of genomic DNA elimination buffer (GE2) was added to 8 µl of RNA and allowed to incubate at room temperature for 10 minutes. Following the incubation, 6 µl of RT Master Mix (BC5) was added. Reverse transcription was carried out using the Mastercycler Gradient PCR machine (Eppendorf's Scientific, Hauppauge,

NY) at 42 °C for 15 minutes then 95 °C for 5 minutes. Molecular grade water was added at a volume of 91 µl and the samples were stored at -20 °C until use.

Quantitative Real-time PCR

Real-time Quantitative PCR (RT-qPCR) is used to amplify and quantify the amount of template (cDNA or DNA) in a sample. RT-qPCR allows for quantification to happen in real time after each amplification cycle and is paired with reverse transcription to quantify messenger RNA (mRNA). The $\Delta\Delta C_T$ (threshold cycle) programming was used in which fluorescence is used to determine if the sample surpasses a given threshold. This technique was performed using SABiosciences primers and RT^2 SYBR Green qPCR master mix reagents to determine gene expression. Samples that have undergone RT were used following manufacturer's instructions. Beta-actin (β-actin) was used as a housekeeping gene while IL-10, INF-γ, and IL-4 primers were used to quantify IL-10, INF-γ, and IL-4 expression. The primers were designed to span introns when possible and were purchased from Sigma (St. Louis, MO). Each reaction was run in triplicate with water blanks set as controls. The reaction mixture for a single reaction was set up as follows in Table 1:

Table 1. qPCR	
Component	Volume (µl)
RT^2 SYB® Green qPCR master mix	12.5
Primer Set	1
Molecular grade H_2O	10.5
Total Volume	24

For each reaction, 1 µl of the sample RNA was added to 24 µl of the above mixture. Assays were performed using StepOnePlus RT-PCR from Applied Biosystems (Carlsbad, CA). Instrumentation was programmed for a hot start of 95 °C for 10 minutes. The amplification ran for a total of 40 cycles which included 95 °C for 15 seconds and 60 °C for 1 minute. A melt curve was programmed for 95 °C for 15 seconds, 60 °C for 1 minute, and 95 °C for 15 seconds. Expression was quantitated via the Pfaffl method and normalized to β-actin. Unlike the ΔΔCT model which assumes 100% efficacy between the primers, the Pfaffl method uses a standard curve of each primer to determine efficiencies to aid in the analysis [71].

Enzyme-Linked Immunosorbent Assay

Cell culture supernatants were acquired at 48, 72, and 96 hours and subjected to cytokine analysis using enzyme-linked immunosorbent assay (ELISA). The cytokines of interest were IL-10, IFN-γ, and TGF-β. ELISAs were performed per manufacturer's instructions using the Ready-Set-Go kit from eBiosciences (San Diego, CA). ELISA's are used to detect and quantify specific proteins that have been secreted from cells.

Using a high binding affinity, the ELISA plate captures the target protein, which is then detected by a protein-specific biotinylated antibody. Quantification is done through a colorimetric reaction based on activity of avidin-horseradish peroxidase (HRP).

Before beginning the ELISA, the 96-well plate was coated overnight with 1x dilution of the capture antibody in 1x coating buffer at 4°C. The wells were then washed 5 times with 100 µl wash buffer (PBS and 0.05% Tween-20) per well. The wells were then blocked with 200 µl 1x Assay Diluent and incubated at room temperature for 1 hour. The plate was again washed and 100 µl of the standards or each sample was added to each well and incubated at room temperature for 2 hours. The wells were washed and 100 µl of detection antibody was added and the plate was again incubated for 1 hour at room temperature. Following incubation, the plate was washed and 100 µl Avidin- HRP diluted in 1x Assay diluents was added to each well and incubated at room temperature for 30 minutes. After a final wash cycle, 100 µl of tetramethylbenzidine (TMB) substrate solution was added to each well and incubated for 15 minutes. After, 50 µl of stop solution (2.5N H_2SO_4) was added to each well and the plate was read at 450 nm within 30 minutes of adding the stop solution.

Flow cytometry

Cells acquired from spleens and cultured and treated as previously discussed were analyzed by flow cytometry to characterize the phenotype of responding cells. After the final light treatment, cells were incubated for an additional 2 hours to allow for transcription to take place. The cells were then counted for viability using trypan blue, as previously described. Using Lymphocyte Separation Medium (9.4 g sodium diatrizoate,

6.2 g ficoll per 100 ml of LSM) the cells were place over a density-dependent gradient to separate mononuclear cells. In this process, the cell suspensions were overlaid on 5 ml LSM and centrifuged for 30 minutes at 500xg at 4°C (with the break turned off). The interface was then removed and diluted 1:2 with HBSS and underwent two additional washes using HBSS. Again, the cells were then counted for viability and aliquotted into 1.7 ml tubes at a cell count of 2.0×10^6.

The aliquotted cells received 5 µl F_C block (eBiosciences, San Diego, CA) to limit the amount of non-specific binding through the F_C receptors and were incubated on ice for 30 minutes. The cells underwent two washes and were resuspended in 50 µl FACS wash buffer (0.5% PBS, 0.1% FBS, and sodium azide) and were incubated on ice for 30 minutes with 1 µl of a designated antibody for cell surface markers.
The cells were stained as follows:

Table 2. Flow Cytometry Cell Markers			
FITC (FL1 channel)	PE (FL2 channel)	PerCP (FL3 channel)	APC (FL4 channel)
CD3	CD19	Foxp3	CD25
CD3	CD4	Foxp3	CD25
CD3	CD8	Foxp3	CD25
Controls			
Rat IgG2a	Rat IgG2b	Rat IgG2a	Rat IgG2b

Cells were labeled with antibodies to identify T cell populations (CD3, CD4, CD8, CD25, and FoxP3), B cell populations (CD19), as well as macrophages (CD11b)

and neutrophils (Ly6G). Species, isotype, and fluorochrome matched antibody controls were also used, as well as single-labeled controls to allow for proper compensation using the flow cytometer. Cells expressing IL-10 were identified by virtue of green fluorescence. The phenotype of IL10-producing (e.g., green fluorescing) cells were identified by virtue of interaction with specific antibodies, as outlined above.

The antibodies were purchased from Biolegend (San Diego, CA) and eBioscience (San Diego, CA). Foxp3 is an intracellular stain in which the cells were first stained using surface markers, then underwent permeabilization for intracellular staining. Following the last wash with the surface markers, the supernatant was discarded and the sample was vortexed to dissociate the pellet. 1 ml of Foxp3 Fixation/Permeabilization working solution (eBiosciences, San Diego, CA; 1 part fixation/permeabilization concentrate with 3 parts fixation/permeabilization diluent) was added to each sample and vortexed. After a 30 minute dark incubation at 4 °C, 2 ml of 1x Permeabilization Buffer (1ml permeabilization buffer in 9 ml distilled water) were added to each sample. The supernatant was then discarded after centrifuging the cells at 400xg at room temperature for 5 minutes. The cells were then resuspended in 100 µl 1x Permeabilization Buffer with 1 µl of PerCP-Cy5.5 Foxp3 antibody and incubated in the dark for 30 minutes on ice. Following the incubation, the samples underwent 2 washes with 2 ml 1x Permeabilization Buffer and resuspended in 500 µl FACS wash buffer, ready for analysis using flow cytometry.

The data were collected using a dual laser BD FACS Calibur (BD Biosciences, San Jose, CA) using the computer program CellQuest Pro (BD Biosciences, San Jose,

CA). After the data were collected, analyses were run using FlowJo software (Tree Star, Ashland, OR).

Microarrays

Complimentary DNA (cDNA) from the two groups (treated vs. sham) were further investigated using microarrays. Microarrays are used to measure the expression levels of genes simultaneously using a fluoriphore-labeled target. Using a hybridization protocol from Qiagen (Valencia, CA), gene expressions from both groups were compared. The use of this technique allows for analysis of genes whose expression has been changed during the course of MS/EAE due to photobiomodulation with 670nm light. Specific time points of sham and treated cells were analyzed using two kits: Th17 for Autoimmunity and Inflammation and Nitric Oxide Signaling Pathway, (Qiagen, Valencia, CA). 96h samples from our sham and treated cell pellets from cell culture were compared using the Th17 kit to look at inflammatory and autoimmune cytokine expression levels. Our 48h samples used the nitric oxide signaling pathway to compare gene expression levels in our sham and treated samples.

Statistical Analysis

Data were analyzed and statistical analyses were carried out using GraphPad Prism 5.0 (La Jolla, CA) or FlowJo software (Tree Star, Ashland, OR). Clinical scores were analyzed using 2-way ANOVA.

CHAPTER III: SPECIFIC AIM I

Investigate the role of immune modulation in the amelioration of EAE by photobiomodulation.

Rationale

While the current paradigm describes MS/EAE as autoimmune diseases mediated by myelin-reactive $CD4^+$ Th1/Th17 cells, recent data suggest that the axonal loss associated with disease progression is mediated by oxidative stress. The currently approved therapies are of limited effectiveness, probably because they fail to prevent the accumulation of oxidative stress leading to axonal loss. Photobiomodulation has proven effective in the treatment of chronic inflammation and neurodegeneration through remediation of oxidative stress. Our published data demonstrated the therapeutic potential of 670nm-mediated photobiomodulation in part through an immune-mediated mechanism. Given the role of the immune response in MS/EAE pathogenesis, a better understanding of the effect of photobiomodulation on the immune response is necessary in order to proceed to clinical studies in the MS patient population.

Through the use of cell supernatants, in which protein are found, ELISAs were performed to look at gene expression. QPCR and microarrays looked at the cell pellet, which contains mRNA to analyze the message for protein synthesis. Preliminary data showed up-regulation of IL-10 with down-regulation of INF-γ protein expression by ELISA when antigen-primed lymph node cells were exposed to NIR light at a wavelength of 670 nm [22]. Transcriptional up-regulation of IL-10 and down-regulation

of IFN-γ by QPCR analysis was also noted in the spinal cord of 670nm light treated mice over the course of MOG-induced EAE. The role of IL-10, and global regulation of inflammation, in the amelioration of EAE by 670nm light phototherapy was further investigated.

Research Design

Female WT and IL-10$^{-/-}$ mice of the C57/BL6 background were immunized with 100μg MOG$_{35-55}$ emulsified in Freud's incomplete adjuvant mixed with *M. turberculosis* H37RA. Treatment began immediately after immunization and continued for 10 dpi. On the first day of treatment the mice were assigned to the 670nm NIR-LED treated or sham group, and were treated accordingly using the suppression treatment protocol. Using a pre-determined clinical scale, disease severity was recorded daily throughout the experiment.

Both treated and sham mice were placed in a polypropylene restraint for the duration of the defined treatment time (90s or 180s). The sham mice, receiving identical treatment without the administration of light, served as the control population. Mice receiving the NIR-LED therapy at 67% intensity for 180s received 10J/cm^2 on the dorsal surface. At the end of the experiment, mice were sacrificed and their clinical grades were normalized to the day of onset for analysis. In order for statistical significance to be achieved, anywhere from 20 to 40 mice were immunized to assure that each treatment group had at minimum 10 mice.

For cytokine and QPCR analysis, draining peripheral lymph nodes and spleens were removed from mice 10 dpi and set up in cell culture receiving 670nm light or no light. The cells were treated and harvested at 24 hour intervals for a total of 96 hours.

Results

Cytokine analyses by ELISA shows a up-regulation of IL-10 protein in cells introduced to photobiomodulation

Lymph node cells cultures receiving 670nm light treatment as previously described show a down-regulation of IFN-γ in comparison to the sham controls at 48h and 96h [22]. In the current study, the largest decrease of IFN-γ was seen at the 96h time point (Figure 10A). This agrees with our published data of spinal cord analysis with ELISA analyzed throughout the course of the experiment [22], particularly with the QPCR data obtained 2 days post secondary treatment (Figure 8). Conversely, IL-10 production was up-regulated in the presence 670nm light, however, maintained the level of production throughout the course of the experiment (Figure 10B).
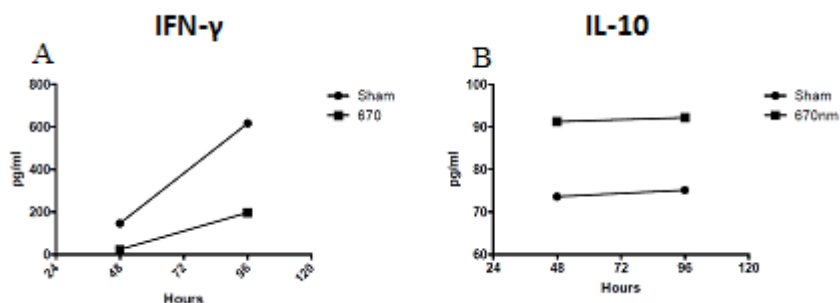
Figure 10. Up-regulation of IL-10 in the presence of 670nm light. ELISA analyses of IL-10 and IFN-γ at 48h and 96h time points show a decrease in IFN-γ production with a subsequent increase in IL-10 levels when 670nm light as administered. IL-10 levels within the two groups remain constant throughout the duration of the experiment. **670nm light impacts the production of pro- and anti-inflammatory cytokines.**
(This experiment must be replicated as these results are compiled from a single experiment, thus accounting for the lack of error bars.)

QPCR analysis indicates a down-regulation of IL-10 mRNA in the presence of 670nm light

QPCR analysis of cDNA from lymph node cells treated as previously indicated showed a down-regulation of message for both IFN-γ and IL-10 cytokines. Contradictory to what was previously shown from *in vivo* analysis of cytokines through QPCR, the *in vitro* study of lymph node cells indicate that the light is not only down-regulating IFN-γ but having the same effect on IL-10 production when normalized to the sham control (Figure 11). This indicates that the light has a different effect on translation by increasing gene expression, than on transcription.
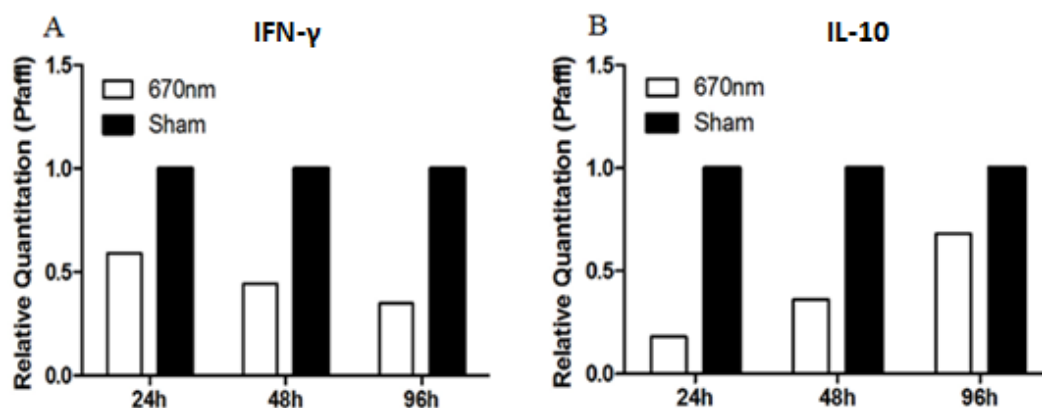
Figure 11. QPCR analysis shows down-regulation of IFN-γ and IL-10. QPCR analysis from cell cultures from spleen cells show a down-regulation of both pro- and anti-inflammatory cytokines in the presence of 670nm light. **670nm light therapy down-regulates IL-10 production *in vitro.***

670nm light globally down-regulates inflammatory markers <u>in vitro</u>

Microarray analysis was performed on 96h cDNA obtained from lymph node cells. This microarray tested 96 different genes and cytokines involved in pro- and anti-inflammatory processes. The 670nm light experimental group, normalized to the sham control, resulted in 2-3 fold increases of anti-inflammatory genes, with a 2-5 fold decrease of pro-inflammatory genes (Table 3). Chemokine ligand 7 (CCL7), a regulator of macrophages, and chemokine ligand 1 (CXCL1), displaying neuroprotective functions, were both up-regulated in the presence of 670nm light. IL-17a, a pro-inflammatory cytokine that enhances the production of NO was also increased.

Figure 12 shows a scatter plot of the genes and cytokines that were analyzed with the microarray. Analysis of 96h 670nm cDNA was normalized to the sham control.

Placing the parameters at a 2-fold increase allows for better visualization of these cytokines affected by the light. A marker has also been placed to indicate IL-10 within the plot. Again, indicating that the transcription of IL-10 is unaffected by the light treatment.
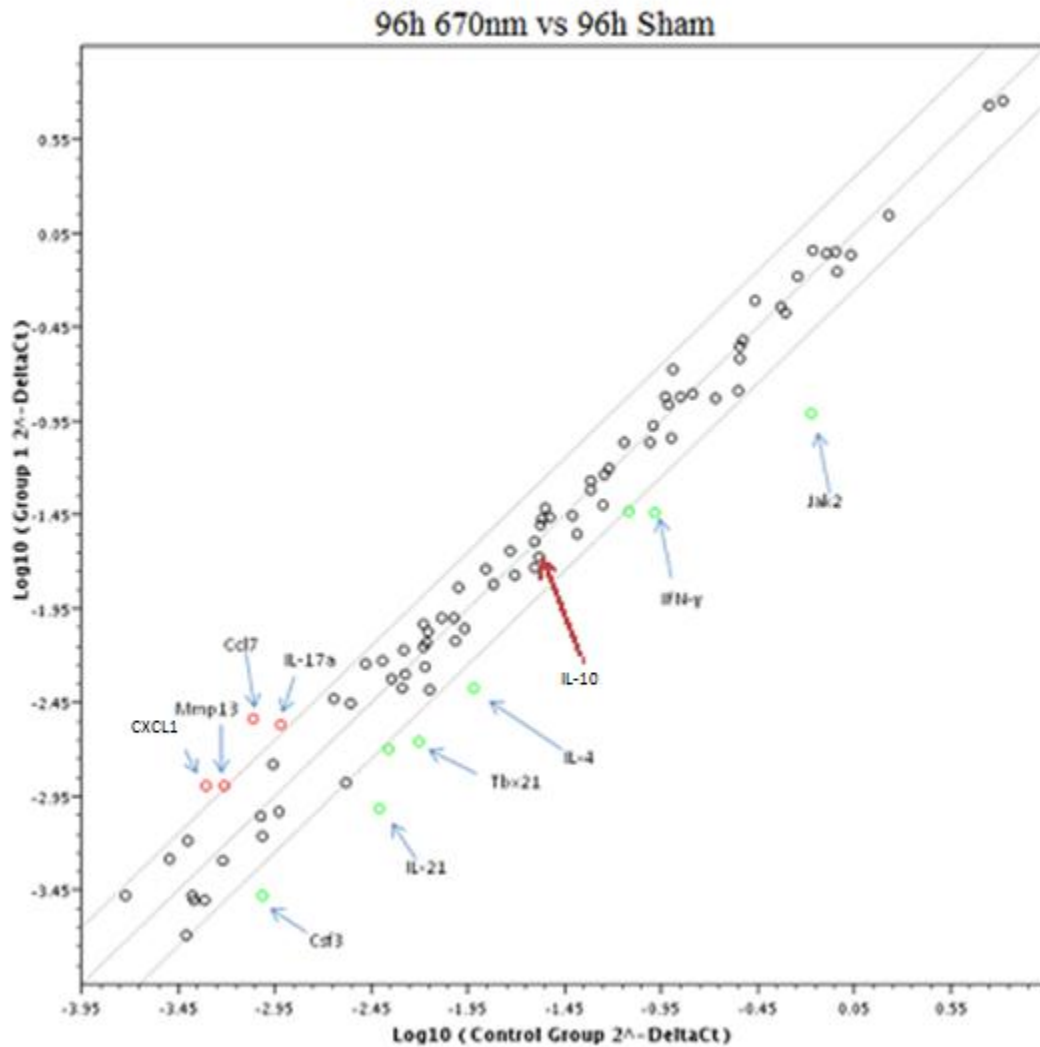
Figure 12. Scatter plot analysis from inflammatory microarrays. Emphasizing a 2-fold increase, the scatter plot displays the cytokines both increased and decreased in the presence of 670nm light. **670nm light decreases IFN-γ production.**

Decreases were found in JAK3 and TBX21, both transcription factors for IFN-γ, IL-21, which is regulated by JAK3, and subsequently IFN-γ. These results agree with the findings from preliminary data that IFN-γ is down-regulated in the presence of the light therapy [22]. Overall, the down-regulations seen agree with the understanding that 670nm light decreases or inhibits pro-inflammatory processes.

96h 670nm vs. 96h Sham			
Up-regulated		Down-regulated	
Gene	Fold Regulation	Gene	Fold Regulation
CCL7	3.3413	JAK2	-5.4823
CXCL1	2.5723	IL-21	-4.0596
IL-17a	2.2075	TBX21	-2.8609
MMP13	2.0626	IFN-γ	-2.8516
		IL-4	-2.8343
		CSF3	-2.0392

Table 3. Gene and cytokine fold changes in microarray analysis of 96h 670nm treated sample in comparison to the 96h sham control.

The nitric oxide signaling pathway microarray, performed on the 48h lymph node samples, was normalized to the sham control. Figure 13 is a scatter plot that highlights the signaling markers that fall outside of a two-fold increase. Up-regulation was seen in signals that produce ROS (NOXA1) and free radicals (NOS2) lending to the mechanism of the light. Up-regulation was also seen in those that display protective qualities such as preventing against peroxidation (GPX5) and oxidative stress (UCP3). Down-regulation of signals were much more prevalent than up-regulation. Down-regulation was seen in CAPNS1, in which activation is triggered by oxidative stress, DYNLL1, which

destabilizes nitric oxide synthesis, GPX1, an antioxidant, as well as PRDX2, which plays

an antioxidant protective role.
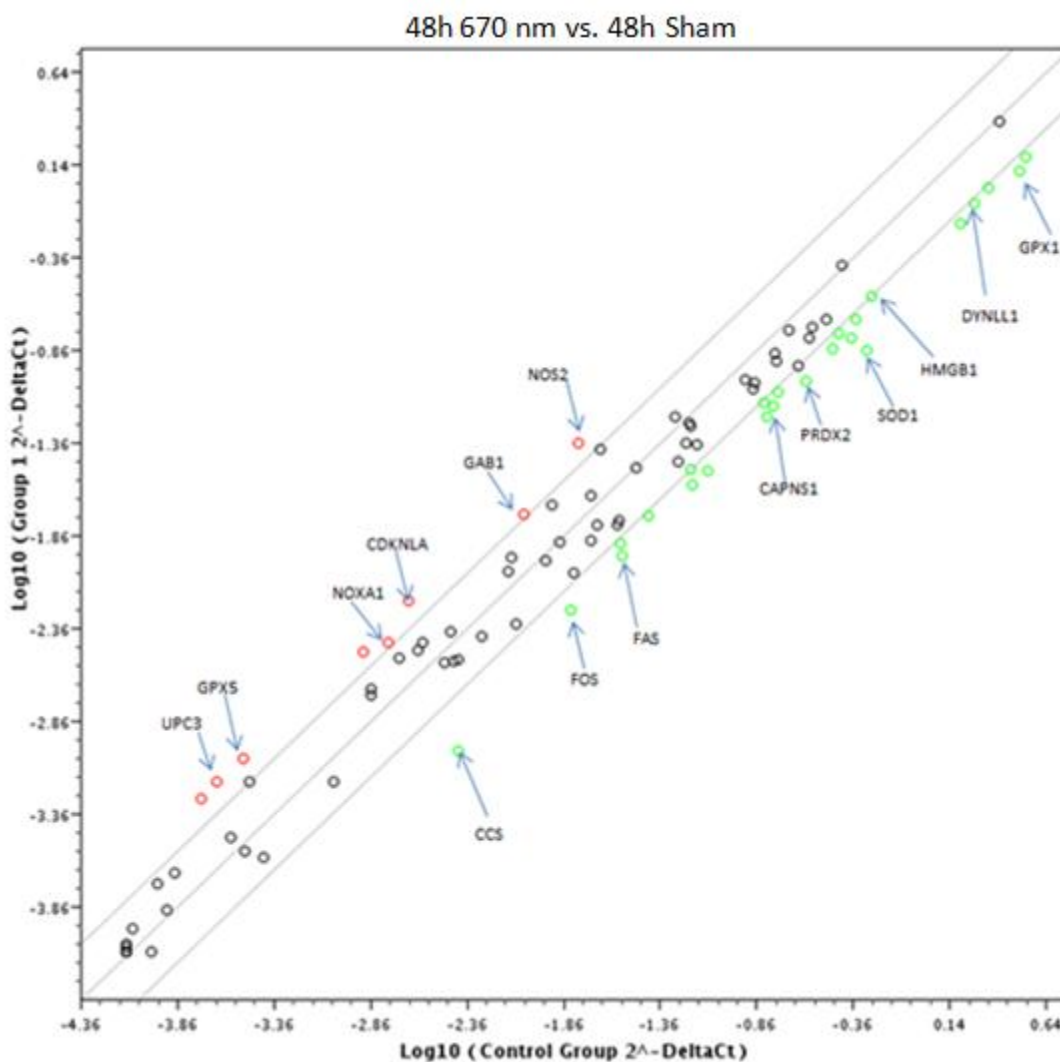
Figure 13. Scatter plot analysis from NO signaling microarrays. Emphasizing a 2-fold increase, the scatter plot displays the signaling molecules both increased and decreased in the presence of 670nm light. **670nm light briefly up-regulates ROS.**

Table 4 shows an itemized list of the signaling molecules that were regulated by at least a two-fold increase or decrease in the presence of 670nm light therapy when normalized to the sham control.

48h 670nm vs. 48h Sham					
Up-regulated		Down-regulated			
Gene	Fold Regulation	Gene	Fold Regulation	Gene	Fold Regulation
UCP3	2.9624	CCS	-4.1009	CAPNS1	-2.4221
CDKNLA	2.8641	SOD 1	-3.6734	DYNLL1	-2.1605
GPX5	2.8576	FOS	-2.7614	HMGB1	-2.0253
NOS2	2.6639	PRDX2	-2.6352		
NOXA1	2.1411	FAS	-2.5479		
GAB1	2.1035	GPX1	-2.4417		

Table 4. NO signaling molecule fold changes in microarray analysis of 48h 670nm treated sample in comparison to the 48h sham control.

Discussion

Previous data from our lab has shown a decrease in disease severity in WT mice receiving 670nm light therapy. Results have also showed that in the presence of light, IL-10$^{-/-}$ mice display the same disease course as their sham counterparts in comparison to the WT treated mice who display a less severe disease course than the WT sham mice (Figure 9) [22]. This is indicative of a protective role afforded through light therapy through the action ofIL-10. This hypothesis is further supported by *in vitro* cytokine analysis. ELISA data demonstrated that 670nm light-treated cultures displayed a

decrease in IFN-γ production and an increase inIL-10 over the course of 96h culture (Figure 10). Agreeing with this data, IFN-γ levels in QPCR as well as microarray analyses were also decreased when treated with 670nm light.

The current studies utilized QPCR microarray to investigate the effect of 670nm light treatment on inflammation at a global level. The microarray utilized allowed for 96 different genes to be analyzed at one time. In addition to a decrease in IFN- γ expression, molecules important the regulation of IFNγ or regulated by IFNγ were also decreased, including TBX21, a transcription factor for IFN- γ, and JAK2, a kinase regulated by IFN-γ. In addition IL-21, a cytokine that uses that JAK/STAT pathway was also down-regulated. Not only is the light therapy responsible for the production of protective cytokines, it is also responsible for a down-regulation of the transcription factor as well as the pathway for IFN-γ production. This is indicative of the protective role afforded by the light therapy. A down-regulation is also seen in colony-stimulating factor 3 (CSF3) which controls the production, differentiation, and function of granulocytes.

Up-regulation of CXCL1 displays beneficial qualities toward the amelioration of MS/EAE. Omari *et al.* found that mice with increased levels of CXCL1 display milder forms of EAE than those at normal or below normal levels [72]. Mice displaying higher CXCL1 had decreased inflammation and demyelination, suggesting a neuroprotective role for CXCL1 [72].

As mentioned earlier, photobiomodulation (PBM) briefly up-regulates ROS and NO, which increases production of NF-κB, involved in the production of protective genes. Due to the findings of this microarray, we can attribute this up-regulation of NO to IL-17a. IL-17a regulates NF-κB through enhanced production of NO [70].

Our previous data demonstrated up-regulation of IL-10 protein expression by ELISA. However, IL-10 mRNA expression was decreased compared to the Sham control, as demonstrated by QPCR and microarray analysis. These apparently contradictory results could be explained by a number of factors. First, could be due to the fact that IL-10 is regulated at the transcriptional and post-transcriptional levels [73]. Transcription is responsible for converting DNA into RNA, and is the first step in the gene-making process. Post-transcription is the regulation of gene expression at the RNA level. In post-transcription, mechanisms are put in place to extend the half-life of the RNA molecule and protect it from degradation. These modifications allow for faster, more complete protein synthesis. In a traditional sense, if transcription is increased, translation would increase resulting in higher gene expression. At the post-transcriptional level, if transcription remains constant, messenger RNA (mRNA) would remain the same. Post-transcriptional modifications can be made at this point which can alter the level of protein expression from single mRNA molecules. Another reason for this discrepancy could be that both QPCR and microarray analyses are merely snapshots of gene expression at a given time. The fact that IL-10 levels are higher in the ELISA may indicate that the 96h time point was too late to capture maximum IL-10 message expression.

The fact that IL-10 production shows an increasing trend in the QPCR analysis may simply indicate that we are not allowing enough time between treatment and the harvesting of cells. In the current experiments, the cells were harvested 2h after treatment to allow for translation to occur. Again, we know that the light temporarily increases NO in order to activate signaling pathways toward protective gene expression. Microarray analysis at 96h is still associated with increased NO, perhaps due to the

production of IL-17a. This indicates that two hours after treatment may not enough time for the transcription factors to respond and generate increased gene expression. Perhaps harvesting the cells at later time points will allow enough time for the message to be translated, because as it stands, two hours disrupts signaling before the translation can have an effect on gene expression.

The decrease in IFN-γ through the down-regulation of its transcription factor is one way the light therapy is offering protection. Although not enough time was allowed for the up-regulation of the protective cytokines to come to fruition, the initial steps alluding to protection are seen, including up-regulation of IL-10.

CHAPTER IV: SPECIFIC AIM II

Characterize the source anti-inflammatory cytokines in response to photobiomodulation by visualizing what cells are releasing IL-10 through the use of GFP reporter mice.

Rationale

It is accepted that anti-inflammatory cytokines ameliorate the severity of MS and EAE. The FDA approved therapies, all active through immunomodulatory or immunosuppressive mechanisms, function in least in part by inducing anti-inflammatory cytokines. Data generated by this lab suggest that photobiomodulation induced by 670 nm light improves the severity of EAE at least in part through the production of IL-10 [22]. Because the production of IL-10 is known to amelioration clinical EAE, it is

important to know which cells are responsible for the synthesis of IL-10 in an infected mouse. Thus, draining peripheral lymph node and spleen cells from MOG_{35-55} immunized mice were analyzed by flow cytometry to identify the immune cells responsible for the secretion of IL-10 induced by 670 nm-induced photobiomodulation.

Research Design

Female mice of the C57/BL6 background expressing GFP under the control of the IL-10 promoter, and WT mice were immunized with 100µg MOG_{35-55} emulsified in Freud's incomplete adjuvant with *M. tuberculosis* H37RA at 4 dorsal sites. Mice were sacrificed 10 dpi and draining peripheral lymph nodes and spleens were removed cultured *in vitro* in the presence of antigen. The cells were subjected to either 3 days of NIR-LED light therapy using a handheld WARP 75 Light Delivery System, or no light at all. After the treatment period, the cells were stained with cell surface and intracellular makers to identify B cells, T cells, and regulatory cells and subjected to analysis using flow cytometry. To assure that a sufficient number of cells were attained for analysis, 4 to 7 mice were immunized for lymph nodes or spleen removal.

Results

Photobiomodulation induces IL-10 production.

Our previous data showed the up-regulation of IL-10 secretion by QPCR and ELISA of cells and tissue isolated from 670 nm light treated, MOG_{35-55}-immunized mice over the course of EAE. Clinical data demonstrated that mice genetically deficient in IL-10 lacked the ability to recover from EAE when treated with 670nm light therapy,

confirming the importance of IL-10 in protection against clinical EAE afforded by 670 nm-mediated photobiomodulation.

   To directly investigate the induction of IL-10 by 670 nm light, flow cytometric analysis of lymph nodes extracted from 7 IL-10/GFP mice was performed. The analysis was gated on the lymphocyte population using a forward vs. side scatter plot. As expected, an increase in IL-10 production was seen in the 670nm treated cells in comparison to the sham controls (Figure 14).
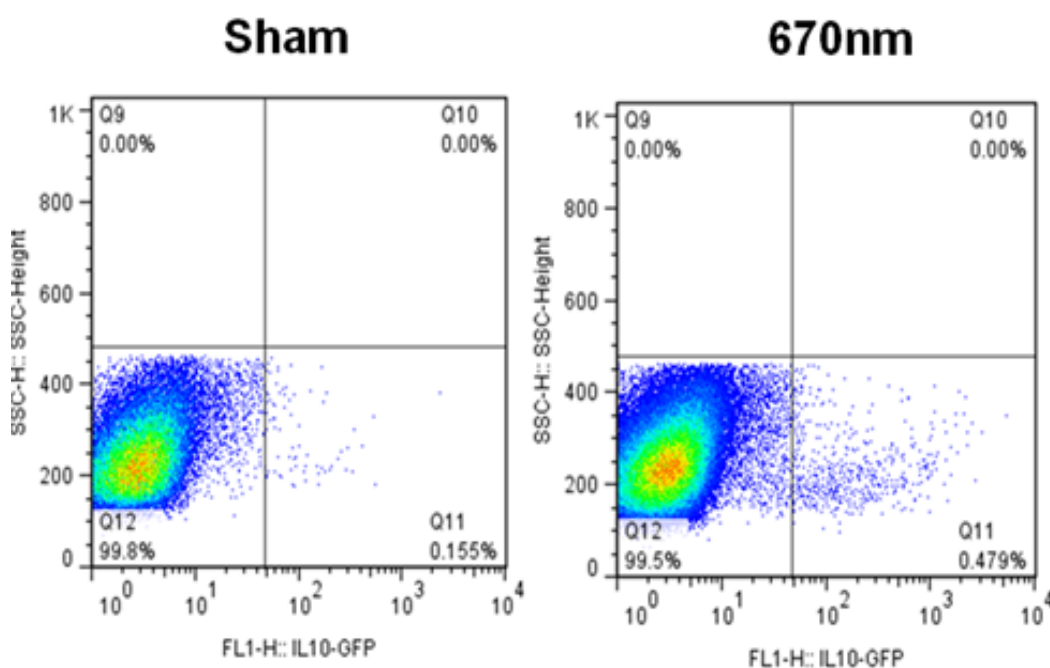
Figure 14:   670nm light induces IL-10 production by lymphocytes. Lymph node cells from MOG35-55 immunized mice were subjected to a 3 day light treatment (670nm) or received no light treatment. Flow cytometry analysis gated on lymphocytes showed an increase in IL-10 production in 670nm light treated cells compared to the sham control. **Induction of IL-10 by 670nm light would be expected to protect against EAE.**

Because it is known that both B and T cells secrete IL-10, further analysis was done to determine which of these lymphocytic populations were responsible for the 670 nm light-induced increase in IL-10 production. The results showed that CD3, not CD19 cells were responsible for the largest increase in IL-10 secretion (Data not shown). Thus, subsequent studies focused on characterization of T cells.

Regulatory CD4$^+$ and CD8$^+$ T cells are known to secrete IL-10. To identify with population of regulatory T cells was induced by 670 nm light, flow cytometric analysis of draining lymph node cells was performed. Cells from IL-10/GFP immunized mice were labeled with CD8, CD4, and CD25 surface markers, and the analysis was gated on the IL-10$^+$ lymphocytes. The highest increase is found in CD8$^+$CD25$^-$ cells (Figure 15B). There was also a modest increase in CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells of the light treated cultures (Figure 15D).
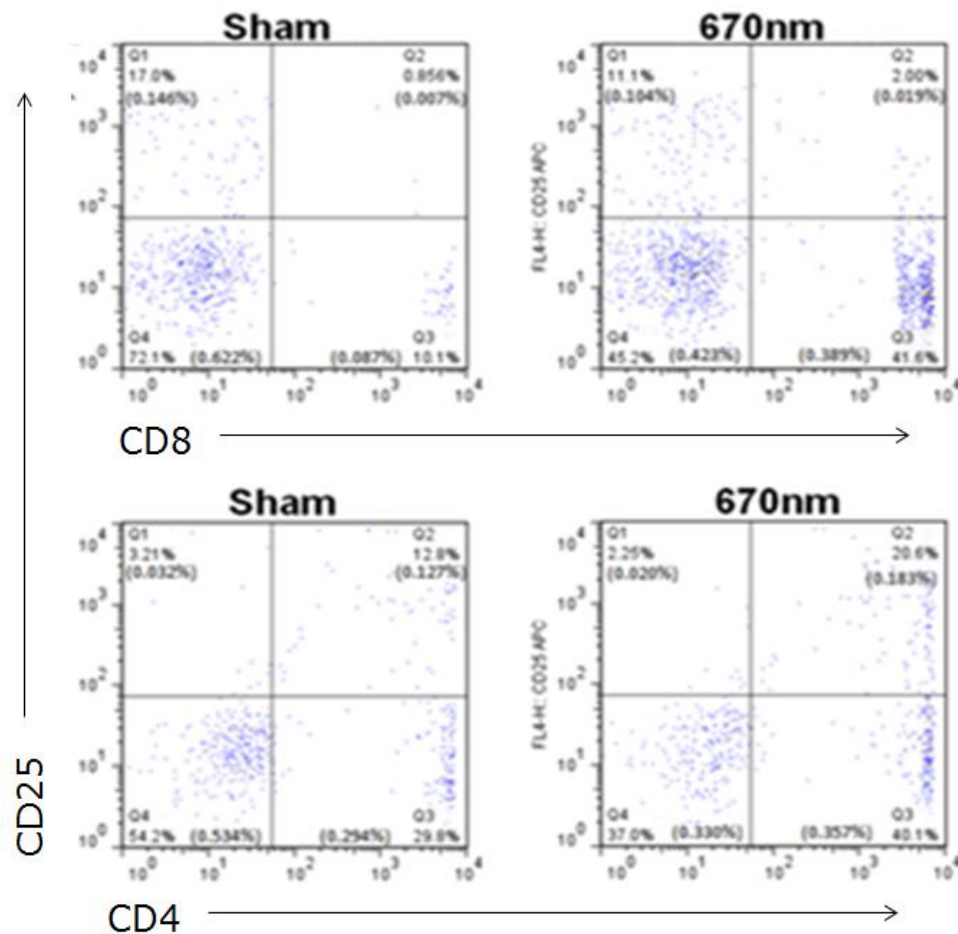
Figure 15: Increased production of IL-10 in lymphocytes induced by 670nm light. Two populations of regulatory T cells – CD8$^+$ and CD4$^+$-- have been implicated in protection against EAE. Lymph node cells isolated from MOG35-55 immunized mice were given a 3 day light treatment (670nm) or no light treatment. Flow cytometry gated on IL-10 revealed an increased population of CD8$^+$CD25$^-$IL-10$^+$ cells induced by 670nm light. A modest increase in IL-10 expression by CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells in the treated group was also noted. **670 nm light induces a population of IL-10 expressing CD8$^+$CD25$^-$ cells which could protect against clinical disease.**

Photobiomodulation increases FoxP3 expression

Production of IL-10 by $CD25^+FoxP3^+$ regulatory T cells is well-characterized [74]. To further investigate the induction of regulatory T cells by 670 nm light, spleen cells from 6 WT immunized mice were labeled with CD8, CD4, and CD25 extracellular markers as well as the intracellular marker, FoxP3, to look at potential Treg involvement. Looking at FoxP3 expression gated on CD3 cells, we noted an increase of FoxP3 in cells cultured with antigen and treated with 670nm light, in comparison to those receiving no light, or not cultured with antigen (Figure 16).
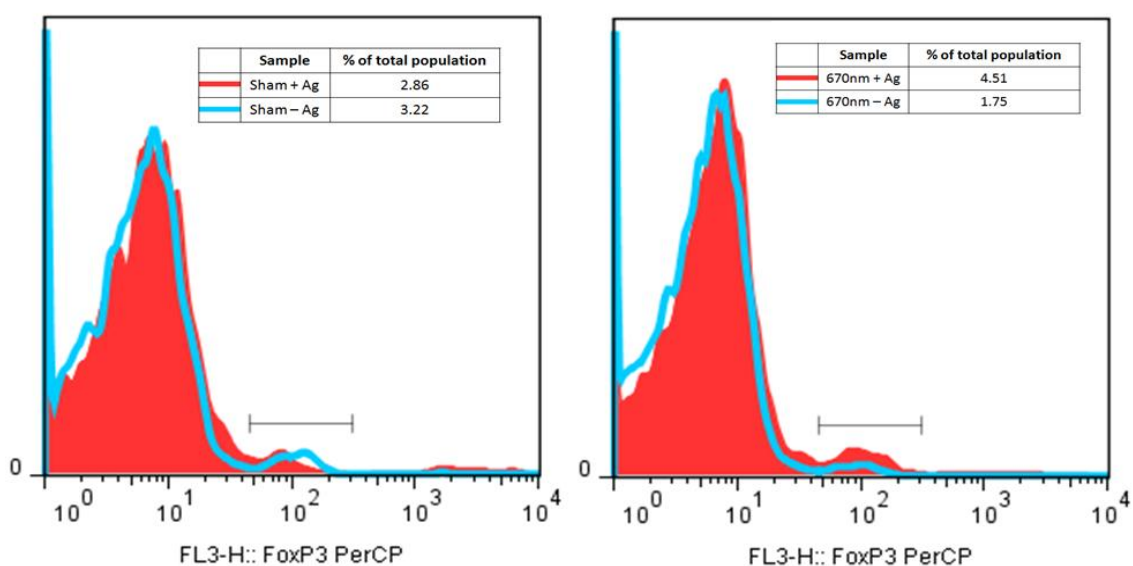
Figure 16: 670nm light in the presence of antigen induces Foxp3 expression. Spleen cells from mice immunized with MOG35-55 were cultured with and without antigen and received 670nm light or no light for 3 days. Flow cytometric analysis gated on CD3^{+} T cells shows an increase of Foxp3^{+} cells when exposed to antigen and 670nm light. **Induction of FoxP3 expression by T cells exposed to 670nm light plus antigen suggests regulatory T cells may play a role in disease protection afforded by 670nm light.**

Due to the fact that the CD8$^+$ cells demonstrated the greatest increase in 670 nm-induced production of IL-10, we wanted to see if this stood true for the cells producing FoxP3. Using the same parameters but gating on CD8 and CD4, analyses show that the FoxP3 producing cells are CD4 and not CD8 (Figure 17).
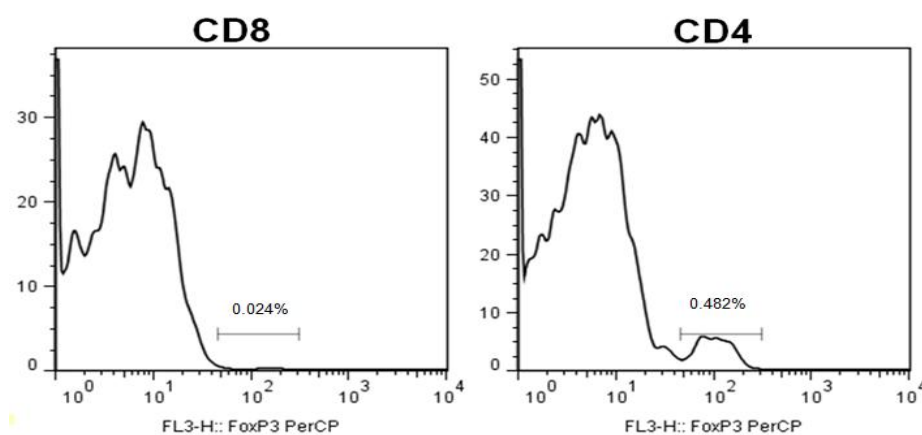
Figure 17: 670nm light induces Foxp3 expression in CD4$^+$ T cells. Spleen cells from mice immunized with MOG35-55 received 670nm light for 3 days. Flow cytometric analysis gated on CD8$^+$ or CD4$^+$ T cells, respectively, shows an increase of CD4$^+$Foxp3$^+$ cells with 670nm light exposure. **CD4$^+$Foxp3$^+$ T cells induced by 670nm light in the presence of antigen could play a role in amelioration of EAE by 670nm light.**

The phenotype of the CD4$^+$ FoxP3$^+$ cells was further analyzed. Gating on FoxP3$^+$, the expression of CD4 and CD25 was investigated in 670 light treated vs. sham treated cells, cultured in the presence or absence of antigen. Analysis of the sham −Ag group (e.g., the immunized control group) revealed a CD4$^+$CD25$^-$Foxp3$^+$ population (Figure 18A). The sham +Ag group has the same CD4$^+$CD25$^-$Foxp3$^+$ population plus a population of CD4^{+hi}CD25$^-$ cells (Figure 18B). 670nm light treatment resulted in the up-regulation of CD4$^+$CD25$^+$FoxP3$^+$ cell population, expected to be regulatory T cells

(Figure 18C). However, in the 670nm +Ag group there is also a CD4^{+hi}CD25$^-$ population (18D).
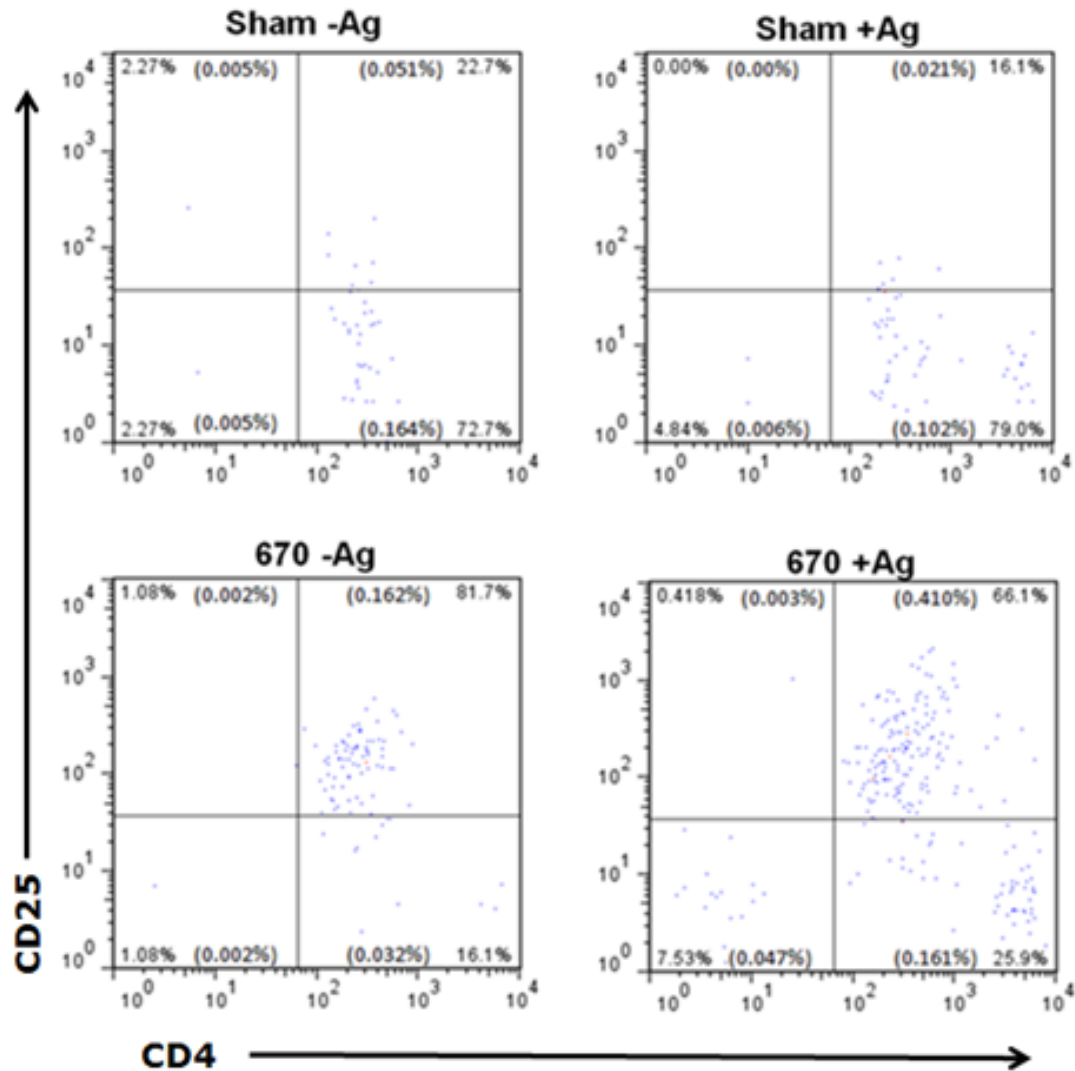
Figure18: Induction of CD4$^+$CD25$^+$Foxp3$^+$ cells by 670nm light treatment. Spleen cells from MOG35-55 immunized mice cultured with and without antigen were treated with light (670nm) or no light for 3 days. Flow cytometric analysis of populations gated on Foxp3$^+$ lymphocytes shows an up-regulation of CD4$^+$CD25$^+$Foxp3$^+$ T cells in the light treated cells compared to the sham controls. There is also an up-regulation of CD4$^+$CD25$^-$Foxp3$^+$ T cells with 670nm light. **Induction of CD4$^+$CD25$^+$Foxp3$^+$ cells, expected to be regulatory T cells, by 670nm light would be expected to reduce autoimmunity in EAE.**

Discussion

Preliminary data has shown the beneficial effect that light therapy has on decreasing the clinical severity of EAE. In the study by Muili *et al.*, as well as our findings from the first specific aim, this decrease in disease is seemingly due to the increase of IL-10 followed by a decrease in pro-inflammatory cytokines afforded through the use of PBM [22]. The next logical step, then, was to determine the cells that, in the presence of the light therapy, were responsible for the secretion of IL-10. The use of GFP aided in this finding because of its interaction with the IL-10 promoter. Therefore, every time IL-10 gene is transcribed GFP will be attached. Because our mice are bred in-house, it is important that a homozygous WT female is bred with a heterozygous GFP/WT male to ensure that the mice are IL-10/GFP heterozygotes.

Through the removal of spleens and lymph nodes of immunized mice, cells could be stained for specific surface markers. This fluorescence-activated cell sorting (FACS) analysis is used as yet another confirmation that the 670nm light is inducing a population of IL-10 producing cells. What differentiates FACS from the other conformational tests is that it allows us to look further into what cells in particular are involved. By staining the cells with the surface markers CD3, CD4, CD8, CD19, and CD25 we were able to distinguish what cells were involved in the highest production of IL-10 in the presence of the light therapy. It is important to note that B cells are involved in the production of IL-10; however, the light therapy has no effect on the up-regulation of these cells. While further analysis on the therapeutic role B cells play in MS/EAE may be looked at down the road, these results ultimately led to the investigation of T cells.

Gating on the IL-10 population, the highest increase of IL-10 production came from CD8$^+$CD25$^-$ cells (Figure 5). CD25 expression is typically linked to regulatory immune cells in which CD25 expression occurs in the thymus (nTreg) or within the periphery (iTreg) [48]. In CD4$^+$CD25$^-$ cells, the expression of CD25 depends on the secretion of IL-2 by antigen-stimulated T cells within the peripheral lymphoid organs [74]. It is important to note, however, that there are circumstances in which Tregs may lack, or lose, CD25 markers. Bonelli *et al.* demonstrated that CD4$^+$CD25$^-$FoxP3$^+$ cells display a suppressive function similar to CD4$^+$CD25$^+$FoxP3$^+$ cells in a model of autoimmunity [75]. To see if this was the case for the CD8$^+$CD25$^-$ population, FoxP3 staining was done. As a reminder, FoxP3 is an intracellular marker typical of most regulatory cells, specifically CD4$^+$ Tregs and some CD8$^+$ Tregs [75, 76]. The results from this finding indicated that these CD8$^+$ cells responsible for such a sharp increase of IL-10 do not contain FoxP3. This does not mean, however, that they are not regulatory CD8$^+$ cells. Because CD8$^+$ Tregs have not been investigated as much as CD4$^+$ Tregs, the specific markers of regulation have not been fully established, and FoxP3 expression may not be a specific marker for CD8$^+$ Tregs [76]. The majority of FoxP3-expression is linked to MHC class II restricted CD4$^+$ cells [76]. In a study by Trandem *et al.,* it was hypothesized that IL-10 production from CD8$^+$ T cells may be another mechanism in place to diminish destruction of tissues in models of acute encephalitis or it may be used as a self-regulation of CD8$^+$ production [77]. Further analysis of these cells would need to be done to determine regulatory functions or the possible relationship between cytotoxic CD8$^+$ T cells and IL-10 production.

While the largest increase of IL-10 was seen in $CD8^+CD25^-$ T cells, we chose to investigate the role of FoxP3 expression in $CD4^+$ T cells in the presence of 670nm light because more research has gone into the expression of these cells. In putative $CD4^+$ regulatory cells, FoxP3 expression is up-regulated in the presence of 670nm light (Figure 7). By gating on the population of FoxP3, Figure 9 looks at CD4 and CD25 markers to further identify these cells. In the sham −Ag group, in which the cells are only exposed to the self-reactive peptide at immunization, $CD4^+CD25^-FoxP3^+$ cells are present. There are conflicting views as to the functionality of this population of cells. The majority of the characterization of these cells has occurred in systemic lupus erythmatosus, a systemic autoimmune disease. In a published manuscript from Yang *et al.* this $CD4^+CD25^-FoxP3^+$ population, as well as $CD4^+CD25^{low}FoxP3^+$ cells, were identified to be distinct from Tregs, not only phenotypically, but through the production of increased IL-2 and are, therefore, activated T cells [78]. Bonelli *et al.*, however, found these cells to resemble functional Tregs *in vitro* [75]. In this study, they also found that this population of putative Tregs did not display increased levels of IL-10 or TGF-β, indicating that these cells are not cytokine mediated [75]. What they concluded is that these cells resemble a population partially functioning of Tregs [75]. Including the Ag in the sham culture resulted in an induced population of $CD4^{low}CD25^-FoxP3^+$ cells.

The exact role and relationship of these two different populations has yet to be established. However, because these cells are acquiring FoxP3 expression in the periphery (i.e. iTregs) there is clearly a maturation process that must occur. In this instance, it is possible that FoxP3 expression is acquired before CD25 expression, giving

us this population of CD4$^+$Cd25$^-$FoxP3$^+$ cells. This would indicate that these cells are immature Tregs waiting for CD25 expression to maturate.

The CD4$^+$CD25$^-$FoxP3$^+$ cells found in the sham group, seemingly acquire CD25 expression in the 670nm treated cells cultured in the absence of Ag (Figure 18). Similarly, the CD4$^+$CD25$^-$FoxP3$^+$ population in the sham +Ag group is less robust and an increase of CD4$^+$CD25$^+$FoxP3$^+$ cells, presumably Tregs, arise. These findings would agree with hypothesis that these are immature Tregs acquiring CD25 expression in the periphery. It would also go to stand that the 670nm light therapy induces this immature population of putative Tregs to become fully functional Tregs.

CHAPTER V: DISCUSSION

Discussion

As the most common cause of neurologic disorders of early to middle adulthood, multiple sclerosis and its complexities are of interest of many researchers [2]. Previous research describes the disease as an immune-mediated demyelinating disease of the CNS, initiated by MHC class II-restricted, auto-reactive T cells due to [32,64]. These T cells are responsible for secreting pro-inflammatory cytokines (INF-γ and IL-17) important in the pathogenesis of disease. The exact mechanisms of the disease are unknown; however, mitochondrial dysfunction [32] and oxidative stress [2] have been shown to play a role in axonal loss. While many anti-inflammatory, immunomodulatory, and immunosuppressive therapeutics demonstrate proven efficacy in the delaying the

progression of the disease, there are no approved therapeutic agents which prevent or reverse the disease [64].

Experimental autoimmune encephalomyelitis (EAE) is the primary animal model for MS, sharing clinical, mechanistic, and histopathological aspects with MS. It should be noted that as an animal model, EAE does have limitations. A major criticism of EAE is that it is not a spontaneous disease. While a spontaneous disease model has been developed with transgenic mice, most EAE activation requires active immunization as well as the use of an adjuvant to further induce disease [20, 79]. EAE has also been criticized because the disease is often studied in in-bred animals, in which much of the genetic heterogeneity has been removed. It can be argued, though, that this homogeny is required for the reproducibility of the disease among generations of the animals [20].

Preliminary data in the EAE model suggests disease amelioration is possible using 670 nm light therapy [22]. This was not only seen through a decrease of clinical scores in 670 nm light-treated animals in comparison to sham treated mice, but also found in pro-inflammatory/anti-inflammatory cytokine ratios [22]. The current studies further investigated the mechanism of immune modulation by 670nm-mediated light therapy. Our ELISA data show up-regulation of IL-10 and a down-regulation of IFN-γ in the 670nm treated group. In the presence of the light, the gene expression of IL-10 is increased. QPCR analysis shows trends of increasing IL-10 production in the presence of light. An increase in IL-10 production was also increased in the presence of 670 nm light in our FACS and ELISA analyses, as well as with ELISA and QPCR analyses in recent published data [22]. Decreased IL-10 expression levels in our 670 nm light group in comparison to the sham group when analyzed using QPCR between the current data and

that previously published by this laboratory may be due to differences in experimental design. Previous QPCR data from Muili *et al.,* demonstrating increased anti-inflammatory and decreased pro-inflammatory cytokines with 670nm light treatment was performed on spinal cords of treated mice extracted throughout the course of the experiment [22]. The current studies were performed on lymphoid cells isolated from immunized mice and treated *in vitro*. It is important to note that IL-10 production is not limited to lymphocytes. Through the removal of spleens and lymph nodes for the *in vitro* analyses, we are targeting B and T cells and looking at the effects the light has on these cells specifically. While there is certainly a case for the regulatory effects of the light within these two cell populations, perhaps the spiked increase in the *in vivo* analyses is due to non-lymphocyte cells, such as astrocytes or microglia, reacting to the light to produce IL-10 as well [80].

The protective nature of the light therapy could be due to either a down-regulatory effect that the light has on pro-inflammatory cytokines, an increase in IL-10 afforded by the light that results in shifting the environment to a more anti-inflammatory disease state, or, most likely, both factors. This is supported by the findings from ELISA data that showed an increase in IL-10 production with a decrease in IFN-γ (Figure 10), and QPCR analysis that shows that even when IL-10 levels are low, IFN-γ levels continue to decrease (Figure 11).

It is reasonable to believe that harvesting cells 2h post treatment does not allow enough time for transcription to take place. This is also supported by the fact that upon light exposure, an initial spike in ROS and NOS occurs in order to prompt transcription factors to produce protective genes [70]. Eventually the ROS species will be decreased

resulting in long term protection. The microarray at 96h (Figure 12) showed increased levels of IL-17A, which is known to be responsible for the up-regulation of NO [81], indicating that transcriptional processes were merely beginning to take place, not allowing enough time for translation to occur. Because it is known that IL-10 is regulated primarily by transcription and post-transcriptional factors [73], allowing more time for the light to take full effect would presumably increase IL-10 expression.

Due to our findings that 670nm light had little effect on B cells we chose to focus on IL-10 production in Tregs. Supported by a study by Matsushita *et al*., up-regulation of Tregs or IL-10 production through Tregs after disease onset will prove to be most beneficial in disease amelioration [42]. The study by Matsushita *et al.* proposed that Bregs reduce disease severity during EAE initiation and Tregs inhibit the late stage effects of MS [42]. The deletion of Bregs before immunization resulted in a more severe disease than when Bregs were deleted during EAE progression. Similarly, the deletion of Tregs had no effect on disease onset, but severely exacerbated the disease in later stages [40].

While further characterization of the putative Treg population is necessary, our data suggest that the light is inducing iTregs to produce IL-10. This is due to the fact that FoxP3 expression levels increased in the presence of the light. The light also affected the production of $CD25^+$ cells as well. A population of $CD4^+CD25^-FoxP3^+$ cells found in the sham controls, presumably immature Tregs, gain CD25 expression when light was administered. Through the induction of CD25 on these cells, the population is thought to mature to become functional Tregs. While more studies are necessary to confirm

findings, the cells responsible for the up-regulation of IL-10 are likely responsible for the decrease in clinical disease severity seen in *in vivo* experiments.

Future Directions

To continue this study, a number of experiments must be performed. More experiments of *in vitro* studies needs to be done to understand the effect the light has on message and protein expression of IL-10. There needs to be further clarification on the potential decrease of message and increase gene expression of IL-10 seen in QPCR analysis. A longer incubation period between treatment and cell harvesting may allow for more complete transcription and translation. From there, characterization of the $CD4^+CD25^-FoxP3^+$ and $CD4^+CD25^+FoxP3^+$ populations must be done to verify suppressive properties. Characterization can be done through co-culture and transwell assays. Further investigation of the IL-10 producing $CD8^+$ T cells and their role in the amelioration of EAE would also be beneficial in understanding disease amelioration through the use of 670 nm light. An adoptive transfer experiment could be done once it is determined what cells are most beneficial in the amelioration of EAE. These cells would be transferred into a recipient host in hopes of transferring the immunologic benefits of the disease, or induce tolerance to the disease.

In vivo studies of $IL-10^{-/-}$ mice must also be repeated. Treatment through a suppression protocol in these $IL-10^{-/-}$ mice would help to further understand disease course. In this protocol, the mice would be treated for 10 consecutive days after immunization. This addresses the issue concerning at which time point is treatment is

most beneficial; treatment before disease onset, or treatment throughout the disease course.

Conclusion

Photobiomodulation has the potential to decrease MS/EAE disease severity through the up-regulation of IL-10 and down regulation of IFN-γ. While the largest increase of this IL-10 production was seen in $CD8^+CD25^-$ cells, increased production of $FoxP3$ led our analyses toward $CD4^+$ T cells. While confirmation of this population of putative regulatory cells is necessary, an increase in $CD4^+CD25^-FoxP3^+$ T cells appear to be responsible for the decreased disease severity found in WT mice receiving 670nm light therapy. With this knowledge, PBM is a promising adjunct therapy in the amelioration of MS.

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