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Amelioration of Experimental Autoimmune Encephalomyelitis Using the Myelin Oligodendrocyte Glycoprotein35-85 Peptide

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AMELIORATION OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS USING THE MYELIN OLIGODENDROCYTE GLYCOPROTEIN$_{35-85}$ PEPTIDE

by

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ABSTRACT

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Abstract: Multiple sclerosis (MS) is a chronic debilitating disease affecting the central nervous system (CNS) in humans. Experimental autoimmune encephalomyelitis (EAE) remains the primary animal model of MS. MS/EAE are considered to be autoimmune diseases mediated by $CD4^+$ T helper (T_H) cells. The role of B cells and antibody is under debate. Previous studies established B cell dependent (induced with recombinant myelin oligodendrocyte glycoprotein, [rMOG]) and B cell independent (induced with the MOG_{35-55} peptide) animal models of EAE. The identification of a unique B cell epitope (MOG amino acids [aa] 46-85) preceding the identified protective epitope (MOG_{61-85}) led to the hypothesis that these antibodies against MOGaa46-85 were important in epitope selection in the rMOG model of EAE. Co-immunization of WT and B cell deficient (B $cell^{-/-}$) mice with MOG_{35-55} and MOG_{61-85} resulted in abrogation (B $cell^{-/-}$ mice) or

amelioration (WT mice) of EAE. Thus, mice were immunized with MOG_{35-85} peptide and observed for EAE induction. Absence of EAE in WT and B cell$^{-/-}$ mice was observed. These results confirmed the protective nature of the MOG_{61-85} peptide but did not support a role for antibodies to MOG_{46-85} in the selection of the protective epitope. Mechanistic studies revealed decreased production of the pro-inflammatory cytokines, interferon (INF) γ and interleukin (IL) 17, when immune cells were primed to MOG_{61-85} *in vivo*. Furthermore, using IL10 deficient (IL10$^{-/-}$) mice, it was demonstrated that IL10 was important in EAE incidence, but not in disease severity, in the presence of the MOG_{61-85} epitope. Flow cytometric analysis of spleen cells from these mice demonstrated an increase in the number of T cells expressing FoxP3 expression and an increase in the $CD4^+$ $CD25^+$ T cell population, but a comparable level of $CD4^+$ T regulatory (T_{reg}) cell population. In addition no changes could be detected in the $CD8^+$ T cell population. These experiments provide a deeper understanding of the B cell-dependent, rMOG model of EAE, demonstrating the role of the MOG_{61-85} epitope in down-regulating the pro-inflammatory response leading to protection from EAE, perhaps mediated by $CD4^+$ T_{reg} cells.

Dedicated to the memory of my father

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LIST OF ABBREVIATIONS

°C: Degree Celsius
Ab: Antibodies
Ag: Antigen
ANOVA: Analysis of variance
APC: Allophycocyanin
AUC: Area under the curve
B cell$^{-/-}$: B cell deficient
B6 mice: C57BL/6 mice
BCR: B cell receptor
CD: Cluster of differentiation
cDNA: Complementary Deoxyribonucleic acid
CFA: Complete Freund's adjuvant
CFSE: Carboxyfluorescein succinimidyl ester
CNS: Central nervous system
CO_2: Carbon dioxide
CSF: Cerebrospinal fluid
Ct: Threshold cycle
CTLA-4: Cytotoxic T-lymphocyte Antigen 4
D/W: Distilled water
DNA: Deoxyribonucleic acid
dpi: Day's post immunization
EAE: Experimental autoimmune encephalomyelitis
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme linked immunosorbent assay
FBS: Fetal bovine serum
F_C: Fragment constant
FITC: Fluorescein isothiocyanate
FoxP3: Forkhead box P3
g: Grams
gDNA: Genomic Deoxyribonucleic acid
GFP: Green fluorescent protein
GITR: Glucocorticoid induced Tumor Necrosis Factor Receptor Family related gene
H_2SO_4: Sulphuric acid
HBSS: Hanks balanced salt solution
HCl: Hydrochloric acid
Hi: High
HPLC: High pressure liquid chromatography

HRP: Horse radish peroxidase
hrs: Hours
IFA: Incomplete Freund's adjuvant
Ig: Immunoglobulin(s)
IgG: Immunoglobulin G
IgM: Immunoglobulin M
IL: Interleukin
$IL4^{-/-}$: Interleukin 4 deficient
$IL10^{-/-}$: Interleukin 10 deficient
$IL17^{-/-}$: Interleukin 17 deficient
IL10-GFP mice: Interleukin 10 Green fluorescent protein mice
INFγ: Interferon gamma
$INF\gamma^{-/-}$: Interferon deficient
IR: Immune response
LAP: Latency associated peptide
lit: Liter
Lo: Low
MBP: Myelin basic protein
MDSC: Myeloid derived suppressor cell
MHC: Major histocompatibility complex
ml: Milliliter
mM: Millimolar
MOG: Myelin oligodendrocyte glycoprotein
MRI: Magnetic resonance imaging
mRNA: Messenger Ribonucleic acid
MS: Multiple Sclerosis
N: Normal (Normality)
nm: Nanometers
OCBs: Oligoclonal bands
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
PE: Phycoerythrin
PerCP-Cy5.5: Peridinin chlorophyll 5.5
pg: Picogram
PLP: Proteolipid protein
PPMS: Primary progressive multiple sclerosis
PT: Pertussis toxin
qPCR: Quantitative polymerase chain reaction
qRTPCR: Quantitative reverse Transcriptase Polymerase Chain reaction
rMOG: Human recombinant myelin oligodendrocyte glycoprotein

RNA: Ribonucleic acid
RPMI medium: Roswell Park Memorial Institute medium
RRMS: Relapsing emitting multiple sclerosis
RT: Reverse transcription
SCID: Severe combined immunodeficiency
SPMS: Secondary progressive multiple sclerosis
TCR: T cell receptor
TE buffer: Tris-EDTA buffer
T_{eff}: T effector celLs
TGFβ: Transforming growth factor beta
T_H1: T helper type 1
T_H2: T helper type 2
TMB: Tetramethylbenzidine
TNFα: Tumor necrosis factor alpha
T_{reg}: T regulatory cells
Tris-HCl: Tris Hydrochloric acid
US: United States
Vh: Heavy chain variable region
WT: Wild type
β-actin: Beta actin
Δ: Delta
µl: Microliter

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CHAPTER I: INTRODUCTION

Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis Model

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). In the United States alone, an estimated 350,000 individuals are affected (Sospedra and Martin, 2005). The etiology of MS is under debate, but it is generally accepted that MS develops in genetically susceptible individuals due to environmental triggers such as viral or bacterial infections (Sospedra and Martin, 2005). Traditionally MS has been considered to be an autoimmune disease characterized by demyelination of the white matter via immune mechanisms. MS is considered to be mediated by $CD4^+$ T helper type 1 (T_H1) cells, with B cells and macrophages contributing to its pathogenesis (Barnett and Sutton, 2006; Raine, 1997).

Some of the most common MS symptoms include paralysis, fatigue, walking and balancing problems, bladder dysfunction, optic neuritis, among others (http://www.nationalmssociety.org). Clinically, MS is classified into 3 subtypes including relapsing remitting (RRMS-the most common disease manifestation), primary progressive (PPMS) and secondary progressive (SPMS) (Sospedra and Martin, 2005). Patients demonstrating the RRMS form of the disease experience periodic episodes or symptoms of the disease followed by periods of remission. About 50% of patients suffering from RRMS often progress to SPMS. If these patients progress to a SPMS disease course, the relapses show a gradual worsening until they merge into a general progression. In this case complete remission never occurs. The PPMS disease course is characterized by a gradual worsening of the symptoms from onset without any remissions (Sospedra and Martin, 2005).

Immune-mediated destruction of myelinated CNS tissue leads to plaque formation, which is s a well-demarcated lesion characterized by inflammation, demyelination and gliosis. These plaques are further classified based on the cellular and soluble elements present as Type I through IV, indicative of at least four mechanism of pathogenesis and are considered to be the hallmark of MS (Lassmann et al., 1994; Sospedra and Martin, 2005). Type I and II lesions have pre-dominantly T cells and macrophages contributing to inflammation, along with blood-brain barrier damage. Type II lesions are distinguished on the basis of dominant immunoglobulins (Igs) and complement deposition at the site of active demyelination. Type III lesions are also mediated by T cells and macrophages along with microglial cells. This type is distinguished from Types I and II on the basis of its diffused inflammation. Type IV is also mediated by T cells and macrophages but has no detectable changes in the myelin components (Lucchinetti et al., 2000).

In humans, myelin basic protein (MBP) and proteolipid protein (PLP) are the most abundant proteins in the CNS, and immune responses to both are noted in MS patients (Greer et al., 1997; Meinl et al., 1993). Myelin oligodendrocyte glycoprotein (MOG) is a minor constituent of myelin. However, it is found on the surface of the myelin sheath and is considered to be the most accessible to the immune system. Hence, MOG is considered a candidate for the induction of MS. Using MOG to induce experimental autoimmune encephalomyelitis (EAE) in the C57BL/6 (B6) mouse, is now a well-established model for studying MS (Bernard et al., 1997). MOG has been known to induce both an encephalitogenic T-cell response as well as a demyelinating antibody response in susceptible mice (Gold et al., 2006; Oliver et al., 2003).

EAE is an animal model resembling MS in humans in its course and histopathology and serves as a primary animal model for elucidating MS (Devaux et al., 1997; Hjelmström et al., 1998; Lyons et al., 1999). As with MS, EAE is characterized by inflammation, paralysis and destruction of the myelin sheath (Gold et al., 2006). EAE is induced by the immunization of susceptible animals (rats, mice, guinea pigs) with any one of the CNS proteins (MBP, PLP, MOG) along with an appropriate adjuvant (Bernard et al., 1997; Devaux et al., 1997; Fritz et al., 1983; Gold et al., 2006; McRae et al., 1992). The particular antigen used for immunization depends on the species and strain of animal utilized. It can also be induced with the passive transfer of $CD4^+$ T cells specific for any of the above mentioned proteins (Pettinelli and McFarlin, 1981).

The immune responses and the clinical picture seen with EAE depends on the animal used and the EAE inducing agent (Tuohy et al., 1988). Female SJL/J and male PL/J mice, when immunized with MBP, developed a relapsing-remitting disease course. Lewis rats, when immunized with MBP, first develop an acute phase of EAE, followed by recovery and then a milder form of the disease about a week later (Fritz et al., 1983; McFarlin et al., 1974). Conversely, MOG immunizations in NOD/Lt mice result in a chronic relapsing disease, whereas B6 mice exhibit a chronic non-remitting disease (Bernard et al., 1997). In conclusion, different EAE models are used to study different disease courses.

Immune responses in MS/EAE

T effector (T_{eff}) cells in MS/EAE

$CD4^+$ T cells

The role of T cells in MS/EAE has been under investigation for several years, with both diseases primarily considered to be mediated by $CD4^+$ T T_H1 cells. (Sospedra and Martin, 2005). MBP specific T cell responses have been demonstrated in the peripheral blood of MS patients, as noted by Meinl et al. (1993) and Olsson et al. (1992). Immune responses were also noted against PLP in MS patients exhibiting a relapsing-remitting type of MS; however, their pathogenic role remains to be elucidated (Greer et al., 1997).

T and B cells reactive to MOG have been demonstrated in the peripheral blood as well as the CNS of MS patients. MOG reactive T cells were present in increased numbers in the peripheral blood and the CNS of MS patients as compared to healthy controls. In addition, antibodies specific for MOG were also detected in the blood and CNS of MS patients. This immune response, when compared to a healthy control, was significantly elevated, implying an active antigen driven process (Sun et al., 1991). MOG is a trans-membrane protein, and it was demonstrated that a T cell response was generated against three extracellular regions of the proteins (amino acids[aa] - 1-22, 34-56 and 64-96). These responses were major histocompatibility complex (MHC) class II restricted, indicating that these responses were mediated by $CD4^+$ T cells (Kerlero de Rosbo et al., 1997). An additional response against another trans-membrane region of the protein, aa146-154, was also noted (Weissert et al., 2002).

In general all three antigens (MOG, MBP, PLP) have been used to induce EAE in several different animal models. Moreover in EAE, the immune responses towards encephalitogenic regions of the antigen are known to play a major role in disease pathogenesis. Specifically in the SJL/J model mice were found to be resistant to relapsing EAE induction when they were first tolerized to an encephalitogenic peptide of PLP, but not when they were tolerized to a non-encephalitogenic peptide of the protein (Kennedy et al., 1990). Thus demonstrating the importance of the encephalitogenic regions of the antigens.

A major criticism of classic EAE models is that it is not a spontaneous disease; it must be induced by active immunization of animals or passive transfer of antigen-activated cells. However, spontaneous EAE was noted in transgenic mice expressing a T cell receptor (TCR) specific for MBP (Goverman et al., 1993; Lafaille et al., 1994). Interestingly Goverman et al. showed that EAE developed only in those mice which were housed in a non-sterile facility, supporting the notion of the disease being induced with the aid of certain environmental triggers. Lafaille et al. further demonstrated that there is a sudden increase in the number of $CD4^+$ T cells specific for MBP immediately preceding EAE induction in these mice. The authors concluded that these cells represent the first burst of interferon gamma (INFγ) that is eventually responsible for EAE progression. The same group also demonstrated that $CD4^+$ T helper type 2 (T_H2) cells failed to protect these mice after the adoptive transfer of $CD4^+$ T_H1 cells (Lafaille et al., 1997). EAE was also noted in mice expressing a TCR specific for PLP (Waldner et al., 2000).

However it was demonstrated that, transgenic mice expressing a TCR for MOG, do not develop spontaneous EAE but develop optic neuritis (Bettelli et al., 2003). Optic

neuritis is the inflammation of the optic nerve and is considered to be an early symptom of MS. Optic neuritis is manifested as an acute blurring or loss of vision in a single eye (http://www.nationalmssociety.org). However, the same group demonstrated that mice expressing both T and B cells specific for MOG, develop spontaneous EAE. The authors demonstrated that the MOG specific B cells effectively presented the antigen to T cells, leading to increased T cell proliferation. The increased MOG specific T cell proliferation led to increased immunoglobulin G (IgG) antibody production by B cells. These T and B cell responses led to EAE in these mice (Bettelli et al., 2006).

$CD8^+$ T cells

$CD8^+$ cells, previously ignored in MS/EAE pathology, have recently been considered as a potential effector cell type. Auto-reactive $CD8^+$ T cells have been demonstrated in the peripheral blood and CNS of MS patients (Crawford et al., 2004). Another group demonstrated that there were equal numbers of myelin specific $CD8^+$ T cells in MS patients and healthy controls. However, there was a functional difference in the $CD8^+$ T cells between the two groups. Specifically, the $CD8^+$ T cells from MS patients were more activated and demonstrated increased expression of CXCR3 (a chemokine receptor), INFγ and Interleukin (IL)10 (Babbe et al., 2000).

It was demonstrated that MBP-specific $CD8^+$ cells, transferred to Wild Type (WT) animals, resulted in EAE induction in these mice. Of note, however, is the fact that these cells could induce EAE in severe combined immunodeficiency mice, demonstrating that they are not dependent on $CD4^+$ T cells for their pathogenesis (Huseby et al., 2001). Similarly, EAE was also observed using MOG specific $CD8^+$ T cells adoptively

transferred into B6 mice. Both these results effectively demonstrate the pathogenic role of $CD8^+$ T_{eff} cells in EAE pathology (Sun et al., 2001). Interestingly, a $CD8^+$ T cell epitope(s) had been identified which overlaps with the known $CD4^+$ T cell epitope MOG_{35-55}. It is comprised of MOGaa37-46 and MOGaa44-54 (Ford and Evavold, 2005; Sun et al., 2003). Using the MOG_{37-46} epitope, it was demonstrated that mice developed a milder form of the disease, with trafficking of the $CD8^+$ T_{eff} cell population to the CNS. These authors hypothesized that these $CD8^+$ T cells then lead to the recruitment of $CD4^+$ T cells within the CNS, resulting in a chronic form of EAE in the B6 animal model. (Bettini et al., 2009).

Role of T Regulatory (T_{reg}) cells

T_{regs} in MS

Evidence now points to a protective role of $CD4^+$ $CD25^{Hi}$ $FoxP3^+$ T_{reg} cells in MS. Various studies have demonstrated the presence of these cells within the CNS of MS patients. However the abundance of these cells in MS patients as compared to healthy controls remains under debate. Some studies have shown a comparable frequency of these cells in the periphery and the CNS of MS patients and healthy controls (Haas et al., 2005), whereas others have shown an increase in their frequencies in MS patients (Feger et al., 2007; Kumar et al., 2006). One aspect consistent with all of these findings is that the suppressive capacity of these T_{reg} cells is diminished (Viglietta et al., 2004). This decrease in the suppressive capacity of these cells is now linked to the development of MS. More specifically, these cells have been shown to be down regulated during relapses of RRMS, when there is an increase in frequencies of $CD4^+$ T_H1 cells (Frisullo et al.,

2009). The development of MS in these patients has also been linked to the decreased expression of Forkhead Box P3 (FoxP3) in these T_{reg} cells (Feger et al., 2007; Venken et al., 2008).

In addition to $CD4^+$ cells, $CD8^+$ T_{reg} cells have also been shown to induce tolerance in MS patients (Frisullo et al., 2010). Alterations in the frequencies of suppressive $CD8^+$ cells have been demonstrated in the peripheral blood of MS patients (Crucian et al., 1995). More specifically, treatment of MS patients with Coapxone has been shown to induce a MHC class I restricted $CD8^+$ T cell population. The same group later demonstrated that these $CD8^+$ T cells had suppressive functions. These cells were found to suppress $CD4^+$ T cell proliferation in a contact-dependent manner. The authors also demonstrated that these cells produced increased amounts of INFγ and tumor necrosis factor (TNF) α, with no production of IL4 (Karandikar et al., 2002; Tennakoon et al., 2006). Therefore Copaxone is thought to prevent relapses of MS, by inducing apoptosis in activated cells, through the production of TNF α and not by shifting the immune response towards a T_H2 cell subset.

T_{regs} in EAE

There is now substantial evidence that points towards a protective role of $CD4^+$ $CD25^+$ $FoxP3^+$ T_{reg} cells in EAE. Lafaille et.al., demonstrated that the transfer of these cells into the previously described mice, expressing a TCR transgenic for MBP, was protective against EAE development (Lafaille et al., 1994; Olivares-Villagomez et al., 1998; Reddy et al., 2004). The presence and action of these T_{reg} cells have also been implicated in those animals that are naturally resistant to EAE induction (Reddy et al.,

2004). Varying results have been obtained for the exact mechanism of T_{reg} cell action. Data implicate the amelioration of EAE though the production of anti-inflammatory cytokines such as IL10 and/or down-regulation of INFγ, along with expression of molecules such as Cytotoxic T-lymphocyte Antigen (CTLA) 4 and Glucocorticoid induced Tumor Necrosis Factor Receptor Family related gene (GITR) (Liu et al., 2006; McGeachy et al., 2005; O Connor et al., 2007; Zhang et al., 2004). Some research has also shown that the presence of $CD4^+$ $CD25^+$ $FoxP3^+$ T_{reg} cells skews the immune response towards a T_H2 dependent mechanism (Kohm et al., 2002). Moreover the outcome of disease has been shown to be dependent on a critical balance between the population of $CD4^+$ T_{eff} cells and $CD4^+$ T_{reg} cells (Stephens et al., 2005). More recently another subset of $CD4^+$ T cells expressing latency associated peptide (LAP) has also been implicated in possessing suppressive activity (Chen et al., 2008). Antigen-specific activation of T_{reg} cell proliferation does not appear to be important, as T_{reg} cells induced due to an active vaccination against an irrelevant antigen such as *Salmonella* vaccine expressing colonization factor antigen I can still protect against EAE (Ochoa-Repáraz et al., 2007).

$CD8^+$ T_{reg} cells were also found to be important in EAE suppression. Najafian et al. (2003) demonstrated the presence of $CD8^+$ $CD28^-$ cells with suppressive function in EAE. The authors demonstrated that these cells suppress INFγ production by MOG-specific $CD4^+$ T cells in a contact-dependent manner. Lee et al. (2008) demonstrated the presence of naturally occurring $CD8^+$ $CD122^+$ T_{reg} cells which are important in the recovery phase of EAE. Chen at al. who demonstrated the presence of a $CD4^+$ LAP^+ suppressive T cell population also identified $CD8^+$ LAP^+ T cells, with suppressive

functions (Chen et al., 2009). Moreover, it was demonstrated that these $CD8^+$ LAP^+ T cells may or may not possess FoxP3 or CD25 and they were found to suppress EAE in a transforming growth factor (TGF) β, INFγ dependent mechanism.

Myeloid derived suppressor cells

Myeloid derived suppressor cells (MDSCs) are defined as a heterogeneous group of immune cells derived from the myeloid lineage and having potent T cell suppressive functions. They are primarily derived from myeloid progenitor cells: immature macrophages, immature granulocytes and immature dendritic cells (Gabrilovich and Nagaraj, 2009). These cells were first described in cancer patients and have recently come under investigation as potent effectors of immune responses (Young et al., 1987). In mice, these cells are characterized by the presence of the surface makers CD11b and GR1 (Kusmartsev et al., 2004) and are further divided based on the expression of the two epitopes of GR1, specifically Ly-6G and Ly-6C. Granulocytic MDSCs are $CD11b^+$ $Ly\text{-}6G^+$ $Ly\text{-}6C^{Lo}$, whereas monocytic MDSCs are $CD11b^+$ $Ly\text{-}6G^-$ $Ly\text{-}6C^{Hi}$ (Youn et al., 2008). In humans MDSCs are defined by the presence of $CD14^-$ $CD11b^+$ or $CD33^+$ cells, lacking the mature cell markers along with an absence of MHC II molecules (Almand et al., 2001; Ochoa et al., 2007). Several different mechanism of suppression by MDSCs have been documented as reviewed by Gabrilovich and Nagaraj (2009).

An increase in the $CD11b^+$ $Ly\text{-}6G^-$ $Ly\text{-}6C^{Hi}$ subset of MDSCs in the spleen, blood and CNS of mice with EAE (Zhu et al., 2007). These cells were demonstrated to suppress the proliferation of $CD4^+$ and $CD8^+$ T cells. Contradictory to these observations, another group demonstrated that these immature cells migrate from the periphery to the CNS

where they mature into dendritic cells and macrophages and in turn contribute to the pathogenesis of the disease (King et al., 2009; Mildner et al., 2009). Thus a definitive role for MDSCs in EAE pathology is unknown. Furthermore, even less is known about the role of MDSCs in MS. The role of this novel regulatory population in MS/EAE pathogenesis remains under investigation.

B cells in MS/EAE

The exact role of B cells in the pathogenesis of MS/EAE is still controversial. Studies have demonstrated that the CNS of MS patients has a higher percentage of memory B cells as compared to the peripheral blood along, with a concomitant reduction in the percentage of naïve B cells. The presence of plasma cells within the CNS is still under debate (Cepok et al., 2005; Corcione et al., 2004). B cells and antibodies have been found in increased concentration, in recently developed plaques as compared to older plaques and also as compared to the surrounding unaffected area, but their exact role is under investigation (Esiri, 1977; Genain et al., 1999).

As previously mentioned, 4 distinct types (I-IV) of demyelination have been identified in MS patients. In this regard Type II plaques are defined by a significant antibody and complement accumulation in active MS lesions, thereby demonstrating at least one distinct mechanism of pathogenesis involving B cells (Lucchinetti et al., 2000). More recently, it was also demonstrated that that MOG specific antibodies were present in active lesions in MS patients (Genain et al., 1999; Lucchinetti et al., 2000)

One aspect that is generally accepted is that the intrathecal production of antibodies in the CNS and the presence of oligoclonal bands (OCBs), particularly of the

IgG subtype, in the cerebrospinal fluid (CSF) of MS patients are associated with a worse prognosis (Olsson et al., 1976; Villar et al., 2005). The absence of these OCBs is associated with a better prognosis (Avasarala et al., 2001; Zeman et al., 1996). These OCBs are composed of antibodies, the specificity of which has been under intense debate. Some studies have indicated that these antibodies are produced as a result of an antigen driven process and are specific to CNS proteins with specific VDJ regions being expressed (Baranzini et al., 1999; Colombo et al., 2000; Genain et al., 1999; Harp et al., 2007; Qin et al., 1998; Villar et al., 2005), while others have shown that the B cell response is not targeted towards any of the CNS proteins (Owens et al., 2009). Alternatively, it is hypothesized that the Ig response could also be directed against an unknown etiological entity such as a virus (Owens et al., 2006). It has been postulated that the identification of the specificities of these antibodies could be used to identify potential antigens in MS (Owens et al., 2003). Whether the subtype of Ig is indicative of a recent relapse or disease activity or which subtype is a better choice as a diagnostic marker is still under debate (Izquierdo et al., 2002; Schneider et al., 2007; Sharief and Thompson, 1991).

Recently, Rituximab, a monoclonal antibody directed against the CD20 antigen on B cells has been approved for treatment of MS. In MS patients this drug was found to transiently deplete the peripheral B cell population, without many alterations in the Ig pool, in terms of the specificity of the Igs produced (Cross et al., 2006). A possible reason for this could be that CD20 is not expressed on plasma cells. Because of the short duration of this study, no improvement in the clinical score was noted in these patients. In another study, Rituximab was found to decrease the number of inflammatory loci in the

CNS of MS patients as demonstrated using magnetic resonance imaging (MRI) (Hauser et al., 2008). Studies using a transgenic mouse model in which animals express human CD20 demonstrated that this drug decreased the B cell population in the periphery, spleen and the CNS, along with clinical scores. Significant alterations in the T cell repertoire were also noted in these mice (Monson et al., 2011). All of these studies support the notion of a pathogenic role of B cells in MS.

In contrast to the accepted pathogenic role of B cells and antibodies, others have demonstrated that some antibodies are effective at promoting remyelination in a model of MS. A group of researchers identified two human monoclonal IgM antibodies isolated from patients with monoclonal gammopathy, which facilitated remyelination in-vitro (Warrington et al., 2000).

The role of B cells in EAE has been found to be dependent on the animal model used and EAE inducing agent used. Early studies demonstrated the importance of B cells and/or antibodies in EAE induced by intact proteins (Gausas et al., 1982; Myers et al., 1992; Willenborg and Prowse, 1983; Willenborg et al., 1986). However further studies demonstrated that EAE could be induced in B cell deficient (B cell$^{-/-}$) animal models with the use of shorter antigenic peptides (Dittel et al., 2000; Hjelmström et al., 1998; Lyons et al., 1999; Wolf et al., 1996). It was also demonstrated that B cell$^{-/-}$ animals failed to recover from EAE indicating that B cells may not be important in disease induction but may play a role in recovery (Wolf et al., 1996). The exact mechanism through which B cells exert their effects is still under investigation.

Some studies have suggested that B cells or its products are responsible for the pathology, as demonstrated by adoptive transfer experiments in B cell$^{-/-}$ mice (Lyons et

al., 2002). Another group (Svensson et al., 2002) has demonstrated that the effects of B cells may be through demyelination rather than inflammation in support of the study by Lyons et al. (2002). In addition, anti-MOG antibodies have been detected within the CNS of MS patients and also in the CNS of the marmoset EAE model (Genain et al., 1999). Another group showed that transgenic mice expressing a B cell receptor (BCR) specific for MOG resulted in an accelerated and exacerbated disease course (Litzenburger et al., 1998). Thus all these experiments support a pathogenic role for B cells in MS.

The demyelinating potential of anti-MOG antibodies has also been shown *in-vitro* by Kerlero de Rosbo et al. The authors used aggregating fetal rat brain cell cultures which develop myelin around themselves to demonstrate this. The authors demonstrated that the amount of myelin generated was inversely related to the amount of anti-MOG antibodies added to the cultures in presence of complement. However, the addition of anti-MBP antibodies did not demonstrate any demyelination (Kerlero de Rosbo et al., 1990). Another group showed that T cells specific for MBP induce EAE in Lewis rats without demyelination. Over time, these animals develop resistance to EAE induction. However, if these mice also receive anti-MOG monoclonal antibodies at the time of T cell transfer, they develop severe relapses with a higher degree of demyelination (Linington et al., 1992). Conversely, other reports suggest that B cells or their products may not play a role in EAE pathology since EAE could be induced in B cell$^{-/-}$ animals with shorter peptides (Hjelmström et al., 1998; Wolf et al., 1996). In conclusion the role of B cells in EAE remains under investigation.

Cytokines in MS/EAE

Most of the disease pathology in EAE has been attributed to the increased production of INFγ. However, studies using transgenic INF deficient ($INF\gamma^{-/-}$) mice demonstrated that the knockout mice developed more severe EAE than WT littermates (Ferber et al., 1996; Krakowski and Owens, 1996) indicating that INFγ may not be necessary for EAE induction but for its progression. Recently, increased INFγ production has been implicated in the up-regulation of T_{reg} cells, which help in disease suppression, thus explaining the increased disease severity in $INF\gamma^{-/-}$ mice (Zhu et al., 2007).

IL10 has also been implicated in suppressing EAE induction and progression. It was demonstrated that the loss of $CD1d^{Hi}$ $CD5^{+}$ regulatory B cells producing IL10, during EAE induction led to a more severe course of the disease (Matsushita et al., 2008). It has also been demonstrated that IL4 deficient ($IL4^{-/-}$) mice on B6 and BALB/c backgrounds were more susceptible to EAE induction. In both strains of mice, there was increased inflammation, increased production of pro-inflammatory cytokines and a higher degree of demyelination. These results indicated that IL4 is important in modulating the severity of the disease (Falcone et al., 1998). Recently, it has also been demonstrated that $CD4^{+}$ and $CD8^{+}$ T cells expressing the LAP molecule work in a TFGβ dependent mechanism to suppress cell proliferation *in-vitro* (Chen et al., 2008; Chen et al., 2009).

Another cytokine implicated in EAE pathogenesis is IL17. EAE severity was reduced upon immunization of IL17 deficient ($IL17^{-/-}$) mice with MOG (Komiyama et al., 2006). Furthermore, $INF\gamma^{-/-}$ mice produced greater levels of IL17 than WT mice when immunized with MOG. These data establish that IL17 is another important pro-inflammatory cytokine important in EAE pathogenesis (Komiyama et al., 2006).

The B cell dependent and B cell independent model of EAE

As previously mentioned EAE could be induced in a B cell$^{-/-}$ mice with shorter antigenic peptides. One such example is the immunization of B cell$^{-/-}$ animals on a B6 background with MOG_{35-55} peptide emulsified in Freud's complete adjuvant. Studies demonstrated that WT B6 mice were susceptible to EAE when immunized with the Human Recombinant MOG (rMOG) or with the encephalitogenic human MOG_{35-55} peptide. Conversely the B cell$^{-/-}$ animals were only susceptible to EAE when immunized with the MOG_{35-55} peptide and not the rMOG protein (Lyons et al., 2002; Lyons et al., 1999). The resistance to EAE in B cell$^{-/-}$ mice was not due to a lack of processing and presentation of the encephalitogenic MOG_{35-55} epitope from the rMOG protein, as indicated by a similar antigen-specific proliferation and cytokine secretion profiles elicited in *in vitro* cell cultures (Lyons et al., 1999). Further studies demonstrated that MOG specific serum or MOG primed B cells transferred to B cell$^{-/-}$ mice reconstituted the susceptibility of the B cell$^{-/-}$ mice to rMOG induced EAE. Non-specific serum or non-specific priming of the transferred B cells failed to induce EAE in the B cell$^{-/-}$ mice. This demonstrated that an antigen–specific factor is involved in the development of EAE in the B cell$^{-/-}$ mice (Lyons et al., 2002).

To determine whether the differences in the role of B cells in rMOG vs. MOG_{35-55} induced EAE would be evidenced by differences in the B cell repertoire of immunized WT mice, characterization of the heavy chain variable region (Vh) repertoire of the CNS-infiltrating B cells was performed and it showed a preferential use of certain regions in both the animal models. Analysis was carried out for the three dominant genes of mouse Vh regions, VhJ558, VhQ52 and Vh7183. PCR analysis revealed a comparable amount

of VhJ558/Jh region transcripts and VhQ52/Jh region transcripts in the CNS of both the animal models. However, Vh7193/Jh region transcripts were observed in the CNS of rMOG-immunized mice, but not MOG_{35-55} immunized mice. Further analysis showed the presence of Vh7193/Jh region transcripts in the draining lymph nodes of both models, indicating that the lack of these transcripts in the CNS was not due to a failure to prime these cells in the periphery. Quantification showed a comparable expression of 7183/Jh2 and 7183/Jh4 transcripts in the periphery of both animal models, but a lack of 7183/Jh3 transcripts in the periphery of MOG_{35-55} immunized mice (Figure 1) (Liu et al., 2012).

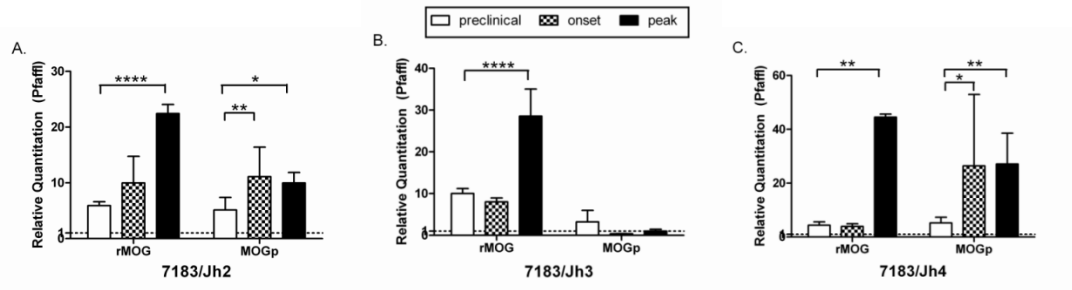

Figure 1. **qRTPCR analysis of the 7183/Jh2, 3, 4 transcript levels in the draining lymph nodes of rMOG and MOG_{35-55} immunized mice.** RNA was extracted from lymph node cells of WT animals immunized with either rMOG or MOG_{35-55}, and was subjected to qRTPCR for determining the transcript levels of 7183/Jh2, 7183/Jh3 and 7183/Jh4 gene segments. **Results demonstrated a comparable level of the 7183/Jh2 and 7183/Jh4 transcripts, but a lack of the 7183/Jh3 transcripts in the periphery.**

As B cells and antibody can direct antigen processing and epitope selection, peptide mapping of the antibody response to linear epitopes of rMOG was carried out. Antibodies were found against the region encompassing the amino acids 21-40 in both animal models (rMOG and MOG_{35-55} peptide immunized mice). In addition, antibodies were also found in the rMOG immunized mice against a group of epitopes comprising the

amino acids 46-85 which were absent in the MOG peptide induced model (Figure 2). Given the location between the two T cell epitopes (aa35-55 and aa61-85 [Discussed below]), it is possible that this antibody epitope could be important in epitope selection in rMOG-immunized WT mice (Liu et al., 2012).

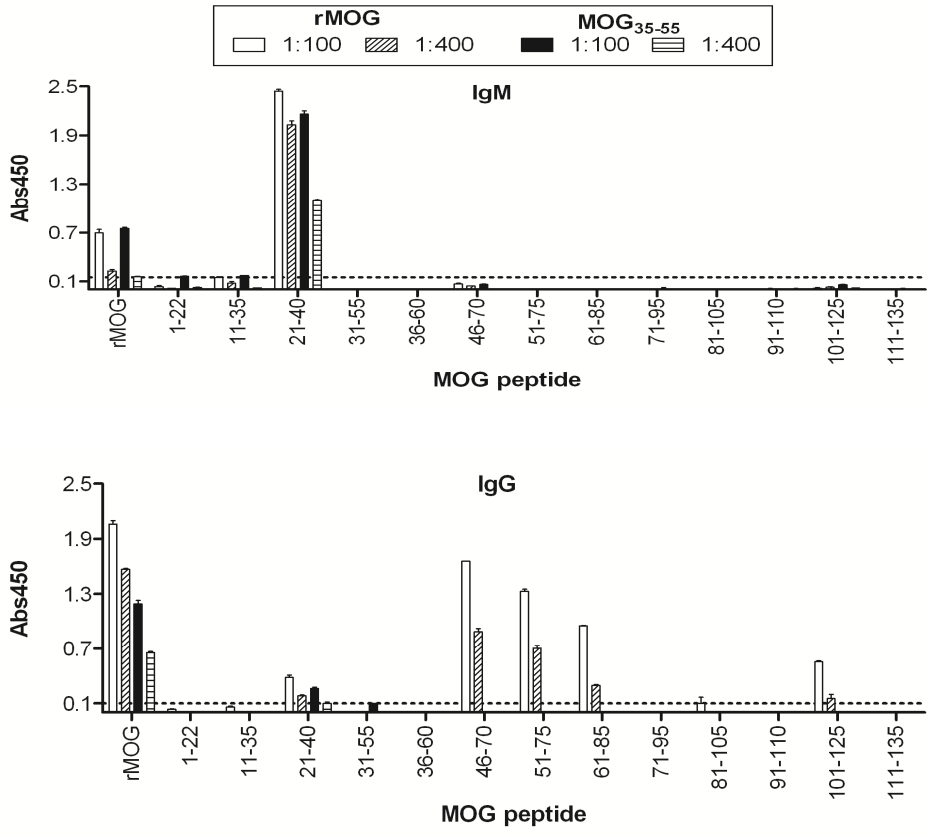

Figure 2. **Serum antibody responses to rMOG or MOG_{35-55} peptide immunizations.** WT animals were immunized with rMOG or MOG_{35-55} peptide and serum was collected from these animals. The serum was then subjected to antibody peptide mapping analysis, using ELISA, to determine the specific antibody responses, seen with each antigen. **The animals immunized with rMOG or MOG_{35-55} demonstrated a response against MOGaa21-40; however, antibodies were also found in the rMOG immunized mice against a group of epitopes comprising the amino acids 46-85.**

The particular set of T cell epitopes generated is dependent on the phenotype of the antigen presenting cell. Furthermore, the unique antibody response detected could play a role in epitope selection in the rMOG model. Thus, peptide mapping analysis of the T cell epitopes generated in rMOG-immunized WT and B cell$^{-/-}$ mice was performed. These studies demonstrated an immune response towards the encephalitogenic epitope, amino acids 31-55, in both strains. In addition, the B cell$^{-/-}$ animals also responded to another region encompassing the amino acids 61-85, which was absent in the rMOG immunized WT mice (Figure 3, Lyons *et al.* unpublished data).

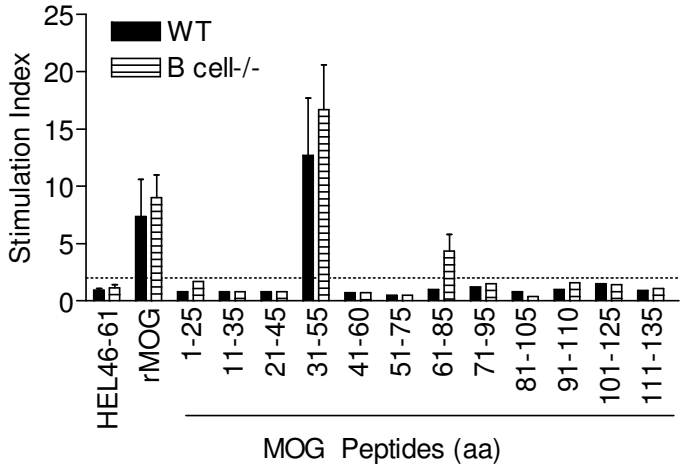

Figure 3. **Peptide Mapping for T cell epitopes.** Mice (WT and B cell$^{-/-}$) were immunized with rMOG and the lymph nodes extracted 10-14days post immunization (dpi). T cells were extracted from the lymph nodes and subjected to proliferation assays in response to shorter peptides of the rMOG protein. **T cell proliferation was observed in response to rMOG as well as MOGaa31-55. In addition, B cell$^{-/-}$ mice also responded to MOGaa61-85, which was absent in the WT animals, suggesting that aa61-85 might be a cryptic epitope in WT animals.**

Thus B cell$^{-/-}$ mice, which are resistant to rMOG-induced EAE, reacted to an additional epitope (MOG_{61-85}) not noted in rMOG-immunzied WT mice. To determine if this epitope could play an active role in disease protection in B cell$^{-/-}$ mice, co-immunizations of the MOG_{35-55} and MOG_{61-85} peptides were performed. Co-immunization with these 2 peptides prevented EAE in B cell$^{-/-}$ animals. A similar effect was also seen in WT animals, although it was less pronounced (Figure 4). This indicated that the MOG_{61-85} was a cryptic epitope in the WT animals and could possibly play a role in the resistance of B cell$^{-/-}$ animals towards rMOG induced EAE (Lyons *et al.* unpublished data).

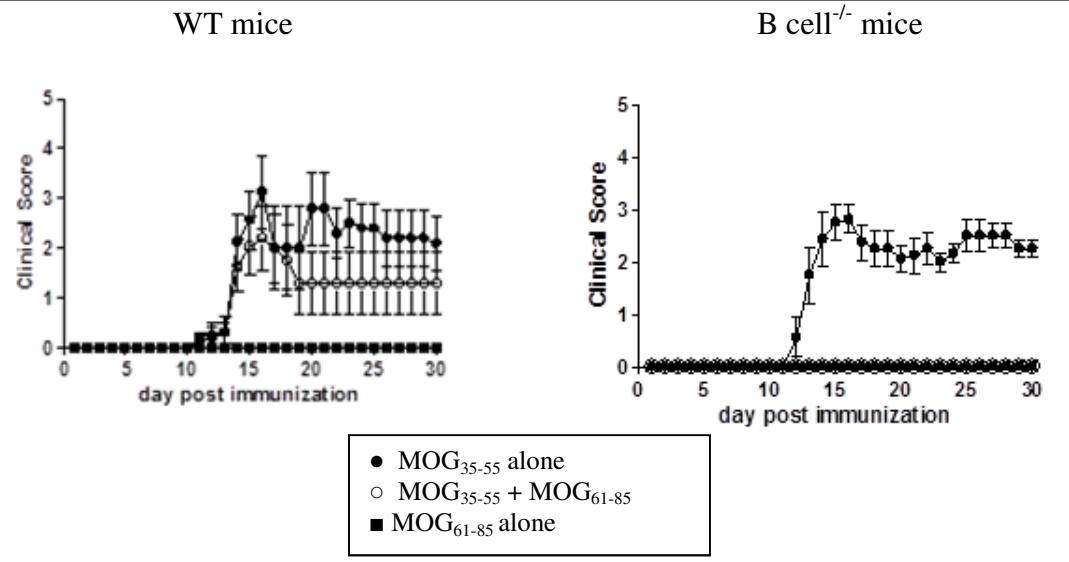

Figure 4. **Co-immunization studies with MOG_{35-55} and MOG_{61-85} peptides.** WT and B cell$^{-/-}$ animals were co-immunized with the encephalitogenic MOG_{35-55} and MOG_{61-85} peptide and observed for EAE induction and progression. WT animals co-immunized with both the peptides showed a decrease in EAE severity when compared to the animals immunized with MOG_{35-55}. Conversely B cell$^{-/-}$ animals co-immunized with both peptides showed no EAE induction. **These results effectively demonstrated the protective nature of the MOG_{61-85} peptide.**

To investigate whether the observed serum response was involved in the generation of the protective immune response, lymph node cells were isolated from B cell$^{-/-}$ mice that received rMOG primed serum at the time of rMOG immunization. Characterization of the cytokine response in these animals demonstrated that the immune response towards the MOG_{61-85} region was abrogated in these animals (Figure 5). These experiments demonstrated that the serum derived factor from the WT animals was involved in the antigen processing and presentation in the B cell$^{-/-}$ animals and was thus involved in the resistance of the B cell$^{-/-}$ animals towards rMOG induced EAE (Lyons et al. unpublished data), leading to the central hypothesis addressed in this thesis below.

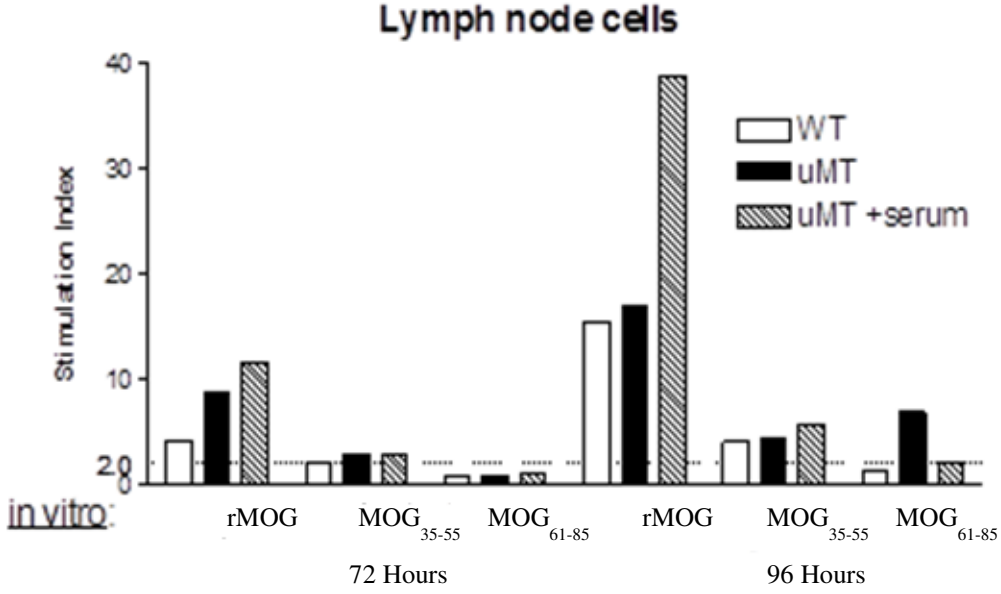

Figure 5. **Transfer of rMOG primed serum abrogates the immune response towards the 61-85 peptide.** WT and B cell$^{-/-}$ animals were immunized with rMOG. At the time of immunizations, half of the B cell$^{-/-}$ animals also received rMOG primed serum from WT animals. 10-14 dpi, lymph node cells were harvested from all three groups of animals and cultured *in-vitro* with rMOG, MOG_{35-55} and MOG_{61-85}. **At 72 hrs, the immune response towards MOG_{61-85} in B cell$^{-/-}$ animals which received serum was completely**

abrogated, thus indicating that a serum derived factor prevented the immune response towards MOG_{61-85}.

uMT: B $cell^{-/-}$ mice

Hypothesis

Previous studies identified B cell dependent (B6 mice immunized with the rMOG protein) and B cell independent (B6 mice immunized with MOG_{35-55}) mouse models of EAE. Subsequent studies showed distinct T cell and B cell responses in WT and B $cell^{-/-}$ mice immunized with these antigens, and identified a protective cryptic T cell epitope (MOG_{61-85}) in WT mice immunized with rMOG. Furthermore, peptide mapping analysis identified an adjacent B cell epitope within amino acids MOG_{46-85} implicated in processing and presentation of the MOG_{61-85} epitope.

This work further investigated the mechanism of protection by the cryptic MOG_{61-85} epitope in rMOG induced EAE and the B cell response in regulating the processing and presentation of this epitope. Thus I hypothesized that immunization with the longer MOG_{35-85} peptide will prevent EAE induction in these mice. It was further hypothesized that a regulatory T cell population to the MOG_{61-85} epitope is responsible for the lack of EAE in rMOG-immunized B $cell^{-/-}$ B6 mice. This hypothesis was addressed by the following Specific Aims.

Specific Aims

1) Identify the role of B cells in the generation of the protective immune response against MOG_{61-85} epitope. Our *working hypothesis* was that a B cell response to MOG, amino acids 46-85, regulates the processing and presentation of the MOG_{61-85} epitope. This was investigated by immunizing B cell$^{-/-}$ B6 and WT B6 mice with a longer peptide encompassing the encephalitogenic MOG_{35-55} epitope, the protective MOG_{61-85} epitope, and the intervening amino acids. Mice were followed for disease induction and progression.
2) Investigate the immune mediators responsible for the protection observed with the MOG_{61-85} peptide. WT and IL10$^{-/-}$ mice were immunized with MOG_{35-85} to determine the role of IL10 in disease induction and progression. B cell$^{-/-}$ B6 and WT B6 mice were immunized with MOG_{35-55} and the MOG_{35-85} peptide and subjected to cytokine analysis by ELISA and gene expression analysis by qPCR utilizing the spleen cells isolated from immunized mice.
3) Identify the immune cell population responding to the protective MOG_{61-85} peptide. The *working hypothesis* was that the regulatory cell population responding should be T cells, since B cell$^{-/-}$ animals lack a functional B cell compartment in their immune system. This was demonstrated by flow cytometry of spleen cells isolated from either B cell$^{-/-}$ B6, WT B6 or IL10$^{-/-}$ B6 mice immunized with the protective MOG_{35-85}.

CHAPTER II: MATERIALS AND METHODS

Mice

Specific pathogen free female WT B6, B cell$^{-/-}$ and mice genetically deficient in Interleukin-10 (IL10$^{-/-}$) on a B6 background at 6-8 weeks of age were used for the experiments. All the mice required for the experiments were bred in-house, from breeding pairs purchased from Jackson Laboratories (Bar Harbor, ME). All animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility on the University of Wisconsin Milwaukee (UWM) campus according to university and National Institutes of Health (NIH) guidelines. All the protocols were certified by the Institutional Animal Care and Use Committee.

Antigens

The MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK), MOG_{61-85} (QAPEYRGRTELLKDAIGEGKVTLRI) and MOG_{35-85} (MEVGWYRSPFSRVVHLYRNGKDQDAEQAPEYRGRTELLKDAIGEGKVTLRI) peptides were obtained from GenScript, Piscataway, NJ. These peptides were commercially synthesized and then purified by High Performance Liquid Chromatography (HPLC).

Immunizations

Mice were anesthetized using a ketamine (100 mg/ml), xylazine (300 mg/ml) mixture (1 ml ketamine + 0.15 ml xylazine + 4.6 ml water) injected intramuscularly and immunized with 50 µg or 100 µg MOG_{35-55} or 100 µg, 125 µg or 250 µg of MOG_{35-85},

emulsified in incomplete freund's adjuvant (IFA, MP Biochemicals, Solon, OH) containing 300 µg *Mycobacterium tuberculosis* strain H37RA (TB, Difco Laboratories, Detroit, MI). Emulsions were prepared using an Omni Mixer Homogenizer mechanical mixer and injected subcutaneously, at 2 sites (base of the tail and between the front forelimbs).

300 ng of pertussis toxin (PT) (List Biological labs, Inc., Campbel, CA) in Phosphate Buffered saline (PBS-200mg/L, potassium phosphate monobasic-0.2g/lit, potassium chloride-0.2g/lit, sodium chloride-8g/lit, sodium phosphate dibasic-1.15g/lit) was also injected intraperitoneally at the time of immunization and 72 hours post immunization. PT injections are necessary for keeping the day of disease onset consistent in this animal model.

Experimental Autoimmune Encepahlomyelitis (EAE) grading and Disease stages

Immunized animals were assessed daily beginning 7 dpi for clinical signs of EAE. Disease severity was scored using a scale from 0 to 5 as follows, 0: no disease; 1: loss of tail tone (failure of the tail to helicopter); 2: hind limb weakness (failure to right itself when placed on its back), wobbly gate; 3: single hind limb paralysis; 4: both hind limbs paralysis; 5: dead or moribund. Mice with a score of >4.0 were euthanized as per protocol requirement.

EAE disease stages were defined as follows: Preclinical: no clinical signs; Onset: the first day of clinical score; Peak: grade 4 or the maximal score reached and stable for 3 days; Recovery: a decrease ≥ 1 grade from the peak score lasting for 3 days; Chronic: recovery from the peak score ≥1 grade with a stable score lasting longer than 3 days.

Cell Culture

The immunized mice were sacrificed 29 dpi by anesthetizing them with the ketamine-xylazine mixture and euthanizing them by cervical dislocation. The mice were pinned down on a tray and a small incision made between the back legs gently separating the fur/skin from the peritoneal cavity and extended along the length of the abdomen and up each leg. The animals were doused with reagent grade ethanol and using sterile forceps and scissors the spleens were extracted. The extracted spleens were suspended in Hanks Balanced Salt Solution (HBSS) until use. The tissue was then homogenized in sterile glass homogenizers and centrifuged to separate the cells from the debris and fat and counted with the help of trypan blue staining (90 µl of 0.4% tyrpan blue solution + 10 µl of the cells) on a haemocytometer. Cell concentration was determined using the following formula:

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

Cells were cultured in complete RPMI 1640 [penicillin (100 U/mL)/streptomycin (100 µg/mL), L-glutamate (2 mM), sodium pyruvate (0.1 mM), 2-mecarptoethanol (50 mM)] supplemented with 10% Fetal bovine serum (FBS) and the appropriate antigen (MOG_{35-55} or MOG_{61-85}) at a concentration of 10 µg/ml at a cell density of 2 X 10^6 cells per ml. A third control group was also set up with no antigen, with the volume being made up using RPMI. The cells were cultured for up to 4 days at 37 °C, at 10% CO_2.

RNA Extraction

The cells harvested from the spleens were cultured as described above. Cells were recovered by centrifugation at respective time intervals (12 hrs, 24 hrs, 48 hrs) and total

mRNA extracted using TRIZOL® reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The cells were homogenized in 1 ml of TRIZOL reagent per $5 \times 10^6 - 1 \times 10^7$ cells and incubated at room temperature for 5 minutes to allow dissolution of nucleoprotein complexes. The cells were then frozen at -80 °C until use. After centrifuging the homogenized tissue samples, 200 µl of chloroform per 1 ml of TRIZOL reagent was added to the supernatant. The tubes were then shaken vigorously for 15 seconds, incubated at room temperature for 2-3 minutes then centrifuged at 12,000g at 4 °C for 15 minutes. RNA from the upper aqueous layer was precipitated using 0.5 ml of isopropanol per 1 ml of TRIZOL. The precipitated RNA was recovered by centrifugation at 12,000 g at 4 °C for 10 minutes and then washed once with 1 ml of 70% ethanol per 1 ml of TRIZOL. The RNA pellet was air-dried and dissolved in molecular grade/RNase free water and stored at -80 °C until use. The RNA yield, concentration, and purity were determined by measuring the absorbance's, at 260 nm and 280 nm. For determining the absorbance the samples were diluted (1:50 or 1:25) in 1x TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). A 260/280 ratio of 1.8-2.1 was considered acceptable for subsequent experiments.

Reverse Transcription (RT)

The RT reactions were carried out using the Mastercycler® Gradient PCR machine (Eppendorf Scientific, Hauppauge, NY). RNA samples were reverse transcribed to cDNA using the RT^2 HT First Strand Kit (SA Biosciences, Valencia, CA) according to the manufacture's instructions. This kit was specifically used because of its compatibility with the RT^2 SYBR Green qPCR master mixes, which were utilized thereafter.

In the first step, 8 µl of RNA was added to 6 µl of genomic DNA (gDNA) elimination buffer and incubated at room temperature for 10 minutes. 6 µl of the RT master mix was added to the above reaction mixture and reverse transcription was carried out at 42 °C for 15 minutes. The reaction was stopped by heating to 95 °C for 5 minutes. Finally, 91 µl of RNase-free or molecular grade water was added to the mixture, which was then stored at -20 °C until use.

Quantitative Real Time PCR (qRTPCR)

qPCR quantifies the initial amount of template DNA present in the sample during the amplification reaction whereas Real Time PCR measures the amount of DNA present after each amplification cycle. This measurement is based on the increase in fluorescence output as the PCR product increases. The point at which the fluorescence crosses a pre-determined threshold, which is based on the background fluorescence, is called the Threshold cycle (Ct). The Ct is further used to determine the amount of the initial template cDNA present in the sample.

The cDNA obtained from the previous step was subjected to qRTPCR analysis to determine gene expression. PCR was run using the RT^2 SYBR Green qPCR master mix from SA Biosciences according to the manufacture's instructions. The primers required for the reaction were also from SA Biosciences and the sequences from the company are propriety and hence cannot be disclosed. The reaction mixture for a single reaction had the following components:

Table 1. PCR components.

Component	Volume in µl
RT^2 SYB® Green qPCR master mix	12.5
Primer set	1
Molecular grade water	10.5

For the reaction, 24 µl of the above soup was added along with 1 µl of the sample. The reaction mixture was then held at 95 °C for 10 minutes and amplified at 95 °C for 15 seconds followed by 60 °C for 1 minute, for a total of 40 cycles. This was followed by a melt curve analysis (95 °C for 15 seconds, 60 °C for one minute and 95 °C for 15 seconds). The melt curve analysis helps determine the specificity of the amplified gene of interest. All the reactions were run in triplicate for statistical analysis with β-Actin as the house-keeping gene. All PCR analysis was carried out using the StepOnePlus™ Real-Time PCR System from AB Biosystems, Carlsbad, CA. Analysis was carried out for the expression of IL17 and INFγ genes AT 12, 24 and 47hrs, using the Pfaffl method (Pfaffl, 2001).

The Pfaffl method utilizes the primer efficiencies for mRNA quantification. For determining the primer efficiency, a standard curve for each primer set was run with the samples. The Ct values obtained were plotted against the concentration for each dilution. The slopes for these standard curves were determined using the equation $E = 10^{[-1/slope]}$. The primer efficiencies were found to be 1.990, 1.936 and 1.988 for INFγ, IL17 and β-actin respectively.

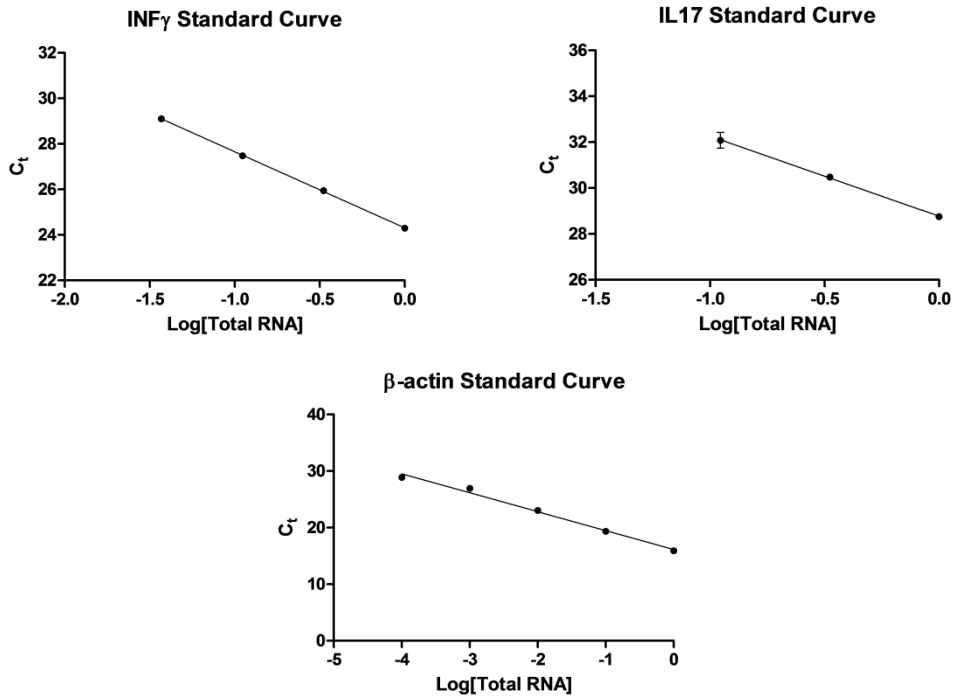

Figure 6. **PCR standard curves obtained for each of the primer sets utilized.** A serial dilution was carried out using the cDNA samples for the amplification. The slope of each line was used to determine primer efficiency. Each standard curve reaction was run in duplicate. Ct: Threshold cycle.

Based on the above primer efficiencies the amount of mRNA was calculated using the following formula.

$$\text{Ratio} = \frac{(E_{target})^{\Delta Ct_{target}\,(\text{control-sample})}}{(E_{ref})^{\Delta Ct_{ref}\,(\text{control-sample})}}$$

Where, E_{target}: PCR efficiency of target gene (IL17 or INFγ)

E_{ref}: PCR efficiency of reference gene (β-actin)

ΔCt_{target}: Difference in the Ct of the control and the sample of the target gene transcript level

ΔCt_{ref}: Difference in the Ct of the control and the sample of the reference gene transcript level (Pfaffl, 2001).

Statistical analysis for the amount of mRNA was carried out using a 2-way ANOVA in GraphPad Prism software.

Enzyme Linked Imunosorbent Assay (ELISA)

The animals were immunized, the spleen cells extracted and cultured as described above. Cell culture supernatants were taken at regular intervals, (48 hr, 72 hr and 96 hr) and subjected to cytokine analysis by ELISA. The cytokines determined included IL10, and INFγ. ELISA's were run using the Ready-Set-Go Kits from eBiosciences (San Diego, CA) according to the manufacture's instructions. The cytokines are detected using a colorimetric reaction based on activity of avidin-horseradish peroxidase (HRP) bound to the biotinylated detection antibody, on a specific substrate.

Briefly, 96 well plates (Corning Costar 9018) were coated over-night at 4 °C with 1x dilution of the capture antibody in 1x concentration of the coating buffer (PBS Coating Buffer). Plates were then washed 5 times with 300 µl of the wash buffer (PBS with 0.05% Tween 20). The plates were blocked at room temperature for 1 hr using 200 µl of the assay diluent. After washing the plates, 100 µl of each sample or the standard was added in duplicate to the wells and the plate incubated at room temperature for 2 hrs. Following another wash, 100 µl of detection antibody diluted in 1x assay diluent was added and the plate incubated at room temperature for 1 hr. After another wash, 100 µl of avidin-HRP diluted in 1x Assay diluent was added and the plate incubated at room temperature for 30 minutes. Following another wash, 100 µl of the tetramethylbenzidine

(TMB) substrate solution was added to the wells and the plate incubated at room temperature for 30 minutes, following which 50 µl of the stop solution (2.5N H_2SO_4) was added and the optical density measured at 450 nm.

For the standards recombinant cytokines at the following concentrations were used.
For INFγ, Standard Range 2000-15.625 pg/ml in a serial two-fold dilution.
For IL10, Standard Range 4000-31.25 pg/ml in a serial two-fold dilution.

The final optical density was measured using a Synergy™ HT Multi-Mode Microplate Reader from Biotek, Winooski, VT. An absorbance of >0.1 was considered positive. Data was analyzed using a 2-way ANOVA in the GraphPad Prism 5.0 software.

Flow Cytometry

Cells were isolated from the spleen and cultured *in-vitro* as previously mentioned. The cells were harvested at 96 hrs and counted using trypan blue as previously described to determine cell count and observe cell viability. These cells were then either subjected to centrifugation using the LSM® Lymphocyte Separation Medium (9.4 g sodium diatrizoate, 6.2 g ficoll per 100 ml of the LSM) to separate living mononuclear cells or directly used for flow cytometry, depending on cell viability. When cells were subjected to density gradient centrifugation, cell suspensions were overlaid on 5 ml of LSM and centrifuged at 500xg for 30 minutes at 4 °C. Viable cells were then collected from the interface and diluted at least 1:2 with HBSS. Cells were then washed twice with HBSS and subjected to staining as follows. When density gradient centrifugation was not employed, cells were recovered from culture using a 5 ml serological pipet, centrifuged at 500xg for 10 minutes to pellet cells, and then washed 2x with 10 ml HBSS. Cells were

resuspended in (1-2 ml) for counting and were aliquotted at 1-2x10^6/tube into 1.7 ml microcentrifuge tubes for antibody labeling.

Cells were first incubated over ice for 30 minutes with F_C block (eBioscience, San Diego, CA) or normal mouse serum (Jackson ImmunoResearch Labs., West Grove, PA), so as to reduce non-specific binding through F_C receptors. The cells were washed and then resuspended in 50 µl FACS wash buffer (PBS - 0.5%, FBS - 0.1%, sodium azide), and incubated with 1 µl of the respective antibodies, for cell surface markers, on ice for 30 minutes. The cells were then washed thrice with the FACS wash buffer and finally suspended in 500 µl of the FACS wash buffer for analysis on the flow cytometer.

Cells were stained with four of the following cell markers conjugated with appropriate fluorescent molecule.

1) FITC conjugated CD4 or CD8 or CD11b (FL1 channel in case of WT or B $cell^{-/-}$ mice), if the IL10-GFP mice were used, the production of IL10 was analyzed through FL1.

2) PE conjugated CD4 or CD8 or CD25 or CD28 (FL2 channel)

3) PerCP-Cy5.5 conjugated FoxP3 (FL3 channel) (Intracellular stain)

4) APC conjugated CD25 or CD3 or Ly6G (FL4 channel)

These antibodies were purchased from either Biolegend (San Diego, CA) or eBioscience (San Diego, CA). In the case of staining cells for the intracellular molecule, FoxP3, the cells were first stained for the surface molecules and then subjected to intracellular staining as follows. After the last wash cycle the supernatant was discarded and the tube was pulse vortexed to dislodge the pellet. 1 ml of 1x fixation/permeabilization solution (eBioscience, San Diego, CA; 1 part

fixation/permeabilization concentrate and 3 parts fixation/permeabilization diluent) was added to each sample and pulse vortexed. The samples were incubated at 4 °C for 30 minutes in the dark. Following the incubation, 1x permeabilization buffer (1 ml of the permeabilization buffer and 9 ml of distilled water was added to the each sample and then centrifuged at 400xg at room temperature for 5 minutes. The pellet was resuspended in 100 µl of 1x permeabilization buffer and 1 µl of the PerCP-Cy5.5 conjugated FoxP3 antibody was added and the samples incubated in the dark for 30 minutes on ice. Following the incubation the samples were washed thrice with 1 ml of 1x permeabilization buffer and the resuspended in 500 µl of the FACS wash buffer for further analysis. Controls were run using species, isotype and fluorochrome matched antibodies along with single labeled controls. Single-labeled controls were included to ensure proper compensation of the flow cytometer. The analysis was performed on a dual laser BD FACS Caliubur (BD Biosciences, San Jose, CA) using Cell Quest Pro (BD Biosciences, San Jose, CA) and the data analyzed using the FlowJo software (Tree Star, Ashland, OR).

Statistical Analysis

All statistical analysis was carried out using the GraphPad Prism 5.0 (La Jolla, CA) or the FlowJo software (Tree Star, Ashland, OR) using the tests indicated.

CHAPTER III: SPECIFIC AIM I

Identify the role of B cells in the generation of the protective immune response against the MOG_{61-85} epitope.

Background

Previous experiments have identified antibody responses in WT animals immunized with rMOG against MOGaa21-40 and to an island of epitopes spanning MOGaa46-85. In addition, a T cell epitope was identified in B cell$^{-/-}$ mice, comprised of MOGaa61-85. Immunizations of WT mice with this MOG_{61-85} identified it as cryptic epitope in WT animals.

Co-immunizations of WT B6 and B cell$^{-/-}$ mice with the encephalitogenic MOG_{35-55} and cryptic MOG_{61-85} peptides demonstrated an amelioration of clinical EAE severity in both strains (Lyons et al. unpublished data). Based on these results I hypothesized that antibodies against MOG_{46-86} were involved in the selection of epitopes in WT mice through the regulation of antigen processing and presentation when immunized with rMOG. These antibodies facilitated the processing of the encephalitogenic MOG_{35-55} and prevented the processing of the protective MOG_{61-85} epitope in the WT animals, leading to clinical presentation of EAE induction when immunized with rMOG. Conversely, the absence of these antibodies in B cell$^{-/-}$ animals led to the processing and presentation of the both the encephalitogenic MOG_{35-55} epitope and the protective MOG_{61-85} epitope, thereby rendering these mice resistant to EAE induction when immunized with rMOG.

Thus the *working hypothesis* is that the WT animals immunized with the longer MOG_{35-85} peptide, which encompasses the encephalitogenic MOG_{35-55} epitope, the

protective MOG_{61-85}, and the identified B cell epitope, should show EAE induction. Conversely the B $cell^{-/-}$ animals immunized with MOG_{35-85} peptide, should not present with EAE induction.

Results

WT and B $cell^{-/-}$ mice are equally susceptible to EAE induced by MOG_{35-85}.

WT and B $cell^{-/-}$ animals were immunized with either MOG_{35-55} or MOG_{35-85} and observed for EAE induction and progression for a period of 29 days. The Aim was conducted in three different experiments and the total number of mice in each group was as follows (Table 2).

Table 2. Groups of mice immunized with either MOG_{35-55} or MOG_{35-85}.

Group	Mice	Antigen used	Number of animals in group
1	WT	MOG_{35-55}	14
2	WT	MOG_{35-85}	13
3	B $cell^{-/-}$	MOG_{35-55}	13
4	B $cell^{-/-}$	MOG_{35-85}	16

In the first experiment, mice received 100 μg of either peptide emulsified in Freud's incomplete adjuvant containing *M. tuberculosis* H37RA. In the second experiment, in order to keep the molar concentration of the antigenic peptide constant, animals received either 125 μg or 250 μg of MOG_{35-85} and 50 μg or 100 μg of MOG_{35-55} respectively. In the third set of experiments, animals received 125 μg of MOG_{35-85} and 50 μg of MOG_{35-55}. The complied results of all three experiments are as follows.

WT and B cell$^{-/-}$ animals immunized with MOG_{35-55} developed EAE which is consistent with previous findings (Lyons et al., 1999). Conversely WT and B cell$^{-/-}$ animals immunized with MOG_{35-85} showed a less severe EAE when compared to the animals immunized with MOG_{35-55}. Analysis using a 2-way ANOVA resulted in a p value of <0.0001 (Figure 7).

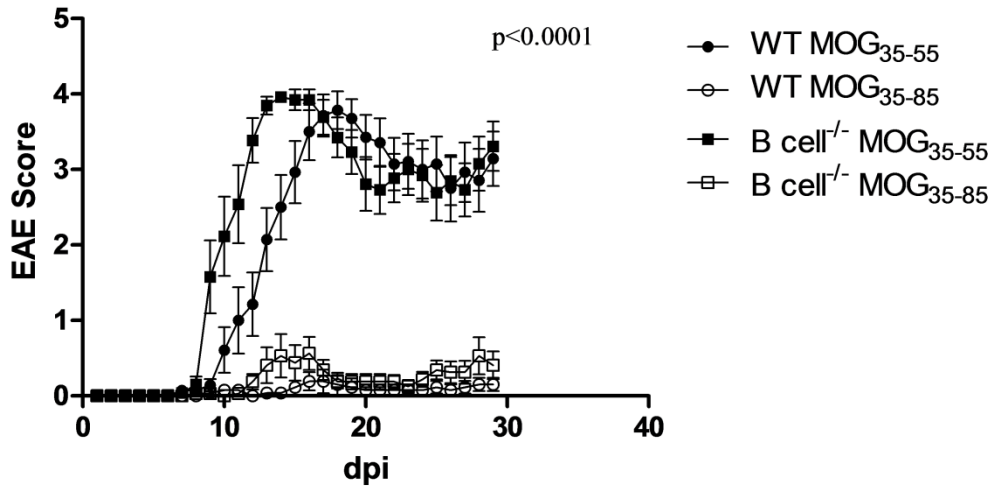

Figure 7. **Immunizations with MOG_{35-85} peptide results in a decreased disease severity in WT and B cell$^{-/-}$ animals as compared to the animals immunized with MOG_{35-55}.** Animals were immunized with the appropriate peptide and observed for a period of 29 days post immunization for EAE induction and progression. These graph indicated the complied results of all three experiments. **Reduced disease severity in WT and B cell$^{-/-}$ animals demonstrated the protective nature of the longer peptide.** Error bars represent the standard range of EAE scores in that group. Statistical analysis, using a 2-way ANOVA in the GraphPad Prism software resulted in a p value of <0.0001.

Among WT animals, significant differences were seen in animals immunized with MOG_{35-55} and MOG_{35-85}. The animals immunized with MOG_{35-85} demonstrated lower

EAE scores and a low incidence when compared to the animals immunized with MOG_{35-55}. A p value of <0.0001 was achieved for both the parameters (Figure 8, Table 3). Statistical analysis on the day of EAE onset and the median maximum score was calculated for only those mice that developed EAE (Table 3 & 4). For this reason statistically significant results could not be achieved, for both parameters, due to a single mouse that developed EAE in the WT group immunized with MOG_{35-85}. However, differences between the WT animals immunized with either peptide were evident as all the mice in the group immunized with MOG_{35-55} developed EAE, whereas only one mouse in the group immunized with MOG_{35-85} developed EAE (Table 3 & 4).

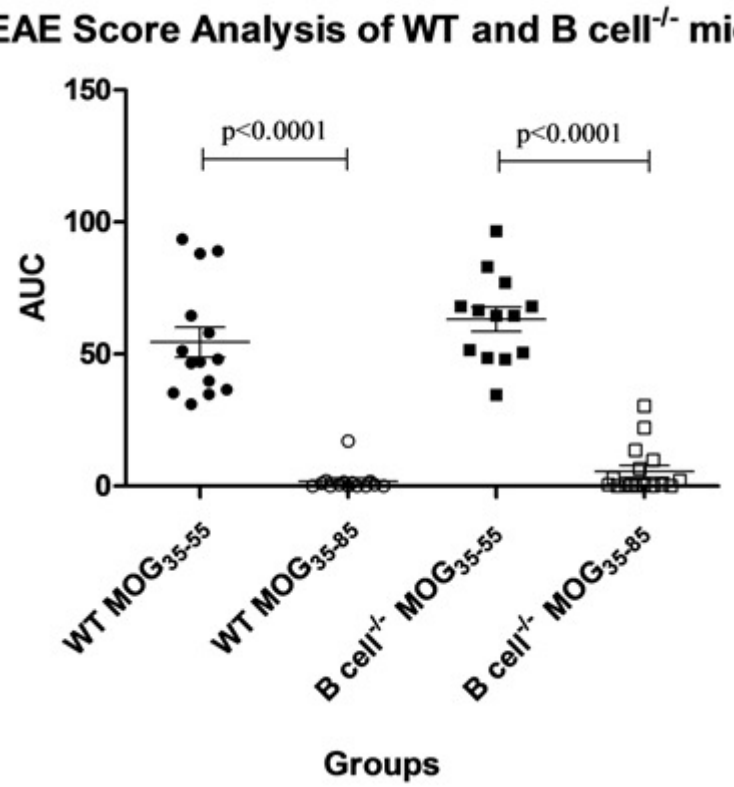

Figure 8. **Analysis of EAE score demonstrated significant differences between animals immunized with MOG_{35-55} or MOG_{35-85}.** Above figure demonstrates the Area under the curve (AUC) analysis for each of the groups. This analysis takes into consideration the area present under each line, which are shown in Figure 7. This analysis was used to determine individual differences in EAE score for each mouse in each group. WT or B $cell^{-/-}$ animals receiving MOG_{35-55} had higher scores when compared to the animals receiving MOG_{35-85} supporting the clinical EAE data. Statistically significant differences were found between animals that received MOG_{35-55} or MOG_{35-85} in both the WT and B $cell^{-/-}$ animals. Error bars indicate the average and the standard deviation in the AUC score in each group. Statistical analysis was carried out using an Unpaired T test (Mann Whitney Test) in the GraphPad Prism software.

Table 3. **Incidence rates of EAE induction and the median maximum score.** Animals were considered positive for EAE induction only if they demonstrated a score of at least 1 for three consecutive days. Median maximum score takes into consideration only those mice which got sick and represents the highest EAE score for each group in terms of its frequency. **Significant differences were found in WT and B cell$^{-/-}$ animals immunized with either MOG_{35-55} or MOG_{35-85}. A 100% incidence was noted in the group of animals immunized with MOG_{35-55}, however a low incidence was seen in the group of animals immunized with MOG_{35-85}. In addition the group of animals immunized with MOG_{35-55} had higher median maximum scores as compared to the group immunized with MOG_{35-85}.** These results were consistent in, both WT and B cell$^{-/-}$ mice.

Group	Antigen	Incidence	p values[A]	Median Max. Score	p values[B]
WT	MOG_{35-55}	14/14	**<0.0001**	4±0.4	*
WT	MOG_{35-85}	1/13		2±0*	
B cell$^{-/-}$	MOG_{35-55}	13/13	**0.0050**	4±0.2	**0.0003**
B cell$^{-/-}$	MOG_{35-85}	7/16		1.7±1.1	

A: Data analyzed by Chi-Square analysis

B: Data analyzed by Mann Whitney T Test

* Only one mouse developed EAE hence the median maximum score and its p value could not be calculated.

Table 4. **Analysis for the day of EAE onset in only those mice that developed clinical EAE.** Values represent the average and the standard deviation for the day of onset. **Differences were seen in WT animals immunized with either MOG_{35-55} or MOG_{35-85}. A significant delay in EAE onset was seen in the group of animals immunized with MOG_{35-85}.** However, because of the presence of a single mouse that developed EAE in the WT mice immunized with MOG_{35-85}, the average day of onset and the p value could not be calculated. **Similarly, in the B cell$^{-/-}$ animals, a statistically significant delay was seen in the day of EAE onset in the group of animals immunized with MOG_{35-85} as compared to the group immunized with MOG_{35-55}.** Statistical analysis was conducted using the Unpaired T test (Mann Whitney Test).

Group	Antigen	Average day of EAE onset	p values
WT	MOG_{35-55}	11.3±3.1	*
WT	MOG_{35-85}	15*	
B cell$^{-/-}$	MOG_{35-55}	9.6±1.5	0.0007
B cell$^{-/-}$	MOG_{35-85}	15.1±4.2	

* Only one mouse developed EAE hence average day of EAE onset and its p value could not be calculated.

Similarly for the B cell$^{-/-}$ animals, immunized with either peptide, significant differences were observed. The group immunized with MOG_{35-85} demonstrated lower EAE score, lower incidence and a low median maximum score, with p values of <0.0001, 0.0050 and 0.0003 respectively (Figure 8, Table 3). In addition, a significant delay in EAE onset was seen resulting in a p value of 0.0007 (Table 4).

Discussion

Previous experiments established two distinct models of EAE. Immunizations with rMOG which required B cells/Ab for disease expression and immunizations with the encephalitogenic MOG_{35-55} peptide which did not (Lyons et al., 1999). Further experiments identified a second cryptic epitope in the WT animals comprised of MOGaa61-85. Co-immunizations with both the MOG_{35-55} and MOG_{61-85} led to an amelioration of EAE in WT and B cell$^{-/-}$ animals (Lyons et al. unpublished data). This effect was more pronounced in the B cell$^{-/-}$ animals when both the peptides were emulsified together as compared to those mice which received two separate emulsions. It was also shown that antibodies were produced against MOGaa46-85 in the WT animals. It was further demonstrated that the immune response towards the MOG_{61-85} region was abrogated by the passive transfer of rMOG primed serum from WT animals into B cell$^{-/-}$ animals (Lyons et al. unpublished data). This led to the hypothesis, addressed in this thesis, that the immunizations with the longer MOG_{35-85} peptide will prevent EAE induction in B cell$^{-/-}$ animals. Aim 1 investigated this hypothesis.

Immunizations with the longer MOG_{35-85} peptide, led to a decrease in EAE severity in WT and B cell$^{-/-}$ mice compared to the mice immunized with MOG_{35-55} (Figure 7 & 8). The disease severity was found to be similar in WT and B cell$^{-/-}$ mice immunized with MOG_{35-85} indicating that the B cell epitope present between MOG_{35-55} and MOG_{35-85} was not involved in the selection of the protective MOG_{61-85} epitope. Had this been the case, WT animals should not have processed the protective MOG_{61-85} peptide and should have developed EAE with MOG_{35-85} immunizations with a severity similar to that noted with the shorter MOG_{35-55} peptide. In agreement with previous data (Lyons et al.

unpublished data), B cells did not seem to play any role in protection from EAE induction since B cell$^{-/-}$ animals were protected. As a result it is highly unlikely that antibodies will have a role to play in this protection observed with MOG_{35-85} immunizations. This needs further investigation.

These results lead to several new questions. Antibody responses were also seen against amino acids immediately preceding the encephalitogenic MOG_{35-55} epitope (Liu et al., 2012). It is possible that these antibodies are the ones which are involved in processing and presentation of the encephalitogenic epitope and thus warrant further investigation. Another possibility involves the 3-dimensional conformation of the MOG_{35-85} peptide. There is a possibility that the chemically synthesized MOG_{35-85} peptide, could not achieve the conformation present in naturally occurring which could have confounded these results.

Another possible explanation for the less severe EAE observed in these experiments, could have been due to the lack of processing the antigen. In order to address these questions, cells from animals immunized with MOG_{35-85} were cultured *in-vitro* with either MOG_{35-55} or MOG_{61-85} and analyzed for cytokine mRNA expression and cytokine levels in cell culture supernatants. These data will be discussed in Chapter 4.

CHAPTER IV: SPECIFIC AIM 2

Investigate the immune mediators responsible for the protection observed with the MOG_{61-85} peptide, with the use of MOG_{35-85}.

Background

The current understanding of MS/EAE is as a $CD4^+$ Th1/Th17 mediated disease: the production of pro-inflammatory cytokines such as IFNγ, TNFα, and IL17 within the CNS leads to the onset of clinical signs and the progression of disease. Conversely, production of anti-inflammatory cytokines such as IL4 and IL10 are protective against disease and lead to recovery from active disease. Preliminary data suggested that antigen-specific production of IL10 in response to MOG_{61-85} immunizations may play a role in the protection of B $cell^{-/-}$ mice to rMOG induced EAE. Thus, these experiments further sought to characterize the cytokine response to the MOG_{61-85} peptide to gain an understanding of the mechanism of protection by this cryptic epitope.

Results

IL10 may play a role in EAE onset

Preliminary data indicated that IL10 may play a role in the resistance to EAE induction using the MOG_{35-85} peptide (Lyons et al. unpublished data). To investigate this possibility $IL10^{-/-}$ and WT animals were immunized with MOG_{35-55} or MOG_{35-85} and observed for EAE induction and progression for a period of 29 days. The total number of animals immunized in each group is as follows (Table 5).

Table 5. Groups of mice immunized with either MOG_{35-55} or MOG_{35-85}.

Group	Mice	Antigen used	Number of animals in group
1	WT	MOG_{35-55}	14
2	WT	MOG_{35-85}	13
3	$IL10^{-/-}$	MOG_{35-55}	11
4	$IL10^{-/-}$	MOG_{35-85}	10

This Aim was conducted as previously described in Aim1 and the complied results of all three experiments are as follows. WT and $IL10^{-/-}$ animals immunized with MOG_{35-55} showed EAE induction as is consistent with previous findings (Lyons et al., 1999). Conversely the WT and $IL10^{-/-}$ animals immunized with MOG_{35-85} showed a less severe EAE induction indicating that IL10 may not play a role in protection with the MOG_{61-85} peptide. Analysis using a 2-way ANOVA resulted in a p value of <0.0001 (Figures 9). The AUC analysis supported these results, wherein the group of animals immunized with MOG_{35-85} had decreased EAE severity as compared to the group immunized with MOG_{35-55} in, WT as well as $IL10^{-/-}$ mice (Figure 10). Statistically significant differences were found between WT and $IL10^{-/-}$ animals immunized with MOG_{35-85}, indicating that IL10 may have a role in EAE severity. In order to confirm this ELISA was used to determine IL10 protein expression as described below (Figure 10).

Similar to previous experiments significant differences were found within the WT animals immunized with either peptide for EAE severity and incidence with a p value of <0.0001 for each parameter analyzed. Statistical analysis could not be conducted for

median maximum score and the day of EAE onset as previously explained (Tables 3, 4, 6 & 7).

In continuation, in the IL10$^{-/-}$ animals immunized with the longer MOG$_{35-85}$ peptide differences were also evident in the analysis of the median maximum score (Table 6). Conversely no significant differences were noted between the IL10$^{-/-}$ mice, immunized with either peptide for incidence and the day of EAE onset indicating that IL10 may be important in incidence, but not in disease severity (Table 6 & 7).

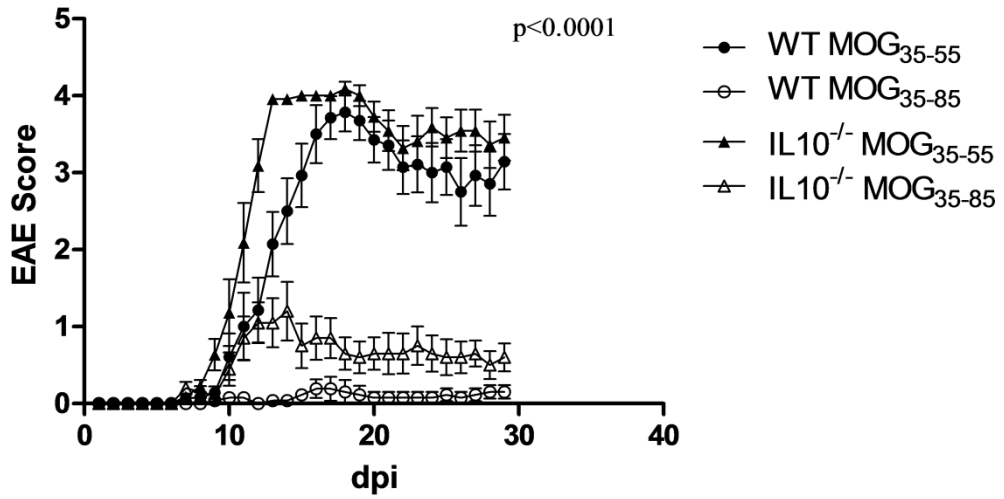

Figure 9. **EAE severity in WT and IL10$^{-/-}$ mice immunized with MOG$_{35-55}$ and MOG$_{35-85}$.** WT and IL10$^{-/-}$ mice were immunized with the appropriate peptide and observed for a period of 29 days for EAE induction and progression. The mice, WT and IL10$^{-/-}$, immunized with MOG$_{35-55}$ exhibited EAE induction and progression. Moreover WT and IL10$^{-/-}$ mice immunized with MOG$_{35-85}$ 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 IL10$^{-/-}$ mice, indicating that **IL10 may not play a role in protection from observed with MOG$_{61-85}$.** Error bars indicate the average and the

standard deviation in EAE score for all mice in that particular group. Statistical analysis, using a 2-way ANOVA in the GraphPad Prism software resulted in a p value of <0.0001.

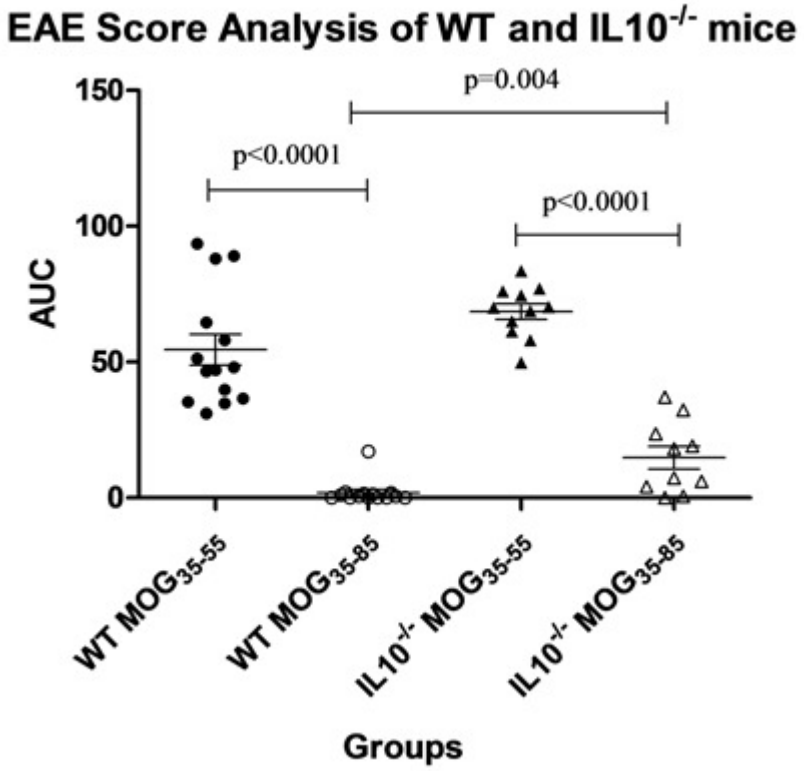

Figure 10. **AUC analysis in WT and IL10$^{-/-}$ mice.** In both groups of animals (WT and B cell$^{-/-}$), mice immunized with MOG_{35-55} had higher EAE scores as compared to the group immunized with MOG_{35-85}. However EAE score analysis of IL10$^{-/-}$ mice, immunized with MOG_{35-85}, showed that these mice had higher EAE scores when compared with WT animals immunized with the same peptide. **This resulted in a p value of 0.004 between WT and IL10$^{-/-}$ mice immunized with MOG_{35-85}.** Error bars indicate the average and standard deviation in the AUC in each group. Statistical analysis was carried out using the Mann Whitney T test to determine individual group differences, with p values as indicated.

Table 6. **Incidence of EAE induction and the media maximum score. In WT animals immunized with either peptide, s**tatistically significant differences were noted for incidence. The p value for the median maximum score could not be calculated as previously explained. However for the $IL10^{-/-}$ mice, differences were only noted between the groups immunized with MOG_{35-55} and the group immunized with MOG_{35-85} for the median maximum score, not for the incidence. **This indicated that IL10 may have a role in EAE incidence, but not severity.**

Group	Antigen	Incidence	p values[A]	Median Max. Score	p values[B]
WT	MOG_{35-55}	14/14	**<0.0001**	4±0.4	*
WT	MOG_{35-85}	1/13		2±0*	
$IL10^{-/-}$	MOG_{35-55}	11/11	0.1459	4±0.3	**0.0007**
$IL10^{-/-}$	MOG_{35-85}	7/10		2±1.0	

A: Data analyzed by Chi-Square analysis

B: Data analyzed by Mann Whitney T Test

* Only one mouse developed EAE hence the median maximum score and its p value could not be calculated.

Table 7. **Analysis for the day of EAE onset in only those mice that developed EAE.** As previously explained statistical analysis could not be conducted in WT animals immunized with either MOG_{35-55} or MOG_{35-85}. **In the $IL10^{-/-}$ mice statistical differences were not noted in the day of EAE onset between the group of mice immunized with MOG_{35-55} as compared to the group immunized with MOG_{35-85}.** This is consistent with the previous observation that differences in incidence were also not significant. Data analyzed using the Mann Whitney T test.

Group	Antigen	Day of Onset	p Values
WT	MOG_{35-55}	11.3±3.1	*
WT	MOG_{35-85}	15*	
$IL10^{-/-}$	MOG_{35-55}	9.3±2.0	0.14440
$IL10^{-/-}$	MOG_{35-85}	10.8±0.8	

* Only one mouse developed EAE hence average day of EAE onset and its p value could not be calculated.

In Figure 10, significant differences in EAE score were noted between WT and $IL10^{-/-}$ mice immunized with MOG_{35-85}. This indicated that IL10 may a partial role in the protection seen with MOG_{61-85}. To further investigate this possibility spleens were harvested from WT mice 29 days post immunization with either MOG_{35-55} or MOG_{35-85} and cultured *in-vitro*, in three separate culture conditions: MOG_{35-55}, MOG_{61-85} and without antigen. The cells were cultured in complete RPMI supplemented with FBS and the respective antigen. Cell culture supernatants were taken for cytokine detection using ELISA at 48, 72 and 96 hrs. The IL10 levels were found to be below the level of detection, i.e. 31.25 pg/ml in all samples. These results further support the clinical

observations of EAE in $IL10^{-/-}$ mice and demonstrate that IL10 does not play a role in the protection observed with MOG_{61-85}.

Cells primed to MOG_{35-85} produce decreased amounts of pro-inflammatory cytokines.

To further investigate a potential mechanism of protection by the MOG_{61-85} peptide, spleens were harvested from WT mice 29 days post immunizations with MOG_{35-55} and MOG_{35-85} and cultured *in-vitro* under three separate conditions. The cells were cultured in RPMI containing either MOG_{35-55}, MOG_{61-85} or no antigen. Aliquots were then taken at appropriate time intervals for qPCR analysis and ELISA.

qPCR analysis demonstrated that the cells primed to MOG_{35-55} *in-vivo* and then cultured with MOG_{35-55} *in-vitro* had the highest level of INFγ mRNA expression as is consistent with previous findings (Lyons et al., 1999). Conversely, there was a decrease in MOG_{35-55}-specific INFγ mRNA expression when the cells were primed to MOG_{35-85} *in-vivo*. There was also a delay in the kinetics of INFγ mRNA expression when the cultures were primed to MOG_{35-85} *in-vivo* and then cultured with MOG_{35-55} *in-vitro*. Statistically significant results were seen at all three time points (Figure 11).

Similarly the highest IL17 mRNA expression was seen in the cultures primed *in-vivo* with MOG_{35-55} and then cultured *in-vitro* with MOG_{35-55}. Conversely a decrease in the IL17 mRNA expression was observed when the cells were primed to MOG_{35-85} *in-vivo* and then cultured with MOG_{35-55} *in-vitro*. Significant differences were observed at 24 and 48 hrs, with p values of <0.01 and <0.001 respectively (Figure 12).

Very little INFγ and IL17 mRNA was observed when the cells were cultured *in-vitro* with MOG_{61-85} irrespective of the *in-vivo* priming agent (Figure 11 & 12).

qPCR Analysis

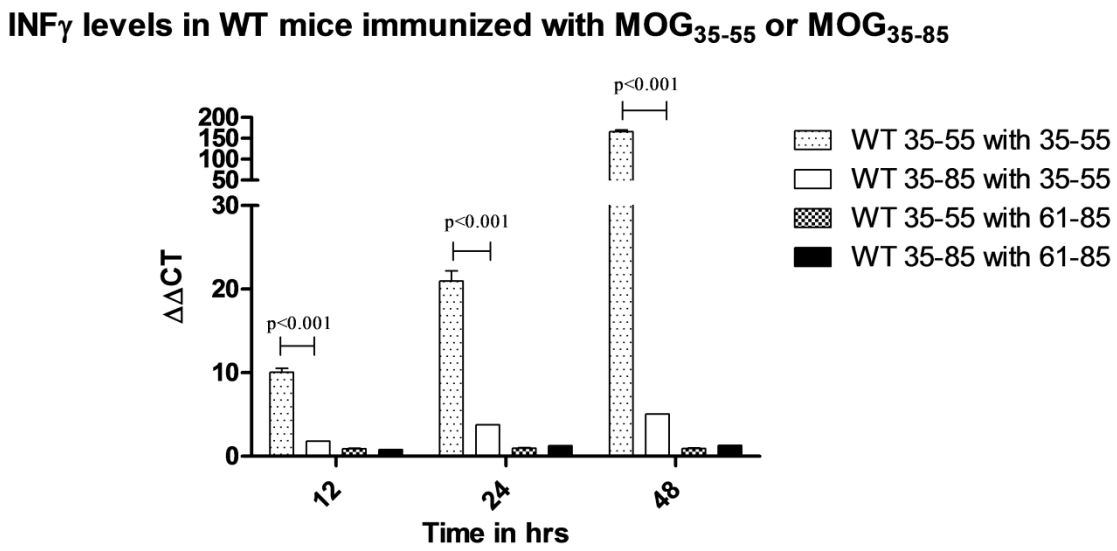

Figure 11. **The graph represents INFγ mRNA levels.** Results were obtained after normalizing it to the culture without antigen with β-actin run as a house-keeping gene using the ΔΔCt method. In general, cells exposed to MOG_{35-55} *in-vivo* and *in-vitro* had increased INFγ mRNA levels. Conversely cells primed to MOG_{35-85} *in-vivo* and then cultured with MOG_{35-55} *in-vitro* showed delayed production of INFγ mRNA. Also cells cultured with MOG_{61-85} *in-vitro* demonstrated low INFγ mRNA levels. Statistical analysis was carried out using a 2-way ANOVA followed by Bonferroni *posthoc* analysis. The error bars indicate the deviations between the replicates. **These resulted indicated that the MOG_{35-85} had anti-inflammatory potential.**

qPCR Analysis

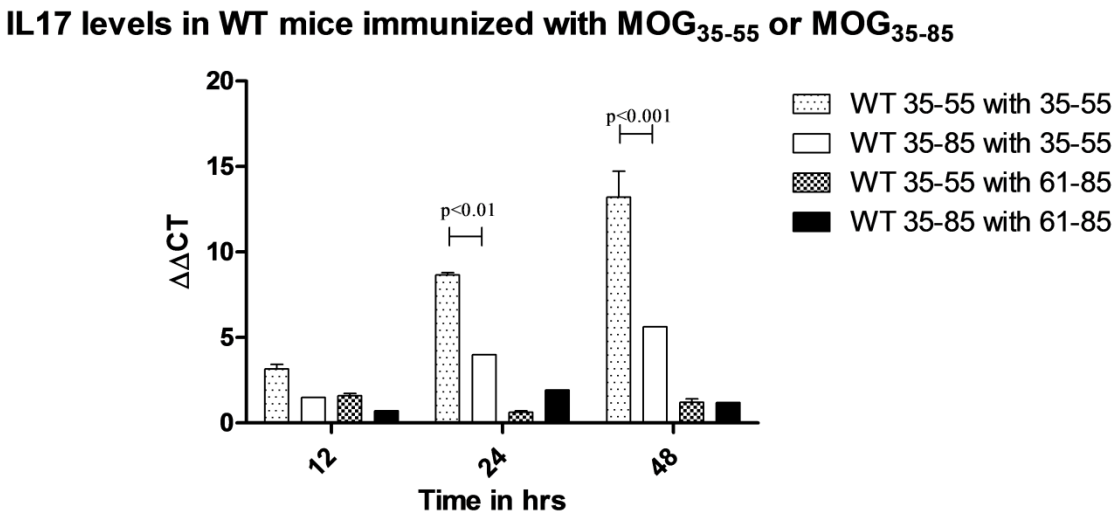

Figure 12. **Analysis for IL17 mRNA expression.** Highest mRNA expression was seen when the cells were primed to MOG_{35-55} *in-vivo* and then cultured *in-vitro* with MOG_{35-55}. A delay in IL17 mRNA expression along with low level of expression was observed when the cells were primed with MOG_{35-85} *in-vivo* and then cultured with MOG_{35-55} *in-vitro*. Very low levels of IL17 mRNA was noted when the cells were cultured with MOG_{61-85} *in-vitro*, irrespective of the *in-vivo* priming agent. Statistical analysis was carried out using a 2-way ANOVA followed by Bonferroni p*osthoc* analysis. Error bars indicate the deviations between the replicates. **These results also support an anti-inflammatory role for the longer peptide.**

The level of protein expression for INFγ was analyzed using ELISA. These results were consistent with the gene expression data obtained from qPCR. The cell primed with MOG_{35-55} *in-vivo* and the cultured *in-vitro* with MOG_{35-55} demonstrated the highest levels of INFγ. There was a decrease in the INFγ levels when the cells were primed *in-vivo* with MOG_{35-85} and then cultured with MOG_{35-55} *in-vitro*. Conversely the

cells cultured with MOG_{61-85} *in-vitro* showed no INFγ production (Figure 13). However further experiments are required to achieve statistical significance.

ELISA - INFγ

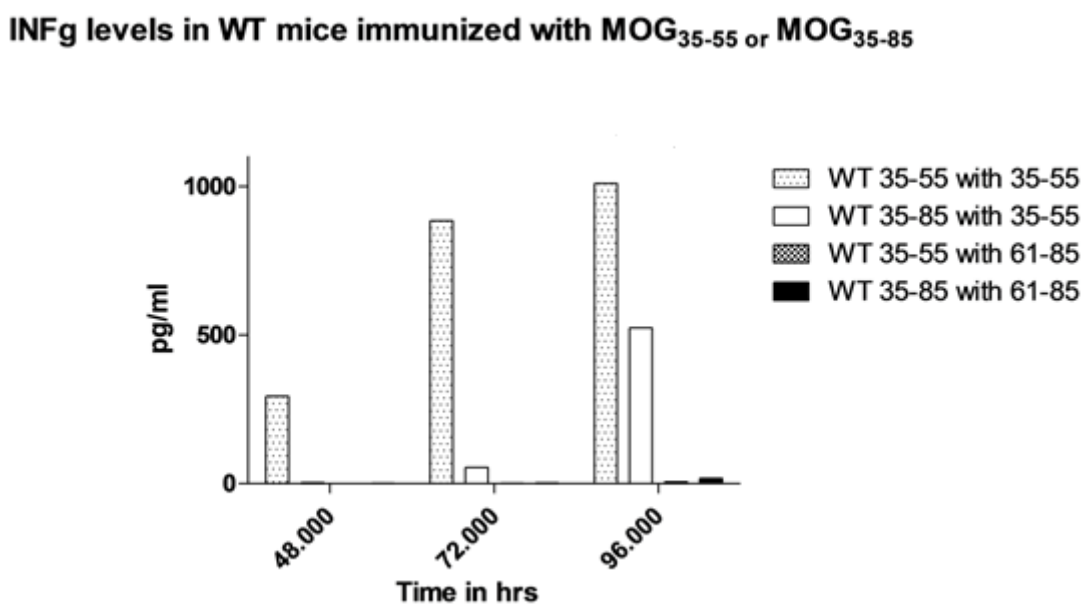

Figure 13. **Cell culture supernatants collected at 48, 72 and 96 hrs, were subjected to INFγ analysis**. Cells exposed to MOG_{35-55} *in-vivo* as well as *in-vitro* demonstrated the highest levels of INFγ. A delay as well as a decrease in INFγ expression was noted when the cells were primed with MOG_{35-85} *in-vivo* and then cultured *in-vitro* with MOG_{35-55}. Conversely cells exposed to MOG_{61-85} *in-vitro* irrespective of the *in-vivo* priming agent produced less INFγ. Statistical analysis was carried out using a 2-way ANOVA followed by Bonferroni p*osthoc* analysis. These results are from a single experiment and thus no error bars could be generated. **Thus these results demonstrated the anti-inflammatory role of MOG_{61-85}.**

INFg: Interferon gamma

Discussion

This Aim was designed to look at some of the potential effector molecules by which mice were protected against EAE induction when immunized with MOG_{35-85}. Preliminary data (Lyons et al. unpublished data) indicated that IL10 may play a role in the protection observed with MOG_{61-85}. Thus, the role of IL10 in EAE amelioration by MOG_{61-85} was investigated further.

$IL10^{-/-}$ mice were immunized with either MOG_{35-55} or MOG_{35-85} and observed for EAE induction and progression. Contrary to our hypothesis, $IL10^{-/-}$ mice presented with less severe EAE when immunized with MOG_{35-85} compared to animals immunized with MOG_{35-55}. A lack of a role for IL10 in disease amelioration was further supported by ELISA for IL10 in cell culture supernatants, as no protein was detected in any of the samples. Thus, these results indicated that there were some other mechanisms involved.

The role of pro-inflammatory molecules in mediating MS/EAE is well-established (Sospedra and Martin, 2005). Given the lack of a role for IL10 in the observed amelioration by MOG_{61-85}, we investigated whether the low level of EAE severity could be due to a down-regulation in pro-inflammatory cytokines. In the experiments, the cells from WT mice, were first primed *in-vivo* by immunization of mice with either MOG_{35-55} or MOG_{35-85} and then cultured with either MOG_{35-55} or MOG_{61-85} or no antigen *in-vitro*. In general, cells primed to MOG_{35-55} *in-vivo* and cultured with MOG_{35-55} *in-vitro* demonstrated greater INFγ and IL17 mRNA levels. This correlated with the clinical data which showed that the mice immunized with MOG_{35-55} had a worse disease course as compared to the ones immunized with MOG_{35-85}. These results support previous results

establishing that the disease is mediated in large part by pro-inflammatory cytokines (Sospedra and Martin, 2005).

A delay in the increase in levels of these pro-inflammatory cytokines was observed when the cells were primed with MOG_{35-85} *in-vivo* and then cultured with MOG_{35-55} *in-vitro*. This demonstrated that the cells were being primed to the MOG_{35-55} peptide *in-vivo* but the presence of the protected MOG_{61-85} epitope inhibited the production of these pro-inflammatory cytokines and possibly suppressed EAE. A similar trend was seen in INFγ levels in the cell culture supernatants using ELISA, supporting the results of the qPCR analysis. ELISA data also indicated that the INFγ mRNA observed with qPCR was being translated into proteins. Very little IFNγ or IL17 mRNA expression was observed when the cells were cultured *in-vitro* with MOG_{61-85}. This is not surprising, given that this epitope is protective against EAE (Lyons et al. unpublished data).

These results demonstrated two different aspects related to the hypothesis. First, a decrease in amounts of pro-inflammatory cytokines was observed in the presence of the protective MOG_{61-85} epitope. However, the mechanism leading to this observation is not clear and necessitates the analysis of other anti-inflammatory cytokines such as IL4 or TGFβ. Analysis of IL4 may provide useful information to the mechanism of protection. Secondly, it was effectively demonstrated that the animals immunized with MOG_{35-85} could process and present the antigen, specifically the encephalitogenic MOG_{35-55} epitope, since these cells responded to this epitope *in-vitro*. Thus, the lack of EAE in these animals was not due to a failure to process and present the antigen. Along with the effector molecules another possible mechanism of protection is directly through cell-to-cell contact. Trans-well assays with similar settings will help answer this question.

CHAPTER V: SPECIFIC AIM 3

Identify the immune cell population responding to the protective MOG_{61-85} peptide.

Background

Previous data demonstrated that B cell$^{-/-}$ mice are resistant to the induction of EAE when immunized with rMOG. Preliminary data suggested that this is because a protective immune response is generated to the MOG_{61-85} region in the absence of B cells. Experiments in Aim 1 demonstrated that both WT and B cell$^{-/-}$ B6 mice were resistant to EAE induced by MOG_{35-85}, encompassing the encephalitogenic MOG_{35-55} epitope, the protective MOG_{61-85} epitope, and a putative B cell epitope, MOG_{41-85}. The protective response was generated in the absence of B cells, and preliminary data suggested that IL10 had no role to play in EAE severity, indicating that there were some other mechanisms involved. Thus Aim 3 tried to identify the cells responsible for EAE resistance seen in these animals. We hypothesized that a MOG_{61-85}-specific regulatory T cell population is the responsible population in protection of B cell$^{-/-}$ mice to rMOG-induced EAE.

Results

An increase in the number of cells expressing FoxP3 is observed when mice are primed with MOG_{35-85} in vivo.

Preliminary data indicated that an antigen-specific immune response to MOG_{61-85} actively suppressed EAE induced by the encephalitogenic MOG_{35-55} peptide (Lyons et al.

unpublished data). Recent data identified a role for T_{reg} cells in controlling autoimmune responses, such as those seen in in MS/EAE (Sospedra and Martin, 2005). To further characterize the MOG_{61-85} response, spleens were harvested 29 dpi from mice which were immunized with either MOG_{35-55} or MOG_{35-85}. Single cell suspensions were prepared and cultured *in-vitro* with either MOG_{35-55} or MOG_{61-85} to increase antigen specific cell proliferation. The cells were harvested at 96 hrs and analyzed by flow cytometry.

Early experiments attempted to remove dead cells via density gradient centrifugation. However, this led to substantial cell loss, with insufficient cells for analysis. Thus, data presented are from cells recovered from culture by centrifugation and washed 2x in media. Cell debris was removed by increasing the threshold of the forward scatter for sample collection and further gating on fluorescence staining during analysis. Using FlowJo analysis software, the cells were first gated on the presence of CD3/side scatter data to isolate T cells and were then analyzed for the expression of the FoxP3 transcription factor, which is expressed by T_{reg} cells.

Similar number of cells expressing FoxP3 was noted when they were cultured with MOG_{35-55} *in-vitro* (2.85% and 2.88%). Conversely, increased percentage of cells expressing FoxP3 was noted when the they were cultured with MOG_{61-85} *in-vitro* (3.32% and 5.89%). This increase was much more evident when the cells were first primed with MOG_{35-85} *in-vivo* and the cultured with MOG_{61-85} *in-vitro*. This was an 80% increase in the number of cells expressing FoxP3 when the cells were primed to MOG_{35-85} *in-vivo* (Figure 14). It was also interesting to note that there was an increase in the number of cells expressing FoxP3 when the cells primed with MOG_{35-55} *in-vivo* and the cultured

with MOG_{61-85} *in-vitro*. These results are based on a background correction of 0.233% in the isotype-matched controls (Figure 15).

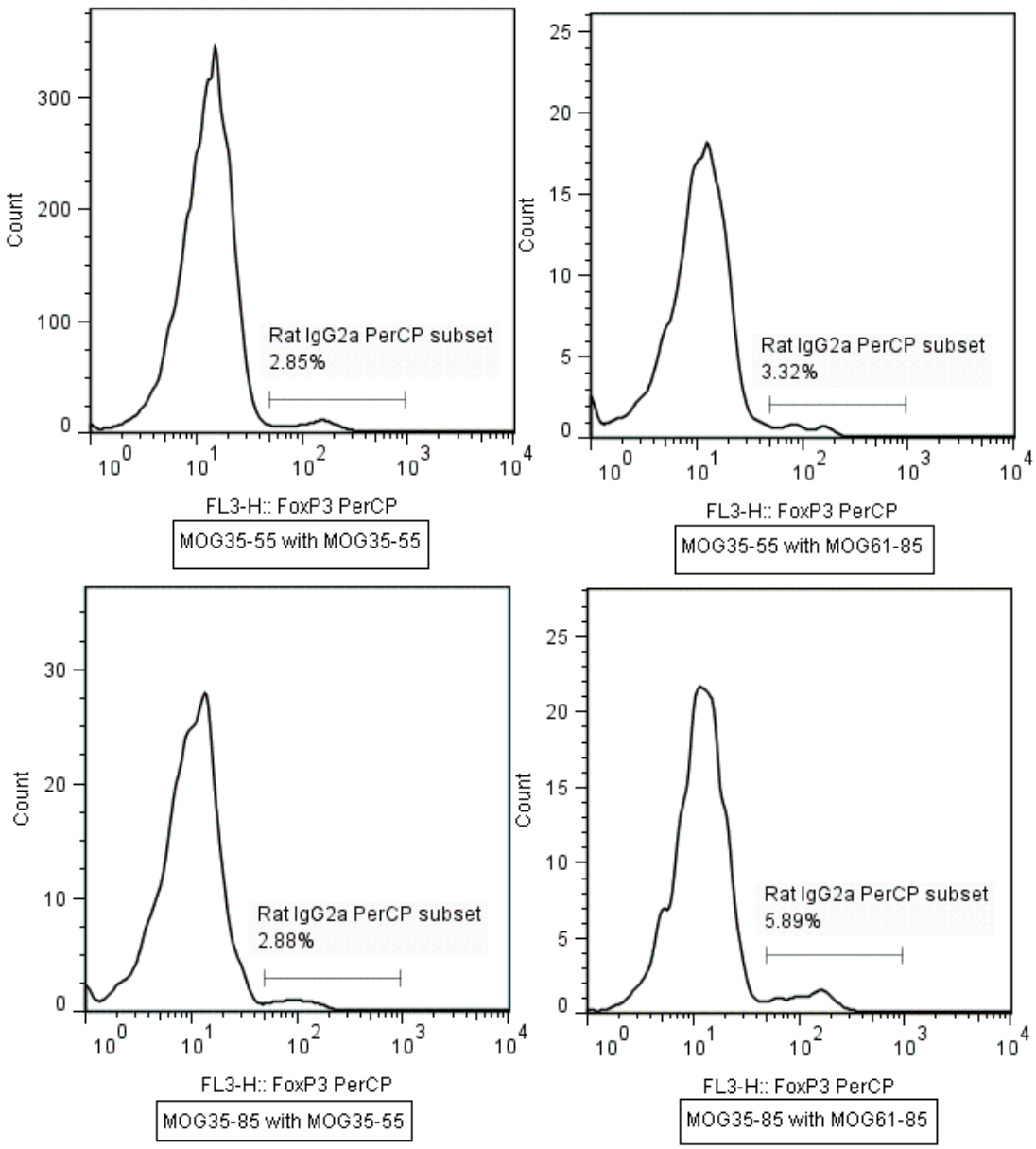

Figure 14. **FoxP3 expression in T cells.** Spleens were harvested from WT mice immunized with MOG_{35-55} or MOG_{35-85} and cultured *in-vitro* with MOG_{35-55} or MOG_{61-85}. **Decreased FoxP3 expression was noted when the cells were cultured with MOG_{35-55} as compared to the cells cultured in presence of MOG_{61-85}. This increase was much**

more evident when the cells were first primed with MOG_{35-85} *in-vivo* and the cultured with MOG_{61-85} *in-vitro*.

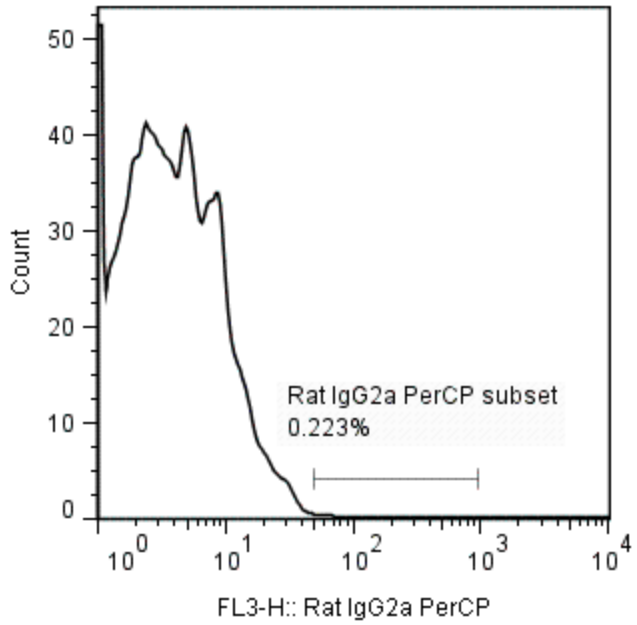

Figure 15. **Control for the analysis of FoxP3 expression in T cells.** The cells were stained with species, isotype and fluorochrome matched antibodies. **The control exhibited a background of 0.233% $FoxP3^+$ cells.**

An increase is also seen in the percentage of $CD3^+$ $CD4^+$ $CD25^+$ T cells, but a comparable level of $CD4^+T_{reg}$ cells.

In addition to FoxP3, T_{reg} cells also express CD4 and CD25. To further characterize the responding populations, expression of CD4 and CD25 on the $CD3^+$ T cells was characterized. Analysis for CD4 and CD25 gated on CD3 & side scatter demonstrated comparable frequencies of $CD3^+$ $CD4^+$ $CD25^+$ T cells in all cultures (2.76%, 2.2.5% and 2.19%) except when the cells were primed with MOG_{35-85} *in-vivo* and then cultured with MOG_{61-85} *in-vitro* (4.00%) (Figure 16). Analysis was also carried

out on the corresponding control so that it could be established that the double positive population was real (Figure 17).

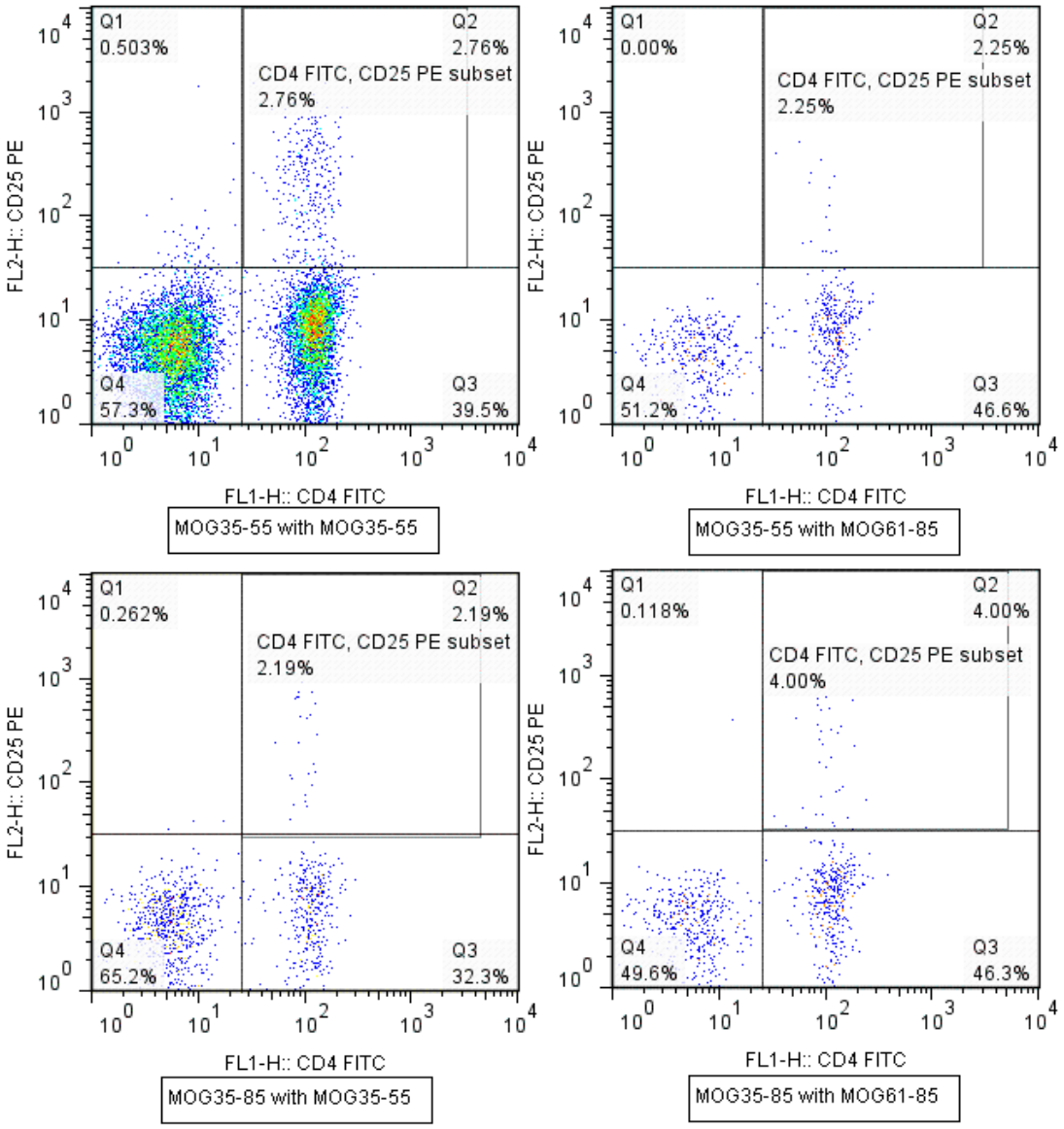

Figure 16. **$CD3^+$ $CD4^+$ $CD25^+$ T cells within the CD3/side scatter gate.** The cells gated on CD3/side scatter were further gated on the presence of CD4 and CD25 on their cell surface. **An increase was seen in those cells which were primed *in-vivo* with MOG_{35-85} and cultured *in-vitro* with MOG_{61-85}.**

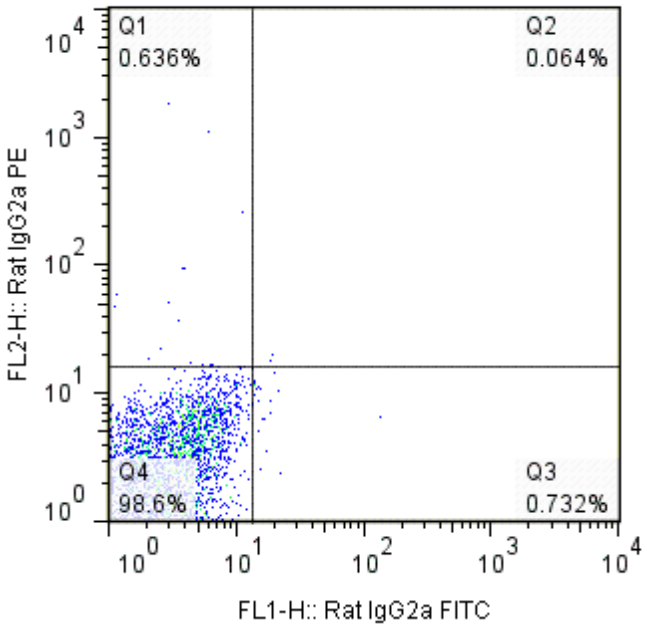

Figure 17. **Control for the analysis of $CD3^+$ $CD4^+$ $CD25^+$ T cells.** The cells were stained with species, isotype and fluorochrome matched antibodies. **The control demonstrated that there was no non-specific staining in the samples.**

Lastly, the expression of FoxP3 by $CD3^+$ $CD4^+$ $CD25^+$ T cells was analyzed. Generally, a lower expression level was seen on cells cultured *in-vitro* with MOG_{35-55} (67.7% and 64.0% of cells expressing FoxP3) as compared with the cells cultured with MOG_{61-85} (76.5% and 76.5% of cells expressing FoxP3) irrespective of the *in-vivo* priming agent. However taking into consideration the low numbers of cells initially present in the $CD3^+$ $CD4^+$ $CD25^+$ T cell gate, further experiments are necessary to confirm observations for these $CD4^+$ T_{reg} cells (Figure 18).

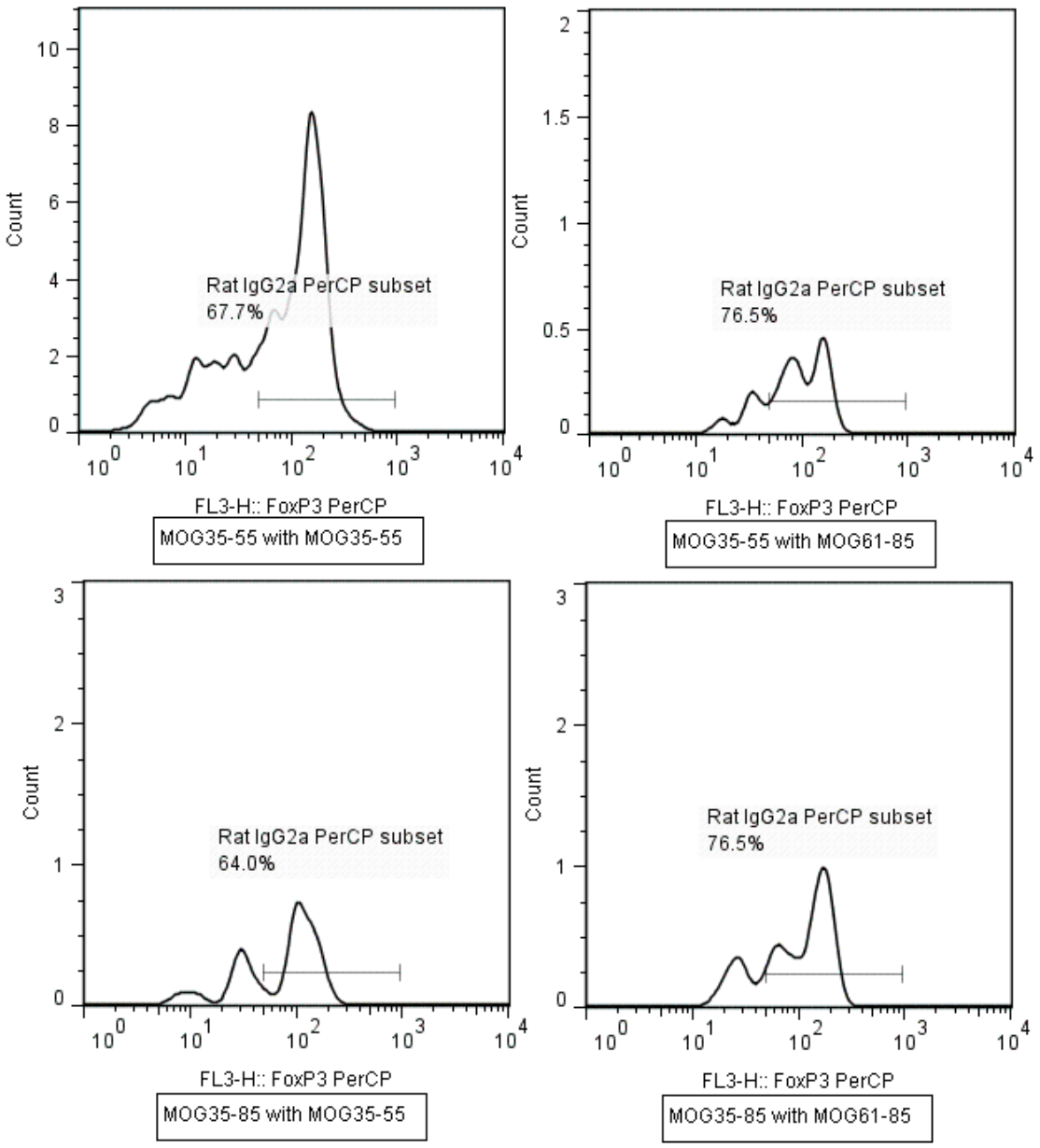

Figure 18. **Percentage of $CD3^+$ $CD4^+$ $CD25^+$ $FoxP3^+$ T_{reg} cells.** The cells within the $CD3^+$ $CD4^+$ $CD25^+$ gate were analyzed for the expression of FoxP3. **A comparable level of $CD4^+$ T_{reg} cells were seen in all the samples.**

The $CD8^+$ T cell numbers varied between the cell cultures.

The cells within the CD3/side scatter gate were also analyzed for the expression of CD8. Levels of $CD8^+$ T cells varied between all the cultures. Moreover very few cells were found which were $CD3^+$ $CD8^+$ $CD25^+$, in all the cell cultures. The low percentage of $CD8^+$ T cells, along with few total events which occurred, make analyzing the CD8 data difficult (Figure 19).

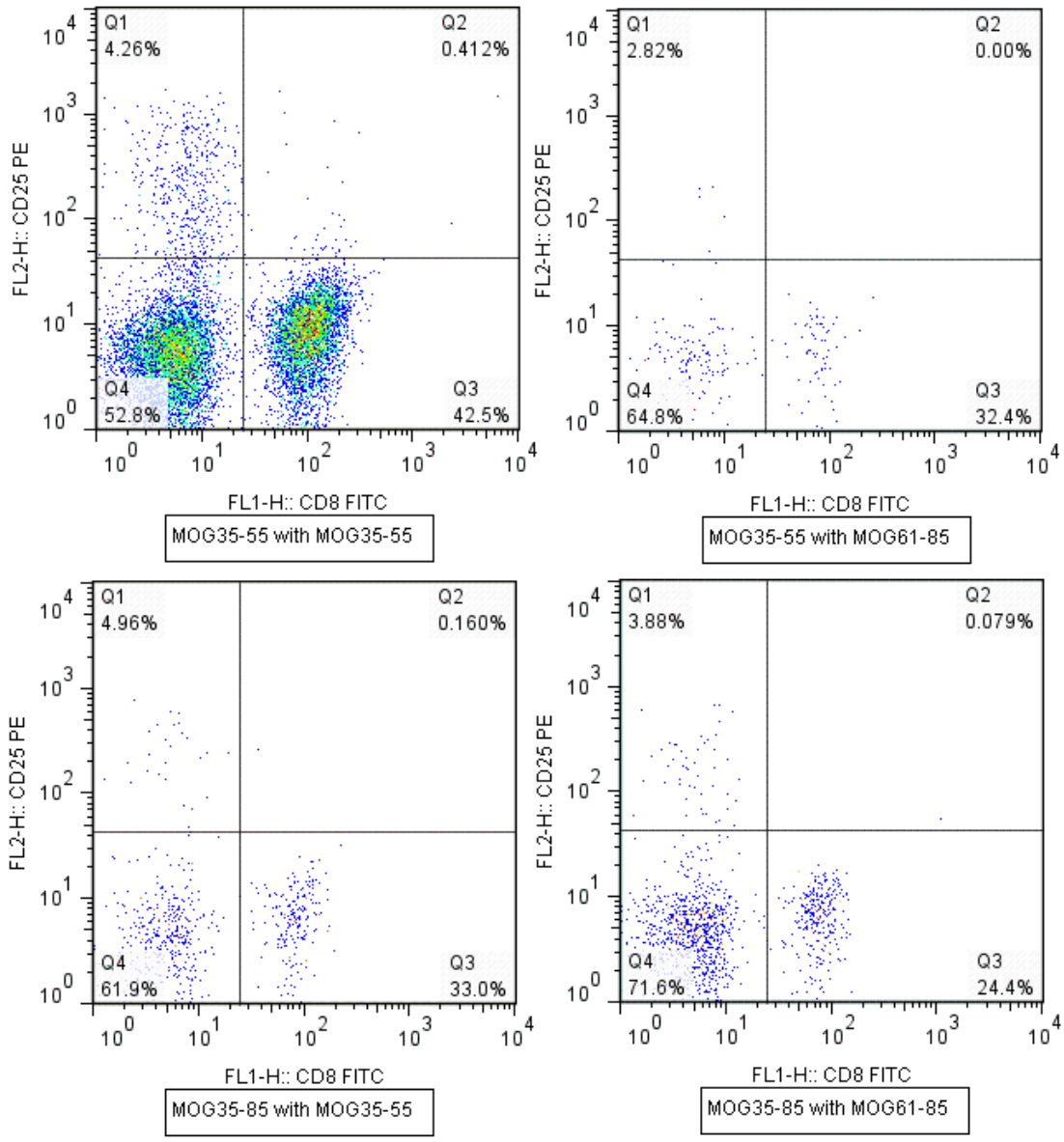

Figure 19. **Percentage of $CD3^+$ $CD8^+$ $CD25^+$ T cells within the CD3/side scatter gate.** The cells within the CD3/side scatter gate were analyzed for the expression of CD8 and CD25. **Very few $CD8^+$ $CD25^+$ T cells were noted in all cultures. This indicated that it maybe the $CD4^+$ T_{reg} cells which are responding to the MOG_{61-85} epitope.**

Discussion

This specific aim investigated the specific cell population that was responding to the protective MOG_{61-85} epitope. Since B $cell^{-/-}$ animals did not present with EAE after immunizing with rMOG, it was hypothesized that the cells responding to the MOG_{61-85} epitope, important to amelioration of MOG-induced EAE, should be other than B cells. Thus, our analysis focused on T_{reg} cells.

For analysis of the T_{reg} cell population, the cells were first gated by virtue of the expression of the pan-T cell marker, CD3, and side scatter characteristics (Figure 20). The traditional lymphocyte gate could not be used because of the high amounts of dead cells and debris that interfered with analysis.

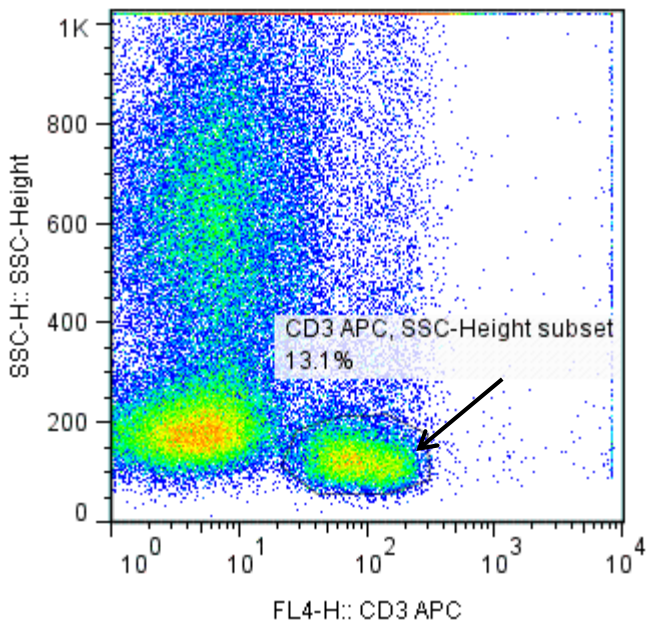

Figure 20. **The CD3/side scatter gate set in FlowJo for data analysis.** The traditional lymphocyte gate could not be set up because of the high amount of dead cells and debris in the samples. **The $CD3^+$ gated cells were further used for analysis.**

Next, FoxP3 expression was analyzed in the above gated population. As shown in Figure 14, there was an increase in the FoxP3 expressing cell population when the cells were cultured with MOG_{61-85}, lending support to the hypothesis that it is a T_{reg} cell population that is responding to the MOG_{61-85} region.

In order to determine which of the T_{reg} cells (CD4 or CD8) were responding, the T cells were further analyzed for the expression of these markers and CD25. As demonstrated in Figure 16, there was an increase in the $CD4^+CD25^+$ double positive cell population when the cells were primed *in-vivo* with MOG_{35-85} and cultured *in-vitro* with MOG_{61-85}. This indicated that it is a $CD4^+$ T cell population that is responding to the MOG_{61-85} epitope. In order to determine if this $CD4^+$ T cells were in fact $CD4^+$ T_{reg} cells, the expression of FoxP3 in these cells was analyzed. Gating on $CD4^+CD25^+$ T cells revealed comparable populations of $FoxP3^+$ cells in all cultures (Figure 18). This, along with the fact that the $CD3^+$ $CD4^+$ $CD25^+$ T cell gate had very few events, makes it difficult to draw conclusions about the role of these $CD4^+$ T_{reg} cells. Another possibility is the fact that the entire $CD4^+$ T cell population (T_{eff} cells as well as T_{reg} cells) were expanded in response to the MOG_{61-85} region. Another point to note is the fact that cells primed to MOG_{35-55} *in-vivo* also seem to respond to MOG_{61-85} *in-vitro*, which was not expected. A possible explanation for this could be the fact the since these animals exhibited EAE, their cells were primed to the endogenous MOG_{61-85}.

Lastly, to determine if it were the $CD8^+$ T cells responding to the MOG_{61-85} epitope, the CD3 gated T cells were gated on the presence of CD8 and CD25 (Figure 19). Surprisingly very few cells expressing both $CD8^+$ $CD25^+$ were noted in all cultures, with most of the cells being $CD8^-$ $CD25^+$, indicating that these could be the $CD4^+$ T cells. The

FoxP3 expression in these $CD8^+$ T cells was also found to be comparable in all cultures (Data not shown). Recent literature has described a unique population of $CD8^+$ LAP^+ T cells with suppressive functions in EAE (Chen et al., 2009). These cells may or may not express CD25 and/or FoxP3. Thus, it would be interesting to see if the small percentage of the $CD8^+$ T cells seen, did express LAP. However, the above data indicated that $CD8^+$ T were not responding to the MOG_{61-85} region.

Another potential population to be looked into is the MDSCs. Preliminary analysis indicated a decrease in MDSCs when the cells are cultured with MOG_{35-85} (Data not shown). However further experiments are required before any conclusions can be made. In summary these results indicated an increase in the number of T cells expressing FoxP3 in response to MOG_{61-85}, however further experiments are required before any conclusions can be made regarding the cell population responding to MOG_{61-85}.

CHAPTER VI: DISCUSSION AND CONCLUSION

Discussion

Previous studies have established B cell dependent (rMOG-induced) and B cell independent (MOG_{35-55}-induced) models of EAE in B6 mice. Previous studies showed also distinct B cell responses in WT animals when immunized with rMOG or MOG_{35-55}, with a group of antibodies directed against MOGaa46-85, present in the rMOG induced model but not in the MOG peptide induced model (Liu et al., 2012). Analysis of the T cell response in rMOG-immunized WT and B $cell^{-/-}$ mice identified an additional T cell epitope in B $cell^{-/-}$ mice comprised of MOGaa61-85 (Lyons et al. unpublished data). Co-immunizations with MOG_{35-55} and MOG_{61-85} demonstrated an amelioration of EAE in these mice. Additionally, it was demonstrated that a serum derived factor from WT animals reconstituted rMOG-induced EAE in B $cell^{-/-}$ mice and abrogated the immune response seen in B $cell^{-/-}$ animals towards MOG_{61-85} (Lyons et al. unpublished data). This project aimed to look into the mechanism of protection against EAE induction when the animals were co-immunized with the encephalitogenic MOG_{35-55} and the protective MOG_{61-85} peptide.

Aim 1 investigated the role of the putative B cell epitope embedded within MOGaa 61-85 in selection of the protective MOG_{61-85} epitope. Immunization of WT and B $cell^{-/-}$ animals with the longer MOG_{35-85} peptide, encompassing the encephalitogenic MOG_{35-55} epitope, the protective MOG_{61-85} epitope, and the putative B cell epitope located within MOGaa41-85, demonstrated a decrease in disease severity in WT as well as B $cell^{-/-}$ mice when compared to the animals immunized with MOG_{35-55}. This led to the conclusion that the intervening region between the MOG_{35-55} and MOG_{61-85} peptides did

not play a role in the processing and presentation of the MOG_{61-85} epitope. However, certain aspects in these results must be taken into account. These results indicated that aa41-86 are not involved in antigen processing and presentation of rMOG, but the three dimensional conformation of the rMOG molecule needs to be in taken into account. Results could be confounded if the chemically synthesized MOG_{35-85} could not achieve the native conformation present in the naturally occurring protein. Serum from WT mice immunized with MOG_{35-55} and MOG_{35-85} has been collected. Peptide mapping analysis can be carried out on these samples to see the responses against linear epitopes. Presence of an antibody response towards MOGaa46-85 will help confirm that this region is not involved in antigen processing and presentation. It can then be determined if the other B cell epitopes (MOGaa21-40, MOGaa101-125) identified are involved in antigen processing and presentation. Conversely, the absence of an antibody response towards MOGaa46-85 will lend credibility to the conformational epitope hypothesis. Better understanding of antigen processing and presentation in these animals might help us gain a better understanding of the immune responses occurring in MS. This is important considering that T cell responses have been noted in MS against aa1-22, 34-56 and 64-96 (Kerlero de Rosbo et al., 1997).

IL10 is considered to be an anti-inflammatory cytokine (Matsushita et al., 2008). Previous results suggested that IL10 may be important in the protection seen with MOG_{61-85} (Lyons et al. unpublished data). Thus, $IL10^{-/-}$ mice were immunized with MOG_{35-55} and MOG_{35-85} peptides and observed for EAE induction and progression. The incidence of EAE in $IL10^{-/-}$ mice was not significantly affected by the longer MOG_{35-85} peptide when compared to the group immunized with MOG_{35-55}, implicating a role for

IL10 in the decreased incidence noted in WT and B cell$^{-/-}$ mice upon immunization with the MOG_{35-85} peptide. However, clinical severity in those IL10$^{-/-}$ mice immunized with MOG_{35-85} that developed clinical signs was significantly decreased to that observed in MOG_{35-55} immunized IL10$^{-/-}$ mice, indicating that IL10 is not important in ameliorating EAE severity by MOG_{61-85}. This was supported by the fact that the cells isolated from MOG_{35-85} primed WT mice and cultured *in-vitro* with MOG_{61-85} did not produce any detectable IL10 as demonstrated by ELISA. This showed that mechanisms other than those mediated by IL10 are important in the decrease in EAE severity seen with MOG_{61-85} peptide and necessitates the identification of other anti-inflammatory cytokines important in this model of MS. However IL10 was found to be important in incidence, indicating that different disease stages in EAE are mediated by different cytokines. Some might be more important in induction, others in progression and disease severity. It would be interesting to look at the cytokine expression of cells harvested from mice at different disease stages, such as onset, peak, recovery and chronic. Thus, it can be hypothesized that MOG_{61-85} prevents EAE induction in an IL10 dependent mechanism, but the decrease in EAE severity is due to some other unidentified effector molecules.

Results also demonstrated that there was a substantial decrease in the levels INFγ and IL17 mRNA in WT animals when the cells were cultured in the presence of MOG_{61-85}. This correlated with the decrease in clinical severity in these animals. Further studies using cells from B cell$^{-/-}$ and IL10$^{-/-}$ mice should be carried out to demonstrate if similar effects are observed in these mice too. The decrease in INFγ mRNA expression was associated with a decrease in protein expression as seen with ELISA. Further experiments are required to establish IL17 protein levels. The identification of other potential effector

molecules could eventually translate into therapies for MS patients and a better understanding about the pathogenesis of the disease.

Lastly it was demonstrated that there was an increase in the expression of FoxP3 following priming with MOG_{61-85}. This is in agreement with previous observations by Feger et al. 2007 that increased FoxP3 expression correlated with amelioration of EAE. There was also an increase seen in the percentage of $CD4^+$ $CD25^+$ $FoxP3^+$ T cells in the cultures with MOG_{61-85}. These results suggest that these $CD4^+$ T_{reg} cells are important in the protection seen with MOG_{61-85}. However this potential role of $CD4^+$ T_{reg} cells in the protection mediated by the MOG_{61-85} epitope warrants further investigation.

Analysis was also carried out for the presence of $CD8^+$ T cells. Very few $CD8^+$ $CD25^+$ T cells were noted in all the cultures. Interestingly, an increase in $CD8^+CD25^-$ T cells was noted in cells cultured with MOG_{35-55} as compared to the cells cultured with MOG_{61-85}. It is interesting to speculate that this increase in $CD8^+$ T cells represents pathogenic $CD8^+$ T cells, as previously described (Bettini et al., 2009). In addition, a very low percentage of $CD8^+$ $CD25^+$ $FoxP3^+$ T cells were noted in the samples and further experiments are required before any conclusions can be drawn regarding the role of these cells in the clinical effect of MOG_{61-85}. The identification of $CD8^+$ T cell populations is important in the context of the recently identified $CD8^+$ LAP^+ T_{reg} cells, which may or may not express the traditional T_{reg} cell markers, CD25 and FoxP3 (Chen et al., 2009).

Limitations and Future Directions

Previous experiments had identified unique B cell epitopes in WT mice immunized with rMOG. These consisted of MOGaa21-40 and MOGaa41-86 (Liu et al.,

2012). These results led to the central hypothesis of this project, that the antibodies against MOGaa41-86 were somehow involved in the antigen processing and presentation of rMOG in WT animals. In the presence of B cells, WT mice fail to develop a response towards the protective MOG_{61-85} and ultimately led to EAE induction in WT mice immunized with rMOG. Conversely, the absence of these antibodies in B $cell^{-/-}$ animals allowed for the selection of the protective MOG_{61-85} epitope and the prevention of EAE when these mice were immunized with rMOG. However, the hypothesis that MOGaa46-85 in the form of the longer peptide (MOG_{35-85}) regulated antigen processing and presentation was disproved by the results of Aim 1. There could possibly be two reasons for this. Firstly, other identified B cell epitopes besides MOGaa46-86 are important in preventing the selection of the protective MOG_{61-85} epitope. These B cell epitopes include MOGaa21-40 and MOGaa101-125 within the rMOG protein. This hypothesis can be further investigated by including these epitopes in immunization experiments. Secondly, the conformation of the chemically synthesized MOG_{35-85} needs to be taken into consideration as previously described. Further investigation of the mechanism of epitope selection by B cells would help us better define the mechanism of EAE induction in this animal model.

In addition, previous data had suggested a role for IL10 in the protection afforded by the MOG_{61-85} epitope. As discussed above, the role of IL10 in the process appears to be in preventing disease onset rather than remediating disease severity. Data do suggest that amelioration of disease may be dependent on decreased production of proinflammatory cytokines, IFNγ and IL17. Further support for this would be obtained from analysis of cytokine secretion in $IL10^{-/-}$ mice. However, qPCR could not be applied

for IL10 quantification due to unexplained difficulty in the isolation of total RNA from tissues and technical difficulties with the designed IL10 qPCR assay. Once these technical issues are resolved, these experiments will be completed.

This work looked at some of the pro-inflammatory cytokines that have been implicated in MS/EAE pathogenesis. However there are several more potential effector molecules which can be analyzed. This becomes important in light of the finding that IL10 is not the only player in the protection obtained with $MOG_{61-85.}$ Characterization of other potential cytokines implicated in EAE pathogenesis, such as IL4 and TGFβ, may shed light on the mechanism of protection by MOG_{61-85} (Chen et al., 2008; Chen et al., 2009; Falcone et al., 1998). In addition, the results of Aim 2 were defined in WT animals, not in B cell$^{-/-}$ animals, and thus experiments can be run to see if these results correlate with each other.

The cell population responding to MOG_{61-85} also needs to be further defined. Assays can be conducted to determine proliferation of cells to MOG_{61-85} using Carboxyfluorescein succinimidyl ester (CFSE) staining. This could help us identify smaller cell populations responding to the protective MOG_{61-85} epitope. Co-culture studies with MOG_{35-55} and MOG_{61-85} can also be carried out to determine if the presence of the protective epitope alters the cellular response to the encephalitogenic epitope. There was an increase seen in the number of cells expressing FoxP3; however, no conclusions could be drawn regarding T_{reg} cells from these experiments. Further experiments could increase the total number of cells used for cell culture and thus remove the problem of losing cells in the density gradient centrifugation step. This would also

help separate out the live cells and help define smaller cell populations, which might have been masked in all the debris and dead cells in the samples.

It can also be studied whether the $CD8^+$ T cells seen are the recently described $CD8^+$ LAP^+ T cells using flow cytometry. The recently published $CD8^+$ LAP^+ T regulatory cells have been shown to act in a contact dependent manner. It can thus be hypothesized that if the $CD8^+$ T cell population were indeed these cells, their effects would be diminished if these cells were cultured in trans-well plates. This too will help us further define the immune cell population responding to MOG_{61-85}.

Conclusion

In conclusion, it can be said that the B cell epitope within MOGaa46-85 does not play a role in the processing and presentation of the antigen. These results also demonstrated the protective nature of the MOG_{61-85} epitope, along with the role of IL10 in disease incidence. Lastly, an increase in the FoxP3 expression was seen when the cells were cultured with MOG_{61-85}, again demonstrating that the protective effects of MOG_{61-85} might at least in part be due to the expansion of a $CD4^+$ T_{reg} cell population.

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