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The Mechanism of Suppression By a Protective Peptide in a Mouse Model of Multiple Sclerosis

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THE MECHANISM OF SUPPRESSION BY A PROTECTIVE PEPTIDE IN A MOUSE MODEL OF MULTIPLE SCLEROSIS

by

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ABSTRACT

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Abstract: Multiple Sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS). MS is characterized by an immune response directed against myelin sheath. This immune response results in demyelination, which leads to the clinical symptoms of MS. It is accepted that MS is mediated by T helper 1/ T helper 17 immune responses. However, the role of B cells and antibodies (Abs) are still under debate. The primary animal model for MS is the experimental autoimmune encephalomyelitis (EAE) that is induced by immunizing animals with one of the myelin components. We previously showed that immunizing mice with the recombinant form of myelin oligodendrocyte glycoprotein (rMOG) results in ameliorated EAE compared to mice immunized with the encephalitogenic peptide MOG_{35-55}. This amelioration was due to the presence of a cryptic epitope of MOG_{61-85} in rMOG as observed in previous peptide mapping analysis. We further investigated the mechanism of EAE amelioration in mice immunized with a longer

peptide MOG_{35-85}, encompassing both MOG_{35-55} and MOG_{61-85} peptides. The mechanism of suppression was shown to be independent of interleukin-10 (IL-10) secretin. This led to the hypothesis that MOG_{61-85} ameliorates EAE through the secretion of transforming growth factor-beta (TGF-β). Therefore, B cell deficient (B cell$^{-/-}$) mice were either co-immunized with both MOG_{35-55} and MOG_{61-85} peptides or immunized with MOG_{35-55} only. Mice were monitored for EAE induction and progression. The cellular response to MOG_{61-85} *in vitro* priming was assessed using flow cytometry. Results showed comparable FoxP3 expression level in $CD4^+CD25^+$ T cells between cell cultures; however a slight increase in FoxP3 expression in $CD8^+CD25^-$ T cell population was observed with MOG_{61-85} *in vitro* priming that was dependent on MOG_{61-85} *in vivo* priming. These results support the suppressive effect of MOG_{61-85} on the immune response and suggest a role of CD8 T regulatory (Treg) cell population in EAE amelioration. Evaluation of MOG_{61-85} specific signaling cytokine showed that MOG_{61-85} induces regulation independent of IL-10 secretion. This was confirmed by immunizing wild-type (WT) and IL-10 deficient (IL-10$^{-/-}$) mice with rMOG and measuring the anti-inflammatory cytokines produced in response to MOG_{61-85} *in vitro* priming. Analysis for TGF-β showed that MOG_{61-85} specific immune response is characterized by high TGF-β secretion. To evaluate the immune response generated with MOG_{61-85} stimulation, TGF-β:IL-6 ratio was mathematically calculated. Data showed that MOG_{61-85} induces an anti-inflammatory immune response characterized by high TGF-β and low IL-6 secretions. These experiments provide understanding of the protective immune response generated in response to MOG_{61-85} priming.

Dedicated to my family

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LIST OF ABBREVIATIONS

AAALAC: association for assessment and accreditation of laboratory animal care

Ab: antibody

Ag: antigen

ANOVA: analysis of variance

APC: antigen presenting cell

APC: Allophycocyanin

Avidin-HRP: Avidin-Horse Radish Peroxidase

BBB: Blood Brain Barrier

B cell$^{-/-}$:B cell deficient

Breg: regulatory B cells

B2 cells: follicular B cells

B10 cells: interleukin-10 producing regulatory B cells

CCK-8: cell counting-8 assay

CFA: complete Freund`s adjuvant

CNS: central nervous system

ConA: Concanavalin A

CO_2: carbon dioxide

cRPMI 1640: complete *Roswell Park Memorial Institute* 1640

CSF: cerebrospinal fluid

dpi: days post immunization

EAE: experimental autoimmune encephalomyelitis

EBV: Epstein-Barr virus

ECAM: endothelial cell adhesion molecules

ELISA: enzyme linked immunosorbent assay

FACS: Fluorescence-activated cell sorting

FITC: Fluorescein isothiocyanate

FoxP3: Forkhead Box Protein P3

g: gram

GITR: glucocorticoid-induced tumor necrosis factor receptor

G-MDSC: granulocytic myeloid-derived suppressor cells

HBSS: Hanks balanced salt solution

HBVP: Hepatitis B Virus Polymerase

HCL: hydrochloric acid

HEPES: hydroxyethylpiperazine-N-2-ethane sulfonic acid

HHV-6: human herpes virus-6

hi: High

HLA: Human leukocytes antigen

hrs: Hours

ICAM-1: intracellular adhesion molecule-1

IFA: incomplete Freund's incomplete adjuvant

IFN- γ: interferon gamma

Ig: immunoglobulin

IL-2R: interleukin-2 receptor

IL-7R: inteluekin-7 receptor

IL-$10^{-/-}$ mice: IL10 deficient mice

Interleukin: IL

IP: intraperitoneal

iTreg cells: induced regulatory T cells

LAP: latency associated peptide

LFA-1: lymphocyte function associated antigen-1

L-Glut: L-glutamate

LN: lymph node

LSM: lymphocyte separation media

MBP: myelin basic protein

MDSC: myeloid-derived suppressor cells

MHC: major histocompatibility complex

MHC II: major histocompatibility complex class II molecule

min: minutes

ml: milliliter

M-MDSC: Monocytic myeloid-derived suppressor cells

MMP: matrix metalloproteinase enzyme

MOG: myelin oligodendrocyte glycoprotein

MRI: magnetic resonance imaging

MS: Multiple Sclerosis

NaOH: sodium hydroxide

Na pyruvate: sodium pyruvate

ng: nanogram

nm: nanometer

NO: Nitric Oxide

nTreg cells: natural regulatory T cells

OB: oligoclonal band

PBS: phosphate buffer solution

PE: Phycoerythrin

PerCP-Cy5.5: Peridinin-chlorophyll proteins-cyanine 5.5

PLP: proteolipid protein

PP-MS: primary progressive multiple sclerosis

PR-MS: progressive-relapsing multiple sclerosis

P/S: Penicillin/Streptomycin

PT: Pertussis Toxin

RCF: relative centrifugal force

rMOG: recombinant human myelin oligodendrocyte glycoprotein

ROR-γt: retinoid-related orphan receptor gamma t

RR-MS: relapsing remitting multiple sclerosis

SC: subcutaneous

SPL: spleen

SP-MS: secondary progressive multiple sclerosis

TCR: T cell receptor

TGF- β: transforming growth factor- beta

Th1: T 1 Helper Immune Response

Th2: T 2 Helper Immune Response

Th17: T 17 Helper Immune Response

TNF-α: tumor necrosis factor-alpha

Treg: regulatory T cells

VCAM-1: vascular cell adhesion molecule-1

VLA-4: very late activation antigen-4

WT: wild-type

α4 β1: alpha 4 beta 1 integrin

µg:micrograms

µl: microliter

2-ME: 2-mercaptoethanol

♀: female

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CHAPTER I: INTRODUCTION

Multiple Sclerosis (MS)

Multiple Sclerosis (MS) is an autoimmune disease of the central nervous system (CNS); the brain and the spinal cord. It is mediated by an inflammatory immune response directed against the myelin sheath insulating neurons. This inflammatory response results in myelin damage or demyelination, which interferes with the transmission of the nerve impulses of the affected neurons. Demyelination exposes neurons to toxic inflammatory elements, which increase the probability of axonal loss leading to permanent neurological disability. These areas of inflammation and neural injury are observed as perivascular brain lesions or plaques within the white matter [1,2].

MS occurs mostly in young adults ranging between 20-40 years old [1,2]; however, recently more pediatric cases are being reported. There is no known explanation of the increase in MS prevalence rate among this population [3]. Additionally, MS is three times more common in females than males. Currently, MS has a global prevalence of 2.5 million people with 400,000 cases solely in the United States [4].

Etiology and Risk Factors

The exact cause of MS is still unknown, however, certain genetic and environmental risk factors were found to increase the susceptibility to MS development. Major histocompatibility complex (MHC) genes are the most common genetic risk factor for MS development, particularly human leukocyte antigens (HLA) such as HLA-DR and HLA-DQ molecules [1,2,5]. Additionally, studies of non-HLA genes, such as interleukin-7 receptor (IL-7R) [6] and IL-2 receptor (IL-2R)[5,7], which have immuno-regulatory functions, indicate that genetic polymorphisms of these genes also play a role in the disease

susceptibility [6,7]. Additionally, some studies suggest the possibility of an infectious agent in initiating MS. Due to seropositivity in MS, latency and their involvement with the nervous system, the most commonly studied infectious agents with MS development are Epstein-Barr Virus (EBV) [8,9] and human herpes virus-6 (HHV-6) [10]. Some of these infectious agents showed a significant association with MS and correlation in the development of MS attacks [11], yet no defined infectious agent has been proven to cause the disease, directly.

Signs and Symptoms

MS is a heterogeneous disease, as the clinical presentation varies between patients [1,2]. The clinical picture of MS chiefly depends on the site of the brain lesions, the type of the affected neuron and the degree of demyelination [1,2]. However, the optic, motor and sensory neurons are the most affected neurons [2]. MS symptoms include general fatigue, walking difficulties, numbness, spasticity, muscle weakness, blurry vision, bladder dysfunction, bowel problems and depression [12,13].

MS is classified into four types based on the clinical progression of the disease. The most common type is the relapsing-remitting (RR) MS, characterized by periods of attacks followed by periods of complete recovery. Relapsing-remitting disease is typically followed by secondary-progressive (SP) MS within 5-10 years of diagnosis. SP-MS is characterized by periods of attacks, but incomplete recovery. Primary-progressive (PP) MS, on the other hand, has no remission phase and is characterized by continuous increase in MS severity. The rare form of MS is called progressive-relapsing (PR) MS, which has a continuous increase of disease severity with some occasional attacks [1,13].

Diagnosis

MS diagnosis is challenging due to lack of specific markers that distinguish MS from other diseases. However, diagnosis is possible based on the unique clinical presentation, the presence of oligoclonal band (OB) in the cerebrospinal fluid (CSF) and the visualization of brain or spinal cord lesions using magnetic resonance imaging (MRI) [1,2,13,14]. Oligoclonal bands are immunoglobulins (Igs) with different specificity to myelin and non-myelin antigens (Ags) found within the CSF but absent from the peripheral blood. The presence of Igs in the CSF is an indicative of intrathecal production, and it was found that the level of these antibodies (Ab) is positively correlated with the disease progression [15]. Additionally, the number of brain lesions is positively correlated with the disease disability.

MS Pathology

MS pathology is not fully elucidated. However, it is accepted that the immune response drives the pathology. The inflammatory immune response in MS is triggered by complex interaction of an unknown agent and genetic susceptibility. Although the etiology is still unknown, it is accepted that the pathology is generated in three crucial steps; activation of T cells in the periphery, infiltration of immune cells into the CNS and demyelination [1,2].

Naïve CD4+ T Cell Activation

It is accepted that the activation of auto-reactive CD4+ T cells is the driving force in initiating MS pathology. The activation of these cells is not fully understood. It was suggested that in the peripheral blood, antigen presenting cells (APC) uptake foreign microbial antigen for processing and present an epitope to naïve CD4+ T lymphocytes. The presented epitope might share some structural similarities with myelin antigens that lead to the generation of auto-reactive CD4+ T lymphocytes. This hypothesis is called

"molecular mimicry" and it suggests the possible cross-reactivity in the immune response between self- and non-self Ags post infection [16]. *Fujinami et al*. showed that Hepatitis B Virus Polymerase (HBVP), a hepatitis virus protein, shares some sequence similarities with the major myelin protein, myelin basic protein (MBP), and is capable of inducing autoimmunity in rabbits similar to MS. The production of Abs that are reactive both to the HBVP and MBP and the histological picture of the rabbit brains with the mononuclear cell infiltration supported this hypothesis [17]. The hypothesis of molecular mimicry is not restricted to viruses as potential activators of T cells. A study by *Wucherpfennig et al.* investigated possible stimuli for T cell receptor (TCR) of MBP-specific T cells derived from MS patients. In their study, they showed that peptides from *Pseudomonas aeruginosa* and *influenza type A virus* stimulate clonal expansion of T cells similarly to MBP_{85-99} peptide [18]. Recent work by *Hughes et al.* showed that bacteria such as *Pseudomonas aeruginosa* and *Acinetobacter sp.* share some structural similarities with myelin components. This was further supported as serum derived from MS patients reacted to the peptide sequences of both myelin and bacteria [19]. These studies introduce possible triggers either viral or bacterial in MS initiation; however, the exact etiology is still to be investigated.

Another hypothesis for the activation of naïve CD4+ T lymphocytes is called "Bystander Activation". This hypothesis suggests that a pathogen-specific inflammatory response might initiate an unrelated autoimmune response [20]. Sensitization of the immune system with a microbe activates innate immune cells or APCs that are capable of activating auto-reactive T cells, hence inducing autoimmunity. Moreover, microbial activation of the immune response might lead to direct damage without activation of auto-

reactive T lymphocytes, due to the release of inflammatory substances such as nitric oxide (NO) [21] or pro-inflammatory cytokines [22]. This is called "Bystander killing" in which the immune response is mounted against infected cells, however un-infected cells might be destroyed as well [20]. *McCoy et al.* suggested that MS pathology might be induced by a combination of both "Molecular Mimicry" and "Bystander Activation". They speculated that a microbe or microbial peptide mimicking one of myelin components prime CD4+ T cells, however an irrelevant infection is crucial in driving the pathology by releasing pro-inflammatory cytokines [23]. Although many studies showed that the clinical and histopathological picture of MS could be generated based on these hypotheses, further studies are needed to support their roles in MS initiation.

CNS Immune Infiltration

Once activated in the periphery, auto-reactive CD4+ T cells migrate to the CNS by crossing the blood-brain barrier (BBB). The CNS is a restricted site [24] in which the vascular structure of the BBB separates circulating blood from the CNS, and permits the entry of nutrients and only limited immune components. The BBB consists of an endothelial lining supported with astrocytes, pericytes and microglia cells [25]. Damage to the BBB integrity aids in immune cell infiltration to the CNS. The cause of BBB damage is not fully known, however, secreted pro-inflammatory cytokines, chemokines and matrix metalloproteinase enzymes (MMP) may affect the cellular junctions of the BBB [26].

Auto-reactive $CD4^+$ T cells circulate in the periphery and slow their movement when they reach the BBB [27]. Selectin molecules on the $CD4^+$ T cells bind to their ligands expressed by the endothelial lining, which tether auto-reactive T cells to the BBB [27, 28, 29]. Tethered lymphocytes roll on the endothelial lining of the BBB [27]. Furthermore,

$CD4^+$ T cell express integrin molecules [30, 31] such as alpha 4 beta 1 ($\alpha 4\ \beta 1$) integrin or very late activation antigen-4 (VLA-4) and lymphocyte function associated antigen-1 (LFA-1). These molecules bind T cells to intercellular adhesion (ICAM-1) and vascular cell adhesion (VCAM-1) molecules on the BBB, respectively [32]. Adhesion of lymphocytes to the BBB lining facilitates extravasation of these cells to the CNS [27, 30]. The expression of endothelial cell adhesion molecules (ECAM) is enhanced with the exposure to pro-inflammatory cytokines, thus facilitating immune infiltration to the CNS. An increase in VCAM-1 and ICAM-1 expression was observed in MS patients and was positively correlated with disease activity [33]. Not only do auto-reactive $CD4^+$ T cells cross the BBB, but also B-lymphocytes and innate immune cells such as natural killer cells, monocytes and dendritic cells [27].

Generation of an Immune Response

The type of the inflammatory immune response generated depends on the cellular signaling of cytokines. Additionally, the type of the immune response generated determines the extent of pathology, hence clinical symptoms. Many immune responses had been described in MS pathology, among those are:

- *T 1 Helper Immune Response (Th1)*

  Interferon gamma (INF- γ) secreted by microglia and interleukin (IL)-12 induces the initiation of Th1 immune response. Th1 immune response is characterized by the production of pro-inflammatory cytokines such as INF-γ and tumor necrosis factor-alpha (TNF-α) and chemokines. In addition, Th1 immune response activates macrophages and recruits more immune cells to the site of inflammation. It is accepted that MS pathology is mediated by a Th1 immune response [1,2].

- *T 17 Helper Immune Response (Th17)*

Recently, Th17 immune response was described to be a major contributor in MS pathogenesis. Signaling of IL-23 [34] and transforming growth factor-beta (TGF-β) [35] and IL-6 initiate a Th17 response by up-regulating the expression of the transcription factor retinoid-related orphan receptor gamma t (ROR-γt) [36]. This pro-inflammatory T helper lineage is characterized by the production of the pro-inflammatory cytokines IL- 17, IL-21 and IL-22. It is accepted that MS pathogenesis is mediated by a Th1/Th17 paradigm [1,2].

- *T 2 Helper Immune Response (Th2)*

IL-4 signaling induces a Th2 immune response. This immune response is considered beneficial as it ameliorates the inflammation through the secretion of anti-inflammatory cytokines such as IL-10 and IL-4. However, a Th2 immune response also leads to the activation of B cells into plasma cells and isotype class switching, leading to the production of antibodies implicated in demyelination [1, 37, 38, 39].

Demyelination

MS is characterized by demyelination mediated by an immune response. Auto-reactive T cells recognize myelin Ags presented by APC through MHC class II (MHC-II) molecules to specific TCR. These myelin Ags are derived from the myelin sheath on axons or oligodendrocytes, myelin producing cells [1]. Additionally, T cells activate macrophages leading to myelin phagocytosis, hence myelin damage. The pro-inflammatory cytokines and NO produced also contribute to myelin and oligodendrocyte damage. Moreover, myelin specific-Abs opsonize myelin Ags that further enhance phagocytosis [40] or

activate the complement cascade [41] as observed in studies in animal models of MS. Demyelination forms the characteristic perivascular plaques located within the white matter that can be observed with the use of MRI [1]. However, cellular trafficking to the CNS result in highly inflamed meninges. This inflammatory infusion leads, in part, to the formation of cortical grey matter plaques in some MS cases [42].

Initially, the immune response is directed against one component of the myelin sheath. Activated cells secrete pro-inflammatory cytokines and chemokines, to activate more immune cells and recruit them to the CNS, respectively. The damage to myelin reveals more of its Ag that lead to the initiation of other immune responses with different target Ags. The subsequent generation of immune responses against other myelin components is called "epitope spreading". Epitope spreading is thought to be responsible for enhancing demyelination and worsening the clinical presentation of MS.

Axonal Loss

The role of myelin is to protect neurons and facilitate the transmission of the nerve impulses. In MS, demyelination exposes neurons to an inflammatory environment containing pro-inflammatory cytokines and NO. Re-myelination may occur, in the remitting forms of MS, however the produced myelin is thinner. Chronic demyelination leads to neural transection or the separation of the distal end of the neuron, which prevents the transmission of nerve impulses. This is followed by the formation of "end bulb" structure and eventual neural degeneration [43].

Gliosis

Gliosis or scarring is a tissue death that results from plaque formation. These scars result from the reactive changes in one of the CNS glial cells such as microglia, astrocytes and

oligodendrocytes in response to demyelination. Demyelination leads to axonal damage and forms plaques. However, pathological destruction of the myelin sheath varies between patients depending on the immunological players employed. Therefore, MS plaques are classified based on their content of immune cells and other immune-related proteins. There are four types of plaques, each with distinct immune composition indicating different types of pathogenesis. Both type I and type II plaques are characterized by damaged BBB, hence the plaques are perivascular in location. Additionally, both types are characterized by a predominant presence of T cells and macrophages and the preservation of oligodendrocytes. However, Type II plaques have more Abs and complement, indicating their possible role in demyelination in this type. Type III plaques are similar in composition to type I and type II. However, oligodendrocytes are damaged, and the brain scarring is more diffuse rather than being limited to the perivascular areas. Similarly, type IV is characterized by the presence of T cells and macrophages. However, more oligodendrocytes are lost with minimum changes in myelin components [44]. How these different histopathologic pictures relate to the clinical presentation of MS is still under investigation.

Immune Regulation

The immune response is regulated through the function of regulatory or suppressor cells such as myeloid-derived suppressor cells (MDSC), regulatory B (Breg) cells and regulatory T (Treg) cells [45]. It is accepted that the adaptive immune response involves the recruitment of both effector and regulatory immune cells to properly control the immune response and maintain self-tolerance [46]. Regulatory cells control the immune response through their suppressive activity either by direct cell-cell contact or by secreting anti-

inflammatory cytokines [47]. It was suggested that the immune regulation in MS is impaired either due to lower number of regulatory cells or to impairment in their activity.

- *MDSC*

The myeloid derived suppressor cell population consists of immature myeloid cells with blocked differentiation. Their role in suppressing the immune response is still to be fully elucidated. However, it was suggested that upon activation they express their suppressive activity by direct cell contact, production of suppressive factors, or by interacting with regulatory T cells. Due to their immaturity, they lack the expression of mature myeloid cell markers [48]. Based on their cellular markers, they are divided into two subpopulations; monocytic and granulocytic MDSCs. Monocytic MDSCs (M-MDSC) are defined as $CD11b^{+}$ $CD14^{-}$ $CD33^{+}$ cells while granulocytic MDSCs (G-MDSC) are defined as $CD11b^{+}$ $CD15^{+}$ $CD33^{+}$ cells [48, 49]. The role of each subset of MDSCs in regulating myelin-specific T cells in MS patients is still under investigation. The role of MDSCs in the animal model of MS is discussed below.

- *Breg cells*

The Breg cell population is a subset of B cell lineage with suppressive functions. The discovery of Breg cells is recent. Thus, their exact phenotype and mechanisms of suppression are still under investigation. Most of the discovered Breg cells are IL-10 producers. Thus, they are referred to as B10 cells [50]. However, non-IL-10 Breg cells may also exist. A study by *Mauri et al.* showed that the human Breg cell population is phenotypically defined as $CD19^{+}$ $CD24^{hi}$ $CD38^{hi}$ cells with the ability to produce IL-10 upon stimulation in healthy individuals [51]. Moreover, a study by *Iwata et al.* to

identify B10 cells in human showed that IL-10 producing subset of B cells is phenotypically defined as $CD24^{hi}$ $CD27^{+}$ B cells. In their study, they showed that B10 cells might also express CD48 and CD148, characteristic markers of cell activation and cell memory, respectively [52]. In addition to IL-10 production, a subset of Breg cells produce TGF- β to regulate inflammation [53]. Lack of unique cellular markers introduces necessities to characterize the Breg cell population along with their suppressive activities.

- *Treg cells*

Treg cell development may occur in the thymus or the periphery yielding the production of thymic-derived or natural Treg (nTreg) and induced Treg (iTreg) cell populations, respectively [54]. Some of Treg cells express Forkhead Box Protein P3 (FoxP3) or Scurfin, a transcription factor that is important for the development of Tregs and in maintaining their suppressive activity [54, 55, 56]. This transcription factor is expressed in the majority of Treg population; however, it is not an exclusive cellular marker [57]. Additionally, it is known that IL-2 is important for maintaining FoxP3 expression and that naïve T cells can differentiate into FoxP3+ Treg cells in a TGF-β-dependent manner. However, TGF-β and IL-6 signaling facilitate the differentiation of naïve T cells into Th17 cells, which mediates a strong pro-inflammatory immune response [46].

The mechanism by which Treg cells control the immune response is not fully understood. However, they may regulate the immune response by direct cell-cell contact or through the secretion of anti-inflammatory cytokines such as TGF-β, IL-10 or IL-35. Recent data suggest that immune regulation in MS is altered which may

contribute to the disease susceptibility. Studies by *Putheti et al.* showed that there is no significant difference in the number of peripheral CD4+ CD25+ Treg cells between MS patients and healthy controls [58]. However, studies by *Huan et al.* showed that there is a decrease in FoxP3 expression in Treg cells derived from MS patients as compared to healthy controls [59]. This suggests that the development of Treg cell population in MS patients might be impaired, hence affecting their suppressive activity. In vitro studies by *Viglietta et al. Hass et al.* and *Kumar et al.* showed that MS derived Treg cells showed impairment in their suppressive activity towards myelin specific auto-reactive T cells [60, 61, 62]. The role of $CD8^+$ Treg cells in suppressing MS was also described. *Crucian et al.* showed that there is a decrease in $CD8^+$ $CD25^-$ Treg cells in the peripheral blood of MS patients [63]. Additionally, *Frisullo et al.* showed that there is a decrease in $CD8^+$ $FoxP3^+$ Treg cells during the relapsing phase of MS and a normal level during the remitting phase [64]. These data support the role of $CD8^+$ Treg cells in suppressing MS pathology.

Experimental Autoimmune Encephalomyelitis as an Animal Model of MS

Representing MS using a single model is challenging as the etiology of MS is still unknown and the clinical picture and disease severity varies between patients [1]. The primary animal model used to study different aspects of MS pathogenesis is experimental autoimmune encephalomyelitis (EAE) [1, 65]. Historically, EAE was induced in a variety of animals including rabbits, guinea pigs and in nonhuman primates like marmoset (Rhesus) monkeys. However, the most commonly used animals are rats and mice due to their smaller size and the availability of genetically altered species [65].

The introduction of self-Ag or auto-reactive T cells to naïve animals is sufficient to induce inflammatory response with a histopathological and clinical picture similar to MS [1]. Specifically, EAE is induced by active immunization of one of the myelin proteins: myelin oligodendrocyte glycoprotein (MOG), MBP or proteolipid protein (PLP) emulsified in complete Freund`s adjuvant (CFA) [65, 66, 67]. This type of immunization also requires the injection of two doses of Pertussis Toxin (PT) [67]. In addition, the adoptive transfer of encephalitogenic T cells specific to one of the myelin proteins also induces EAE in naïve animals [67]. However, EAE shows heterogeneity in the immune response, clinical symptoms and disease progression depending on the myelin peptide used, the animal's genetic susceptibility and its species [1, 67]. Therefore, the immunological process under investigation determines the immunization protocol and the animal strain used to induce EAE [67]. For instance, immunizing C57BL/6 mice with MOG_{35-55} induces a chronic progressive EAE while immunizing SJL mice with $PLP_{139-151}$ result in a relapsing remitting EAE [68].

EAE Immunopathology

The inflammatory response initiated with the immunization drives the pathology of EAE. Thus, studying EAE provides insight to the possible immune players in MS pathology. *Ben-Nun et al.* showed that EAE is T cell mediated disease by inducing EAE upon the passive transfer of MBP-specific T cells [69]. Further studies of the involvement of T cells in MS pathology supported their pathogenic role. It is accepted that Th1 and Th17 immune responses contribute the most to EAE pathology. Th2 immune response, on the other hand, is involved with the activation of B cells. The role of B cells and Abs in

EAE pathology is still under debate [1]. However, recent data suggest that B cells and Abs play protective [70] and pathogenic roles [71] in EAE/MS pathogenesis.

The protective role of B cells is supported by the function of regulatory B cells (B10 cells) that were found to suppress clinical symptoms during recovery periods of MS [71]. Data by *Wolf et al* supported the role of regulatory B cells during the recovery period of EAE by immunizing B10.PL mice with MBP_{AC1-11} [72]. However, *Dittel et al.* immunized B10 X SJL/J mice with the same peptide and failed to support the protective role of regulatory B cells [73]. This variability in the results might be due to the different animal strains used. Moreover, *Zhong et al.* showed that follicular B (B2) cells induce the generation of iTreg cells from $CD4^+$ $FoxP3^-$ T cells derived from FoxP3-GFP knock in C57BL/6 mice in vitro upon the addition of IL-10 and TGF- β. [74]. Additionally, *Lyons et al.* showed that B cells ameliorate EAE by limiting epitope spreading in BALB/c mice immunized with PLP. This was supported as T cells isolated from B $cell^{-/-}$ mice and cultured with different PLP peptides showed an increase in the production of pro-inflammatory cytokines such as IFN-γ and TNF-α [75]. Aside from the current data present in the literature, the protective role of B cells and the mechanism of EAE regulation are still under investigation.

In contrast, *Willenborg et al.* first supported the pathogenic role of B cells by depleting B cells in rats using an anti-rat antiserum treatment therapy. In their study, the rats were resistant to EAE induction both with whole spinal cord homogenate immunization and with purified MBP. However, T cell function was intact. The failure of EAE induction in these rats was thought to be due to the lack of B cell-T cell interaction or the lack of Ab secretion [76]. A recent study by *Mannara et al.* showed that B cells contribute to neurological

inflammation in EAE induced by a passive transfer of auto-reactive T cells in C57BL/6J mice. They supported the pathogenic role of B cells in demyelination as B cells secreting Abs were localized in brain and spinal cord lesions [77]. Beside Ab secretion, B cells may contribute to EAE/MS pathology through Ag processing and presentation, cytokine production or secretion of chemoattractant agents [78]. Though, their exact pathological roles remain elusive.

Appreciation of the pathological role of B cells resulted in the development of monoclonal Ab called Rituximab®. This therapy depletes mature B cells by blocking CD20 cell molecule. The success of B cell depletion therapy in ameliorating the clinical symptoms in EAE/MS further support the of B cells in EAE/MS pathogenesis [1,2]. However, depletion therapy does not target plasma cells, thus the level of auto-Ab remains intact [1,79].

Similar to B cells, the role of Abs in EAE pathology remains unknown. However, their pathological role is more accepted. Abs may damage the myelin sheath and oligodendrocytes by activating the complement cascade or opsonizing myelin Ags for phagocytosis. *Linington et al.* supported the pathogenic role of Abs in demyelination, as the clinical severity and lesion formation were increased upon the administration of monoclonal anti-MOG Abs in a passive transfer model of EAE in Lewis rats [80]. *Urich et al.* showed that Ab-dependent demyelination is mediated by activating the complement cascade [81]. However, *Piddlesden et al.* showed that Ab-mediated demyelination is independent of activating complement [82]. An *in vitro* study by *Van der Goes et al.* supported the involvement of auto-Abs in demyelination. They showed that anti-MOG Ab enhance phagocytosis by macrophages by increasing the level of MOG opsonization [83].

Moreover, another *in vitro* experiment by *Sommer et al.* showed that the addition of myelin-specific Abs isolated from CSF of immunized rabbit to macrophage culture enhance the phagocytosis of myelin Ags. The phagocytic activity was positively correlated to the amount of anti-myelin Abs administered [84].

The possible protective role of Abs remains undefined. *MacPhee et al.* studied the recovery period of EAE in Lewis rats immunized with MBP. They investigated the possible effect of serum-derived factor in suppressing EAE and found a positive correlation between anti-MBP Ab level and EAE suppression [85]. Additionally, *Hughes et al.* supported a possible protective effect of Abs in the protection against EAE. In this study, rats were treated with guinea pig spinal cord antisera pre- and post-immunization. *Hughes* showed that anti-CNS Ab treatment reduced the clinical score of EAE [86]. Recently, *Bieber et al.* described a polyclonal IgM with high affinity to oligodendrocyte epitopes that are capable of promoting myelin repair in a viral model of MS [87].

The contradictions in these results emphasize a role of Abs in EAE pathology; either protective or pathogenic. However, the involvement of Abs in EAE pathology is still to be elucidated.

EAE Immunoregulation

It was suggested that autoimmunity is caused by a lack or impairment in the regulation of the inflammatory response rather than over sensitization of the immune system. Thus, enormous effort has been made to clarify the cellular and humoral components in immune regulation. Based on EAE studies, it is suggested that MS might result from loss of tolerance to self-Ag caused by impaired regulation of inflammation. The main regulatory cells include Breg cells, MDSC and Treg cells.

- *Breg Cells*

Matsushita et al. showed that B cell depletion before EAE induction exacerbates EAE clinical course as a result of depleting $CD1^{hi}CD5^{+}$ Breg cells. These Breg cells ameliorate EAE symptoms by secreting anti-inflammatory cytokines such as IL-10. However, B cell depletion therapy post EAE induction was found beneficial in subsiding the symptoms [70]. *Ray et al.*, on the other hand, showed that Breg regulatory function is independent of IL-10 secretion but depends on maintaining the number of Treg cells balanced. In their study, they showed that B cells contribute to EAE recovery by enhancing Treg cell proliferation and that this function is dependent on the expression of glucocorticoid-induced tumor necrosis factor receptor (GITR) by B cells [88].

- *MDSCs*

Murine MDSCs express CD11b and Gr-1 molecules. Granulocytic and monocytic MDSCs are characterized by the expression of Ly-6C and Ly-6G, respectively. Thus, G-MDSCs have a $CD11b^{+}$ $Ly\text{-}6G^{+}$ $Ly\text{-}6C^{lo}$ phenotype while M-MDSCs have a $CD11b^{+}$ $Ly\text{-}6C^{hi}$ $Ly\text{-}6G^{-}$ phenotype [48]. The suppressive activity of MDSCs in EAE is still under investigation. *Ioannou et al.* showed that G-MDSCs suppress the immune response in EAE by activating programed cell death in auto-reactive Th1 and Th17 immune cells [89]. However, *Yi et al.* showed that MDSCs promote the differentiation of naïve $CD4^{+}$ T cells into Th17 cells and increase EAE severity [90].

- *Treg Cells*

Murine Treg cells also express FoxP3 transcription factor. Treg cells may regulate EAE by the secretion of anti-inflammatory cytokines such as IL-10, TGF-β and IL-35 [91]. Additionally, Treg cells may induce suppression by direct cell-cell contact. The role of those anti-inflammatory cytokines in ameliorating EAE is based on their effect in limiting the activation and the proliferation of $CD4^+$ T cells towards Th1 and Th17 immune cells. Those anti-inflammatory cytokines direct the immune response towards an immuno-suppressive immune response that results in immunological balance. *Podojil et al*. studied the effect of blocking B7-H4, a co-stimulatory molecule, on EAE. They found that blocking this co-stimulatory molecule inhibits $CD4^+$ T cell differentiation into Th1 and Th17 cells. This was correlated with EAE amelioration and was dependent on Treg cell expansion and IL-10 production [92]. *Chen et al*. described the role of TGF- β in suppressing EAE. In their study, they characterized the regulatory cell population as $CD8^+$ that express latency associated peptide (LAP). The adoptive transfer of this population ameliorated EAE in a TGF-β dependent manner. Additionally, in vivo blocking of TGF-β worsened EAE, thus supporting its role in regulating the immune response [93]. The role of IL-35 in ameliorating EAE is not fully elucidated; however, it is suggested that IL-35 inhibits $CD4^+$ T cell proliferation [94]. It was accepted that IL-35 is a novel cytokine of Treg cells; however, recent studies showed that plasma cells also secrete IL-35 and contribute to immune regulation [95].

These studies showed the efficacy of Treg cell population in controlling the autoimmunity seen in EAE. *Kohm et al.* showed that $CD4^+$ Treg cells suppress MOG_{35-}

$_{55}$ induced EAE by switching the immune response towards anti-inflammatory Th2 immune response [91]. Moreover, *Nishibori et al.* showed that impairment in the development of CD4+ CD25+ Treg cells worsen the disease progression in MBP induced EAE [96]. However, the exact mechanisms of EAE regulation by $CD4^+$ Treg cells are still under study.

The role of $CD8^+$ Treg cells in EAE amelioration is being investigated. *Chen et al.* showed that $CD8^+$ Treg cell population expressing LAP regulates EAE in TGF-β and IFN-γ mediated manner. They showed that these cells may express FoxP3 or CD25 and that the passive transfer of $CD8^+$ Treg cells induces the expansion of $FoxP3^+$ cell population and suppresses EAE [93]. Moreover, *Lee et al.* identified a $CD8^+$ $CD122^+$ Treg cell population and supported its ability to suppress EAE. Depleting $CD8^+$ $CD122^+$ Treg cells supported the suppressive ability of this population. CD122 depletion resulted in increased T cell infiltration and cytokine production. The passive transfer of $CD8^+$ $CD122^+$ Treg cells, on the other hand, reduced EAE severity [97].

B Cell Dependent and B Cell Independent EAE Models

The role of B cells in EAE is dependent on the animal model used. Our laboratory previously described B-cell-dependent and B-cell-independent models of disease using the C57BL/6 mice strain [98, 99]. The B-cell-independent model is induced by active immunization with the encephalitogenic MOG_{35-55} peptide. In this model, B cells do not contribute to EAE pathogenesis, as both wild-type (WT) and B cell deficient ($B^{-/-}$) mice were equally susceptible to EAE induction [98, 99]. B cell dependent EAE is induced by active immunization with recombinant human myelin oligodendrocyte glycoprotein (rMOG) peptide. This peptide contains an extracellular human MOG peptide sequence

(amino acid 1-121), encompassing the encephalitogenic MOG_{35-55} peptide. In this model, B cell$^{-/-}$ mice were resistant to rMOG-induced EAE compared to WT mice. Therefore, B cells or antibody have a role in the pathogenesis of rMOG-induced EAE. In this model, the resistance to EAE induction in B cell$^{-/-}$ mice may be due to the difference in antigen processing and presentation leading to different immunological response or due to the lack of antigen opsonization by Abs. Moreover, passive transfer of rMOG-primed serum and B cells into B cell$^{-/-}$ mice previously immunized with rMOG results in clinical EAE in recipient mice [98, 99].

Using peptide-mapping analysis (Figure I) *Lyons et al.* revealed an epitope, myelin oligodendrocyte glycoprotein $_{61-85}$ (MOG_{61-85}), to which rMOG immunized B$^{-/-}$ mice responded to but not WT mice (*Lyons et al., unpublished data)*. These data indicate that MOG_{61-85} peptide might be responsible for EAE resistance seen when B cell$^{-/-}$ mice are immunized with rMOG.

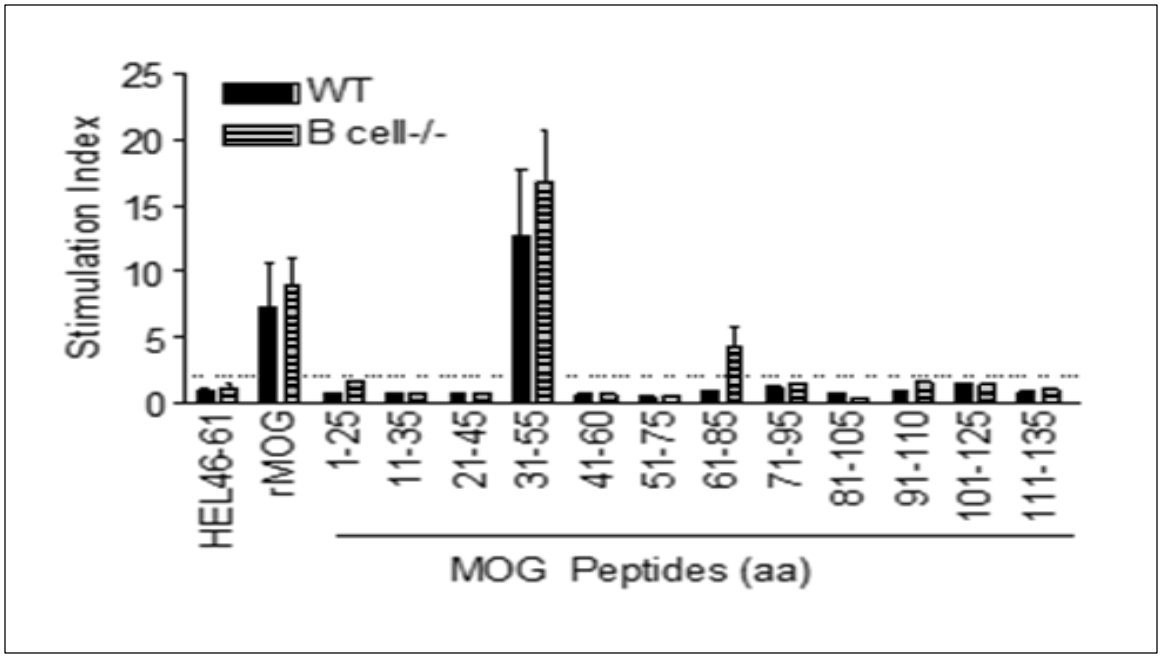

Figure I: Peptide Mapping Analysis using LN isolated from rMOG immunized B cell$^{-/-}$ and WT C57BL/6 mice (*Lyons et al., unpublished data)*. In this experiment T cells were cultured with

a panel of MOG peptides spanning the length of rMOG. Both B cell$^{-/-}$ and WT- derived T cells responded to the encephalitogenic epitope MOG_{31-55}. However, B cell$^{-/-}$ -derived T cells responded to MOG_{61-85}.

Additionally, co-immunization of B-cell-independent model with MOG_{61-85} induces ameliorated form of EAE (Figure II). This supports the protective effect of MOG_{61-85} peptide in suppressing EAE. Subsequent data suggested that MOG_{61-85} induces a population of CD4+ CD25+ T cells (Figure III). Therefore, MOG_{61-85} might be a cryptic epitope that when presented ameliorates EAE severity by generating a regulatory cell population. Also, this cryptic epitope might be the cause of the resistance in EAE induction when B cell$^{-/-}$ mice immunized with rMOG. Thus, antibodies specific to rMOG may play a role in EAE pathogenesis by means of epitope selection that results in clonal expansion of specific CD4+ T cell repertoires.

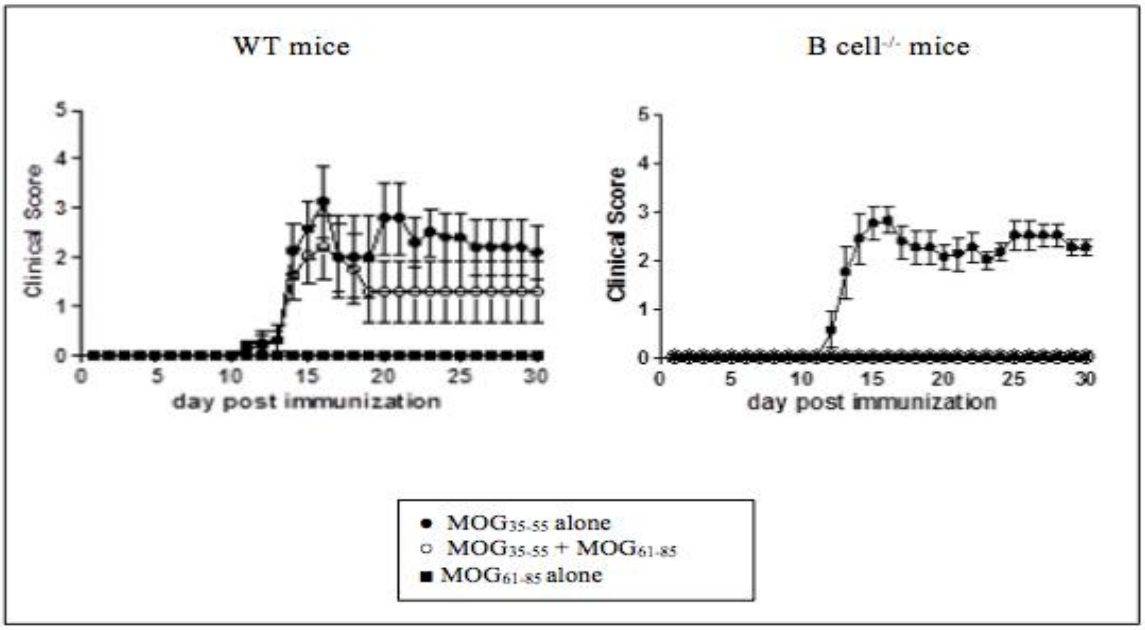

Figure II: Co-immunization studies with MOG_{35-55} and MOG_{61-85} peptides *(Lyons et al, unpublished data).* WT and B cell$^{-/-}$ animals were co-immunized with the encephalitogenic MOG_{35-55} and MOG_{61-85} peptide and were observed for EAE induction and progression. WT animals co-immunized with both peptides showed a decrease in EAE severity when compared to the animals immunized with MOG_{35-55} only. Conversely B cell$^{-/-}$ animals co-immunized with both peptides showed no EAE. **These results effectively demonstrated the protective nature of the MOG_{61-85} peptide.**

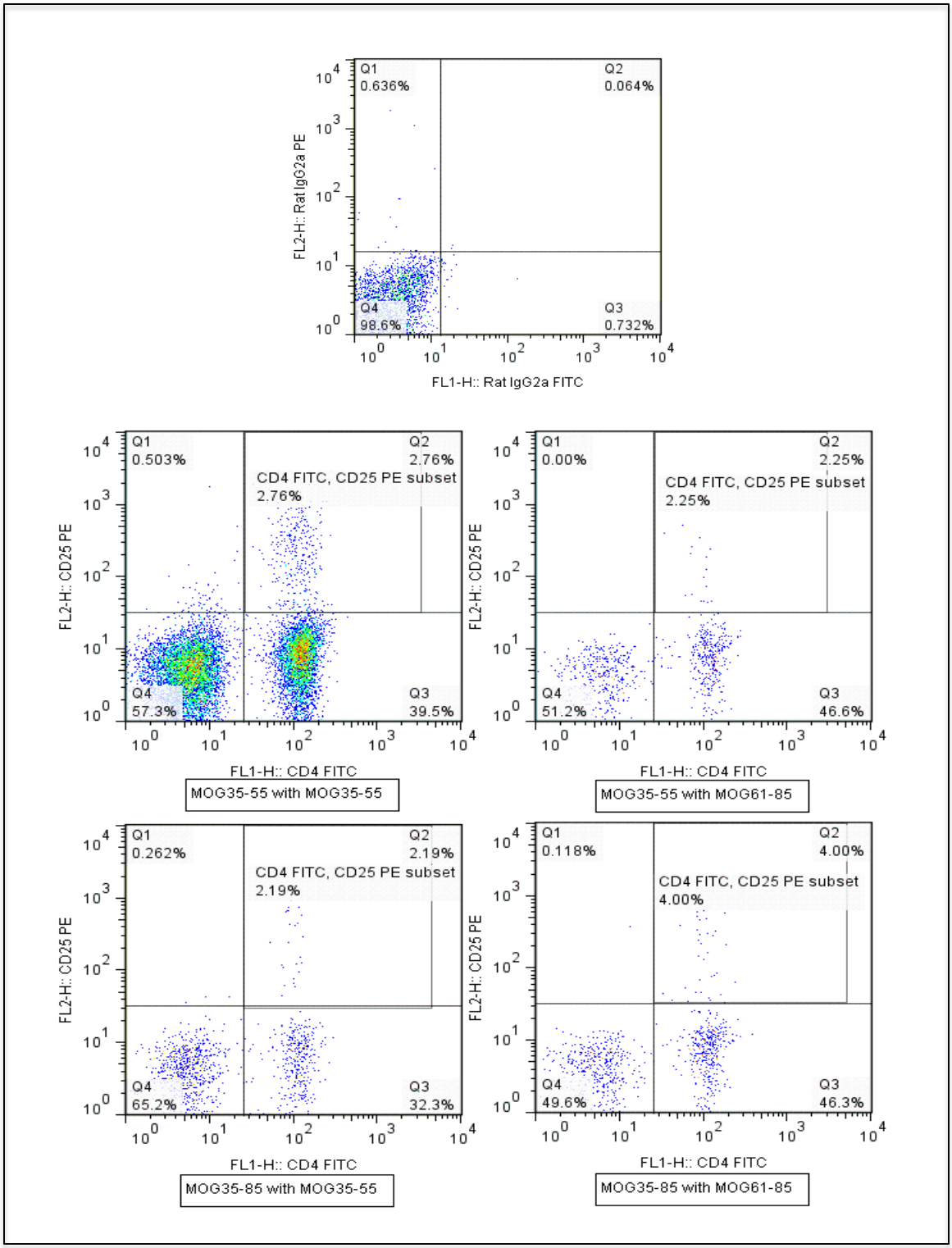

Figure III: Flow Cytometric Analysis to Characterize MOG_{61-85} Specific Cell Population (*Agashe et al, unpublished data).* Spleens were harvested from WT mice immunized with MOG_{35-55} or MOG_{35-85} and cultured *in vitro* with MOG_{35-55} or MOG_{61-85}. An increase in $CD3^+$

$CD4^+$ $CD25^+$ T cell population was observed when the cells were primed *in vivo* with MOG_{35-85} and cultured with MOG_{61-85} as compared to the cells cultured with MOG_{35-55}.

To further investigate the mechanism of suppression by MOG_{61-85}, a longer peptide MOG_{35-85} encompassing both the encephalitogenic MOG_{35-55} epitope and the protective MOG_{61-85} was synthesized. Immunization of WT mice with the longer peptide resulted in less severe EAE than immunization with the MOG_{35-55} peptide (Figure IV). The role of IL-10 as an anti-inflammatory cytokine was tested with immunization of IL-10 deficient ($IL\text{-}10^{-/-}$) mice on the B6 background. Results indicated that amelioration of disease was independent of IL-10 secretion (Figure IV). This was evident as both WT and $IL\text{-}10^{-/-}$ mice immunized with the longer peptide MOG_{35-85} showed low EAE clinical severity as compared to WT and $IL\text{-}10^{-/-}$ mice immunized with the encephalitogenic short peptide MOG_{35-55}.

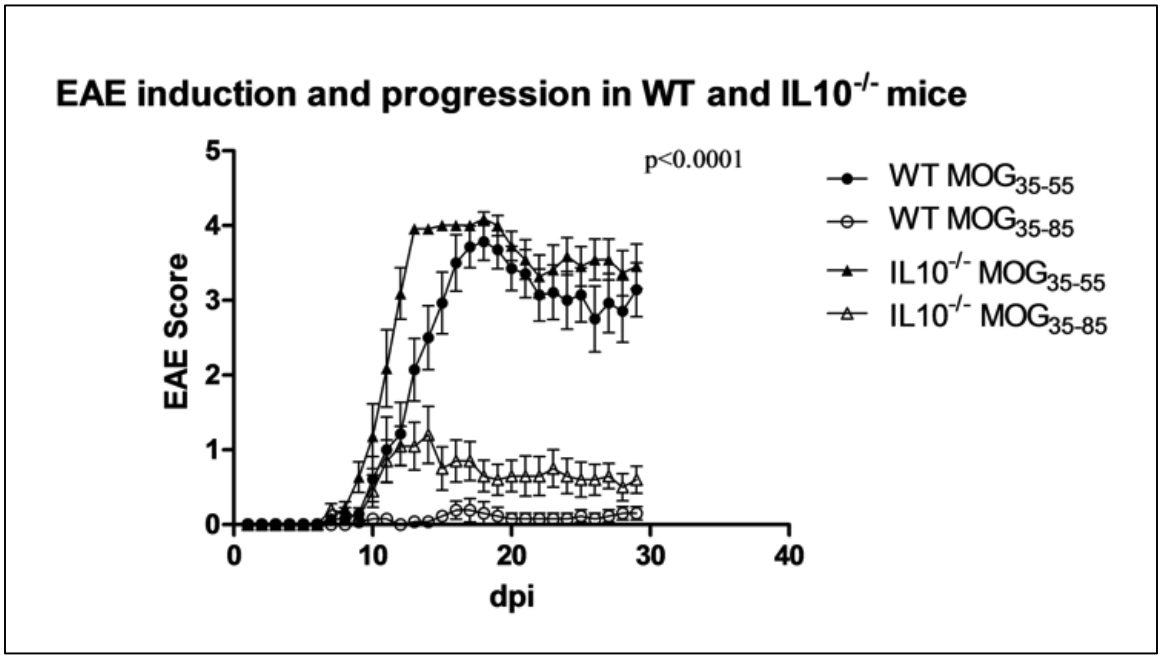

Figure IV: EAE severity in WT and $IL\text{-}10^{-/-}$ mice immunized with MOG_{35-55} and MOG_{35-85} (*Agashe et al, unpublished data).* WT and $IL\text{-}10^{-/-}$ mice were immunized with the appropriate peptide and were observed for a period of 29 days for EAE induction and progression. WT and $IL\text{-}10^{-/-}$ mice, immunized with MOG_{35-55} exhibited EAE induction

and progression. Moreover, WT and IL-$10^{-/-}$ mice immunized with MOG_{35-85} (to observe MOG_{61-85} peptide) exhibited low EAE severity as compared to the group of animals immunized with MOG_{35-55}. However, this degree of severity was found to be similar in WT and IL-$10^{-/-}$ mice, indicating that IL-10 may not play a role in protection from observed with MOG_{61-85}.

Our previous data of EAE resistance in the B-cell-dependent model and the amelioration of EAE severity with the co-immunization of MOG_{61-85} lead to the central hypothesis addressed herein.

Hypothesis

Previous work identified two EAE models; B-cell-dependent and B-cell-independent models. Data from peptide mapping analysis from B-cell-independent model showed a response towards MOG_{61-85} peptide. Further experiments suggested that an immune response to MOG_{61-85} protected $B^{-/-}$ mice from rMOG-induced EAE and was able to ameliorate MOG_{35-55}-induced EAE. Furthermore, previous experiments indicated that this protection to be mediated by the induction of a regulatory T cell population based on observing an increase in $CD4^+$ $CD25^+$ T cell population. EAE amelioration by MOG_{61-85} peptide was shown to be independent of IL-10 secretion.

The experiments described herein further investigated the protective effect of MOG_{61-85} in EAE amelioration in rMOG immunized C57BL/6 mice and in mice co-immunized with MOG_{35-55} and MOG_{61-85}. Therefore, we hypothesized that regulatory T cells specific for MOG_{61-85} suppress the activation of pathogenic MOG_{35-55} T cells in EAE in $B^{-/-}$ C57BL/6 mice through the secretion of TGF-β. This hypothesis was addressed by investigating the following Specific Aims.

Specific Aims

I. ***Specific aim I:*** **Phenotypic characterization of MOG_{61-85} specific regulatory cell population.** *Our working hypothesis was* that the MOG_{61-85}-specific T regulatory cell is a $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cell population, and it regulates the pathogenic activity of MOG_{35-55}-specific $CD4^+$ T cell population. This aim was tested by co-immunizing B $cell^{-/-}$ C57BL/6 mice with both the encephalitogenic MOG_{35-55} and the protective MOG_{61-85} peptides. Mice were

followed for EAE clinical progression and the Treg population was characterized using cells derived from spleens and lymph nodes and cultured in vitro with MOG_{61-85} peptide. Phenotypic characterization of MOG_{61-85} specific Treg was performed using Flow Cytometry.

II. ***Specific aim II:*** **Determine the mechanism controlling the suppressive activity of MOG_{61-85}-specific regulatory cell population.** *Our working hypothesis* was that the MOG_{61-85} Treg cell population ameliorates EAE through the secretion of TGF-β. This aim was tested by immunizing WT and IL-$10^{-/-}$ C57BL/6 mice with rMOG protein. Mice were followed for EAE induction and progression. Then, lymph nodes and spleen cells harvested from rMOG-immunized mice were used for *in vitro* study. In this study, cells were cultured with MOG_{61-85} peptide. The working hypothesis was tested by measuring TGF-β level in the culture supernatants by enzyme linked immunosrbent assay (ELISA).

CHAPTER II: MATERIALS AND METHODS

Mice

Pathogen-free female WT, B cell$^{-/-}$ and IL-10$^{-/-}$ C57BL/6 mice aged 6-8 weeks were used for these experiments. All mice were bred from the breeding pairs purchased from Jackson Laboratories (Bar Harbor, ME). All mice were housed in micro-isolator cages in an animal facility accredited by an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) on the University of Wisconsin-Milwaukee campus. Mice were maintained on a 12-hour light/dark schedule and provided food and water *ad libitum*.

Antigens

Commercially available myelin oligodendrocyte glucoprotein 35-55 (MOG_{35-55}) (MEVGWYRSPFSRVVHLYRNGK) and myelin oligodendrocyte glucoprotein 61-85 peptides (MOG_{61-85}) (QAPEYRGRTELLKDAIGEGKVTLRI) (GenScript, Piscataway, NJ) were used for these experiments. The recombinant human myelin oligodendrocyte glucoprotein rMOG (amino acid 1-121), was produced in collaboration with Dr. Nancy Monson from a baculovirus-expressed construct.

Immunizations

The emulsion was prepared by mixing 100 or 200 micrograms (µg) MOG peptide or rMOG, respectively, emulsified in incomplete Freund's Incomplete Adjuvant (IFA; MP Biochemicals, Solon, OH) and 200 or 300 µg *Mycobacterium tuberculosis* strain H37RA TB (Difco Laboratories, Detroit, MI) in Phosphate Buffer Solution (PBS) (Dulbecco's Phosphate Buffered Saline in 1 liter deionized water) (Sigma, St. Louis, MO, USA) using an Omni Mixer Homogenizer mechanical mixer (Omni International, Kennesaw, GA). For

co-immunization studies, MOG_{35-55} -MOG_{61-85} emulsion was prepared using the same preparation protocol but both MOG peptides (MOG_{35-55} and MOG_{61-85}) were mixed together. Each mouse was immunized with 0.2 milliliter (ml) of emulsion, with 0.05 ml subcutaneously (SC) at each flank.

Also, mice were injected with *Pertussis Toxin* (PT) (List Biological Labs, Inc., Campbel, CA). 300 nanogram (ng) of PT in PBS was used for these injections. All mice were injected intraperitoneally (IP) with 0.1 ml of PT twice; first at the day of immunization and then two days post immunization (dpi).

Experimental Autoimmune Encephalomyelitis (EAE) Grading

Immunized mice were assessed daily for clinical signs at the beginning of the 7^{th} day post immunization. Clinical signs of EAE were graded using a 0 to 5 scale as following:

Grade or Level	Clinical Sign
0	No obvious clinical signs
1	Flaccid paralysis in the tail observed as the mouse fail to helicopter its tail
2	Impairment in the righting reflex observed as Limp tail and weakness of hind legs in which the animal is unable to grasp with its hind legs
3	Limp tail and complete paralysis of hind legs
4	Limp tail and complete hind leg and partial front leg paralysis
5	Moribund or dead

Mice scored as 5 on the EAE scale were euthanized by carbon dioxide (CO_2) and cervical dislocation.

Cell Culture

All mice were anesthetized with 0.1 ml Ketamine-xylazine cocktail injection or by isoflurane inhalation (all from Midwest Veterinary Supply, Sun Prairie, WI) and sacrificed with cervical dislocation before sample collection. At 14 or 30 dpi, spleens (SPL) and lymph nodes (LN) were collected into ~ 45 ml of Hanks balanced salt solution (HBSS; Lonza, Walkersville, MD) and kept on ice. Organs of the same strain and immunization were pooled together, with LN and SPL separated. SPLs and LNs were homogenized using glass homogenizer and centrifuged for 10 minutes (min) at 1250 relative centrifugal force (RCF) at 21 ^{o}C. The supernatant was decanted and the pellet was re-suspended in HBSS (~10 ml). For spleen samples, 10 ml of lymphocyte separation media (LSM) (MP Biomedicals, LLC, Solon, OH) was added to the bottom of the tube and samples were centrifuged at 500 RCF and 21^{o}C for 30 min with no break. Then the interphase layer was collected and was re-suspended in HBSS solution (~30 ml). Alternatively, 5 ml of red blood cell lysing buffer (Sigma, St. Louis, MO) was added to re-suspend the pellet and kept on ice for 3 min. After that, 30 ml of HBSS was added. Both LN and SPL samples were centrifuged again and the pellets were re-suspended in HBSS for a second wash (with ~15 ml). Supernatants were decanted again and samples were re-suspended in HBSS for cell counting (LN in ~15 ml, spleens in ~30 ml).

The cells were counted using a *Neubauer Counting Chamber* (hemacytometer) and trypan blue 0.4% solution (MP Biomedicals, LLC, solon, OH) to check the cell viability. For cell counting; 90 microliter (µl) of trypan blue was mixed with 10 µl well-mixed cell suspension, then each side of hemacytometer was charged with 10 µl trypan blue-stained

cells. Cells were counted in the middle square and cell concentration was calculated using the following formula:

$$\text{Cell concentration} = \frac{(\text{number of cells counted}) \times (\text{dilution factor}) \times (10^4)}{\text{number of squares counted}}$$

Cells were cultured at 2.5 X 10^6 per ml (/ml) in complete *Roswell Park Memorial Institute* 1640, 1X solution (cRPMI 1640) (Mediatech, Inc, Manassas, VA).

Complete RPMI (cRPMI) 1640 was prepared by adding 5 ml N-2 hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) buffer solution (GIBCO, Grand Island, NY, USA), 5 ml L-glutamate (L-Glut) (2 mM) (Mediatech, Inc, Manassas, VA), 5 ml Sodium Pyruvate (Na pyruvate) (0.1 mM) (Cellgro, Manassas, VA, USA), 0.5 ml 2-mercaptoethanol (2-ME) (50 mM) (Sigma-Aldrich, St. Louis, MO) and 5 ml Penicillin (100 U/ml) /Streptomycin (100 µg/ml) (P/S) Mediatech, Inc, Manassas, VA) to 500 ml of RPMI 1640.

Cells were cultured with antigens according to the experiment. Antigens used for this study included: MOG_{35-55} (10 or 20 µg/ml), MOG_{61-85} (10 or 20 µg/ml), Concanavalin A (ConA) (Sigma-Aldrich, St. Louis, MO) (10 µg/ml).

Cells were cultured in 24 tissue culture test plates (Techo Plastic Products, MidSci, St. Louis, MO) and cultures were incubated at 37ºC, 10% CO_2 for three time points; 24 hours, 72 hours and 96 hours.

Cell Counting-8 Assay (CCK-8)

This assay was performed to measure cellular proliferation to the different Ags used in cell culture by measuring the metabolic activity of dehydrogenase enzymes. Cell culture preparations were cultured in sterile 96 well cell culture plates (Techo Plastic Products, MidSci, St. Louis, MO) and were incubated at 37^{o}C, 10% CO_2 for the desired time according to the experiments. On the testing day, 10 µl of CCK-8 staining reagent (Dojindo Molecular Technologies, Rockville, MD, USA) was added to each well and plates were incubated at 37^{o}C, 10% CO_2 for 3 hours. After incubation, plates were read at 450 nanometer (nm) wavelength using BioTek plate reader (BioTek, Winooski, VT).

Flow Cytometric Analysis

Cell suspensions from cell cultures were used for phenotypic characterization by flow cytometry. Cells were harvested from culture and were centrifuged at 500 RCF for 3 min to remove the culture media. Supernatants were collected into sterile tubes and pellets were re-suspended in 1 ml sterile filtered Fluorescence-activated cell sorting (FACS) wash buffer. FACS wash buffer was prepared by mixing 970 ml PBS with 30 ml FBS and 1 gram (g) Sodium Azide (Sigma Chemical CO., St. Louis, MO, USA). Then, FACS wash buffer was filtered using vacuum filtrations system 1000 ML (Techo Plastic Products, Trasadingen, Switzerland).

Each sample was divided equally into a number of tubes for different staining. Samples were centrifuged at 500 RCF for 5 min and supernatant were decanted leaving ~50 µl of FACS buffer to re-suspend pellets. Then, 1 µl of anti-mouse CD16/CD32 or 50 µl of heat inactivated FBS was added and the samples were incubated on ice for 10 min or 30 min, respectively. After incubating the samples, 1 ml of sterile filtered FACS wash buffer was

added to the samples and they were centrifuged again at 500 RCF for 5 min. Supernatants were decanted leaving ~50 µl of FACS buffer to re-suspend pellets. Samples were stained with 1 µl fluorochrome-conjugated Ab (ebioscience, San Diego, CA) for different cell markers using the following conjugated Abs:

FL1 Channel FITC	FL2 Channel PE	FL3 Channel PerCP-Cy5.5	FL4 Channel APC
CD3	CD4	CD19	CD25
CD3	CD8		CD25
CD3	CD25		CD4
CD4	FoxP3 (intracellular marker)	CD8a	CD25
Rat IgG2a	Rat IgG2b	Rat IgG2a	Rat IgG2b

Samples were incubated for 30 min on ice in the dark. To phenotype T cell populations we used CD3, CD4, CD8 and CD25 cell markers. To phenotype B cell populations we used the CD19 cell marker and to phenotype Treg cell populations we used CD4, CD8, CD25 cell markers and FoxP3 intracellular marker. For the control tube we used FITC-Rat IgG2a, PE-Rat IgG2b, PerCP-Cy5.5-RatIgG2a and APC-Rat IgG2b isotype controls.

After incubating, 1 ml of sterile filtered FACS wash buffer was added and samples were centrifuged at 3000 RCF for 5 min. Supernatants were decanted leaving ~50 µl of FACS buffer to re-suspend pellets and samples were washed again with 1 ml of sterile filtered FACS wash buffer. Samples were centrifuged at 500 RCF for 5 min and supernatant were decanted. Finally, 500 µl FACS wash buffer was added to the samples.

When the samples were not run on the same day of preparation; samples were centrifuged at 3000 RCF for 5 min. Supernatants were decanted and 500 µl of 0.5% paraformaldehyde solution (10 ml of paraformaldehyde with 30 ml of PBS) was added. Samples were incubated on ice for 20 min and the centrifuged at 3000 RCF for 5 min. Supernatants were decanted and 1 ml of PBS was added. Samples were centrifuged at 3000 RCF for 5 min, supernatants were decanted and 1 ml of PBS+ 0.5% FBS (49.75 ml PBS with 250 µl of FBS) was added. Then, samples were centrifuged at 3000 RCF for 5 min, supernatants were decanted and 500 µl of PBS+ 0.5% FBS was added. Samples were kept in the refrigerator until the day of testing.

For FoxP3 intracellular staining, after the last washing step, 1 ml of FoxP3 fixation/permeabilization working solution was added to each tube and mixed using force vortex. The tubes were incubated at 4^{o}C for 30 min in the dark. After incubation, 1 ml of 1X permeabilization buffer was added to the tubes, then the tubes were centrifuged at 3000 RCF at 21^{o}C for 5 min. The supernatants were decanted and 100 µl of 1X permeabilization buffer was added to re-suspend the pellet. After that the control tubes were stained with Rat IgG2b isotype control and the experimental tubes were stained with FoxP3-PE conjugated antibody. The tubes were incubated in the dark at room temperature for 30 min. After incubation, 2 ml of 1X permeabilization buffer was added to all tubes and were centrifuged at 3000 RCF at 4- 21^{o}C for 5 min. The supernatants were decanted and 2 ml of 1X permeabilization buffer was added and the tubes were centrifuged again at 3000 RCF at 4- 21^{o}C for 5 min. Then, the supernatants were decanted and 500 µl of flow cytometry staining buffer and data were collected.

To prepare FoxP3 fixation/permeabilization working solution, 1 part of the FoxP3 fixation/permeabilization concentrate was diluted with 2 parts of FoxP3 fixation/permeabilization diluent. To prepare 1X permeabilization buffer, 20 ml of 10X permeabilization buffer was diluted with 180 ml deionized water.

Cytokines levels by Enzyme-Linked Immunosorbent Assay (ELISA)

The culture supernatants were collected at three time points (24 hours, 48 hours, 72 hours, 96 hours) during flow cytometry preparation and were aliquoted into 250 µl per tube. Cytokine level was detected according to the manufacture`s instructions using the Ready-Set-Go ELISA kit (ebioscience, San Diego, CA). A plate was coated with capture Ab in 1X coating buffer and was incubated overnight at 4^{o} C. Then, the plate was washed with PBS+ 0.05 % Tween 20 (Sigma, St. Louis, MO) using ELISA washer instrument (BioTek, Winooski, VT). After washing the coating buffer, the plate was blocked using 1X assay diluent (200 µl/well) and incubated for 1 hour. After that, the standard (prepared according to manufacturer`s instructions, 100 µl/well) and samples (100 µl/well) were plated in duplicate and the plate was incubated for 2 hours. The plate was washed again. After that, the detection Ab was added and the plate was incubated for 1 hour. Then, the plate was washed. The Avidin-Horse Radish Peroxidase (Avidin-HRP) enzyme was added (100 µl/well) and the plate was incubated for 30 minutes. The plate was washed again and the substrate solution was added (100 µl/well) and the plate was incubated for 15 minutes then the stop solution was added (50 µl/well) to stop the reaction. The plate was read using a BioTek ELISA reader at a range of 450 to 562 nm.

ELISA was used to measure three cytokines; IL-10, IL-6 and TGF-β. The minimum detection limits for these ELISA kits are the following:

IL-10	32 pg/ml
TGF- β	8 pg/ml
IL-6	4 pg/ml

Samples for TGF-β detection were activated prior to loading to the ELISA plate. Samples were activated by adding 20 µl of hydrochloric acid (HCL) and the samples were incubated for 10 min at room temperature. Then, 20 µl of sodium hydroxide (NaOH) was added. 100 µl/well of the activated sample was loaded into the plate.

The number of washes varies between cytokine types. The plate was washed according to the manufacturer's instructions for each cytokine ELISA.

Statistical Analysis

All data were analyzed using two-way analysis of variance (ANOVA) or student's t-test, as indicated. $P < 0.05$ was considered significant. These data were analyzed using GraphPad Prism 5.0 (La Jolla, CA). Flow cytometry results were analyzed using the FlowJo software (Tree Star, Ashland, OR).

CHAPTER III: SPECIFIC AIM I

Phenotypic characterization of MOG_{61-85} specific T regulatory cell population.

Background

Previous work showed amelioration of the EAE clinical score in WT and $B^{-/-}$ mice co-immunized with MOG_{35-55} and MOG_{61-85} peptides compared to mice immunized with MOG_{35-55} peptide only. The amelioration of EAE was further studied to identify MOG_{61-85}-specific regulatory cell populations. Preliminary data showed an increase in $CD4^+$ $CD25^+$ T cells and an increase in the $CD8^+$ $CD25^-$ $FoxP3^+$ T cell population in response to MOG_{35-85} immunization, suggesting the generation of a protective immune response. Therefore, we further investigated the MOG_{61-85}-specific regulatory cell population responsible for suppressing the pathogenic MOG_{35-55}-specific T cell population.

Results

A decrease in $CD4^+$ $CD25^+$ cell population was observed with MOG_{61-85} priming

Previous experiments showed generation of protective immunity in response to MOG_{61-85} stimulation. This was evident as mice co-immunized with the encephalitogenic epitope MOG_{35-55} and the cryptic epitope MOG_{61-85} showed an ameliorated form of EAE (*Lyons et al., Unpublished data*). This raised the possibility that MOG_{61-85} induces the generation of an anti-inflammatory immune response by increasing the number of Treg cells. The role of T regulatory cells in alleviating EAE/MS pathogenesis is accepted [1, 2, 45, 46]. To further characterize the MOG_{61-85}-specific T regulatory cell population, B $cell^{-/-}$ mice were immunized with MOG_{35-55} only or with MOG_{35-55} and MOG_{61-85} peptides. EAE induction and progression were monitored for 30 dpi. This study was conducted in one experiment and the total number of B $cell^{-/-}$ mice was as shown in table I. LNs were harvested 30 dpi

from $B^{-/-}$ C57BL/6 mice immunized with MOG_{35-55} and mice co-immunized with MOG_{35-55} and MOG_{61-85}. Cell suspensions were stimulated *in vitro* with either MOG_{35-55} or MOG_{61-85} peptides and were incubated for 48 hrs to increase cell proliferation. The cells were harvested at the 48 hrs time point and were prepared for flow cytometry.

Group	Mice	Number of animals	Peptide
1	♀ B $cell^{-/-}$ C57BL/6	5	MOG_{35-55}
2	♀ B $cell^{-/-}$ C57BL/6	5	MOG_{35-55} and MOG_{61-85}

Table I: Groups of mice immunized with both peptides or with MOG_{35-55} only. In this experiment, mice received 100 μg of MOG_{35-55} and MOG_{61-85} peptides emulsified in complete Freund's adjuvant containing *Mycobacterium tuberculosis* H37RA.

Using FlowJo software, the cell population was first gated on lymphocytes using forward scatter/side scatter (Figure V). Then, the cells were gated on the presence of CD4 or CD8 to isolate T cells.

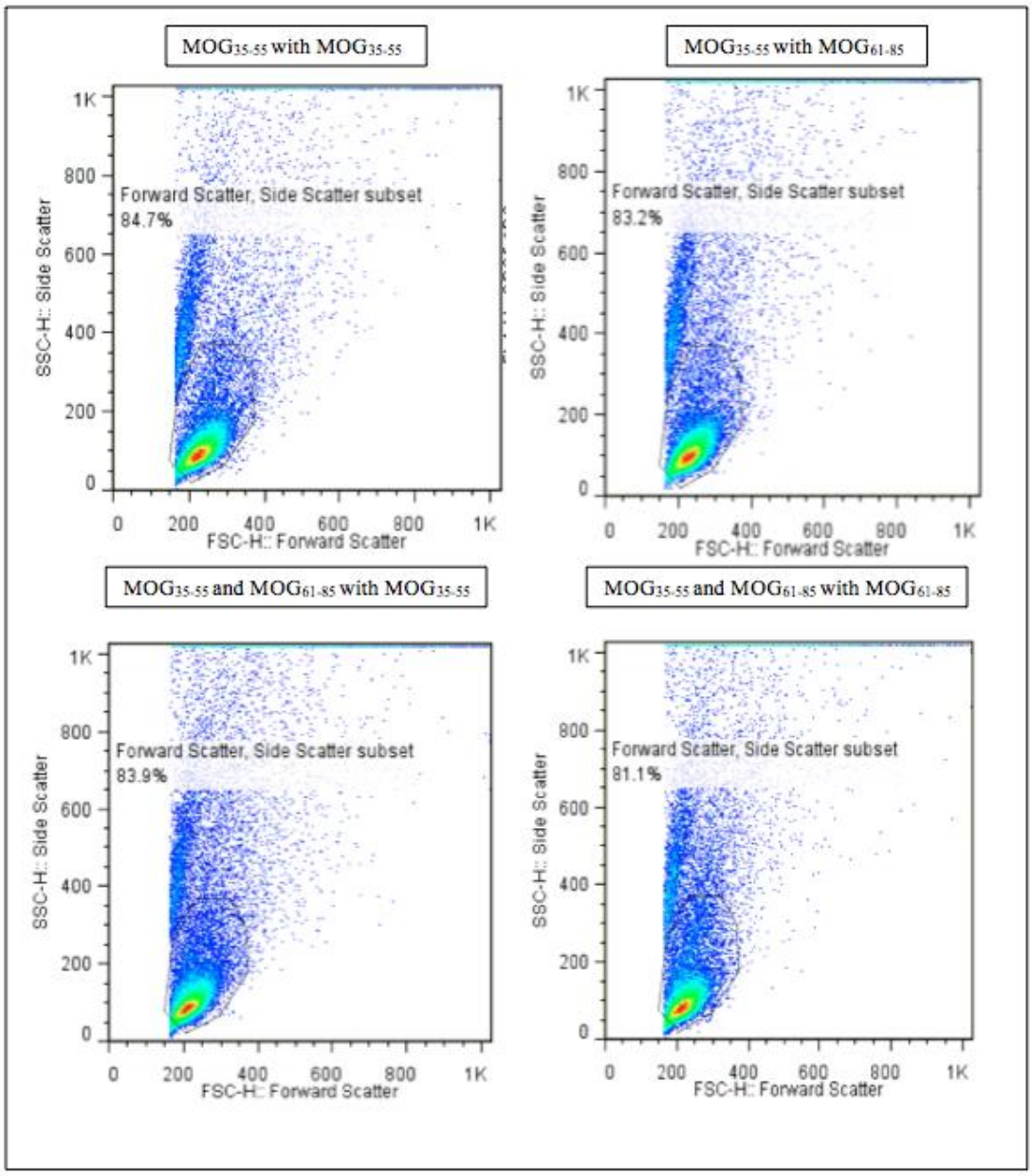

Figure V: The forward scatter/side scatter gate set in FlowJo software to analyze the Treg cell population. LNs were harvested from $B^{-/-}$ mice immunized with MOG_{35-55} and MOG_{61-85} or MOG_{35-55} only. Cell suspensions were cultured *in vitro* with MOG_{35-55} or MOG_{61-85} for 48 hrs. Cells were harvested and prepared for flow cytometry. The gate was based on the lymphocyte size and the lack of granularity. **These forward scatter/side scatter were further used to characterize the Treg cell population.**

Interestingly, the percentage of $CD4^+$ $CD25^+$ T cell population generated in response to MOG_{61-85} was similar between cells primed in *vivo* with MOG_{35-55} only and in cells primed with both MOG_{35-55} and MOG_{61-85} (0.965% and 0.595%) as shown in figure VI. Additionally, there was no significant difference in the cell percentage of cells stimulated *in vitro* with MOG_{35-55} between the two types of immunization (2.82% and 3.36%). The percentage of $CD4^+$ $CD25^+$ T cell population was decreased in cells cultured with MOG_{61-85} as compared to $MOG_{35-55.}$ This was observed in both types of immunization; in cells primed *in vivo* with MOG_{35-55} only (2.82% and 0.965%) and in cells primed *in vivo* with both MOG_{35-55} and MOG_{61-85} (3.36% and 0.595%). However, the decrease was more evident when the cells were primed with MOG_{61-85} *in vivo* and cultured with MOG_{61-85} *in vitro*.

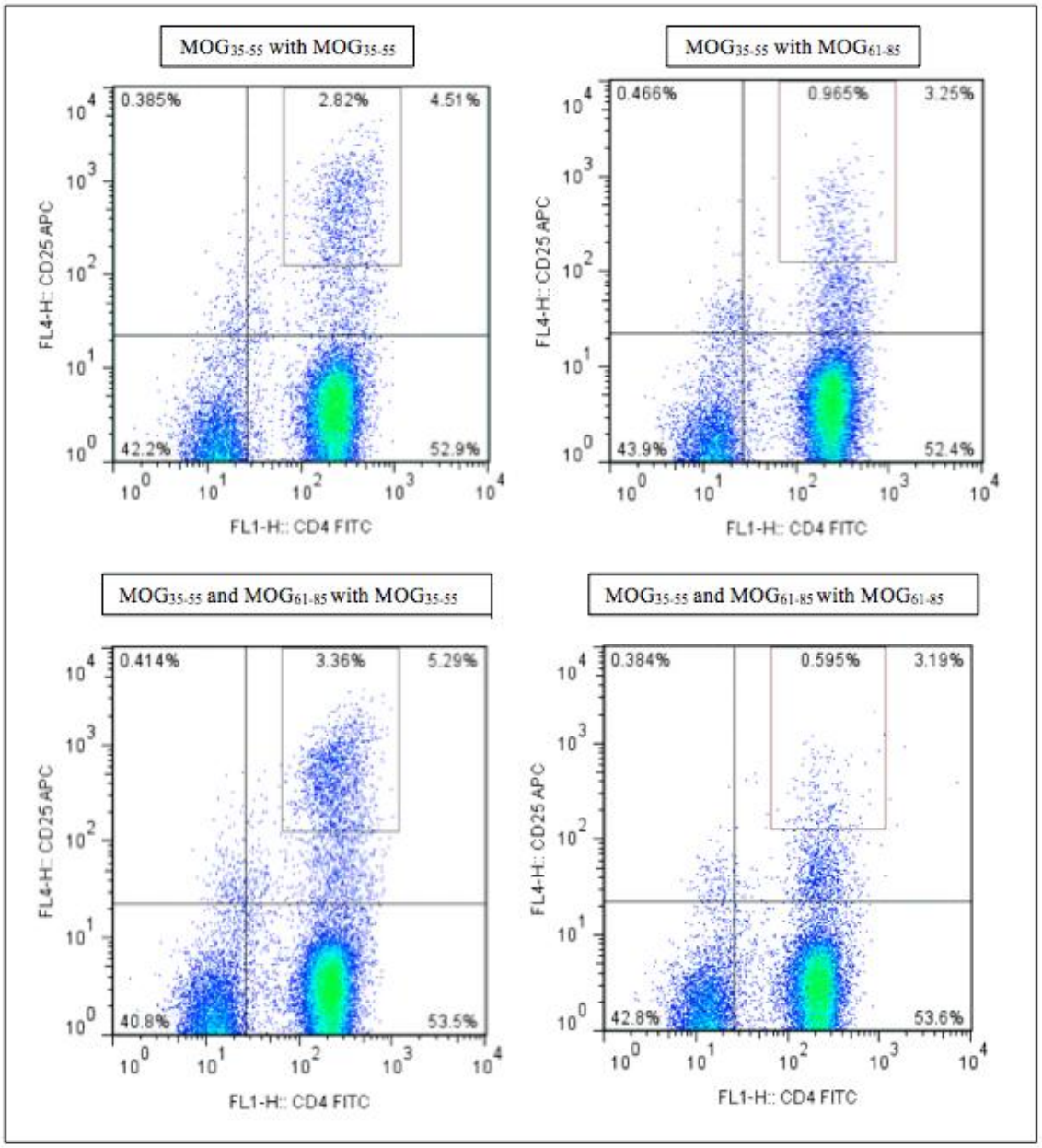

Figure VI : $CD4^+$ $CD25^+$ T cells within the forward scatter/ side scatter gate. The cells gated on forward scatter/ side scatter were further gated on the expression of CD4 and CD25 cellular markers. **Similar cell percentage was noted with *in vitro* stimulation with MOG_{61-85} with both types of immunization. Also, a decrease in $CD4^+$ $CD25^+$ cell percentage was noted when cells were cultured with MOG_{61-85}.**

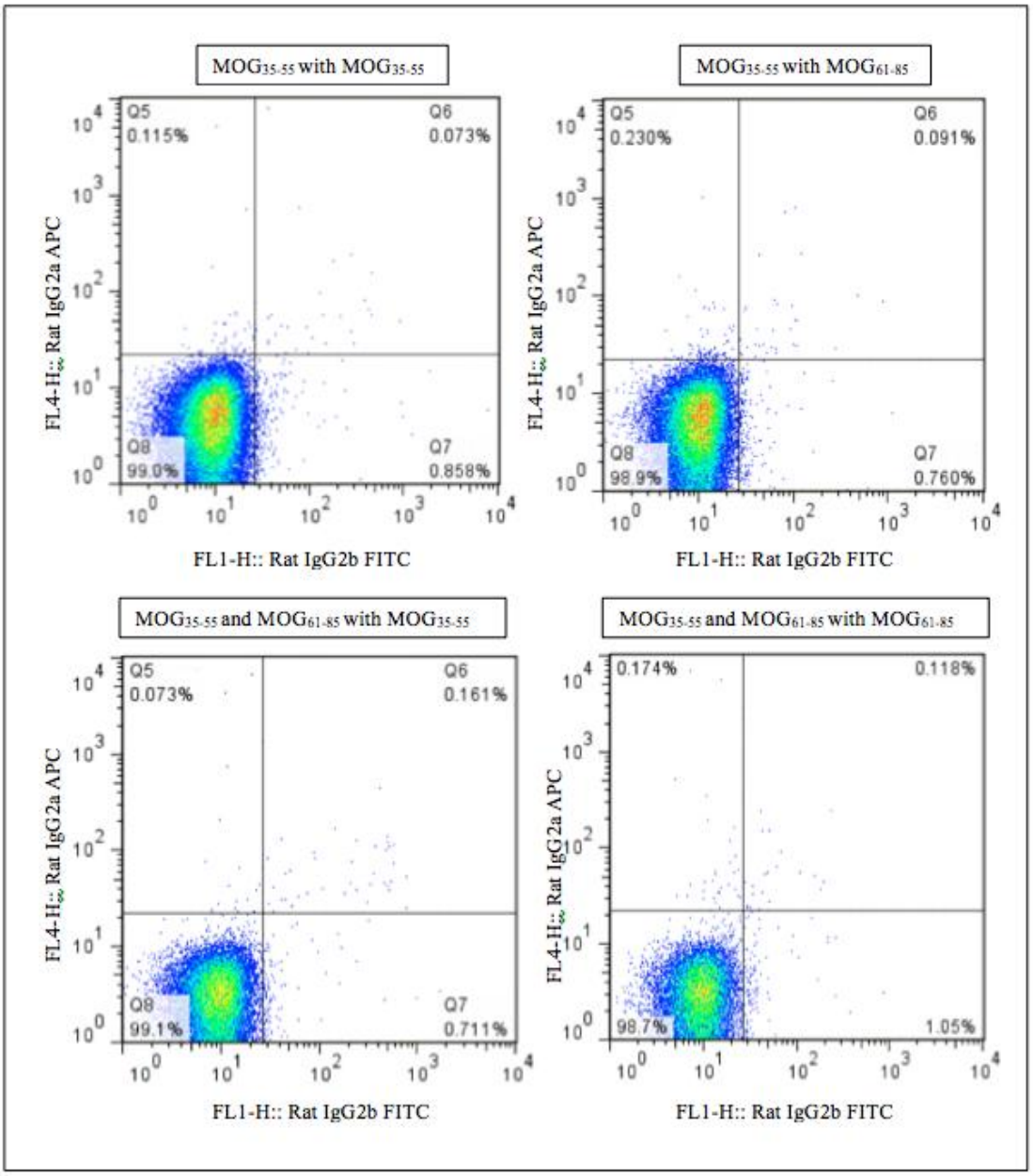

Figure VII: Control for the analysis of $CD4^+$ $CD25^+$ T cells. The cells were stained with isotype and species controls with fluorophores that matched the marker antibodies. **The sample showed that there was no non-specific staining in the sample.**

An increase in $CD8^+$ $CD25^-$ T cell population was observed with MOG_{61-85} priming

The percentage of $CD8^+$ $CD25^-$ T cell population generated in response to MOG_{61-85} *in vitro* stimulation was increased as compared to cells stimulated with MOG_{35-55} peptide (figure VIII). The increase in this population with MOG_{61-85} *in vitro* priming was observed with both types of *in vivo* priming; cells primed with MOG_{35-55} only (43% and 41.6%) and cells primed with MOG_{35-55} and MOG_{61-85} (40.6% and 39.2%).

An increase in $CD8^+$ $CD25^+$T cell population was observed with MOG_{61-85} in vivo and in vitro priming

We also noted an increase in $CD8^+$ $CD25^+$ T cell population in response to MOG_{61-85} priming (figure VII). The increase was observed in LN cell culture primed *in vivo* and *in vitro* with MOG_{61-85} as compared to LN cell culture primed with MOG_{35-55} *in vivo* and MOG_{61-85} *in vitro* (0.094% and 0.046%). As the increase in this population was dependent on the *in vivo* priming with MOG_{61-85}, this population might be MOG_{61-85}-specific Treg cell population. However, the small number of events of this population prevented the accurate analysis of FoxP3 expression by this population. Therefore, this population will be analyzed in future experiments.

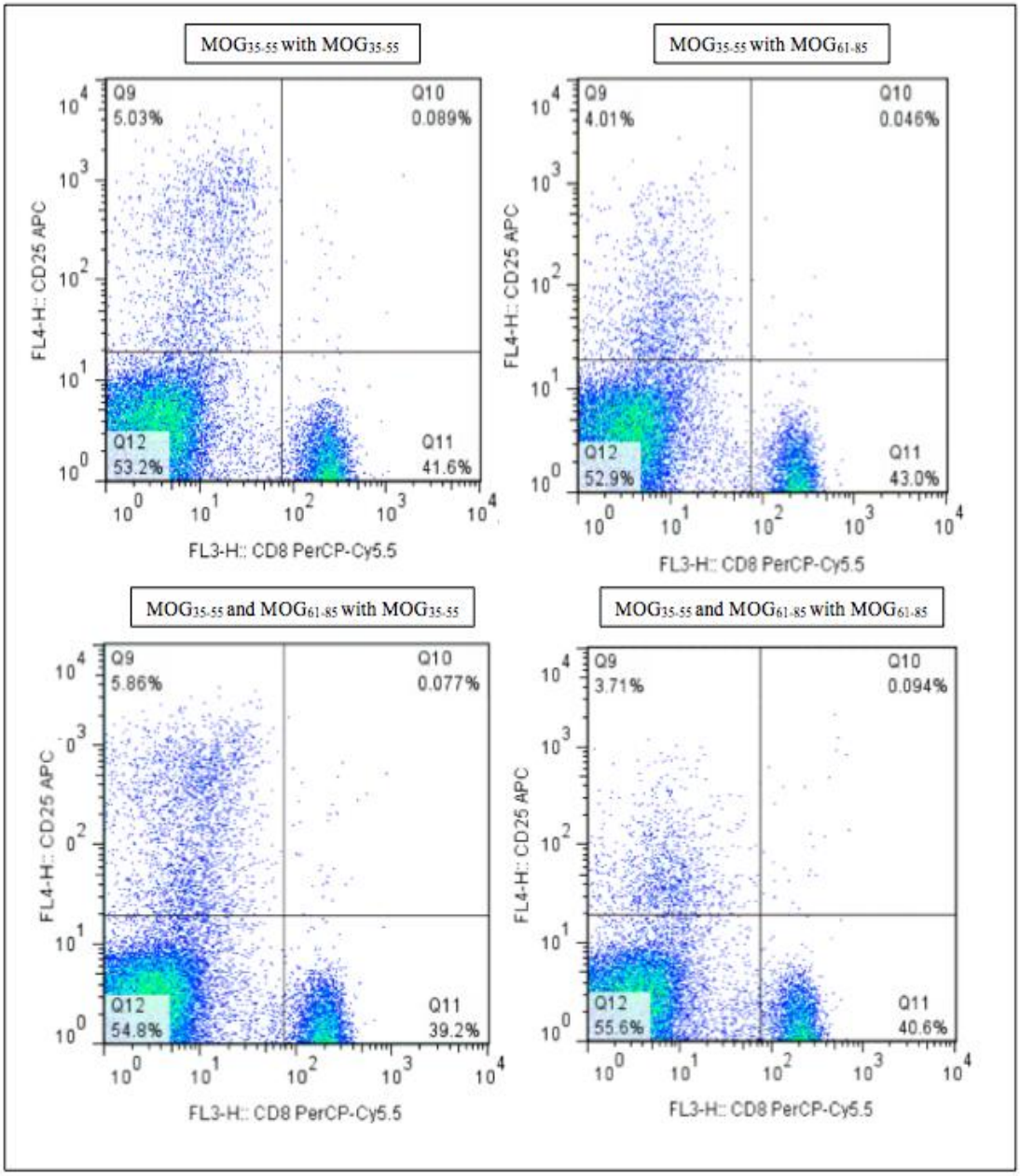

Figure VIII: $CD8^+$ $CD25^-$ T cells and $CD8^+$ $CD25^+$ T cells within the forward scatter/side scatter gate. The cells gated on forward scatter/side scatter were further gated on the expression of CD8 and CD25 cellular markers. **Slight increase in the $CD8^+CD25^-$ T cell population was observed with MOG_{61-85} *in vitro* stimulation; however, this increase was independent of the type of *in vivo* priming. Additionally, there was an increase in $CD8^+$ $CD25^+$ T cell population that was dependent on the *in vivo* priming of MOG_{61-85}.**

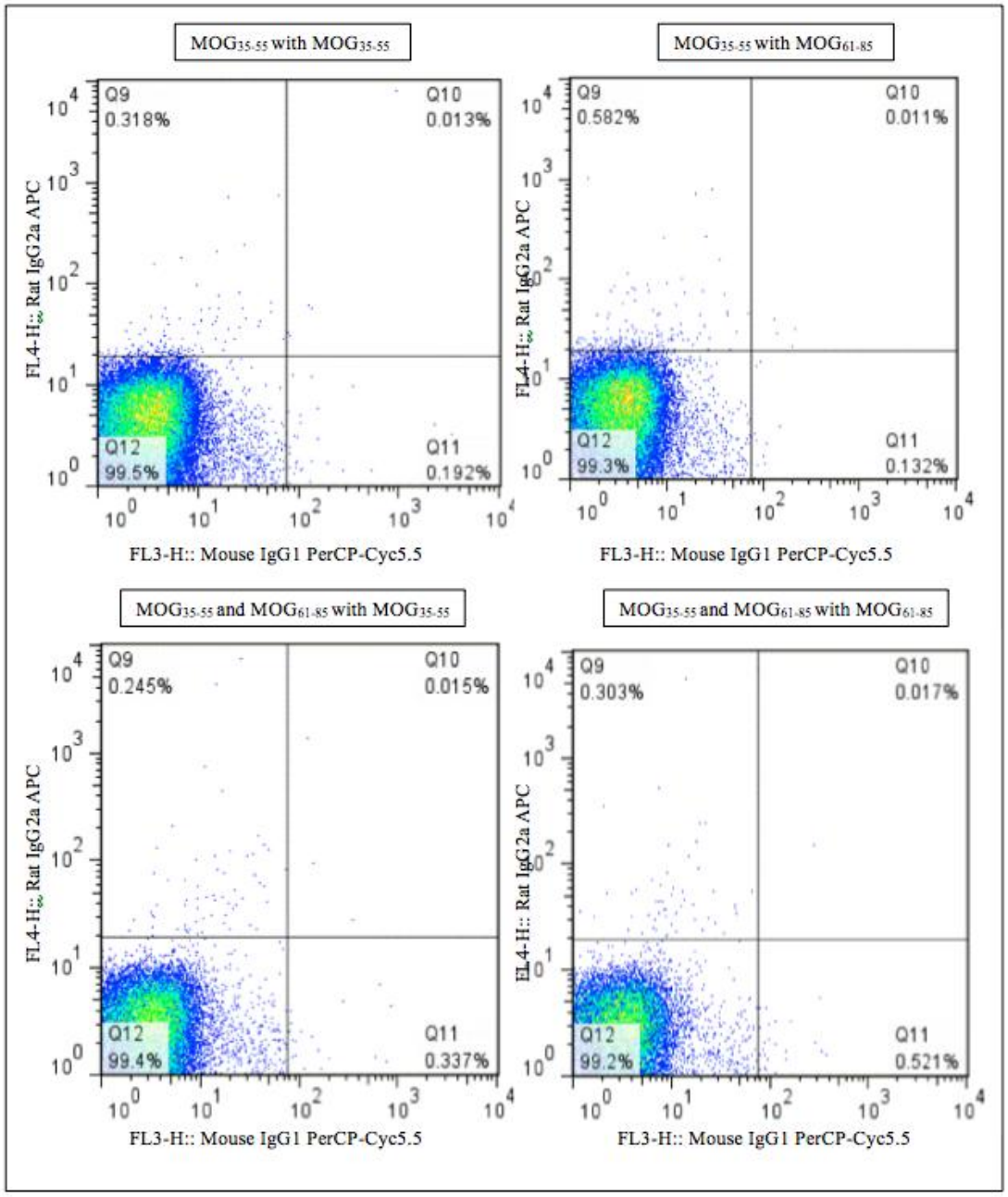

Figure IX: Control for the analysis of $CD8^+$ $CD25^-$ T cells and $CD8^+$ $CD25^+$ T cells. The cells were stained with isotype and species controls with fluorophores that matched the marker antibodies. **The sample showed that there was no non-specific staining in the sample.**

An increase in FoxP3 expression was observed with MOG_{61-85} priming.

Using FoxP3, a transcription factor expressed by T regulatory cells, the Treg cell population was characterized. Cells gated on CD4/CD25 or CD8/CD25 were analyzed for FoxP3 expression.

Gating on the $CD4^+$ $CD25^+$ T cell population, a decrease in FoxP3 expressing cells was noted with MOG_{61-86} *in vitro* stimulation as compared to MOG_{35-55} stimulation (figure X). This decrease was noted with both types of *in vivo* priming. Cells primed with MOG_{35-55} *in vivo* showed 18.9% decrease with MOG_{61-85} *in vitro* stimulation while cells primed *in vivo* with MOG_{35-55} and MOG_{61-85} showed 22.6% decrease. The significant decrease in FoxP3 expression observed with MOG_{61-85} *in vitro* stimulation indicates that the MOG_{61-85}-specific Treg cell population is not a $CD4^+$ $CD25^+$ $FoxP3^+$ T cell population.

Gating on $CD8^+$ $CD25^-$ T cell population, an increase in the percentage of $FoxP3^+$ expressing cells was observed with MOG_{61-85} *in vitro* stimulation as compared with MOG_{35-55} stimulation. Interestingly, this increase was observed only if cells were primed *in vivo* with MOG_{35-55} and MOG_{61-85} (3.5% and 2.9%). In contrast, cells primed *in vivo* with MOG_{35-55} showed a decrease in the percentage of cells expressing $FoxP3^+$ when cells cultured with MOG_{61-85} as compared to MOG_{35-55} (2.7% and 4.2%) (figure XI). This indicates that the MOG_{61-85}-specific cell population might be $CD8^+$ $CD25^-$ $FoxP3^+$ Treg cells and that the expansion of this population is dependent on the *in vivo* and the *in vitro* priming with MOG_{61-85}. However, further experiments are needed to support that MOG_{61-85}-specific Treg population is $CD8^+$ Treg cell population.

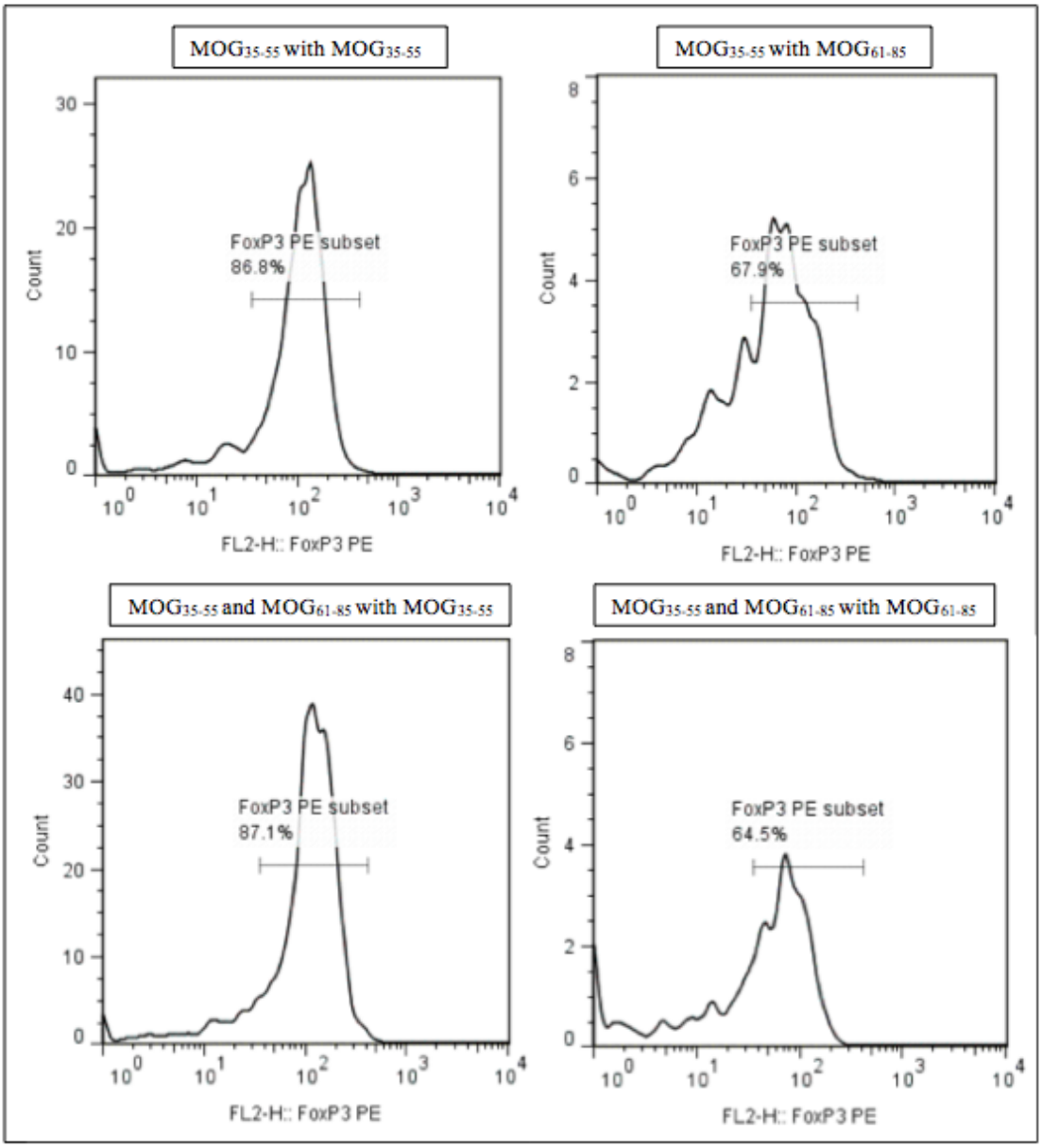

Figure X: Percentage of $CD4^+$ $CD25^+$ $FoxP3^+$ T cells. Cells gated on $CD4^+$ $CD25^+$ were analyzed for FoxP3 expression. **A decrease in $CD4^+$ $CD25^+$ $FoxP3^+$ T cell population was observed with MOG_{61-85} *in vitro* stimulation as compared with MOG_{35-55} stimulation.**

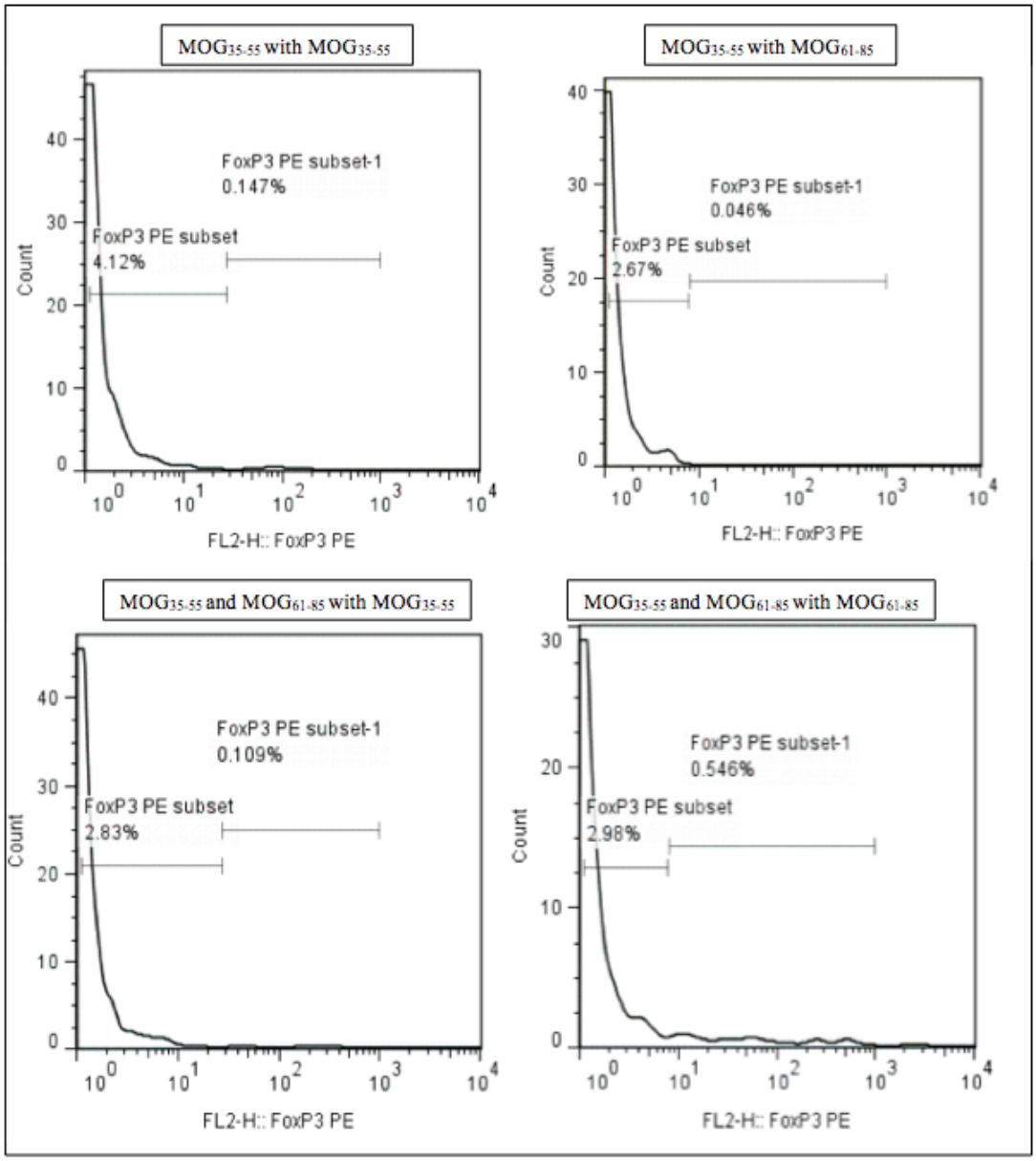

Figure XI: Percentage of $CD8^+$ $CD25^-$ $FoxP3^+$ T cells. Cells gated on $CD8^+$ $CD25^-$ were analyzed for FoxP3 expression. **An increase in $CD8^+$ $CD25^-$ $FoxP3^+$ T cells was observed with cells primed *in vivo* with MOG_{35-55} and MOG_{61-85} and cultured *in vitro* with MOG_{61-85}.**

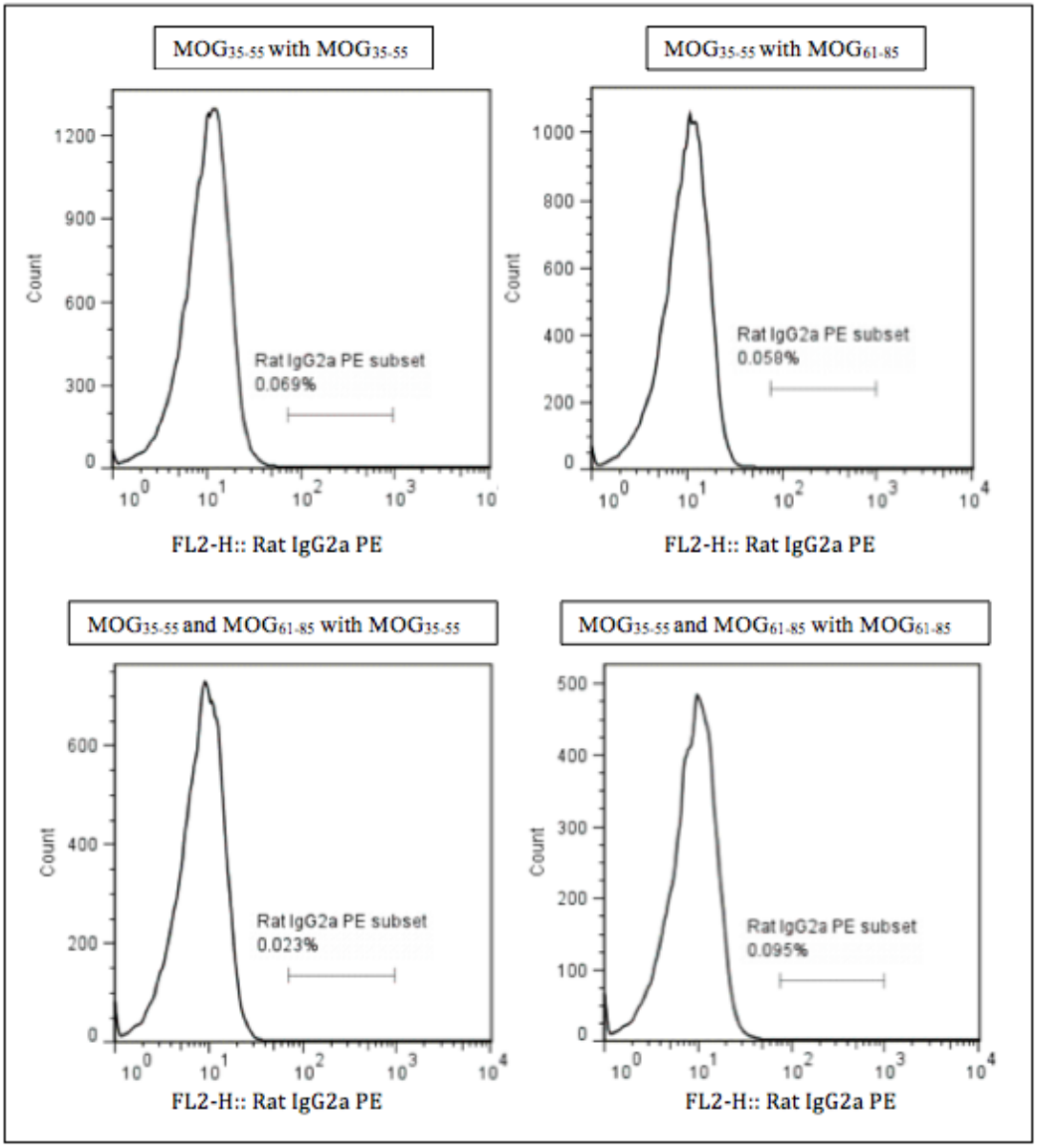

Figure XII: Control for the analysis of FoxP3 expression of cells gated on lymphocytes. The cells were stained with isotype and species controls with fluorophores that matched FoxP3 intracellular staining antibody. **The control showed that there was no non-specific staining in the samples.**

Discussion

This specific aim characterized the phenotype of the MOG_{61-85}-specific T regulatory cell population. As previous co-immunization experiments showed ameliorated EAE, we

investigated the protective effect of MOG_{61-85} on the immune response. We hypothesized that MOG_{61-85} induces a protective immune response by generating T regulatory cell populations. We investigated T regulatory cell populations; CD4 and CD8 as possible MOG_{61-85}-specific Treg cells.

To analyze Treg populations, cells were analyzed by forward and side scatter and cells were gated based on the lymphocyte size and the lack of granularity (figure V). Treg cell populations were analyzed in the gated cell population. To determine the type of Treg cells specific to MOG_{61-85} stimulation, the gated cells were analyzed using both CD4 and CD8 T cell cellular markers. In addition to CD4 and CD8, cells were analyzed for the presence of the CD25 surface marker. As shown in figure VI, there was a decrease in the $CD4^+$ $CD25^+$ T cell population in response to MOG_{61-85} as compared to MOG_{35-55} *in vitro* priming. This finding contradicts previous data that showed an increase in $CD4^+$ $CD25^+$ T cell population in response to MOG_{61-85} stimulation. Although, the cell percentage of $CD4^+$ $CD25^+$ T cell population was comparable with MOG_{35-55} and MOG_{61-85} priming, there was a decrease in CD25 expression in response to MOG_{61-85} priming. This might suggests loss of suppressive activity by this population in response to MOG_{61-85} priming. Analysis for FoxP3 expression by this population as seen in figure X showed a decrease in the $CD4^+$ $CD25^+$ $FoxP3^+$ T cell population in response to MOG_{61-85}. Lack of an increase in the percentage of $CD4^+$ $CD25^+$ $FoxP3^+$ T cell population in response to MOG_{61-85} stimulation indicates that MOG_{61-85}-specific cell population might not be $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells hypothesized in this thesis.

Based on the small number of animals used in this experiment, the MOG_{61-85}-specific Treg cell population might still be $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells. This population might

have expanded with the *in vivo* or *in vitro* priming, however the small number of animals used might interfere with their identification. Additionally, as the *in vitro* culturing requirements for this population are still not exactly described, this population might be lost with *in vitro* culturing or during the preparation for flow cytometry.

Analysis of the $CD8^+$ T cell population as a possible MOG_{61-85}-specific Treg cell population showed a slight increase in $CD8^+$ $CD25^-$ T cell population in response to MOG_{61-85} *in vitro* priming; however, this increase was independent of *in vivo* priming with the same peptide (figure VIII). The increase in this population with the comparable percentage between all cells agrees with previous findings. Further analysis of this population using FoxP3 staining showed an increase in $CD8^+$ $CD25^-$ $FoxP3^+$ T cell population in response to MOG_{61-85} *in vitro* priming (figure XI). The increase in FoxP3 expression was dependent on *in vivo* priming with MOG_{61-85}, which suggests that the MOG_{61-85}-specific Treg cell population might be a $CD8^+$ $CD25^-$ $FoxP3^+$ Treg cell population. Additionally, there was a small increase in $CD8^+$ $CD25^+$ T cell population in response to MOG_{61-85} *in vitro* and *in vivo* priming. However, the small number of events interfered with measuring FoxP3 expression by this population. This population might be MOG_{61-85}-specific Treg cell population and will be analyzed in future experiments.

Chen et al. characterized a Treg cell population with suppressive effect on MOG-induced EAE as $CD8^+$ $CD25^-$ LAP^+ Treg cell population. This population may or may not express FoxP3 and CD25. They showed that this population suppresses EAE by inducing the expansion of FoxP3+ Treg cell population [93]. Additionally, *Lee et al.* described a $CD8^+$ $CD122^+$ Treg cell population as a suppressive population that controls the immune response at later stages of EAE [97]. *Zheng et al.* identified another CD8+ Treg cell

population. They characterize it as $CD8^+$ $FoxP3^-$ $CD103^+$ Treg population that with suppressive effect on EAE [100].

The diverse $CD8^+$ Treg cell populations with the unknown cell surface markers might limit the identification of a MOG_{61-85}-specific Treg cell population. Also, we identified a $CD8^+$ Treg population as FoxP3 expressing cells; however, the comparable FoxP3 expression introduces the possibility that the MOG_{61-85}-specific Treg cell population does not express FoxP3.

Future work may include characterization of MDSCs in response to MOG_{61-85} priming. Previous work showed a decrease in this population; however, the decrease was with MOG_{35-85} priming instead of MOG_{61-85}. Therefore, further work is required to investigate the effect of MOG_{61-85} priming on this population.

In summary, the results illustrated above showed comparable cell percentages of $CD8^+$ $CD25^-$ $FoxP3^+$ and $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cell populations in response to MOG_{61-85} priming. However, there was a decrease in the $CD4^+CD25^+$ cell population in response to MOG_{61-85} priming. Moreover, we noted an increase in $CD8^+$ $CD25^+$ T cell population that was dependent on the *in vivo* priming of MOG_{61-85}.

CHAPTER IV: SPECIFIC AIM II

Determine the mechanism controlling the suppressive activity of the MOG_{61-85} specific regulatory cell population.

Background

It is accepted that CD4 Th1/Th17 cells specific for myelin proteins mediate the pathogenesis of MS/EAE [1,2]. The immune responses initiated by these cells are characterized by the mass production of pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-17. Conversely, switching the immune response towards an anti-inflammatory immune response leads to the amelioration of disease [1, 2]. Anti-inflammatory immune responses are characterized by the by the production of anti-inflammatory cytokines such as IL-10 and TGF-β. In a previous work, we showed that EAE clinical score was ameliorated in $B^{-/-}$ mice immunized with rMOG [98]. Further investigation suggested that the MOG_{61-85} epitope is responsible for this amelioration. Additionally, immunization with MOG_{35-85} peptide, encompassing both the disease inducing MOG_{35-55} and the cryptic epitope MOG_{61-85}, showed that EAE amelioration was independent of IL-10 production (*Agashe et al., unpublished data)*. Therefore, the following experiment aimed to determine the MOG_{61-85}-specific anti-inflammatory cytokine responsible for EAE amelioration.

The working hypothesis is that a MOG_{61-85}-specific regulatory cell population suppresses the activity of MOG_{35-55}-specific $CD4^+$ T cell by secreting TGF-β. This aim was tested in one *in vitro* study using rMOG-immunized mice.

Results

IL-10 may play a role in EAE onset and severity in rMOG-induced EAE

IL-$10^{-/-}$ and WT mice were immunized with rMOG protein and EAE induction and progression was monitored for 29 days post immunization. This study was conducted in one experiment and the total number of WT and IL-$10^{-/-}$ mice was as shown in table II.

Group	Mice	Number of mice
1	♀ WT C57BL/6	5
2	♀ IL-$10^{-/-}$ C57BL/6	5

Table II: Groups of mice immunized with rMOG protein. In this experiment, mice received 200 µg of rMOG protein emulsified in complete Freund's adjuvant containing *Mycobacterium tuberculosis* H37RA.

Both IL-$10^{-/-}$ and WT mice developed EAE, however IL-$10^{-/-}$ mice showed severe EAE as compared to WT mice (figure XII and XIII). Comparing EAE clinical scores, WT mice showed ameliorated EAE as compared to previous immunization with rMOG (data not shown). Statistical analysis of EAE severity between WT and IL-$10^{-/-}$ mice using the area under the curve (AUC) showed that IL-$10^{-/-}$ mice had significantly more severe EAE as compared to WT mice ($p = 0.0079$) (figure XIII). Analysis of the day of EAE onset showed that IL-$10^{-/-}$ mice developed EAE earlier than WT mice ($p = 0.0238$) (figure XIV). This indicates that IL-10 might have a role in delaying and ameliorating EAE in rMOG-immunized mice.

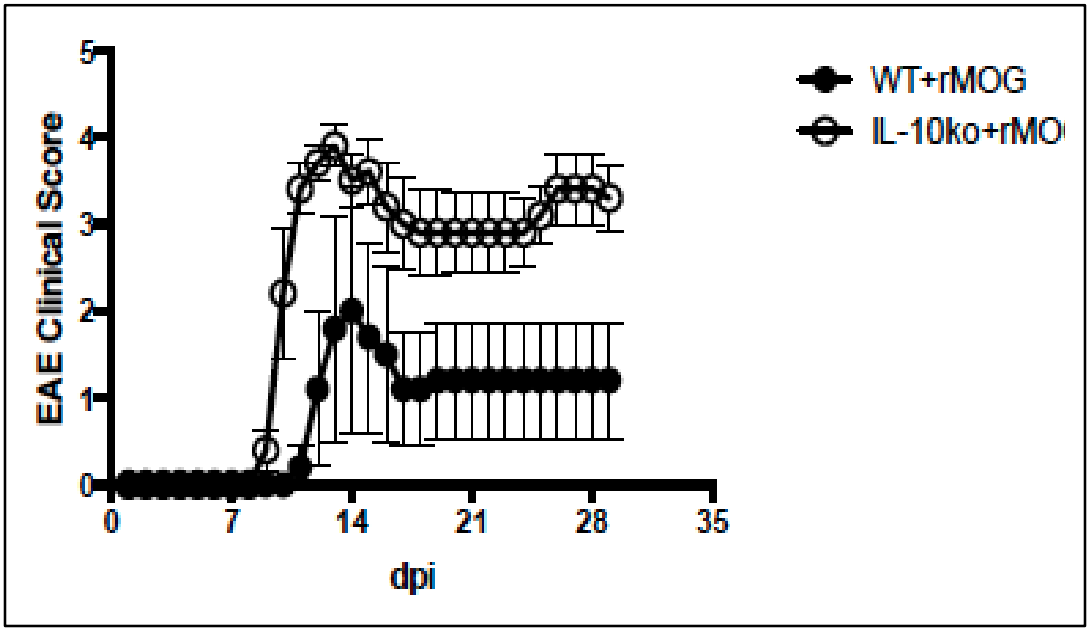

Figure XIII: EAE clinical score in WT and IL-10$^{-/-}$ mice immunized with rMOG protein. WT and IL-10$^{-/-}$ mice were immunized with the above-described amount of rMOG protein and were observed for 29 dpi. **IL-10$^{-/-}$ mice showed more severe EAE as compared to WT mice.** Error bars represent the clinical score average and the standard error of the mean for all the mice in that group. Statistical analysis was performed using non-parametric student t-test to compare the mean of the two groups. A p value of < 0.05 was considered significant.

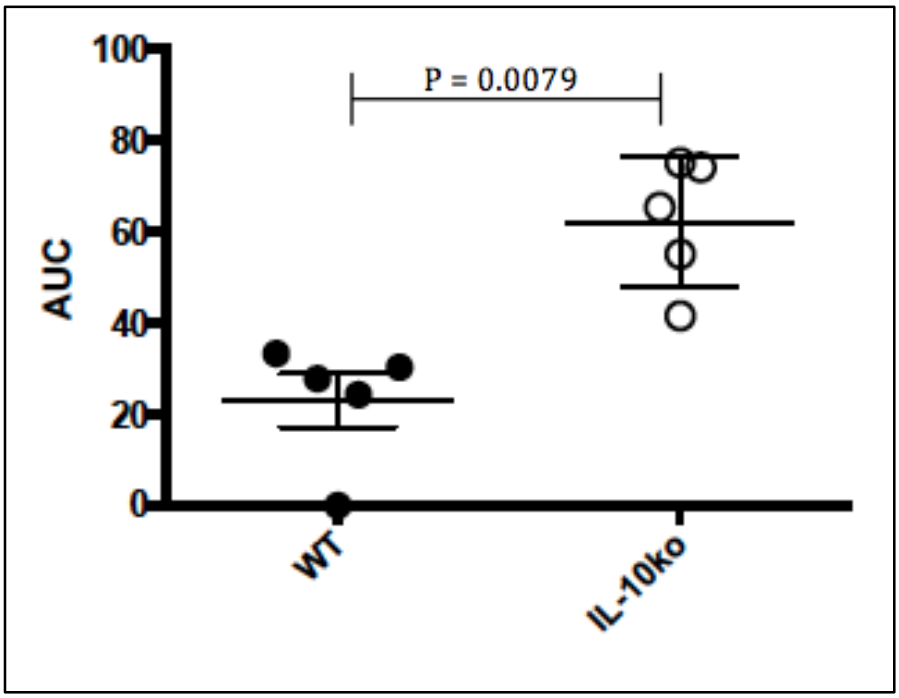

Figure XIV: AUC analysis of WT and IL-10$^{-/-}$ mice immunized with rMOG protein. IL-10$^{-/-}$ mice immunized with rMOG protein had higher EAE score as compared with WT mice immunized with the same protein. Error bars represent the average and the standard error of the mean in the AUC in that group. Statistical analysis was performed using the Mann Whitney T test and resulted in a p value of 0.0079. **This indicates that IL-10 may have a role in controlling rMOG-EAE severity.**

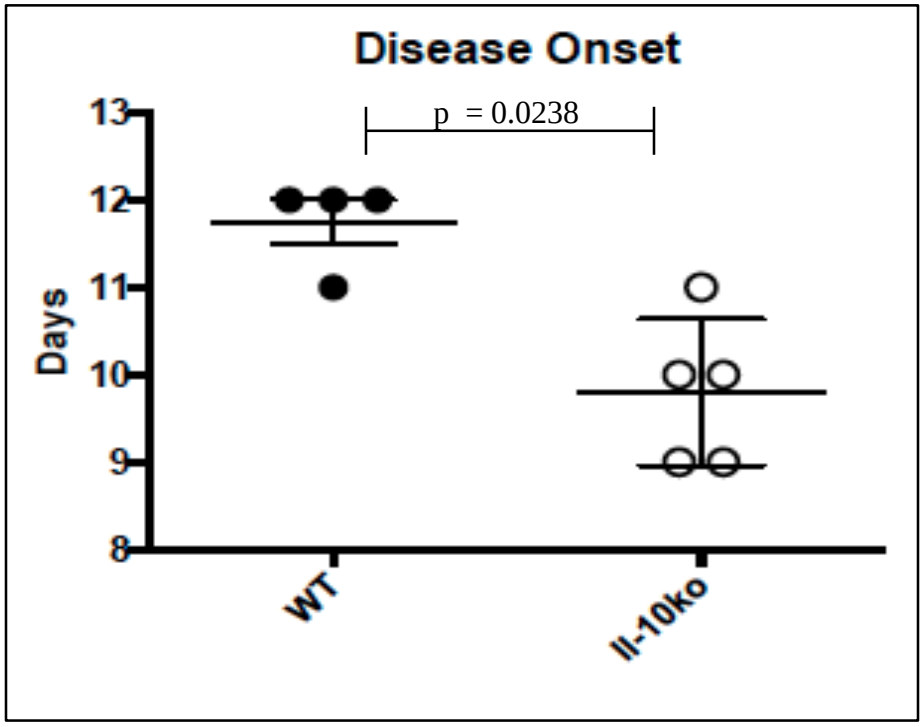

Figure XV: Disease onset in WT and IL-10$^{-/-}$ immunized with rMOG protein. IL-10$^{-/-}$ mice developed EAE earlier than WT mice. Error bars represent the average and the standard error of the mean in the disease onset in that group. Statistical analysis was performed using the Mann Whitney T test and resulted in a p value of 0.0238. **This indicates that IL-10 might have a role in delaying the disease onset in rMOG-EAE.**

MOG_{61-85} induce cell proliferation in rMOG-EAE

To determine if rMOG-primed cells proliferate in response to MOG_{61-85} we performed the CCK-8 cell proliferation assay to measure cell proliferation in response to different *in vitro* stimuli. LN and spleen cells isolated from WT and IL-$10^{-/-}$ mice were cultured in the presence of MOG_{61-85} and MOG_{35-55}, separately. We found that both MOG_{35-55} and MOG_{61-85} induce LN cell proliferation in comparable levels. This indicates that rMOG-primed cells recognize MOG_{61-85} *in vitro* and respond to its stimulation. Interestingly, both WT and IL-$10^{-/-}$ derived LN cells responded to MOG_{61-85} in comparable levels (figure XV). However, WT spleen cells responded stronger than IL-$10^{-/-}$ spleen cells (figure XVI).

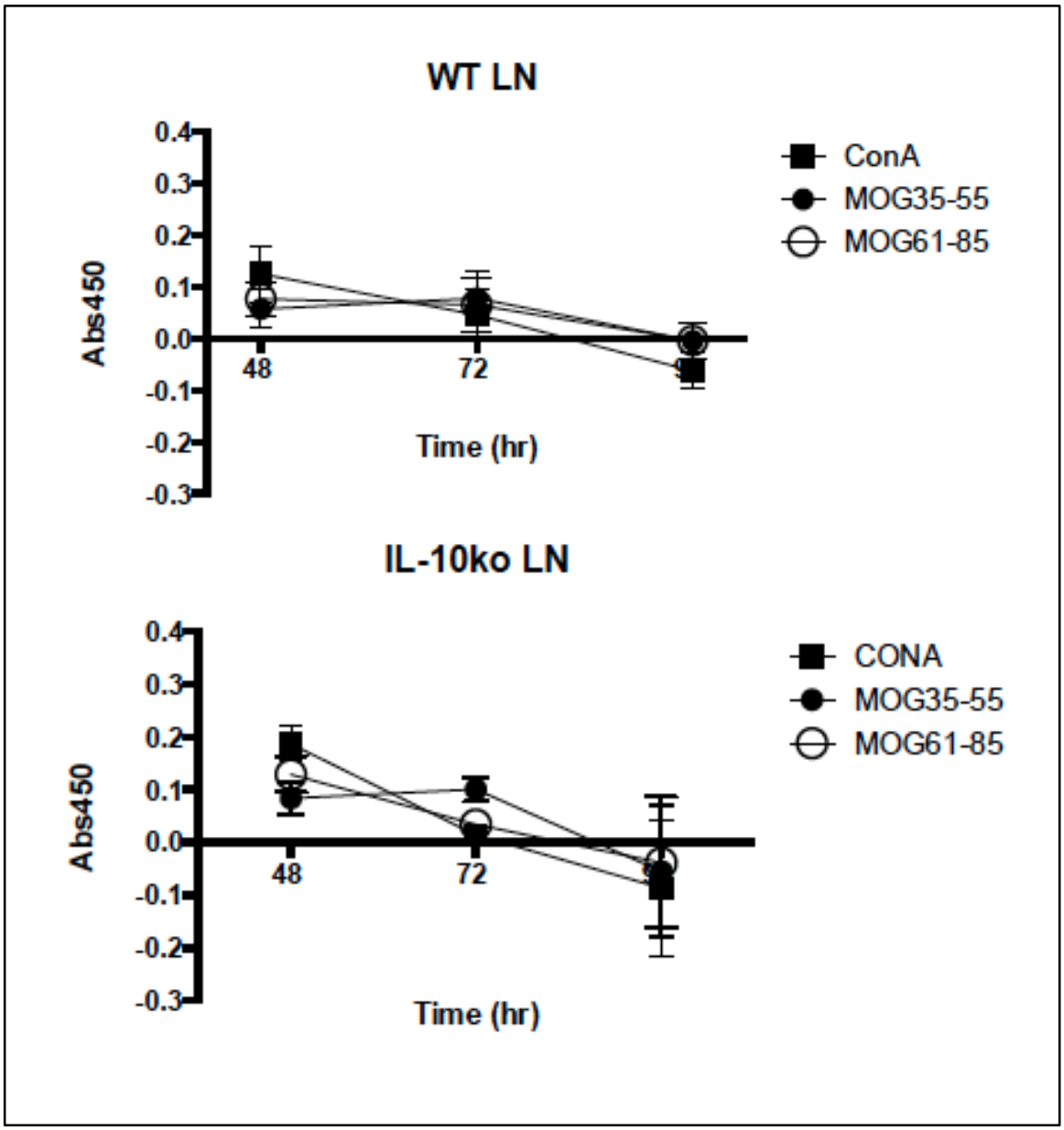

Figure XVI: WT and IL-10$^{-/-}$ LN cell proliferation in response to different stimuli. Cells isolated from WT and IL-10$^{-/-}$ mice were cultured with the above labeled stimuli. **Cells responded to both MOG$_{35-55}$ and MOG$_{61-85}$ with comparable values. This indicates cell recognition to MOG$_{61-85}$.**

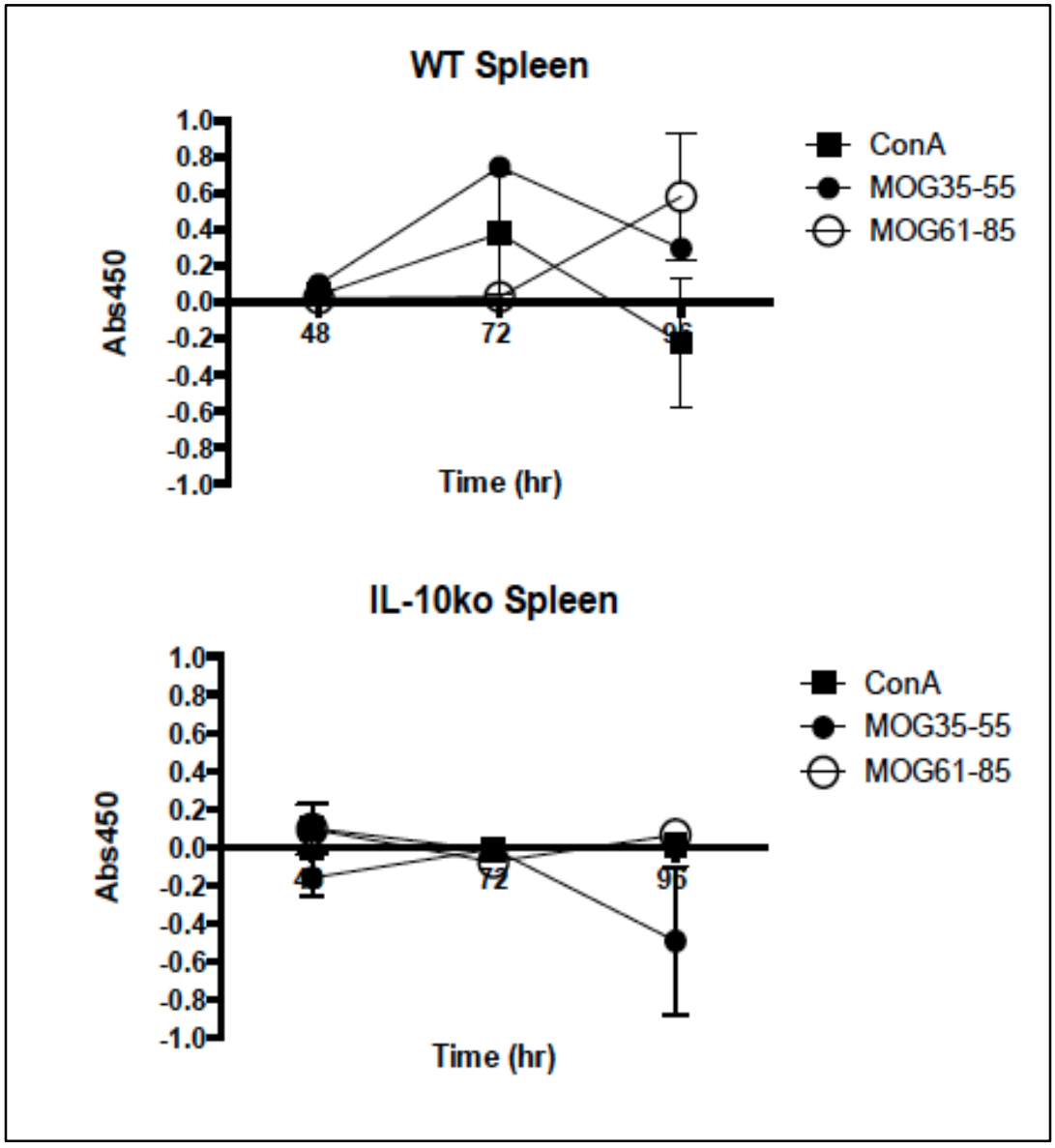

Figure XVII: WT and IL-10$^{-/-}$ spleen cell proliferation in response to different stimuli. Cell isolated from WT and IL-10$^{-/-}$ mice were cultured with the above labeled stimuli. **Cells responded to both MOG$_{35-55}$ and MOG$_{61-85}$ with comparable values. This indicates cell recognition to MOG$_{61-85}$.**

EAE amelioration is independent of IL-10 secretion in rMOG-induced EAE

It is accepted that IL-10 ameliorates EAE disease severity, an observation supported in previous experiments. To determine if MOG$_{61-85}$ induced IL-10 secretion, spleen and LN

cells from rMOG-immunized mice were cultured *in vitro* with MOG_{61-85} and cytokine secretion was measured in cell culture supernatants by ELISA. We failed to detect IL-10 in cell culture supernatants, indicating that this anti-inflammatory cytokine was not responsible for disease amelioration by MOG_{61-85} peptide (figure XVII). This observation further supports previous findings that MOG_{61-85} protective effect is independent of IL-10 secretion (*Agashe V., unpublished data*).

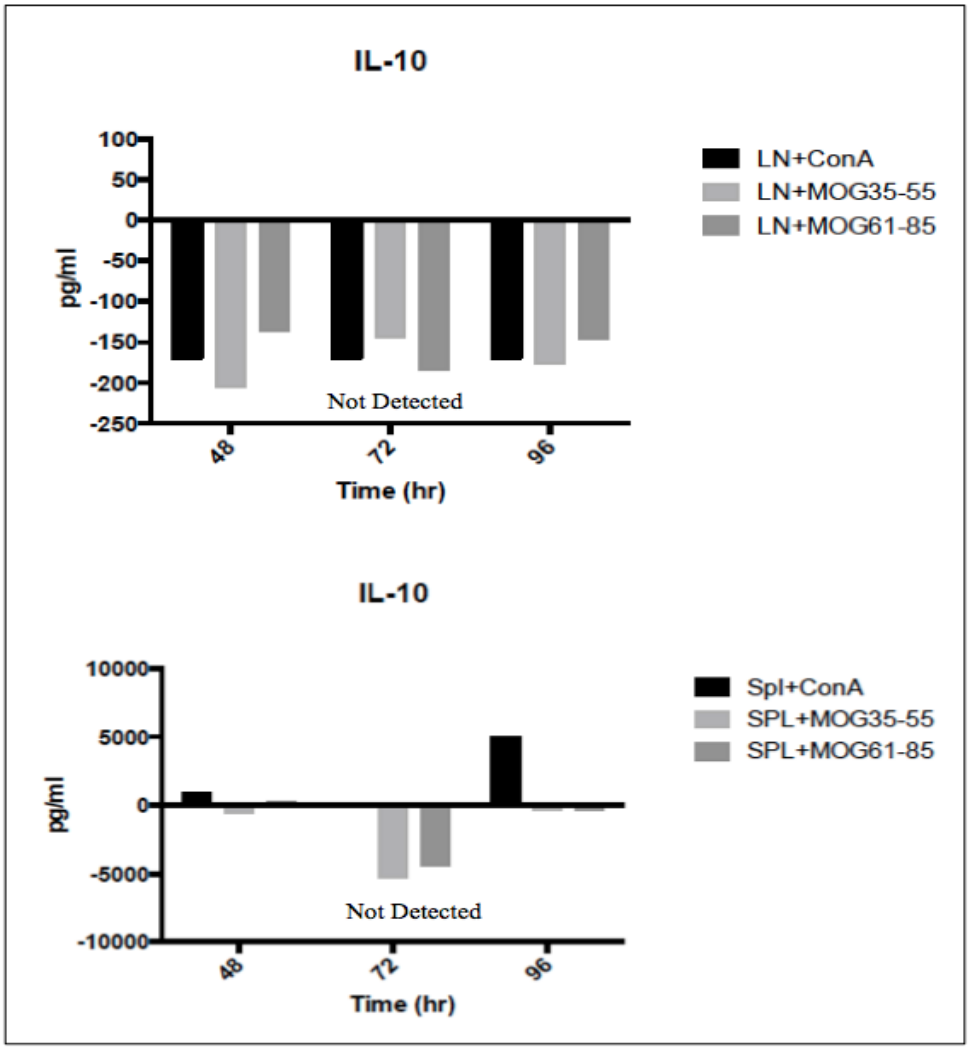

Figure XVIII: IL-10 level in WT cell culture supernatants at three time points. ELISA was used to detect the level of IL-10 secretion in the cell supernatants from the *in vitro* study. The level of IL-10 was below the limit of assay detection in MOG_{61-85} cell cultures. This indicates that MOG_{61-85} protective effect is independent of IL-10 secretion.

MOG_{61-85} induces TGF-β secretion

To further investigate the mechanism by which MOG_{61-85} suppresses EAE, we measured TGF-β levels in the supernatants collected from the cell cultures described above. Data showed an increase in TGF-β level in LN cell cultures stimulated with MOG_{61-85} as compared to MOG_{35-55}. This increase was more evident in cells derived from WT mice as compared to IL-$10^{-/-}$ mice (figure XVIII). In contrast, spleen cell cultures showed comparable values of TGF-β with MOG_{61-85} and MOG_{35-55} stimulation (figure XIX). To further characterize the immune response initiated with MOG_{61-85} stimulation, we measured IL-6 in the same cell cultures.

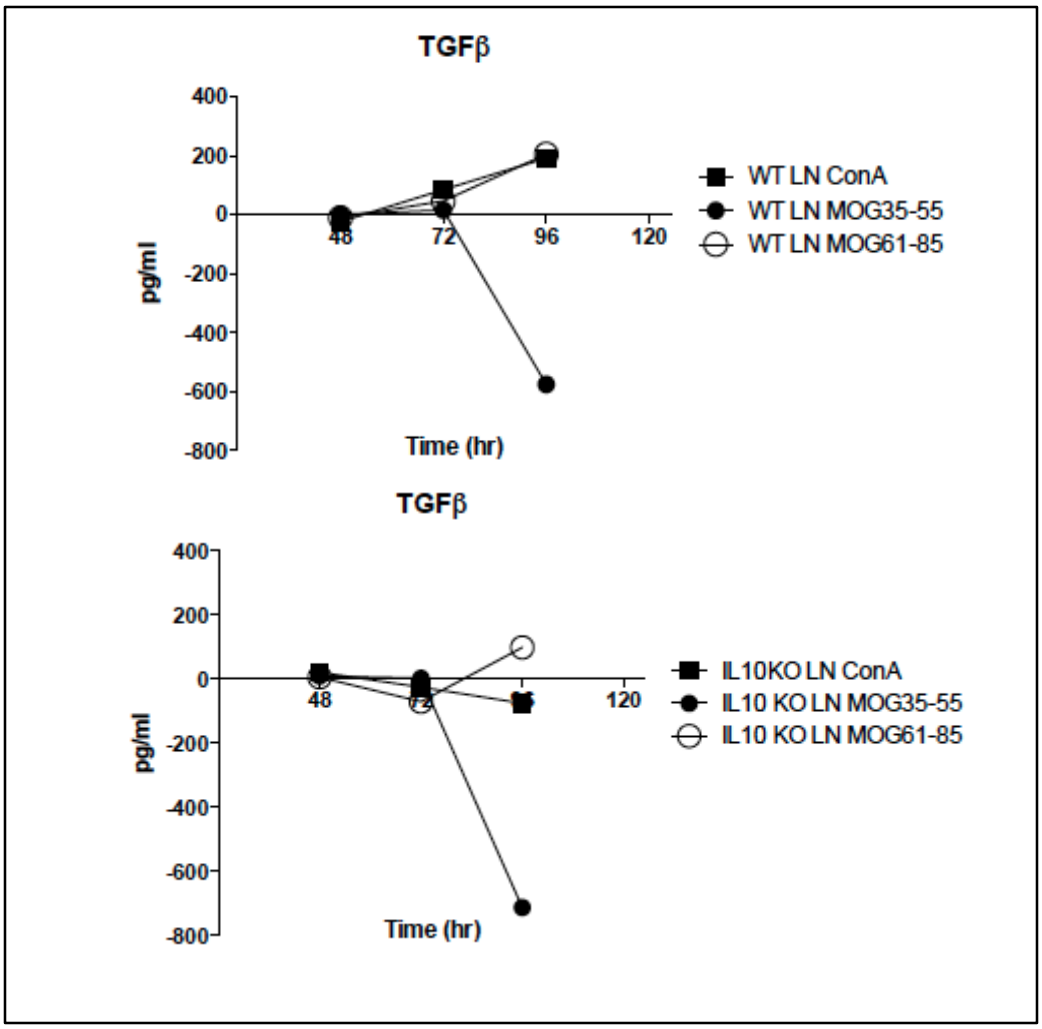

Figure XIX: TGF- β level in LN cell cultures. ELISA was used to detect the level of TGF- β secretion in the cell supernatants from the in vitro study. **The level of TGF-β was higher in MOG_{61-85} cell cultures as compared to MOG_{35-55} cell cultures.** This supports the anti-inflammatory effect of MOG_{61-85} in EAE.

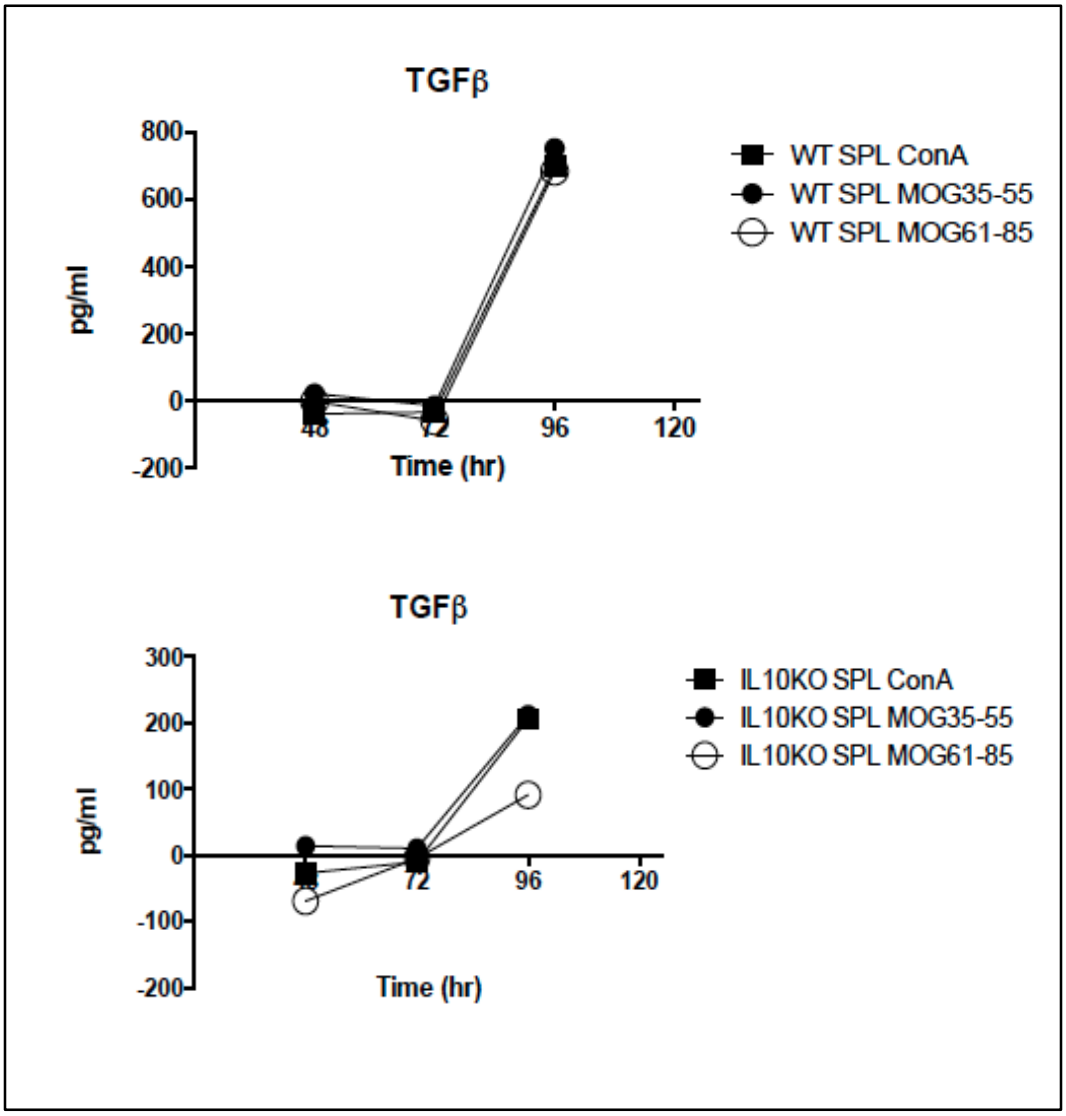

Figure XX: TGF-β level in spleen cell cultures. ELISA was used to detect the level of TGF-β secretion in the cell supernatants from the in vitro study**. Results showed comparable values of TGF-β level with all culture conditions.**

IL-6 secretion is reduced with MOG_{61-85} stimulation

To determine whether TGF-β secretion induces immune regulation or Th17 immune response, we measured IL-6 secretion in the cell culture supernatants. The level of IL-6 was reduced in MOG_{61-85} LN cell cultures as compared to MOG_{35-55} cell cultures (figure XX). However, the IL-6 level in spleen cell cultures was higher than the assay limit and could not be measured (data not shown).

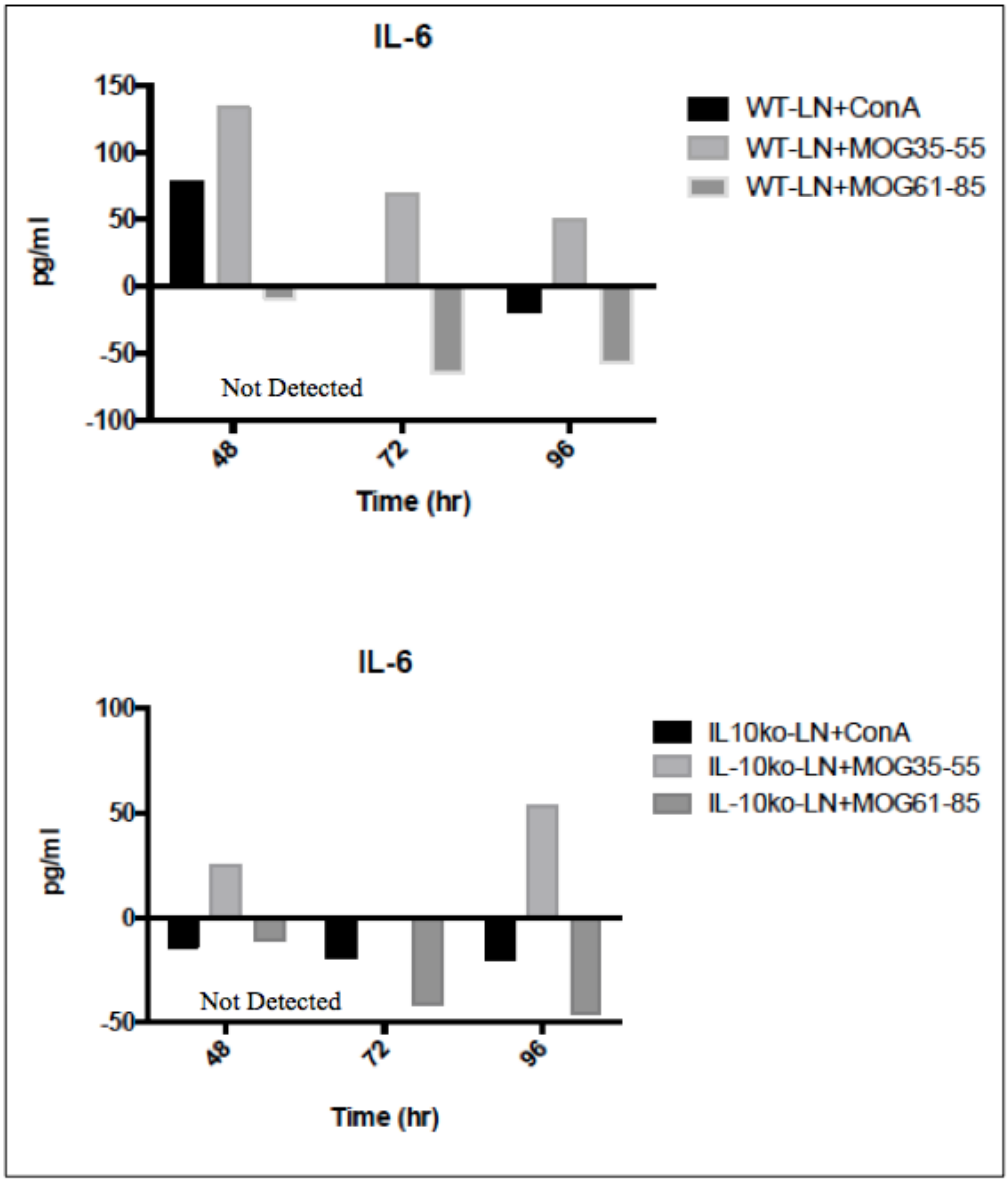

Figure XXI: IL-6 level in LN cell cultures. ELISA was used to detect the level of IL-6 in the cell supernatants from the in vitro study**. Lower IL-6 secretion was observed in cultures stimulated with MOG_{61-85}.**

MOG_{61-85} induce anti-inflammatory immune response

The type of immune response generated depends on the signaling cytokines. Signaling with TGF-β and IL-2 induces an anti-inflammatory or regulatory immune response while signaling with TGF-β and IL-6 initiates a pro-inflammatory Th17 immune response. We observed high TGF-β and low IL-6 secretion in response to MOG_{61-85} *in vitro* culturing. By mathematically calculating TGF-β to IL-6 ratio (TGF-β:IL-6) we determined the type of immune response generated in response to MOG_{61-85} culturing. We found that MOG_{61-85} *in vitro* culturing is characterized by higher TGF-β:IL-6 ratio as compared to MOG_{35-55} culturing. The ratio showed two-fold increase in cell cultures isolated from WT as compared to IL-$10^{-/-}$ LN cell cultures (figure XXI). This supports our hypothesis that MOG_{61-85} priming induces an anti-inflammatory immune response characterized by high TGF-β secretion. As the level of IL-6 in spleen cell cultures was too high, we failed to calculate TGF-β:IL-6 ratio in those cell cultures.

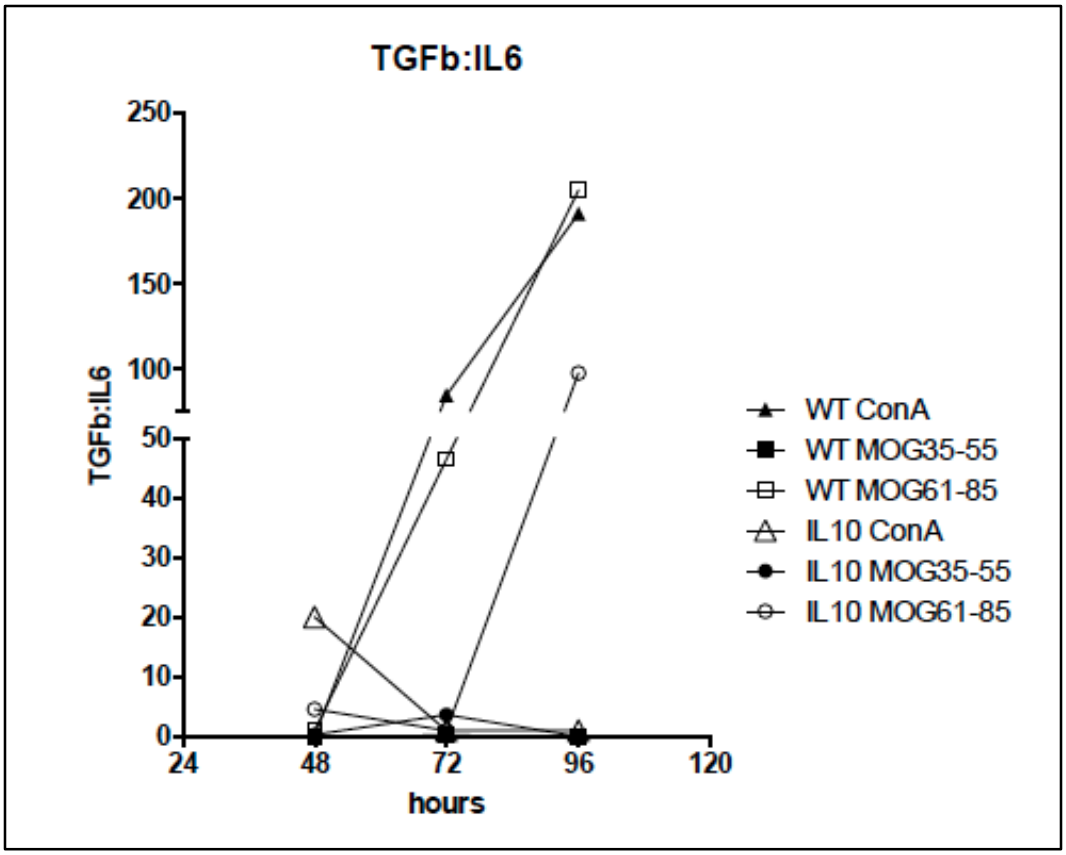

Figure XXII: TGF-β:IL-6 ratio in LN cell cultures. Mathematical calculation of TGF-β:IL-6 ratio showed a higher ration in MOG_{61-85} cell cultures as compared to MOG_{35-55} cultures. This further supports the anti-inflammatory effect of MOG_{61-85} stimulation.

Discussion

This specific aim investigated the mechanism of MOG_{61-85} induced amelioration. Previous experiments revealed a protective peptide within rMOG protein that induces EAE amelioration (*Lyons et al., unpublished data).* Further work to investigate the anti-inflammatory effect of MOG_{61-85} showed that MOG_{61-85} amelioration was independent of IL-10 secretion in MOG_{35-85}-EAE model (*Agashe et al., unpublished data)*. Moreover, MOG_{61-85} induced cellular expansion, of a possible Treg cell population that was investigated in specific aim I. Taken together, this led to our hypothesis that MOG_{61-85} induces an anti-inflammatory immune response characterized by TGF-β secretion.

Immunizing WT and IL-$10^{-/-}$ mice with rMOG protein showed an ameliorated EAE in WT as compared to IL-$10^{-/-}$ mice (figure XII). Therefore, we conducted an *in vitro* study to investigate the anti-inflammatory effect of MOG_{61-85}. By using a cell proliferation assay, we found comparable response to both MOG_{35-55} and MOG_{61-85} in LN cell cultures. However, WT spleen cell culture showed a response to the two MOG peptides while the IL-$10^{-/-}$ cell culture responded weakly. Cell proliferation in response to MOG_{61-85} *in vitro* stimulation indicates that immune cells recognize this peptide and it might have a role in EAE amelioration seen in WT mice. However, it is interesting that IL-$10^{-/-}$ cells in culture also responded to this peptide. Therefore, this requires further investigation.

To support that the anti-inflammatory effect of MOG_{61-85} is independent of IL-10 secretion with rMOG immunization, we measured IL-10 levels in both LN and spleen cell cultures. We failed to detect IL-10 in MOG_{61-85} cell cultures, which indicates that MOG_{61-85} protection is independent of IL-10 production.

To further characterize the immune response induced with MOG_{61-85} stimulation we measured both TGF-β and IL-6 in the cell cultures and calculated TGF-β:IL-6 ratio. Higher ratio indicates that the immune response is characterized by higher TGF-β secretion and low IL-6, a possible regulatory immune response. Lower ratio; in contrast, indicates that the immune response generated has lower secretion of TGF-β as compared to IL-6, a possible Th17 pro-inflammatory immune response. We found that MOG_{61-85} *in vitro* stimulation induced a high TGF-β:IL-6 ratio, suggesting an anti-inflammatory immune response. This supports our hypothesis that MOG_{61-85} induces an anti-inflammatory immune response with high TGF-β production.

Karpus et al. described TGF-β as a suppressor cytokine of the immune response directed against MBP. In their *in vitro* study they showed that TGF-β reduce IFN-γ level in cell cultured with MBP, however IFN-γ level increased by neutralizing TGF-β [101]. Additionally, our previous work showed that cells primed with MOG_{61-85} showed decreased IFN-γ levels which support ameliorating Th1 immune cells (*Lyons et al,* unpublished data). Another study by *Santos et al.* studied immune tolerance induced by oral administration of MBP into SJL mice. They found that immune regulation is mediated by TGF-β secretion and that the adoptive transfer of TGF-β producing T cells suppresses active EAE [102].

Further work should include measuring other anti-inflammatory cytokines such as IL-35 and pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-17 to further describe MOG_{61-85} induced immune response. Moreover, the effect of *in vivo* priming of MOG_{61-85} on the immune response should be also described. In addition to EAE suppression via cytokine secretion, regulating auto-reactive T cells might be mediated by direct cell-cell contact. This possibility should be investigated.

In summary, this aim showed that cells derived from WT mice responded to MOG_{61-85} *in vitro* priming which was not observed in previous experiments. Also, we showed that MOG_{61-85} induces an anti-inflammatory immune response characterized with high TGF-β production. This study only used WT and IL-$10^{-/-}$ mice, therefore future study will include B $cell^{-/-}$ mice to investigate the suppressive cytokine of MOG_{61-85} peptide.

CHAPTER V: DISCUSSION & CONCLUSION

Discussion

Prior studies described a B cell dependent model of EAE. In this model, we observed that B cell$^{-/-}$ C57BL/6 mice were resistant to rMOG-induced EAE [96]. Further studies to investigate this protection revealed a cryptic peptide of MOG_{61-85} within rMOG to which $B^{-/-}$ mice responded but WT mice did not (*Lyons et al., unpublished data)*. We found that co-immunizing mice with the encephalitogenic peptide MOG_{35-55} and the cryptic peptide MOG_{61-85} ameliorates EAE (*Lyons et al., unpublished data)*. Therefore, we studied the effect of MOG_{61-85} on the immune response. Our studies characterizing the MOG_{61-85} response suggested that MOG_{61-85} stimulation induced an anti-inflammatory immune response. This response was independent of IL-10 secretion in MOG_{35-85}-induced EAE (*Agashe et al, unpublished data).* This project aimed to characterize the MOG_{61-85}-specific regulatory cell population and determine its anti-inflammatory mechanism in controlling EAE pathology.

Specific aim I characterized MOG_{61-85}-specific regulatory T cell populations in B cell$^{-/-}$ mice. Previous immunization of B cell$^{-/-}$ mice with both MOG_{35-55} and MOG_{61-85} produced an ameliorated EAE as compared to immunizing B cell$^{-/-}$ mice with MOG_{35-55} only. However, in this study we could not re-produce the same established clinical EAE score. This might be due to accidental difference in the peptide concentration used at the time of emulsion preparation as less MOG_{61-85} peptide used might show less effect when combined with higher amount of MOG_{35-55} peptide. Characterizing the MOG_{61-85}-specific Treg cell population using flow cytometry showed comparable cell percentage of $CD4^+CD25^+FoxP3^+$ T cells between MOG_{35-55} and MOG_{61-85} cell cultures. We also noted

a decrease in CD25 expression in cells primed with MOG_{61-85}, which suggest a loss of suppressive activity. This opposes our hypothesis, however, based on the clinical score seen with this immunization, a MOG_{61-85}-specific Treg population might still be $CD4^+CD25^+FoxP3^+$ T cells. This might be due to unsuccessful *in vivo* priming with MOG_{61-85}, as shown with the clinical score. Insufficient *in vivo* priming with MOG_{61-85} might diminish expansion of the Treg cell population due to lack of processing and presentation of this peptide. Moreover, we observed a slight increase in $CD8^+CD25^-FoxP3^+$ T cells with the *in vitro* priming of MOG_{61-85}. The increase in this population agreed with previous findings, which support a role of $CD8^+$ Treg cell populations in the induced protection seen with MOG_{61-85}. However, the increase in this population was independent of the *in vivo* priming with MOG_{61-85}. We also noted an increase in $CD8^+CD25^+$ T cell population with MOG_{61-85} *in vitro* priming. The increase in this population was shown to be dependent on the *in vivo* priming with MOG_{61-85} peptide. These results support a role of Treg cell populations in the protection induced with MOG_{61-85} immunization and it characterized $CD8^+$ Treg cells as a possible MOG_{61-85}-specific regulatory cell; however, it does not exclude the role of $CD4^+$ Treg cells or MDSCs in EAE amelioration. These results could be confounded if the clinical score was significantly ameliorated with MOG_{61-85} immunization and the cell percentage of $CD8^+$ Treg was significantly higher with MOG_{61-85} *in vivo* and *in vitro* priming. Another co-immunization study is currently being analyzed for EAE clinical score and RNA samples are available to evaluate FoxP3 expression. Characterizing the protective immune response generated in response to MOG_{61-85} stimulation may elucidate a protective mechanism by which we can overcome the auto-reactive immune response seen in MS pathology.

Specific aim II investigated the suppressive mechanism of MOG_{61-85}. This was accomplished by using an *in vitro* study. WT and IL-$10^{-/-}$ mice were immunized with rMOG to study the role of IL-10 versus TGF-β in response to MOG_{61-85} *in vitro* priming. Previous work showed that only cells harvested from B $cell^{-/-}$ mice immunized with rMOG respond *in vitro* to MOG_{61-85} as compared to WT mice immunized with rMOG (*Lyons et al., unpublished data).* However, in this study we showed that WT LN cells respond to MOG_{61-85} *in vitro* priming. This was evident as cells proliferated *in vitro* in response to this peptide. However, this observation could have resulted from protein degradation prior to use and is worth investigating by repeating the immunization with newer protein. As expected, IL-$10^{-/-}$ mice showed severe EAE; however, they also responded to MOG_{61-85} *in vitro* priming. Measuring the anti-inflammatory cytokines IL-10 and TGF-β in the cell cultures determined the suppressive activity of MOG_{61-85}. As shown by ELISA results, MOG_{61-85} *in vitro* priming of WT cells support that MOG_{61-85} suppresses pathology independently of IL-10 secretion. However, lack of IL-10 secretion in IL-$10^{-/-}$ mice and the severe EAE observed further support the role of IL-10 in EAE protection but not as an effect of MOG_{61-85} stimulation. This was evident, as MOG_{61-85} primed cultures did not show a detectable amount of IL-10. Analysis of TGF-β level as a possible MOG_{61-85} specific anti-inflammatory cytokine showed that *in vitro* priming with this peptide increase TGF- β level. Additionally, TGF-β levels was correlated with EAE amelioration. This was evident as WT mice showed ameliorated EAE and high TGF-β level as compared to IL-$10^{-/-}$ mice. To assess the immune response induced in response to MOG_{61-85} *in vitro* priming, the TGF-β:IL-6 ratio was calculated and we found that the MOG_{61-85}-specific immune response switched the pro-inflammatory immune response generated against

rMOG towards an anti-inflammatory immune response characterized by high TGF-β and low IL-6 levels. This suggests that rMOG induce an *in vivo* Th17 immune response and MOG_{61-85} *in vitro* priming is sufficient to control this pro-inflammatory immune response. However, further studies with pro-inflammatory cytokine evaluation must be conducted before concluding this theory. Supernatants from this study are available for pro-inflammatory cytokines evaluation. Additional to anti-inflammatory cytokines, MOG_{61-85} suppressive activity might be induced by direct cell-cell contact. Thus, future studies must investigate this possibility. rMOG protein contains additional peptides that are being processed and presented; therefore, it will be interesting to investigate the effect of MOG_{61-85} *in vivo* priming on the cytokine level. This is currently being conducted as cytokines and RNA samples are available for analysis. Understanding the mechanism by which the cryptic peptide MOG_{61-85} ameliorate EAE by influencing the immune response is crucial as it might introduce potential targets to use for future MS therapy.

Conclusion

In conclusion, MOG_{61-85} suppressive activity is characterized by TGF-β secretion and showed an expansion in the $CD8^+$ Treg cell population, which supports a role of induced cellular immunity in response to MOG_{61-85} priming.

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