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The Effects of Antibodies in Disease Progression of MOG-induced Experimental Autoimmune Encephalomyelitis

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THE EFFECTS OF ANTIBODIES IN DISEASE
PROGRESSION OF MOG-INDUCED
EXPERIMENTAL AUTOIMMUNE
ENCEPHALOMYELITIS

by

Melissa Marie Riter

A Thesis Submitted in
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ABSTRACT

THE EFFECTS OF ANTIBODIES IN DISEASE PROGRESSION OF MOG-INDUCED EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

by

Melissa Marie Riter

The University of Wisconsin-Milwaukee, 2015
Under the Supervision of Professor Jerri-Annette Lyons

Abstract: Multiple sclerosis (MS) is an auto-inflammatory disease of the central nervous system (CNS), affecting over 400,000 people in the US. MS is primarily studied in the animal model experimental autoimmune encephalomyelitis (EAE). MS is a T cell mediated disease but there is mounting evidence for a role for B cells in MS. Previous studies have established that rMOG Induction depends on the presence of B cells, while induction using the MOG peptide covering amino acids 35-55 does not require B cells to cause disease. When plasma from the rMOG and MOG35-55 immunized WT mice was analyzed by ELISA there binding at MOG21-45, covering the encephalogenic epitope, and binding covering the MOG46-85 amino acids, which was not expected. This epitope was observed again in T cells when comparing the WT and B cell -/- mice. WT T cells only bound MOG35-55 but T cells from B cell-/- mice also bound MOG61-85. The same epitope observed in WT antibodies. This led to the conclusion that this was a cryptic epitope and seemed to produce an anti-inflammatory response. Mice coimmunized with both MOG35-55 and MOG61-85 similar results were observed as in rMOG immunization. Coimmunized mice had less severe disease than those immunized with just MOG35-55 giving further evidence that MOG61-85

produces a protective response. When rMOG immunized B cell $-/-$ mice were given serum from rMOG primed WT mice clinical disease could be observed. This was also true for coimmunized mice. Meaning it was B cell products in the serum not the B cells themselves that was altering the immune response. When serum from MOG35-55 and MOG61-85 primed rabbit serum was used after heat inactivation, WT and B cell $-/-$ mice showed more severe disease. B cell $-/-$ mice given the immune serum also had an earlier onset of disease. This demonstrated that the most likely cause of the altered response was antibody. Cells from mice coimmunized injected with either immune rabbit serum or preimmune rabbit serum were cultured with MOG35-55 and MOG61-85, proliferation was measured. The mice in the preimmune group had greater proliferation to both peptides. This may be because the encephalogenic cells had already migrated to the CNS in the immune group. When T cells were cultured again with purified antibody, from rMOG primed mice, proliferation decreased in all the wells with antibody added, but when injected into coimmunized mice similar disease was observed as seen in the mice given immune serum. Over all these results indicate a role for antibody in the processing and presentation of MOG in EAE. This may be through the suppression of the presentation of MOG61-85 when antibody is bound to MOG when it is taken up by antigen presenting cells.

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Dedicated in memory of my Grandmother Ardel Jensen

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LIST OF ABBREVIATIONS

AAALAC association for assessment and accreditation of laboratory animal care

ANOVA analysis of variance

APC antigen presenting cell

aa amino acid

B-/- B cell deficient

BCR B cell receptor

C Celsius

CNS central nervous system

Con A concanavalin A

CSF cerebral spinal fluid

EAE experimental autoimmune encephalomyelitis

EDTA Ethylenediaminetetraacetic acid

ELISA enzyme-linked immunosorbent assay

FBS fetal bovine serum

FoxP3 forkhead box p3

µg microgram

GA glatiramer acetate

HBSS Hanks Balanced Salt Solution

Ig immunoglobulin

IFN interferon

IVIG intravenous immunoglobulin

IL-10-/- interleukin-10 deficient

IL interleukin

LSM lymphocyte separation media
LT lymphotoxin
 μ L microliter
MBP myelin basic protein
MHC major histocompatibility complex
mg milligram
mL milliliter
mM milimolar
M molar
MOG myelin oligodendrocyte glycoprotein
MS multiple sclerosis
ng nanogram
nm nanometer
NIH national institute of health
PBS phosphate buffer saline
PLP proteolipid protein
PPMS primary progressive multiple sclerosis
PT pertussis toxin
rpm rotations per minute
RPMI Roswell Park Memorial Institute medium
RRMS relapsing remitting multiple sclerosis
SPMS secondary progressive multiple sclerosis
TCR T cell receptor
TGF transforming growth factor
Th1 type 1 helper T cell

Th2 type 2 helper T cell

Th17 type 17 helper T cell

TMB 3,3',5,5'-Tetramethylbenzidine

TNF tumor necrosis factor

WT wild type

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Chapter I: Introduction

Multiple Sclerosis and Animal Model of Disease

Multiple sclerosis (MS) is a neurodegenerative, inflammatory disease of the central nervous system (CNS). MS affects 400,000 people in the United States and over 2.5 million worldwide (Smyth, 2011; Steinman, 2014). The etiology of MS is not fully understood, but studies have demonstrate that both genetic and environmental factors are important for disease development. Twin studies reveal a 25% concordance (Compston & Coles, 2008; Willer, et al., 2003) and there are studies linking 52 different genes to the development of MS, such as those for the major histocompatibility complex (MHC) (Gourraud, Harbo, Hauser, & Baranzini, 2012), and cytokines like interleukin-18 (Celik, et al., 2014). On the other hand, environmental factors associated with MS have ranged from diseases, such as Epstein Barr virus and measles (Sundqvist, et al., 2012), to cigarette smoking (Jafari & Hintzen, 2011) and vitamin D deficiency (Berlanga-Taylor, Disanto, Ebers, & Ramagopalan, 2011). How these different factors lead to the development of MS is unknown.

MS patients can suffer from a variety of symptoms including pain, paralysis, balance problems, and bladder dysfunction (Fletcher & Mills, 2012). How the disease progresses will depend on the type of MS, and which symptoms occur will depend on where in the CNS the lesions occur (Noonan, et al., 2010). The two main types of MS are primary progressive MS (PPMS) and relapsing remitting MS (RRMS) (Noonan, et al., 2010; Smyth, 2011). 85% of MS patients present with RRMS, while only 15% present with PPMS (Noonan, et al., 2010).

PPMS exhibits a linear disease course. These patients may have plateaus in disease progression but once a symptom occurs, it becomes permanent. RRMS patients have intermittent disease and early in the disease course will fully recover from all symptoms during a remission. These relapses occur once every few years and last days to weeks (Steinman, 2014). Relapses are defined as a neurological change that lasts for at least 24 hours; not brought on by changes in body temperature (Steinman, 2014). Eventually these patients will no longer fully recover and symptoms become permanent. Patients may still have periods of remission, but disease progression may also change to a more linear disease course. At this point these patients would be considered secondary progressive MS patients (SPMS). About 50% of RRMS patients will progress to this stage (Smyth, 2011). There are treatments available for patients with RRMS, which slow disease progression, however, there is no effective treatment for PPMS or SPMS patients currently available (Cross & Waubant, 2011; Leary & Thompson, 2005).

Clinical signs and symptoms of MS are caused by the development of plaques, or lesions, within the CNS. These plaques can be classified into four different types based on the cells and soluble proteins present. Types I, II, and III are characterized by the predominance of macrophages and T cells contributing to inflammation near the blood brain barrier (Lucchinetti, et al., 2000). Type I and II are separated by the presence of immunoglobulins and complement found primarily in type II plaques (Lucchinetti, et al., 2000; Serres, et al., 2009; Quintana, et al., 2008). Microglia contribute to inflammation in type III

plaques and are further separated by the diffuse inflammation observed with oligodendrocytes undergoing apoptosis (Lucchinetti, et al., 2000; Marik, Felts, Bauer, Lassmann, & Smith, 2007). Type IV also has diffuse inflammation and like type III, and DNA fragmentation is observed, but other signs of apoptosis are not seen (Lucchinetti, et al., 2000). Type III and IV do not show remyelination, unlike type I and II lesions where remyelination is observed (Lucchinetti, et al., 2000; Marik, Felts, Bauer, Lassmann, & Smith, 2007). The T cells found in different lesions of MS patients have different V-D-J junctional regions of the T cell receptor (TCR), which determine the specificity of the T cell (Friese & Fugger, 2009; Huseby, Huseby, Shah, Rebecca, & Stadinski, 2012). This indicates that each lesion has the expansion of a different T cell clone producing a new population of specific T cells causing the damage within a single lesion.

The myelin sheath protects the axons of nerve cells and increases conductivity of nerve signals. In MS the myelin sheath is damaged, exposing the axon to damage and decreasing the conductivity of nerve signals. The sheath is a complex mixture of lipids (70%) and protein (30%). Three myelin proteins relevant to MS include: myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG). MS patients produce an immune response to all three of these proteins (Greer, Cameron, McCombe, Good, & Pender, 1997; Meinl, et al., 1997; Hjelmström, Penzotti, Henne, & Lybrand, 1998). MBP and PLP are the most abundant proteins in the CNS but they are not exposed to the environment (Greer, Cameron, McCombe, Good, & Pender, 1997; Meinl, et al., 1997; Goverman J. M., 2011). Unlike MBP and PLP, MOG is

a transmembrane protein, which means that part of the protein is readily available for recognition by the immune system before any damage has been done to the myelin sheath, making it a prime candidate for the initiation of the autoimmunity characteristic of MS (Weber, Hemmer, & Cepok, 2011; Kursula, 2008).

All three of these myelin proteins are used to induce experimental autoimmune encephalomyelitis (EAE), an animal model of MS. EAE is used to study the immune mechanisms of MS (van der Star, et al., 2012). The EAE model produces a disease similar to MS. The disease can be triggered by a single T cell clone specific to an epitope on a myelin protein, but the response can spread to other epitopes on the same protein or to epitopes on other myelin proteins (Zamvil, et al., 1985; Lehmann, Fursthuber, Miller, & Sercarz, 1992). The protein or peptide of protein is used to induce disease in susceptible animals (i.e. mice, marmosets, and rats) with the appropriate adjuvant. The protein or peptide used depends on the species and strain of animal being utilized (Lyons J.-A. , Ramsbottom, Mikesell, & Cross, 2008; van der Star, et al., 2012). Disease may also be induced using the passive transfer of immune cells or immunization with appropriate protein and serum from animals immunized with the appropriate protein in susceptible animals (Lyons, Ramsbottom, & Cross, 2002; van der Star, et al., 2012). This biggest criticism of the EAE model is that the disease is not spontaneous, but must be induced. Neither MS nor an MS-like disease occurs naturally in animals, but disease can develop spontaneously in mice genetically modified with transgenic T cell receptors specific for MBP, but disease only

occurs under non-sterile conditions (Goverman, et al., 1993; Lafaille, Nagashima, Katsuki, & Tonegawa, 1994)

The Immune Response in MS/EAE

Effector CD4+ T cells

The immune response observed in MS was originally thought to be mediated by type 1 helper T (Th1) cells but this has been challenged with the discovery of Th17 cells. Th1 cells produce a T cell-mediated proinflammatory response through the production of proinflammatory cytokines like interferon (IFN)- γ . IFN- γ induces relapses in MS patients suggesting that an increase in Th1 cells may induce relapses in relapsing remitting MS (Steinman, 2014). Th1 cells infiltrate the CNS and attract macrophages and natural killer cells to the CNS where they are activated, causing the release of more proinflammatory cytokines such as IFN- γ and tumor necrosis factor- α (TNF- α), which is toxic to oligodendrocytes (Hedegaard, et al., 2008). However, mice lacking IFN- γ or TNF- α had worsened disease in EAE this information, led to the discovery of a role for Th17 cells in the pathology of disease (Steinman, 2014). Th17 cells also infiltrate the CNS in MS and have been observed in the cerebral spinal fluid of MS patients along with elevated levels of interleukin (IL)-17, the main cytokine produced by Th17 cells (Komiyama, et al., 2006; Matusevicius, et al., 1999). These cells are also implicated in the pathology of EAE (Kreymborg, et al., 2007; Komiyama, et al., 2006). IL-17 is known to disrupt the blood brain barrier (Compston & Coles, 2008). Furthermore Th17 cells recruit neutrophils to the CNS which, and like macrophages, can cause further damage (Fletcher, Lalor, Sweeney, Tubridy, & Mills, 2010). Both Th1 and Th17 cells are able to induce

EAE through the passive transfer of cells from immunized mice, but appear to do so through independent but complementary mechanisms (Fletcher, Lalor, Sweeney, Tubridy, & Mills, 2010). IFN- β ameliorates Th1 induced disease but will worsen Th17 induced disease (Fletcher, Lalor, Sweeney, Tubridy, & Mills, 2010). The ratio of Th1 and Th17 cells differ in the spinal cord of mice with EAE, but infiltration of the brain occurs when the Th17 cells outnumber the Th1 cells (Steinman, 2014). It's thought that Th1 cells enter the brain first and these cells drive the MS-like disease and Th17 cells come later in the disease (Steinman, 2014).

Cytotoxic T cells

Cytotoxic T cells (CD8+) like CD4+ helper T cells are found in the plaques of MS patients (Friesse & Fugger, 2009; Fletcher, Lalor, Sweeney, Tubridy, & Mills, 2010). CD8+ T cells produce cytotoxic products, such as perforin and granzyme B; produce proinflammatory cytokines, such as TNF- α and IFN- γ ; have cytolytic activity; and activate myelomonocytic cells (Friesse & Fugger, 2009). CD8+ T cells cannot induce lysis in oligodendrocytes, but the myelomonocytic cells, with both monocytic and granulocytic characteristics can induce lysis in the oligodendrocytes, which produce the protective myelin sheath (Friesse & Fugger, 2009). The amount of axonal damage in acute MS lesions has been correlated to the number of cytotoxic T cells infiltrating the lesions (Friesse & Fugger, 2009; Huseby, Huseby, Shah, Rebecca, & Stadinski, 2012; Saxena, Martin-Blondel, Mars, & Liblau, 2011). There is also evidence that specific cytotoxic T cell clones migrate to the CNS in patients with MS, which then undergo further expansion (Friesse & Fugger, 2009; Huseby, Huseby, Shah, Rebecca, & Stadinski, 2012). In

EAE cell transfer experiments, cytotoxic T cells have demonstrated the ability to induce disease (Frieze & Fugger, 2009). It is unknown if cytotoxic T cells are part of the initiation of MS, but they appear to have a role in disease progression through their cytokine production in active MS lesions (Frieze & Fugger, 2009).

Regulatory T cells

Several different subsets of CD4⁺ and CD8⁺ regulatory T cells have been identified (Correale & Villa, 2010). Both types of regulatory T cells suppress the immune response through cell-to-cell interaction and the production of anti-inflammatory cytokines (Filaci, Fenoglio, & Indiver, 2011). CD8⁺ regulatory T cells can also regulate the immune response through cytotoxic mechanisms that are not fully understood (Tejera-Alhambra, et al., 2012; Filaci, Fenoglio, & Indiver, 2011). These cells are difficult to identify as they are present in smaller numbers than effector T cells; do not proliferate as much as effector T cells in response to stimulation; and there is no marker that identifies all regulatory T cells (Fallarino, et al., 2003; Filaci, Fenoglio, & Indiver, 2011). Both CD4⁺ and CD8⁺ regulatory T cells can be identified through the expression of the transcription factor forkhead box p3 (FOXP3), but not all CD8⁺ regulatory T cells are FOXP3 positive (Filaci, Fenoglio, & Indiver, 2011; Hu, Weiner, & Ritz, 2013). Only about half of the identified CD8⁺ regulatory T cell subsets are FOXP3 positive (Filaci, Fenoglio, & Indiver, 2011). Two separate populations of CD4⁺ regulatory T cells produce the anti-inflammatory cytokines IL-10 (inducible regulatory T cells) and transforming growth factor (TGF)- β (Th3 cells) (Fletcher, Lalor, Sweeney, Tubridy, & Mills, 2010). TGF- β is a pleiotropic cytokine, the function of which is dependent on the context in which it is expressed. On its

own, TGF- β inhibits T cell proliferation, promotes regulatory T cell differentiation and may promote cell death (Travis & Sheppard, 2014; Bettelli, et al., 2006). However, in the presence of other cytokines like IL-2 cell death is inhibited and in the presence of IL-6, TGF- β promotes Th17 cell differentiation (Travis & Sheppard, 2014). CD4⁺ regulatory T cells suppression of effector T cells through cells-to-cell contact appears to be antigen specific, but this does not appear to be true for the CD8⁺ regulatory T cells (Sakaguchi, Wing, Onishi, Prieto-Martin, & Yamaguchi, 2009; Hu, Weiner, & Ritz, 2013).

It has long been known that healthy individuals harbor myelin-reactive T cells. Why these cells remain quiescent in these individuals but are activated and lead to pathology in MS patients has long been a topic of investigation. Recent studies have found that CD4⁺ regulatory T cells from MS patients appeared to have less suppressive power than healthy controls when challenged *in vitro* and *in vivo* (Fletcher, Lalor, Sweeney, Tubridy, & Mills, 2010; Steinman, 2014; Korn, et al., 2007). Viglietta, Baecher-Allan, Weiner, and Hafler found that an ongoing immune response in healthy controls did not affect CD4⁺ regulatory T cells ability to suppress effector T cells *in vitro*, while MS patients CD4⁺ regulatory T cells were unable to suppress T cell activation *in vitro* (2004). This demonstrates that the ability of the regulatory T cells to suppress is altered in MS patients and that an active immune response *in vivo* does not affect the *in vitro* results.

In EAE, the regulatory T cells were not observed to expand in the CNS, but the regulatory T cells that were able to enter the CNS produced IL-10 in response to stimulation with MOG35-55 (Korn, et al., 2007). Regulatory T cells

collected from mice immunized with MOG35-55 can suppress specific proliferation better than naïve cells, though both are equally able to suppress non-specific CD3 stimulated cells (Korn, et al., 2007). Effector T cells from the CNS were unable to be suppressed by regulatory T cells from the CNS, which appeared to be due to the combination of high IL-6 and TNF- α levels found in culture supernatants (Korn, et al., 2007). This may indicate that it is not just the activation of the immune system that is stopping the suppression of the effector T cells, but a problem with the CD4⁺ regulatory T cells ability to respond to the immune response.

CD8⁺ regulatory T cells do not appear to be dysfunctional in MS, but are not present in normal numbers compared to healthy controls during relapse (Filaci, Fenoglio, & Indiver, 2011; Fletcher, Lalor, Sweeney, Tubridy, & Mills, 2010; Correale & Villa, 2008). This is especially true in the CNS of MS patients (Correale & Villa, 2008). Correale and Villa found that the cloning frequency of CD8⁺ regulatory T cells was not altered in MS patients (2010). CD8⁺ regulatory T cells that are also Foxp3⁺ are able to reduce dendritic cells ability to present myelin antigens to effector T cells through the downregulation of costimulatory molecules *in vitro* (Correale & Villa, 2010). Cunnusamy et al. found that suppression of CD4⁺ effector T cells by CD8⁺ regulatory T cells was decreased in transwell culture compared to co-cultures with cells from MS patients (2014). This demonstrated that some of the suppressive ability of CD8⁺ regulatory T cells is through cell to cell contact but they also have some suppressive ability through cytokine production. CD8⁺ regulatory T cells in MS are reduced in

numbers but do not lose their ability to suppress, indicating that it may be that CD8⁺ regulatory T cells are not able to enter the CNS effectively or their numbers are not high enough to suppress the effector T cell response (Correale & Villa, 2008).

Type 2 helper T cells

Th2 cells, like regulatory T cells, are anti-inflammatory cells in MS. Th2 cells are normally part of the humoral immune response and are implicated in allergies. Th2 cells produce IL-10, IL-4, IL13 and IL-5, which suppress the differentiation of Th1 cells and direct the differentiation of B cells and isotype switching in antibodies (Oreja-Guevara, Ramos-Cejudo, Stark Aroeira, Chamorro, & Diez-Tejedor, 2012; Celik, et al., 2014; Levings, et al., 2001). Th2 cell differentiation is blocked by TGF- β (Travis & Sheppard, 2014). In *in vitro* experiments, neurons incubated with Th2 cells were protected from damage caused by a Th1 response (Yong, Giuliani, Xue, Bar-Or, & Metz, 2007). This is hypothesized to be through the production of cytokines such as IL-10 (Yong, Giuliani, Xue, Bar-Or, & Metz, 2007).

Th2 cells do not have a role in the progression of MS but Th2 cytokines are up regulated during a relapse along with Th1 cytokines in RRMS patients (de Andrés, et al., 2004). The cytokines produced by Th2 cells inhibit the differentiation of Th1 cells. There has been research to determine how to shift the T cell response toward Th2 cells and away from Th1 and Th17 response, through the modulation of the immune response (Smolders, et al., 2009; Oreja-Guevara, Ramos-Cejudo, Stark Aroeira, Chamorro, & Diez-Tejedor, 2012). This

is how many of the treatments for MS currently work though this is not enough to stop disease progression.

Research on treatments for MS that push the immune response toward to a Th2 response are under development. It has been observed that vitamin D can promote Th2 cell differentiation and increase Th2 cytokine levels in MS (Smolders, et al., 2009; Sloka, Silva, Wang, & Yong, 2011). Vitamin D deficiency has also been implicated as one of the environmental factors associated with the development of MS, as many MS patients are vitamin D deficient (Berlanga-Taylor, Disanto, Edbers, & Ramagopalan, 2011; Correale, Ysrraelit, & Gaitán, 2011). Vitamin D in its bioactive form, 1,25-dihydroxyvitamin D3, produces Th2 cells in culture of human cells through the induction of the STAT6 transcription factor, which is important for Th2 cell differentiation (Sloka, Silva, Wang, & Yong, 2011).

Another treatment is peptide vaccine. Myelin protein peptides are injected in high doses to induce a Th2 response (Pearson, van Ewijk, & McDevitt, 1997). In the EAE model, it has been found that injections of high doses of peptide with low T cell binding affinity can induce the apoptosis of Th1 cells and cause new T helper cells to differentiate into Th2 cells (Pearson, van Ewijk, & McDevitt, 1997; Katsara, et al., 2009). More recently, an analog of MBP, glatiramer acetate (GA), has been used to treat MS. GA induces Th2 cytokines and reduces Th1 cytokines in MS patients (Oreja-Guevara, Ramos-Cejudo, Stark Aroeira, Chamorro, & Diez-Tejedor, 2012; Chen, et al., 2001). These patients also had a reduced number of relapses and a reduction in the number of lesions seen on

MRI scans (Oreja-Guevara, Ramos-Cejudo, Stark Aroeira, Chamorro, & Diez-Tejedor, 2012). The exact mechanism of GA is still under investigation.

B cells in MS/EAE

The role of B cells in the pathogenesis of MS has been controversial. Scientists agree that B cells play a role in the pathogenesis but what that role is, is still under debate. This is due to inconsistent results observed between studies because of differences between animal models and methods used (Lyons J.-A. , Ramsbottom, Mikesell, & Cross, 2008; Liu, Muili, Agashe, & Lyons, 2012; Lyons, San, Happ, & Cross, 1999; Wilson, 2012; Abdul-Majid, et al., 2002; Urich, Gutscher, Prinz, & Becher, 2006; Cross, Trotter, & Lyons, 2001; Dittel, Urbania, & Janeway, 2000; Hjelmström, Penzotti, Henne, & Lybrand, 1998; Wolf, Dittel, Hardardottir, & Janeway, 1996). There are several possible roles for B cells in MS including: antigen presentation, production of proinflammatory cytokines, secretion of antibodies, and regulation of the immune response.

B cells as APCs

Activated B cells are considered professional antigen presenting cells (APC). B cells are potent APCs to T cells specific to the same antigen as the B cell. For a B cell to present antigen, it must first bind antigen via the B cell receptor (BCR). The antigen is broken down through antigen processing, followed by presentation on the major histocompatibility complex (MHC) class II molecule. The MHC/antigen complex interacting with T cell receptor (TCR) must come together with the interaction of the B7 costimulatory molecule on the APC with CD28 expression by the T cell. This leads to T cell activation and cytokine production. Which cytokines are produced is dependent on the APC and the

environment in which T cell activation is occurring, leading to the generation of either a pro- or anti-inflammatory response (Nakae, Iwakura, Suto, & Galli, 2007).

Other antigen presenting cells like dendritic cells can present antigen taken up non-specifically (i.e. pinocytosis, phagocytosis), while B cells need to bind antigen specifically by the BCR to efficiently present antigen (Chesnut & Grey, 1981). B cells can perform pinocytosis and bind antibody via the Fcγ-receptor but these are not presented efficiently to T cells (Chesnut & Grey, 1981). This means that B cells only efficiently present antigen that they bind via the BCR and T cells activated by B cells can only be presented epitopes in the MHC molecule from the antigen bound by cross-linking on the BCR before processing (Grey, Colon, & Chesnut, 1982). Once the BCR bound antigen is taken in to the cell the antigen is transported to the early endosome compartment and then quickly transported to the late endosome compartment where MHC class II molecules are assembled (Cheng, Steele, Gu, Song, & Pierce, 1999).

There is evidence that interactions with B cells are needed for T cells to become activated and enter the CNS (Ireland & Monson, 2011; Harp, Lovett-Racke, Racke, Frohman, & Monson, 2008). Furthermore, Duddy et al. (2007) found that the maturity of the B cell may affect the cytokines produced in response to activation, creating either a pro- or anti-inflammatory response. They observed that naïve and memory B cells produced different cytokines on activation and that these cytokines differed depending on if B cell activation was antigen specific or by bystander mechanisms (Duddy, et al., 2007). This observation, along with the success of the B cell depletion therapy for some MS

patients (Harp, Lovett-Racke, Racke, Frohman, & Monson, 2008; Morris & Yiannikas, 2012; Naismuth, et al., 2010), has led to the belief that T cell-B cell interactions are an important part of disease progression in MS. Harp et al. (2010) found that B cells from some RRMS patients could induce T cell proliferation and IFN- γ production when stimulated with MBP and/or MOG, this was not true for B cells from Healthy controls. They also demonstrated both memory B cells and naïve B cells could induce T cells proliferation in response to MOG, but MBP could only induce proliferation when presented by memory B cells (Harp, et al., 2010). The effectiveness of B cells as APCs to T cells may be a reason that B cell deletion therapy has been an effective treatment for some MS patients (Ireland & Monson, 2011).

There are several things that can effect which antigens are presented to T cells including the amino acid sequence of the antigen, the presence of antibody bound to the antigen at the time of internalization, and regulating proteins in the MHC assembly compartment (Alfonso, et al., 2003). In other antigen presenting cells DM, a regulatory protein for MHC assembly which favors epitopes that stably bind to MHC, is found as free heterodimers, while in B cells DM is closely associated with DO, another MHC associated heterodimer protein found only in B cells and some subsets of dendritic cells (Alfonso, et al., 2003; Kropshofer, Hämmerling, & Vogt, 1999). This association means that antigen loading, mediated by DM-DO, can only occur in a more acidic environment compared to that of DM alone (Roucard, et al., 2001). DO is upregulated after BCR mediated antigen up take and alters the presentation of some epitopes by either

suppressing or promoting them through the acidic environment of the endoplasmic reticulum, where MHC is assembled (Alfonso, et al., 2003; Roucard, et al., 2001).

Another factor that can alter epitope presentation is the presence of antibody bound to the antigen at the time of internalization (Simitsek, Campbell, Lanzavecchia, Fairweather, & Watts, 1995). Like the binding of DO, the binding of soluble antibody to antigen can suppress or promote selection of epitopes (Simitsek, Campbell, Lanzavecchia, Fairweather, & Watts, 1995). The antibody to antigen ratio determines if the T cells response to the antigen is promoted or suppressed (Manca, Fenoglio, Pira, Kunkl, & Celada, 1991). This has been observed in all antigen presenting cells (Manca, Fenoglio, Pira, Kunkl, & Celada, 1991; Watts & Lanzavecchi, 1993).

Cytokine production by B cells

B cells can produce several different proinflammatory cytokines including IL-6, IFN- γ , Lymphotoxin (LT)- α , and TNF- α (Barr, et al., 2012; Ireland & Monson, 2011; Christensen, et al., 2012; Serafini, Rosicarelli, Magliozzi, Stigliano, & Aloisi, 2004). Barr et al. demonstrated that B cell produce 86% of IL-6 in secondary lymphoid tissue in EAE and another study found that neutralization of IL-6 reduced disease in EAE (Erta, Quintana, & Hidalgo, 2012). IL-6 is a proinflammatory cytokine important for the differentiation of Th17 cells and plasma cells (Erta, Quintana, & Hidalgo, 2012). IFN- γ is also produced by B cells which pushes the immune response toward a Th1 response (Ireland & Monson, 2011).

Another cytokine produced by activated B cells is LT- α , which is important for the formation of memory B cells, and along with TNF- α is associated with the formation of ectopic follicles in the CNS (Ireland & Monson, 2011; Christensen, et al., 2012; Serafini, Rosicarelli, Magliozzi, Stigliano, & Aloisi, 2004). Ectopic follicles have been observed post mortem in MS patients and in EAE, and are made up of follicular dendritic cells and proliferating B cells (Serafini, Rosicarelli, Magliozzi, Stigliano, & Aloisi, 2004). The formation of these ectopic follicles is associated with an increase in production of pro-inflammatory cytokines.

The role of antibody in MS/EAE

B cells that differentiate into plasma cells produce antibodies, which can affect disease through multiple mechanisms including, activation of the complement cascade, Fc receptor binding, and epitope selection. Antibody bound to a cell's surface can initiate the complement cascade, which can lead to phagocytosis of the cells by macrophages or the formation of the membrane attack complex causing damage to the cell membrane (Piddlesden, Lassmann, Zimprich, Morgan, & Linington, 1993). Phagocytosis can also occur through binding of the Fc region of the antibody when antigen is bound (Dharmasaroja, 2003; Abdul-Majid, et al., 2002). Furthermore, antibody-dependent cell-mediated cytotoxicity is mediated by the binding of the Fc region by macrophages or natural killer cells, inducing the release of inflammatory molecules, which leads to the lysis of the target cell (Dharmasaroja, 2003; Piddlesden, Lassmann, Zimprich, Morgan, & Linington, 1993). All of these actions have been observed in MS lesions (Breij, et al., 2006; Dharmasaroja, 2003).

Antibody specific to myelin proteins has been observed postmortem in lesions of MS patients, the cerebral spinal fluid (CSF), and serum, but these antibodies have also been observed in the serum of healthy controls, indicating that the presence of myelin antibodies does not indicate disease when it is only present in the periphery (Lucchinetti, et al., 2000; Villar, et al., 2005; von Büdingen, et al., 2004). The bands found in the CSF and not the serum is a sign of inflammation and antibody production in the CNS (Weber, Hemmer, & Cepok, 2011). These antibodies are termed oligoclonal bands and are found in about 90% of MS patients (Compston & Coles, 2008). The presence of these bands increases the likelihood that the patient will develop clinically definite MS, as opposed to a clinically isolated episode (Miller, Chard, & Ciccarelli, 2012). Those patients that do not develop oligoclonal bands are thought to have a more mild disease course (Huttner, et al., 2009; Rojas, Patrucco, Tizio, & Cristiano, 2012). The presence of more oligoclonal bands indicates a more severe disease course (Weber, Hemmer, & Cepok, 2011; Miller, Chard, & Ciccarelli, 2012; Cross, Trotter, & Lyons, 2001). The presence of oligoclonal bands is used as a diagnostic tool to help determine if the patient has MS, but oligoclonal bands are not specific to MS (Miller, Chard, & Ciccarelli, 2012). Oligoclonal bands can also be found in patients with late stage Lyme disease and neurosyphilis (Pachner & Steere, 1985; Vartdal, Vandvik, Michaelsen, Loe, & Norrby, 1982).

Epitope spreading is a process by which responses to new epitopes are developed during an immune response. These new epitopes are not overlapping with the original epitope and may or may not be on the same protein as the

original epitope (Vanderlugt, et al., 1998). This can occur in both the T cell and antibody repertoire. Epitope spreading could follow tissue damage, making new epitopes available for binding, or through the processing of antigen allowing different epitopes to be presented. Epitope spreading of antibodies to several myelin proteins has been correlated with the rate of relapse in three different models of EAE and is thought to proceed in an ordered progression (Lehmann, Fursthuber, Miller, & Sercarz, 1992; Robinson, et al., 2003; Yin, et al., 2001; Yu, Johnson, & Tuohy, 1996). In EAE and at least some MS patients, the original immunodominant epitope loses its capacity to stimulate the immune response, but new epitopes take the place of the original epitope progressing the autoimmune response (Tuohy, Yu, Yin, Kawczak, & Kinkel, 1999). This could be due to the damage in the CNS allowing APCs like B cells to bind new epitopes and present them to T cells, leading to the activation of a new set of autoreactive T cells (Bischof, et al., 2004). The T cells then in turn would activate new autoreactive B cells (Bischof, et al., 2004).

Antibodies specific to conformational epitopes are known to cause demyelination in animal models using the extracellular MOG sequence, while antibodies to linear epitopes do not, which is hypothesized to be because they cannot bind native MOG (Breithaupt, et al., 2008; von Büdingen, et al., 2004; Brehm, Piddlesden, Gardinier, & Linington, 1999). In other models where MOG peptide is used to induce EAE, antibodies to linear epitopes are found to be pathogenic (von Büdingen, et al., 2004). Antibodies binding conformational epitopes have also been found in MS patients, while antibodies to linear epitopes

have been found in both MS patients and healthy controls (Breithaupt, et al., 2008). Conformational epitopes are thought to come first, activating B cells. The B cells activate T cells using linear epitopes, which then activate B cells reactive to these linear epitopes.

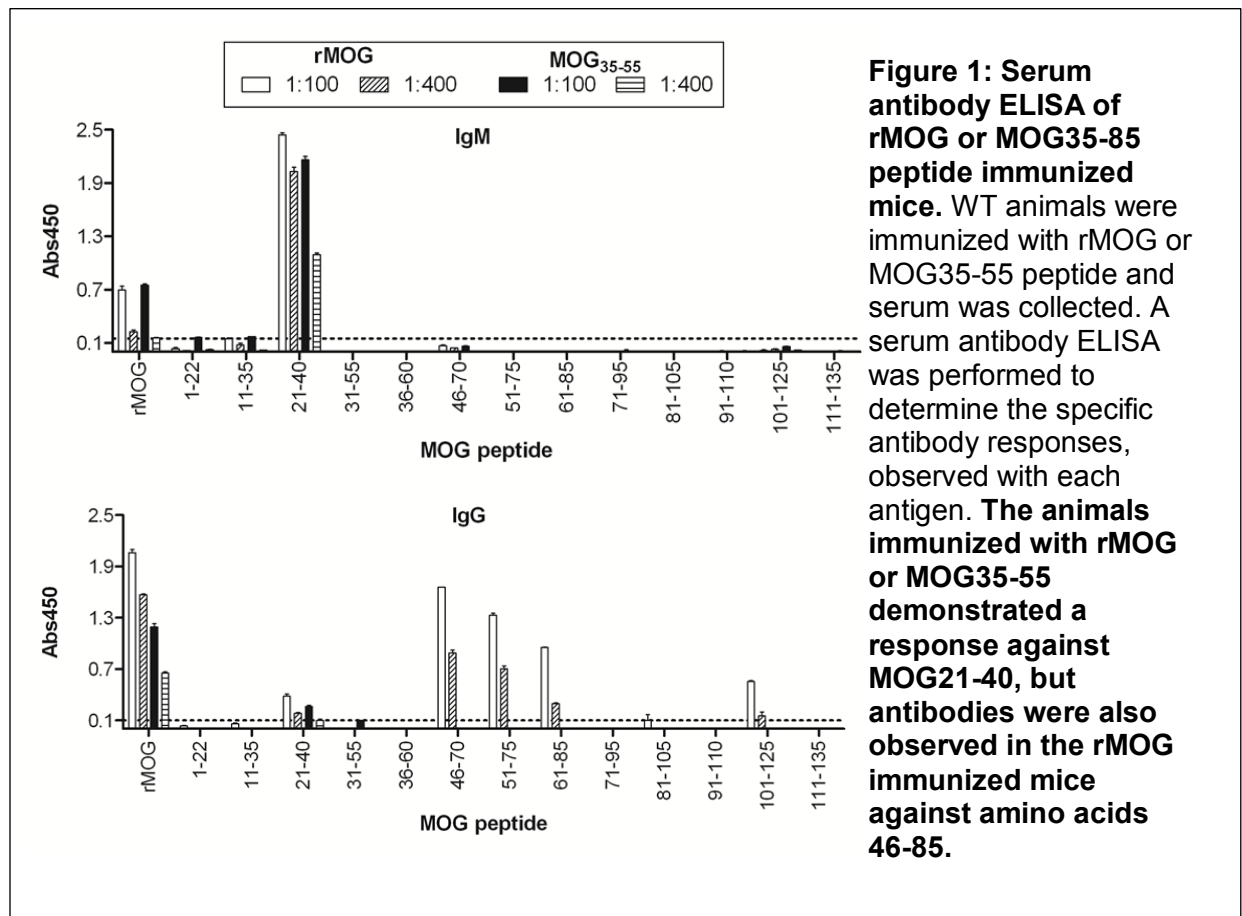
Regulatory B cells

There is a subset of B cells that have properties similar to those seen in regulatory T cells. In EAE, regulatory B cells produce IL-10 in response to toll-like receptor (TLR) 2 and 4 signaling (Ray, Mann, Basu, & Dittel, 2011). TLRs are proteins found on the membrane of cells and in cells that activate the immune response through the binding of conserved structural motifs found in bacteria and viruses. In EAE, regulatory B cells are required for recovery mediated by regulatory T cells (Marín, et al., 2014). Regulatory B cells also regulate the immune response through cell-to-cell interactions with T cell by binding of the B7 and CD80/CD86 proteins (Ray, Mann, Basu, & Dittel, 2011; Mauri, 2010). Unlike regulatory T cells, regulatory B cells do not appear to be defective in MS patients and when stimulated produced significantly more IL-10 than healthy controls (Ray, Mann, Basu, & Dittel, 2011). Furthermore, there is also evidence that direct regulatory B cell interaction with T cells through CD28 and B7 is required for recovery in EAE (Ray, Mann, Basu, & Dittel, 2011; Mizoguchi, Mizoguchi, Preffer, & Bhan, 2000). This interaction may cause the activation of regulatory T cells, since the regulatory T cell response is delayed in EAE when B cells are removed (Ray, Mann, Basu, & Dittel, 2011; Mann, Ray, Basu, Karp, & Dittel, 2012; Matsushita, Horikawa, Iwata, & Tedder, 2010). Additionally, B cell depletion before induction of EAE with MOG35-55 increases disease severity, while

depletion after disease onset decreases disease severity (Yanaba, et al., 2008; Matsushita, Yanaba, Bouaziz, Fujimoto, & Tedder, 2008). This indicates that regulatory B cells are more important early on in EAE.

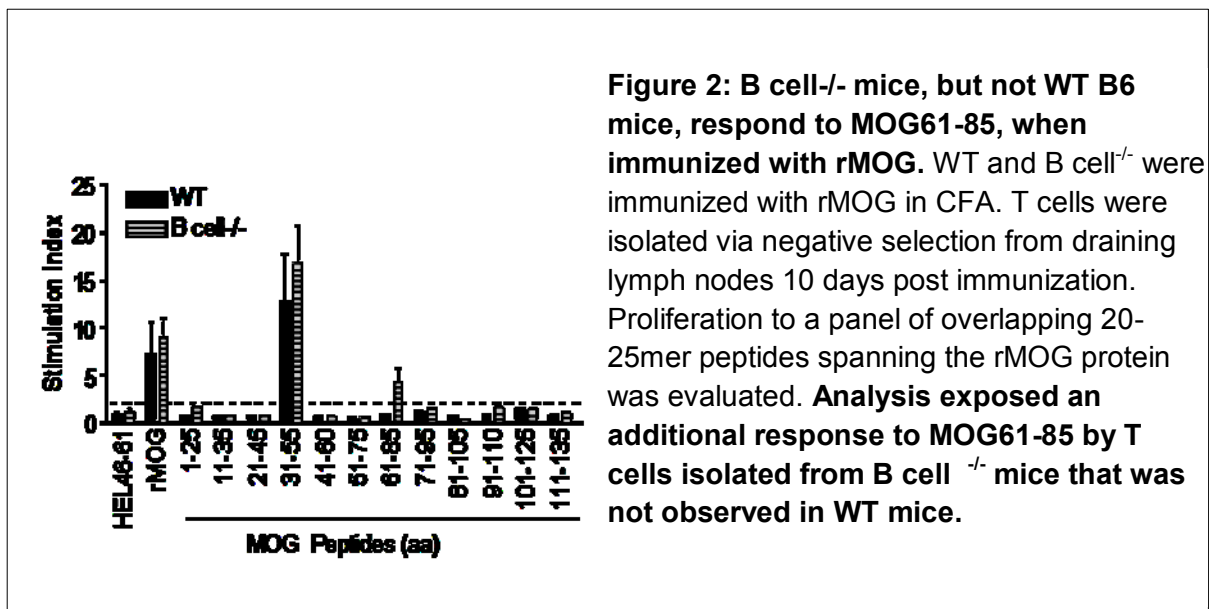
Previous Studies

Our Previous data demonstrated that B cell-deficient (B cell-/-) mice are not susceptible to EAE induction using the whole rMOG protein, but WT mice are susceptible rMOG induction. This indicates that B cells are required for EAE induction using rMOG. But when B cell-/- mice were injected with the MOG35-55, the encephalogenic epitope of the MOG protein, the mice developed EAE like the WT mice (Lyons et al., 1999). We also found that disease could be reconstituted



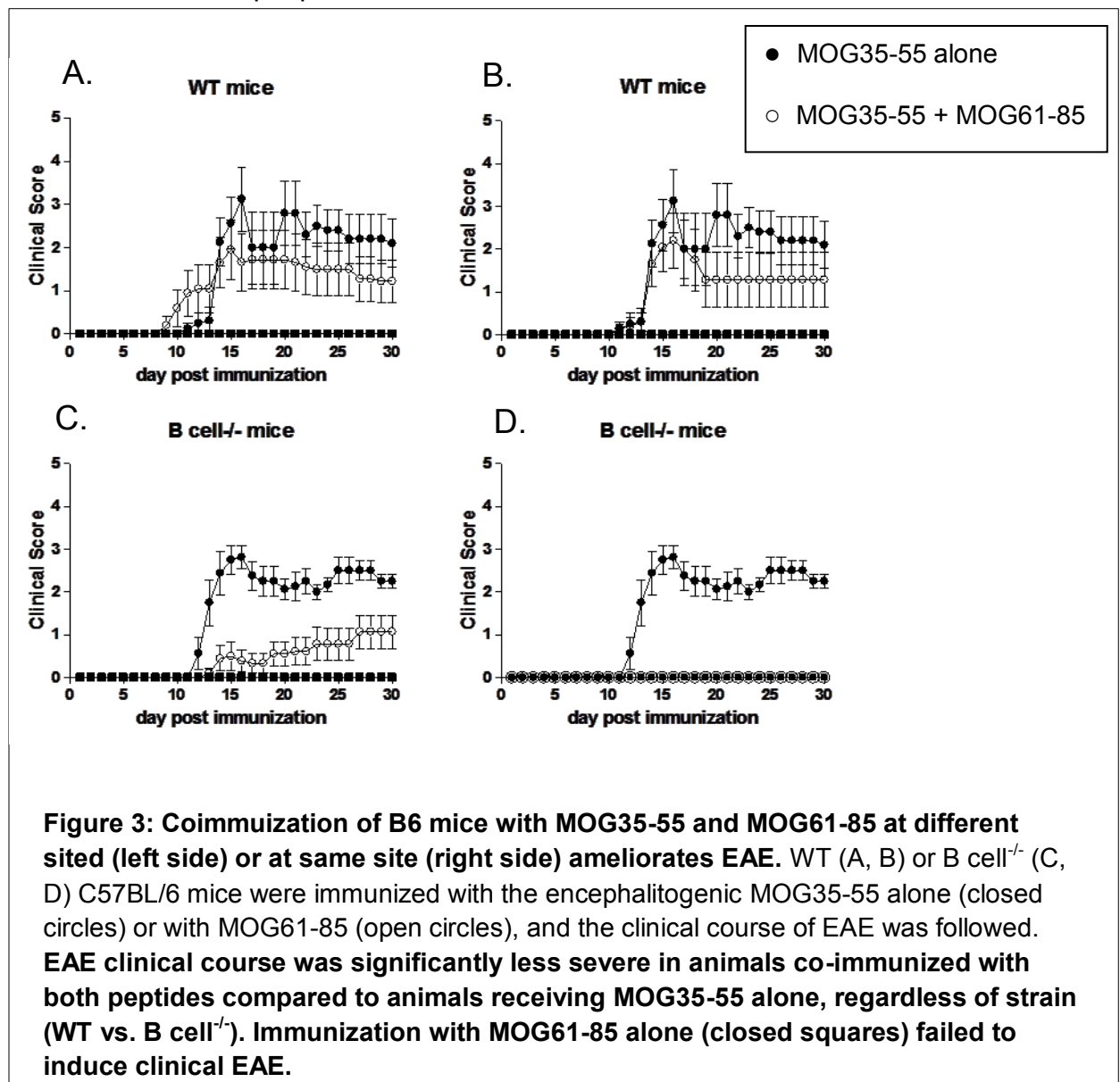
in the B cell^{-/-} through the transfer of B cells or serum from rMOG primed WT mice (Lyons, Ramsbottom and Cross, 2002). Plasma ELISA of the rMOG induced WT mice revealed antibodies to linear epitopes between aa46-85 that were not observed in the MOG35-55 induction (Figure 1) (Lyons et al., unpublished data; Liu et al., 2012).

Next T cell epitopes were studied. The expected encephalitogenic aa35-55 epitope was observed in the WT and B cell^{-/-} mice primed with rMOG. In addition, a novel epitope at aa61-85 was observed in B cell^{-/-} but not WT mice (Figure 2) (Lyons et al., unpublished data; Liu et al., 2012). When B cell^{-/-} mice were co-immunized with MOG35-55 and MOG61-85 epitopes, disease severity was decreased when compared with a MOG35-55 induction. Similar results were observed in WT mice, which led to the conclusion that MOG61-85 is a cryptic



epitope, which is protective against the development of EAE (Figure 3) (Lyons et al., unpublished data; Liu et al., 2012). This amelioration was observed whether the peptide immunizations occurred at the same or different locations (Figure 3)

(Lyons et al., unpublished data). This was corroborated by EAE induction with MOG35-85 peptide, containing both the protective and encephalogenic epitopes. Immunization of B cell^{-/-} or WT mice with this longer peptide resulted in reduced disease severity and incidence compared to MOG35-55 induction (Figure 4) (Lyons et al., 1999; Lyons et al., unpublished data). This indicates that B cell products have a role in disease initiation and perhaps in regulating the selection of the MOG61-85 epitope.



EAE induction and progression in WT and B cell^{-/-} mice

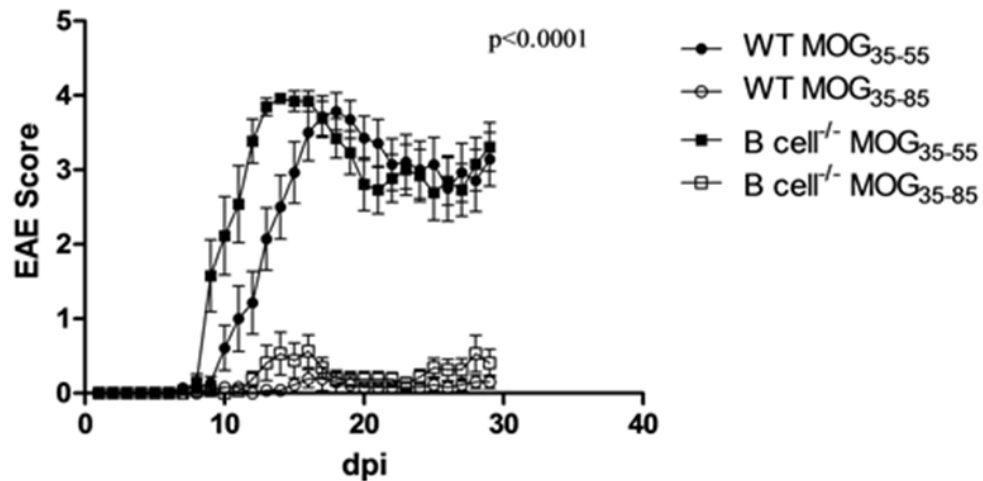


Figure 4: Immunization with MOG35-85 peptide results in a decreased disease severity in WT and B cell^{-/-} animals as compared to the animals immunized with MOG35-55. Animals were immunized with the appropriate peptide and observed for a period of 29 days post immunization for EAE induction and progression. This graph shows the compiled results of all three experiments. **Reduced disease severity in WT and B cell^{-/-} mice demonstrated the protective nature of the longer peptide.**

Hypothesis and Aims

Previous studies in the EAE model have demonstrated that B cells are required when rMOG is used for induction but not with the MOG35-55 induction. Later studies demonstrated B- and T-cell responses unique for specific epitopes in WT and B cell^{-/-} mice immunized with MOG35-55 and MOG61-85. In particular, a cryptic epitope was identified in the 61-85 amino acid sequence which reduced severity of disease in WT and B cell^{-/-} mice.

This work was to determine the effects of serum in disease progression of EAE induction with both MOG35-55 and MOG61-85 epitopes on the disease progression and cellular response to primed serum or purified antibody from primed serum. I hypothesized that antibody specific for linear epitopes of rMOG

in primed serum alter the immune response toward a proinflammatory response.

I addressed this with the following aims.

Specific Aims:

1. Determine the effect of antigen primed serum on disease severity and proliferation in response to antigen. Working Hypothesis: was that the response to the cryptic MOG61-85 epitope is blocked by the presence of serum primed with the MOG35-55 and MOG61-85.
2. Determine the effect of purified antibody on disease severity and proliferation in response to antigen. Working Hypothesis: was that the response to the cryptic epitope MOG61-85 will be blocked by the presence of purified antibody primed with rMOG.

Chapter II: Materials and Methods

Mice

Specific pathogen free, female, WT C57bl/B6 mice and B cell^{-/-} on a B6 background that were 6-8 weeks old were used for these experiments. All mice used were bred in-house from breeding pairs purchased from Jackson Laboratories (Bar Harbor, ME). 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 protocols used were approved by the Institutional Animal Care and Use Committee.

Antigens

The MOG35-55 (MEVGWYRSPFSRVVHLYRNGK), and MOG61-85 (QAPEYRGRTTELLKDAIGEGKVTLRI) peptides were obtained from GenScript (Piscataway, NJ). These peptides were commercially synthesized and then purified by High Performance Liquid Chromatography (HPLC). A 120 amino acid sequence that makes up the extra cellular domain of human MOG, rMOG, was isolated from culture supernatants of High-5 insect cells infected with a recombinant baculovirus expressing the rMOG protein (Devaux, Enderlin, Wallner, & Smilek, 1997).

Immunizations

Mice were immunized with 100 µg of rMOG or 50 µg of MOG35-55 and MOG61-85 each emulsified in incomplete Freund's adjuvant (IFA, MP Biochemicals, Solon, OH) with 300 µg Mycobacterium tuberculosis strain H37RA (TB, Difco Laboratories, Detroit, MI). Emulsions were prepared using Omni

Homogenizer mechanical mixer and were injected subcutaneously at 4 sites, near each limb. 300 ng of pertussis toxin (PT) (List Biological labs, Inc., Campbel, CA) in Phosphate Buffered saline (PBS-200mg/L, KCl-200mg/ml, KH₂PO₄-8000 mg/L, NaOH-1150 mg/ml Na₂HPO₄) was also injected intraperitoneally at the time of immunization and 72 hours post immunization. Mice were injected with 100 µL of heat inactivated serum, from rabbits immunized with MOG35-55 or MOG61-85 in a 1:100 dilution in normal mouse serum, or serum from rabbits before immunization, preimmune serum, at the time of immunization and three days following immunization. The serum was heat inactivated by incubating serum at 55C overnight, this was done for all rabbit serum used. This was done to inactivate complement present in the serum samples, which could harm the mice if the complement was still active at the time of injection. Purified antibody injections followed the same injection schedule as the serum. The antibody was injected as a 1:100 dilution in PBS.

EAE grading

Mice were graded for the signs of clinical disease starting at 10 days post immunization. The mice are graded on a scale from 0-5. Mice receiving a score of 0 are free of clinical signs of disease; a score of 1 reflects a loss of muscle tone in the tail (tail fails to “helicopter” when picked up); a score of 2 indicates mice are unable to right themselves when placed on their backs; a score of 3 signifies paralysis or weakness in one hind limb; a score of 4 represents the paralysis of both hind limbs; a score of 5 indicates the mice are dead or moribund. Mice observed for clinical disease were graded for up to 30 days.

Cell culture

For *in vitro* experiments, mice were euthanized 26-30 days post immunization (dpi). Mice were anesthetized using ketamine (100 mg/ml), and xylazine (300 mg/ml) mixture (1 ml ketamine + 0.15 ml xylazine + 4.6 ml water) injected intramuscularly or inhalation of isoflourane. If serum was to be collected then the mice were pinned down on a tray and cardiac puncture was performed to collect serum and then cervical dislocation was performed. If no serum was collected then after anesthetization cervical dislocation was performed before mice were pinned to tray. The mice were then squirted with reagent grade ethanol and an incision was made between the back legs of mouse then the skin was separated from the peritoneal cavity. The incision was then extended up the front of the mouse and each leg. The skin was then pinned open and squirted with ethanol to remove any loose fur. The lymph nodes (LN) were removed using tweezers and suspended in Hanks Balanced Salt Solution (HBSS). The peritoneal cavity was then opened using scissors and the spleen (SPL) removed using tweezers and suspended in HBSS. The LN and SPL were then homogenized in sterile glass homogenizers and centrifuged to separate the cells from the debris and fat. The SPL also had the red blood cells removed using Lymphocyte Separation Media (LSM;MP Biomedical, Aurora, HO) or red blood cell lysing buffer (Sigma, St Louis, MO). The cells were treated with tyrpan blue (90 μ l of 0.4% tyrpan blue solution + 10 μ l of the cells) and counted using a hemocytometer to determine viability and concentration of cells. 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)] with 10% Fetal bovine serum (FBS) added. The cells were also cultured with the appropriate antigen (MOG35-55, MOG61-85) at a concentration of 20µg/mL, or Con A at 10µg/mL at a cell density of 2.5×10^6 cells/mL. A fourth group was set up with no antigen present as a control. Cells were cultured up to four days at 37° C with 10% CO₂.

Serum collection

Mice were sedated using either isoflourane or a cocktail of molecular grade water, ketamine, and zylazine. The mice were then pinned at each limb on a dissection tray. A cardiac puncture was performed to remove to blood to tubes containing 10µL of EDTA. The blood was kept on ice until all samples were collected. The blood was centrifugated for 3 min 30 seconds at 2500rpm. The serum was pipetted off into new tubes, pooled, aloquoted to 100µL per tube and frozen at -80C until used.

Purification of IgG Antibody

IgG antibody was purified from mouse serum primed with rMOG. The purification was done using magnetic bead purification (generous gift of Dr. Susan Frackman, Promega, Madison, WI). Briefly, the beads were added to the tubes and placed in magnet allowing the storage solution to be removed. The beads were washed using the bind/wash buffer (25mM sodium acetate, pH 6). The beads were mixed and then replaced in the magnet and the bind/wash buffer

removed. Then 50 μ L of sample was added to 50 μ L of bind/wash buffer. This was added to the beads and mixed continually for 30 minutes. The tube was then placed back in the magnet and the supernatant removed and saved, this is the serum in bind/wash buffer minus the IgG. The beads are washed three times in the bind/wash buffer. 50 μ L of Elution buffer (100mM glycine-HCl, pH 2.7) was added to the beads and mixed continually for 5 minutes. The beads were placed in the magnet and the elutant was removed to a new tube and 10 μ L of the neutralization buffer (2M Tris buffer, pH7.5). The elution was repeated. The titer and purity of the elutant was determined using a serum antibody ELISA.

Antigen Presenting Cell Preparation

APCs were obtained from the spleen (SPL) of naïve WT mice. The SPL were collected and prepped for culture as stated above with no antigen. Cells were spun (500g 10min) and resuspended to a concentration of 8.1×10^6 cells/mL. Then 100 μ L of mitomycin C (MP Biomedical, Aurora, OH) was added to the cells. The cells were incubated in a water bath at 37C for 20 minutes, protected from light. Cells were washed 3 times in HBSS with 5% FBS. Cells were cultured in complete RPMI with 10% FBS at the concentration of 8.1×10^6 cells/mL in a 96 well plate, 100 μ L per well and were incubated at 37C with 10% CO₂ for 1 hour. The cultures were mixed and the media removed and discarded. 100 μ L of complete RPMI with 10% FBS was added to the wells. The APCs will adhere to the wells so that the non-APCs were removed with the media after the wells were mixed.

T cell purification

T cells were purified using magnetic beads (STEMCELL, Vancouver, BC).

Lymph nodes (LN) from immunized mice were prepped as stated above. The cells were spun (500g, 10min) and resuspended at a concentration of 1×10^8 cells/mL in 1mL HBSS with 2% FBS. 50 μ L of normal mouse serum was added to the cells. Then 50 μ L of EasySep™ mouse CD4+ T cell isolation cocktail was added and mixed well. This was incubated at room temperature for 10 minutes. While cells incubated EasySep™ streptavidin RapidSpheres™ for 30 seconds. Then 75 μ L of the RapidSpheres™ was added to the cells and mixed well. This was incubated for 2.5 minutes at room temperature. The total volume was brought up to 2mL by adding HBSS with 2% FBS and mixed well. The cells were then placed in the magnet for 2.5 minutes. The tube was then picked up in the magnet and the supernatant containing the purified T cells was poured into a new tube. The cells were recounted using the same method as before. The T cell purity was checked using flow cytometry using CD4, CD8 and CD19 as markers. The percent purity was determined using Flow Jo software. The purified T cells were then plated with the naïve APCs at a concentration of 2.5×10^6 cells/mL with either no antigen; Con A (10 μ g/mL); MOG35-55 (10 μ g/mL); or MOG61-85 (10 μ g/mL). All of these were plated for 48 and 72 hour readings with the CCK-8 protocol along with a set of the cells with a 1:100 dilution of purified IgG antibody read at 72 hours.

Proliferation

Cells were obtained from LN and SPL were suspend in RPMI+10% FBS in tubes to bring the cell starting concentration to 2.5×10^6 cells/mL. The antigens were

added to the cells at the appropriate concentrations. The cells were then plated in duplicate in a 96 well plate 100µL per well. Wells with just RPMI+10% FBS were used as a control. CCK-8 stain (Mayflower, St Louis, MO) was added (10µL) per well and incubated at 37° C with 10% CO₂ for four hours. The optical density was measured using a Synergy™ HT Multi-Mode Microplate Reader (450nm; Biotek, Winooski, VT). The cells were cultured for 48, 72, or 96 hours. The standard used to determine cell concentrations at the time the optical density was read was read at 24 hours. The standard cells were cultured with no antigen and diluted 5 times using a 1:2 dilution starting at 2.5x10⁶ cells/mL as the top of the standard.

Serum Antibody ELISA

The specificity of the serum collected was determined using a peptide antibody ELISA. Peptides spanning the length of the rMOG protein were used to verify the antibody specificity of IgG and IgM was consistent between collections. 96 well plates (Corning Costar 9018) were coated using rMOG as a positive control and just the bicarbonate coating buffer (sodium carbonate, sodium bicarbonate, sodium azide, in deionized water pH9.6) as a negative control. Each of the peptides was diluted to 10µg/mL in the coating buffer. These were incubated over night at 4C. The wells were washed four times in a solution of PBS and 0.05% Tween 20. Blocking buffer (1.5g of BSA in 50mL of PBS) was added. This was incubated for 2 hours. The plate was washed four times. The serum was then added to the wells in two dilutions, 1:100 and 1:400, for each peptide and immunoglobulin. The serum was diluted in dilution buffer (0.05% PBT, 0.5%BSA

in PBS). This was incubated for one hour before washing four times. The secondary antibody specific to either murine IgG or murine IgM was diluted to 1:40,000 or 1:20,000, respectively, and added to the wells. This incubated for one hour. The plate was then washed once more and TMB substrate solution from BD bioscience. The plate was incubated for 15 minutes before the stop solution (2.5N sulfuric acid) was added to the wells before reading on the Synergy™ HT Multi-Mode Microplate Reader (450nm and 562nm; Biotek, Winooski, VT).

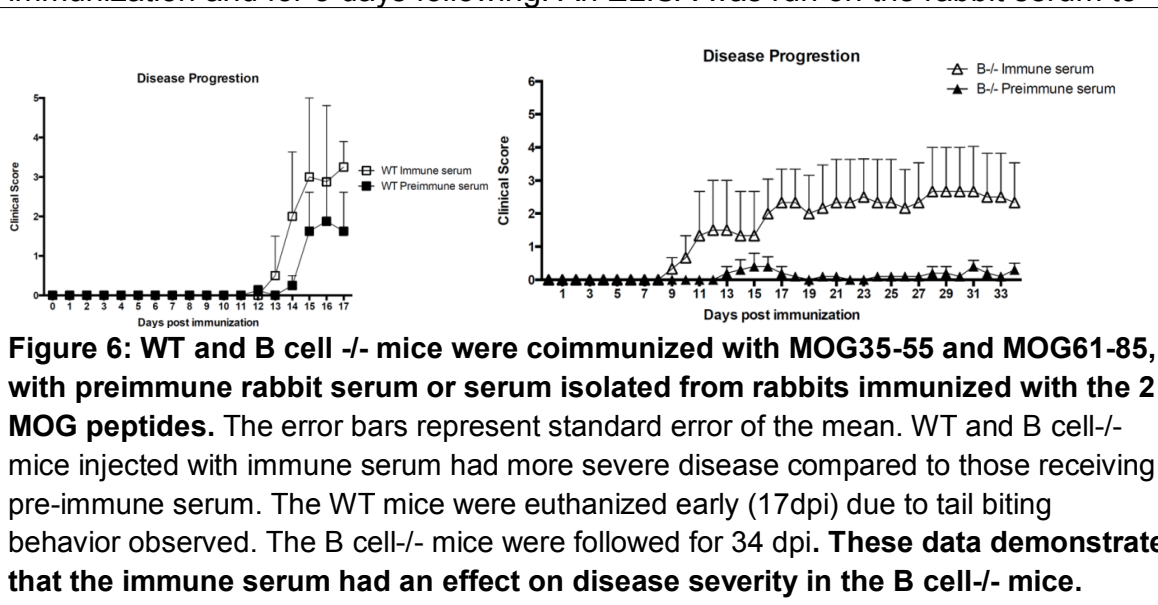
Statistics

All statistics were run using the computer program prism graphpad. All p-values were set to 0.05 for significance. Differences in disease severity were determined by a two-tailed T-test of the area under the curve for all mice in each group. Day of onset was day first clinical signs of disease were observed no number was given if mouse never displayed signs of disease. Proliferation experiments were tested for significance using two-way ANOVA.

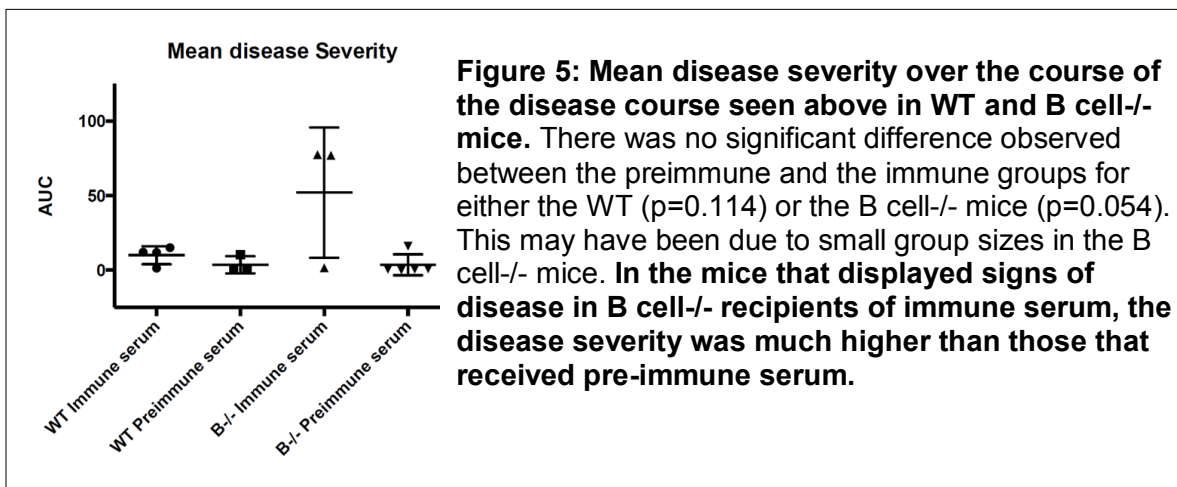
Chapter III: Results

WT and B cell^{-/-} mice were coimmunized with MOG35-55 and MOG61-85.

The mice were given two PT injections, at the time of immunization and day two post immunization. Preimmune or immune serum was injected on day of immunization and for 3 days following. An ELISA was run on the rabbit serum to

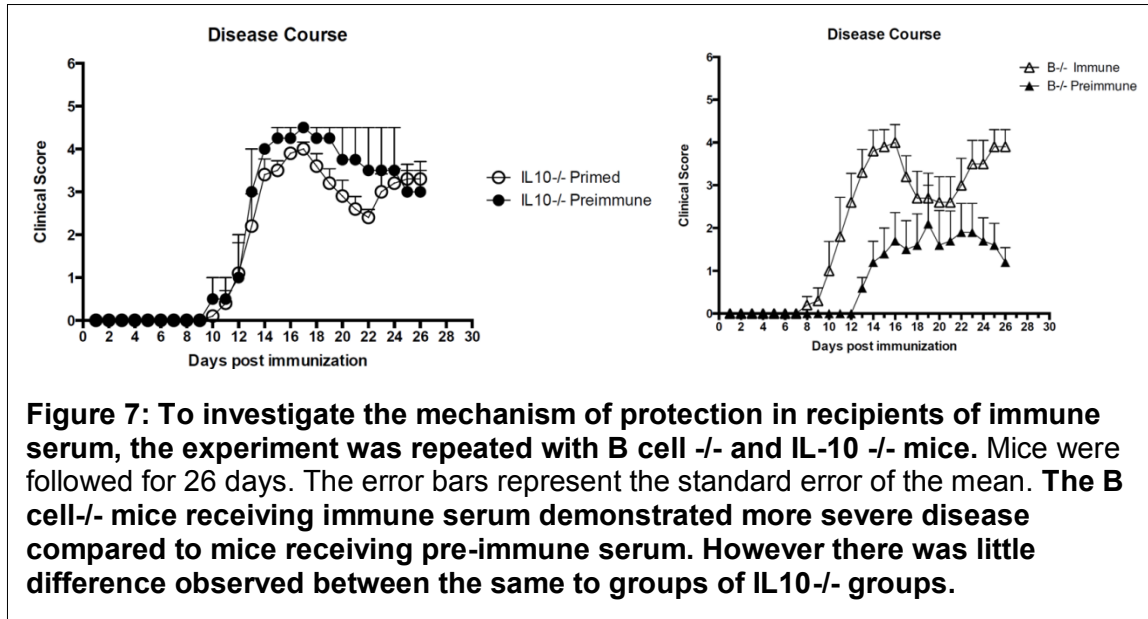


verify the specificity of the antibodies in the serum. The mice were followed for clinical signs of disease. Both WT and B cell^{-/-} mice injected with the immune



serum showed more severe signs of disease compared to mice given the preimmune serum in the B cell^{-/-} groups but not enough of the preimmune B

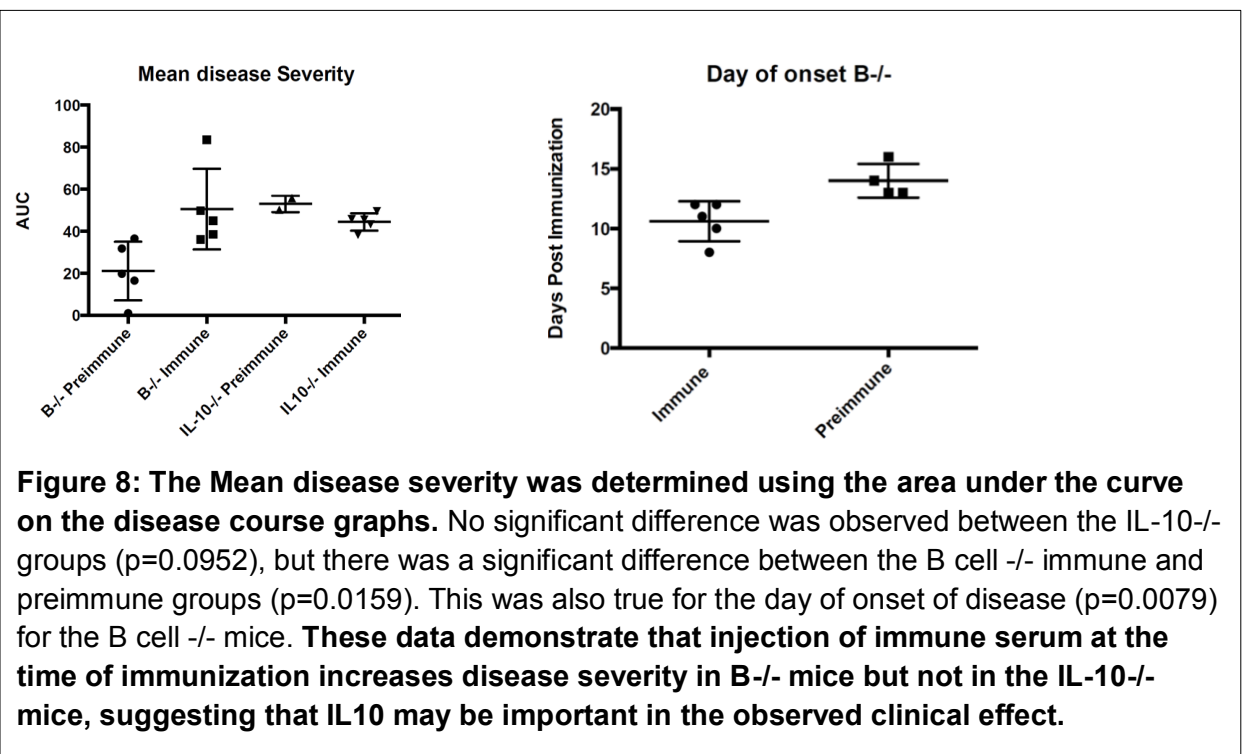
cell-/- mice became sick to run a t-test (figure 5). This may be due to the introduction of primed antibody to the immune system allowing the adaptive immune response to be activated earlier in the disease



course. The day of onset was not significant in the WT mice (p-value=0.99). This may have been because the WT mice produce their own antibody response. The B cell -/- mice in the immune group had severe disease, while the preimmune group had only minimal disease severity and incidence. The disease mean disease severity was higher in the immune groups compared to the preimmune groups in both the WT (p-value=0.114) and the B cell -/- (p-value=0.054) (figure 6). Significance was not reached in either group but both tended toward significance and the group size was small. Both groups of WT mice were sacrificed 17 dpi due to tail biting observed in the immune serum group. Serum antibody ELISAs were run on serum taken from the WT mice to determine if the rabbit antibodies could still be found. I was able to observe the presence of rabbit IgG in the serum of the WT mice in the immune group. B cell -/- mice were not

sacrificed until 34 dpi serum, spleens, and lymph nodes were collected. The Lymph nodes from the preimmune B cell $-/-$ group were observed to be larger than expected from B cell $-/-$ mice.

IL-10 deficient (IL-10 $-/-$) mice and B cell $-/-$ mice were coimmunized with MOG35-55 and MOG61-85 as previously described. These mice were also split into immune and preimmune serum groups. These mice were followed for 26 dpi for clinical signs of disease (Figure 7). The day of disease onset was significantly different in the B cell $-/-$ mice in the preimmune and immune groups (p-value=0.0079) (Figure 4). No significant difference in the day of onset was observed in the IL-10 $-/-$ mice (p-value=0.7919). The mean disease severity was significantly higher in the B cell $-/-$ immune mice compared to the preimmune group (p-value=0.0159) (Figure 8). The IL-10 $-/-$ mice mean severity did not reach significance comparing the immune and preimmune groups (p-value=0.0952). Once again the Lymph nodes from the B cell $-/-$ mice in the preimmune group were observed to be larger than expected.



The Lymph nodes and spleens were homogenized and cultured for CCK-8 testing at 48, 72, and 96 hours, with no antigen, Con A, MOG35-55, MOG61-85, or both MOG35-55 and MOG61-85 (Figure 9). The absorbance was measured to determine the proliferation in response to the stimuli. Proliferation was observed to all three MOG cultures but the proliferation was higher in the preimmune group. The difference in proliferation did not reach significance possibly due to the small number of mice in each group. This along with the observation of the larger than normal lymph nodes in the preimmune group led to the conclusion that MOG61-85 presentation leads to the trapping of T cells in the lymph nodes stopping their migration to the CNS.

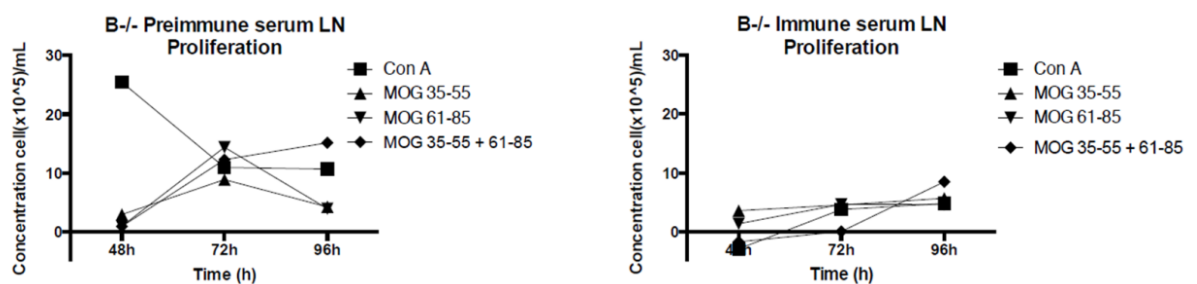
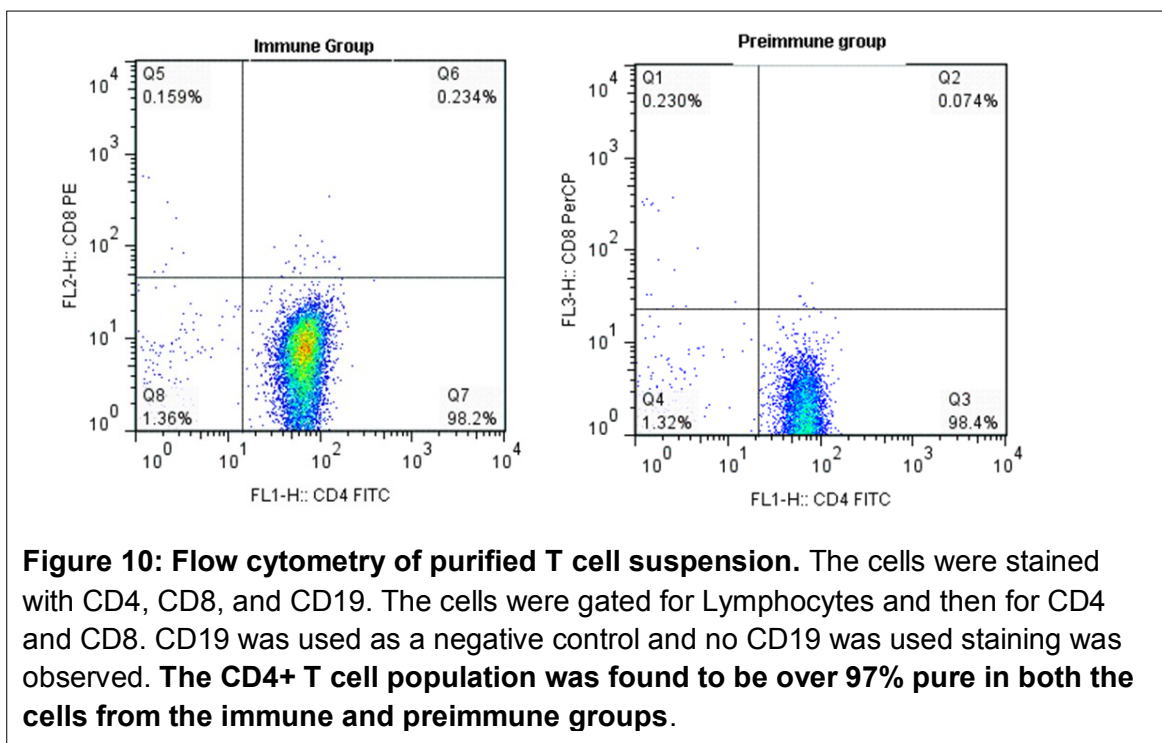


Figure 9: The immune response to MOG35-55 and MOG61-85 in mice receiving pre-immune or immune serum at the time of immunization was investigated using the CCK-8 proliferation assay. Lymph node (LN) cells were isolated from B cell-/- mice receiving pre-immune or immune serum and cultured with the indicated peptides *in vitro* for 48, 72, or 96 hours. Con A was used as a control for cell viability. **Increased proliferation was observed by cells from mice receiving pre-immune serum 72 hours for the MOG35-55 and MOG61-85 groups, and for the MOG35-55+61-85 the same between the immune and preimmune but the preimmune group was higher at 72 and 96 hour but this did not reach significance. This suggests that the immune serum may be directing the development of the T cell response, thus affecting disease severity in the EAE model.**

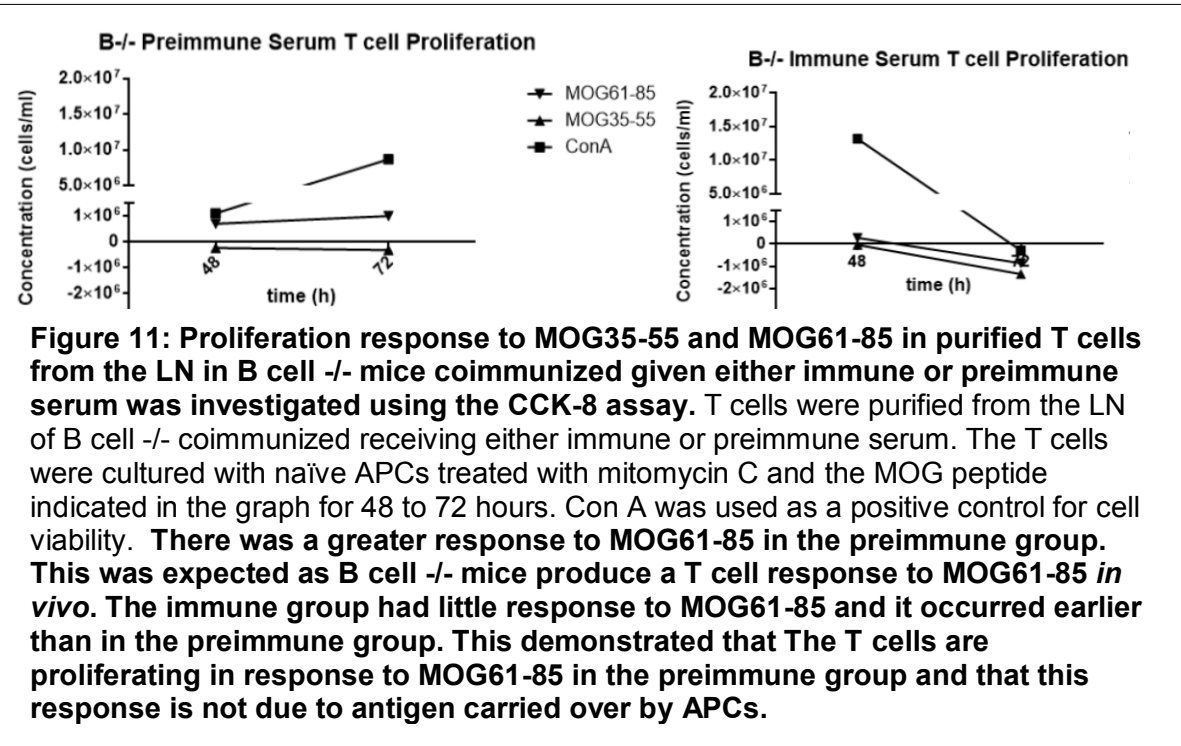
To investigate the response of T cells to MOG35-55 and 61-85 with naïve APCs, SPLs were collected from naïve WT mice and treated with mitomycin C to stop the cells from proliferating. To ensure the culture only contained APCs the cells were incubated for an hour at culture conditions, then the cultures were

mixed and the media removed. This allowed the APCs to adhere to the culture plate so that only the T and B cells were removed with the media. The APCs were then incubated over night at culture conditions in RPMIc with 10% FBS. The next day LNs were collected from B cell -/- mice coimmunized given either immune or preimmune serum. The LNs were prepped for culture and then T cells were purified from the cell suspension. The T cells were checked for purity using flow cytometry staining for CD8, CD4, and CD19. Purity was determined to be

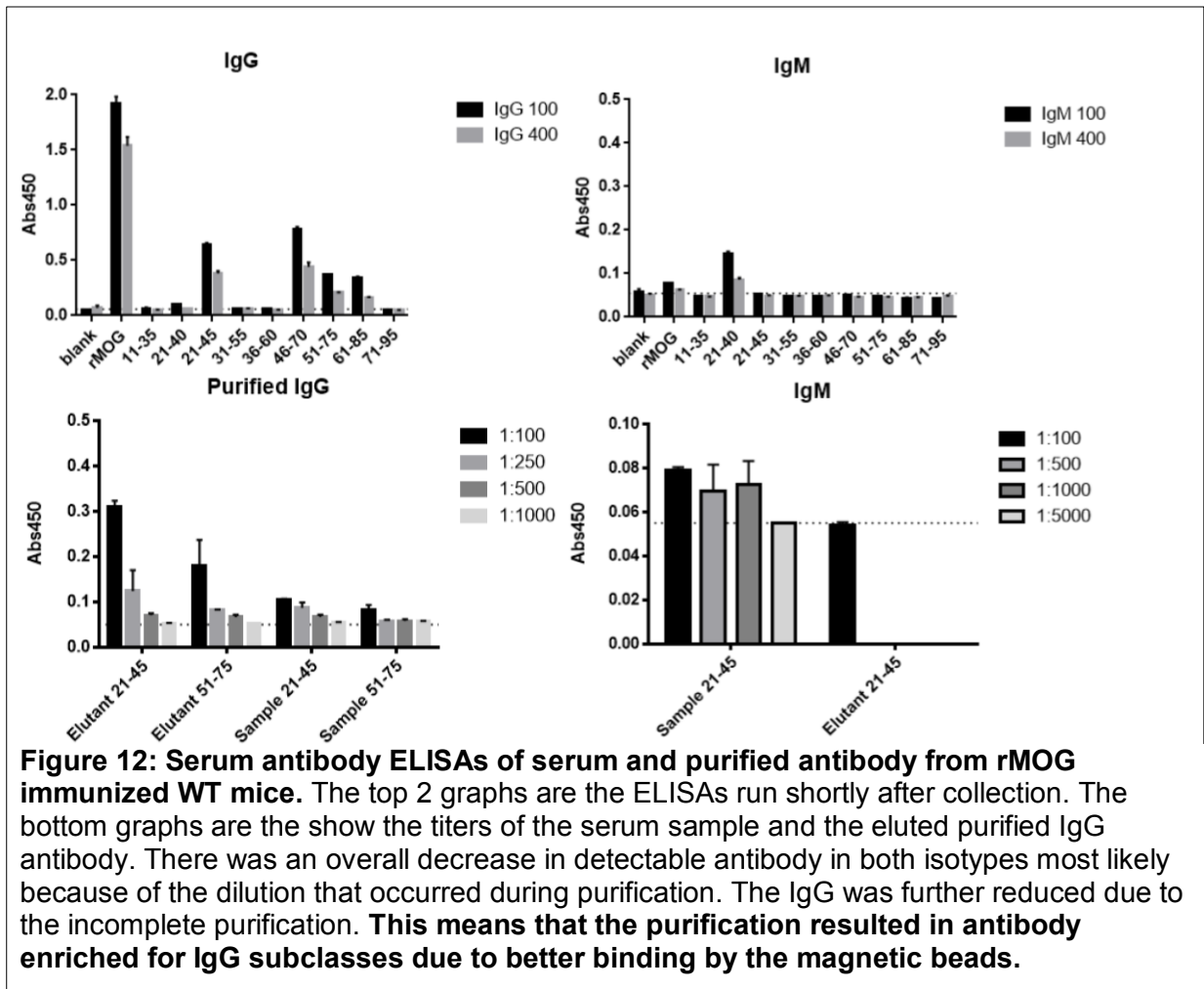


greater than 97% (figure 10). Purified T cells were cultured with the mitomycin C treated APCs. These were cultured for 48 or 72 hours. At 24 hours some of the cells were treated with purified IgG from rMOG primed mice which were read at 72 hours. The purified antibody was tested with an ELISA to determine purity. The ELISA showed that only IgG was in the eluent and that this was enriched for IgG. There was little IgM seen even in the serum after the IgG was purified from

it, though some of the IgG remained behind. These cells were read with the CCK-8 dye. I found a higher response in the preimmune group to MOG61-85 at both the 48 and 72 hour time point (Figure 11). There was very little if any response to the MOG35-55 this may be due to the number of freeze thaws the peptide underwent. This experiment demonstrated that there is a T cell specific

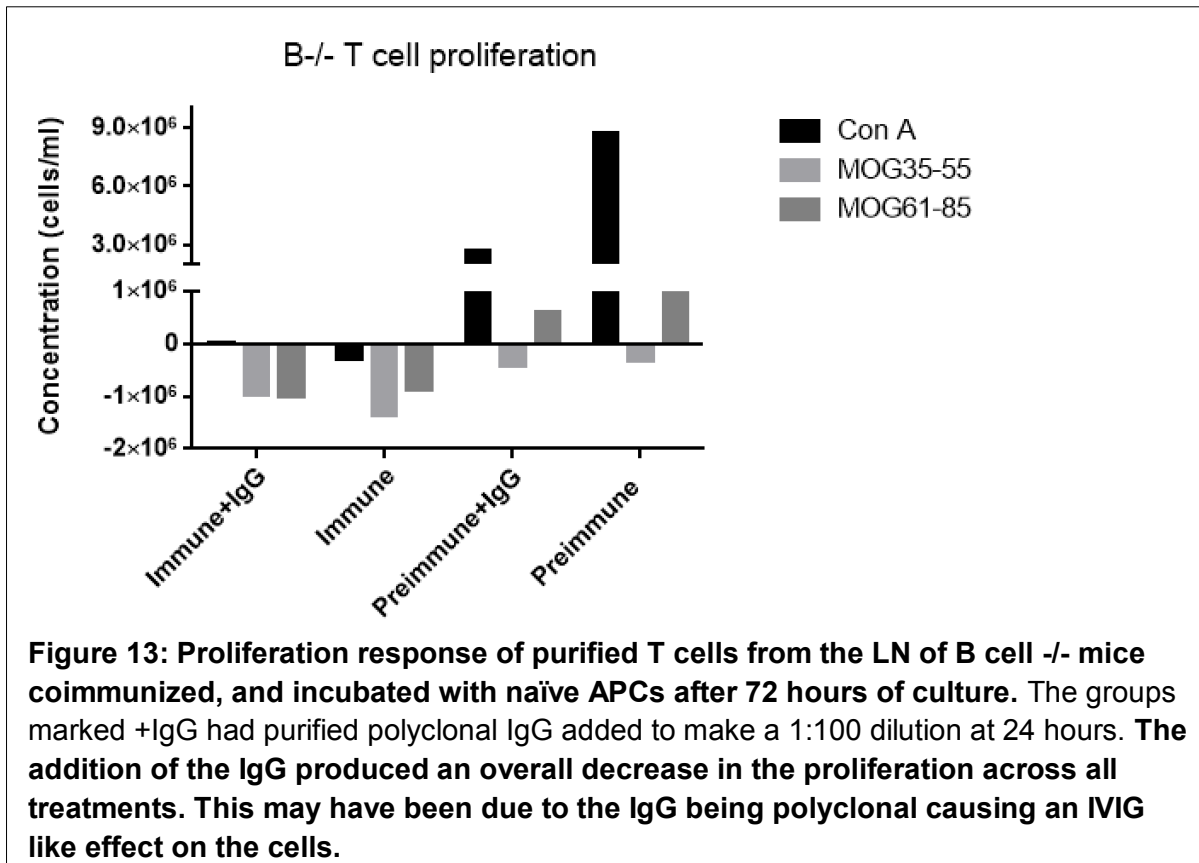


response to MOG61-85 in the preimmune group that is lost with the addition of the immune serum. The proliferation seen also shows that this response is not due to the carryover of antigen in APCs from the *in vivo* response as the APCs used were naïve and did not contribute to the proliferation observed due to the treatment of mitomycin C. Some of the wells had purified IgG from rMOG primed WT mice added to the wells at 24 hours; these were read at 72 hours. The IgG was tested for purity using ELISA and this was also used to determine the titer of the antibody (figure 12). The titer of the purified IgG was



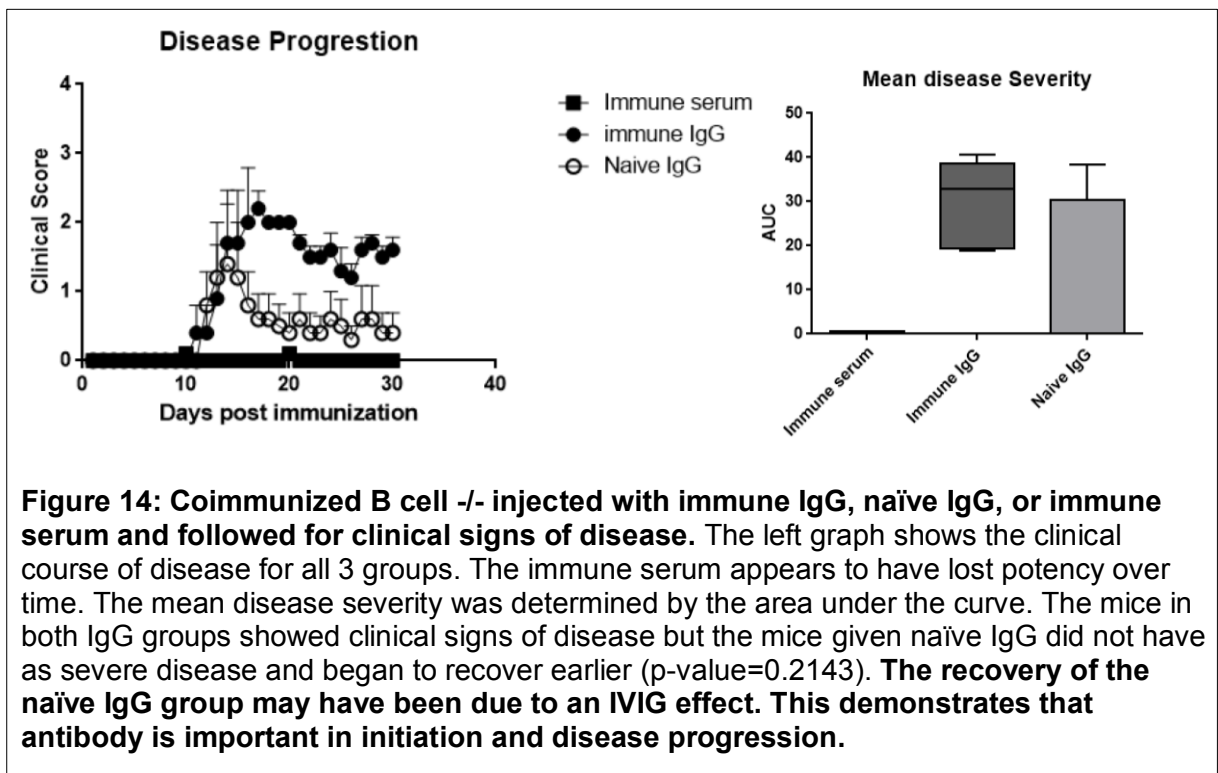
determined to be about 1:500 while the IgM left in the sample was 1:1000 but the IgM did not dilute out as expected. The purified IgG was free of IgM but the serum sample still had IgG though much less than the purified IgG of the elutant. This may mean that the purification beads bind some IgG subclasses better than others making the purified IgG enriched with those subclasses. It is also important to note that the antibody and serum was diluted 1:2 in the purification process. There was also a reduction in the absorbance measured in both IgG and IgM was reduced overall. This is most likely due to the purification process and possibly the freezing and thawing of the samples. The addition of the

antibody was expected to increase the response to the MOG35-55 while



decreasing the response to MOG61-85. This was not observed, instead there was an overall decrease in the response observed in the wells treated with IgG (Figure 12). This may have been because the IgG was polyclonal and not specific for MOG peptides. The polyclonal IgG may be producing a regulatory response, which may be similar to that of Intravenous immunoglobulin (IVIG). IVIG produces a regulatory response and is used to treat many diseases including autoimmune and inflammatory diseases. This means that to produce the expected proinflammatory response the antibody may need to be purified down to those antibodies specific to MOG61-85 and MOG35-55.

To determine if the same response would be observed *in vivo* mice were injected with IgG purified from the serum of WT mice immunized with rMOG. IgG from naïve mice was used as a negative control using the same purification method as the primed IgG. Rabbit serum was used in earlier experiments was used as a positive control. The antibody and rabbit serum was diluted to a 1:100 solution in PBS, or normal mouse serum respectively and injected in the coimmunized B cell $-/-$ mice at the time of immunization and the following 3 days. The mice were followed for clinical signs of disease (figure 14). The day of onset



was not significant comparing the immune IgG and naïve IgG. The disease severity trended toward significance between the 2 groups (p-value=0.2729). The mice in the immune serum showed no signs of clinical disease this may be due to a loss of potency of the serum because of the length of time since the serum had been defrosted. Both the immune IgG and naïve IgG groups started to show

signs of disease about the same time but naïve IgG group began to recover faster than the immune IgG group. This may mean that antibody is important in the initiation disease but specific antibody is important for progression of disease. It is also possible that the naïve IgG produced an IVIG effect leading to the recovery of the mice that did show clinical signs of disease.

Together this data demonstrates that in EAE the protective effects of MOG61-85 produced in B cell -/- mice coimmunized with MOG35-55 and MOG61-85 is blocked by the addition of B cell products in serum, specifically antibody. Evidence here suggests that IgG, possibly a specific subclass of IgG, is altering the T cell response in B cell -/- mice most likely by affecting the processing and presentation of MOG protein causing the MOG61-85 epitope to be suppressed in WT mice. I also found evidence of how MOG61-85 may be stopping the pro-inflammatory response. LN in the B cell-/- mice given the preimmune serum were larger than that of the mice injected with immune serum, which indicates that MOG61-85 may act by sequestering the T cell in the LN. This supports other work in this lab suggesting that MOG61-85 induces the production of TGF- β , which can alter immune cells ability to migrate (Wright, et al., 2003).

Chapter IV: Discussion and Conclusion

Discussion

This lab previously found that when EAE is induced with rMOG, B-/- mice do not show clinical signs of disease, while disease severity increased to be similar to that of the WT mice when immunized with MOG35-55 alone. This demonstrated that B cells are required for rMOG induction of EAE in this model. Another observation in rMOG induction was that B-/- mice produce T cells specific to MOG61-85, which are not found in WT mice immunized with rMOG. WT mice were found to produce antibodies to the MOG61-85 epitope. This lab has also shown that mice coimmunized with the encephalogenic epitope, MOG35-55, and the protective MOG61-85 epitope have reduced clinical disease in WT and B-/- mice. When I immunized mice with both peptides and injected primed rabbit serum, disease severity increased. This further indicated that the change in clinical disease is caused by products produced by the B cells present in the serum. The most likely candidate for this product is antibody. This also demonstrated that it is most likely a response to MOG61-85 that is causing the change in EAE severity because the severity of disease increased when serum was added that was primed to MOG35-55 and MOG61-85 but not serum from naïve rabbits indicating that antibody to other peptides was not involved in this response.

It is accepted that antibodies to conformational epitopes of myelin proteins contribute to demyelination in MS and in some models of EAE (Lucchinetti, et al., 2000; Villar, et al., 2005; von Büdingen, et al., 2004; Piddlesden, Lassmann,

Zimprich, Morgan, & Linington, 1993; Dharmasaroja, 2003). Our analysis demonstrated antibodies specific for linear epitopes in WT mice immunized with rMOG also play a role. The presence of these antibodies was consistent between serum samples over several different experiments, and were consistently demonstrated to be predominantly of the IgG subclass. This was also true of the slight difference observed in the binding of the IgG and IgM in the peptides covering the MOG35-55 epitope. The IgM bound MOG21-40 and the IgG bound MOG21-45. This could just be an alteration in the binding between isotypes but this may be important for how the antibodies affect disease in EAE. In fact the only time these results significantly differed was when the serum sample was from mice only 11dpi. In this case the antibodies spanning peptides covering amino acids 46-85 were IgM antibodies instead of the IgG previously seen. This indicates that isotype switching occurs early on in disease progression around when the first signs of clinical disease can be observed. This important to note since this occurs early on in the progression the class switching may be important for how antibody is affecting the presentation of MOG61-85. This has been observed in experiments investigating the processing and presentation of tetanus toxoid and hen egg lysozyme are used as antigen (Simitsek, Campbell, Lanzavecchia, Fairweather, & Watts, 1995; Amigorena, et al., 1998). This will require a careful study of the time-course of the development of the antibody response with rMOG immunization. Another possible study would be to determine if the MOG35-55 and MOG61-85 antibodies work together or independently in disease.

When clinical disease was observed in WT mice, mice in the immune group had more severe disease than that of the preimmune group. While this did not reach significance it demonstrates a trend that may have continued to reach significance, if the biting behavior had not occurred or if repeated as it represents a trend that was also observed in the B-/- mice. In the B-/- mice the disease severity did reach significance when repeated. The B-/- mice also show a significant difference in the day of onset of clinical disease not observed in the WT mice. This may be due to the lack of B cells and therefore antibody in the B-/- mice. The antibody response in WT mice develops quickly so that the addition of immune antibody does not affect disease onset just the severity of disease. While in B cell-/- mice the addition of antibody that would not normally be present cause clinical signs to develop earlier and increases severity of disease. The rabbit antibody was observed to still be present in the serum of WT mice 17 dpi. Further tests should be run to determine if this is also true in the B-/- mice.

I also looked at the proliferation in cell culture to determine the effects of serum on the response of immune cells to MOG35-55 and MOG61-85. While these results were not significant, a trend can be observed in the responses in the immune and preimmune groups, which may become significant when repeated. There was more proliferation in the preimmune group compared to the immune group in both the MOG35-55 and MOG61-85 conditions at 72 hours but by 96 hours the numbers had dropped back down to similar to the levels seen in the immune group. This could indicate that the activated cells are sequestered in the LN in the preimmune group, while in the immune group these cells have

migrated to the CNS by the time the LN were collected. Another indication of this was the observation that the LNs in the preimmune group were larger than those in the immune group. This could be determined by comparing the lymphocytes in the CNS and the periphery. This would make sense, since previously this lab found that IL-10 is not involved in the protection produced by the MOG61-85 epitope and the next cytokine candidate currently being investigated is TGF- β . TGF- β is known to be able to alter cell migration in several organs including bone marrow, gut, and endothelium (Wright, et al., 2003; Zhang & Bevan, 2013; Godefroy, Guironnet, Jacquet, Schmitt, & Staquet, 2001).

Although there was no significant difference observed in the clinical disease course in IL-10^{-/-} mice when injected with immune or preimmune serum at immunization, this could be due to the small number of mice in these groups and may reach significance if repeated. One difference to note in the IL-10^{-/-} mice was that the preimmune group had more severe disease than that of the immune group. This indicates that IL-10^{-/-} is not a factor in the effect produced by the MOG61-85, which is consistent with all the other data collected by this lab that indicates that IL-10 is not important in the MOG61-85 response. Another test that could help determine if IL-10 is important would be an IL-10 ELISA on cell culture supernatants and previously when ELISAs were run on cell culture supernatants only very low amounts if any IL-10 were present.

When the proliferation of T cells was measured from cultures with naïve APCs, I found the preimmune group had a stronger response to MOG61-85 at both the 48 and 72 hour time points than the immune group. There was only a

small response to MOG61-85 in the immune group. The small response to MOG61-85 observed in the immune group could be explained by results previously demonstrated by this lab that when the peptides are injected in a single emulsion there is a response produced to both peptides in both WT and B-/- mice (Liu, Mulli, Agashe, & Lyons, 2012). There was no response to MOG35-55 in either the preimmune or immune group. This may have been due to the high number of freeze thaws that the peptide had gone through. This could have caused the peptide to denature losing the reactivity in culture.

At 72 hours culture there were also wells with purified IgG added to some of the wells these were compared to the wells without IgG added. The wells with the IgG added had a decreased response to both peptides and the positive control Con A. This could be explained by the IgG producing a regulatory response similar to that of intravenous immunoglobulin (IVIG), is a polyclonal pool of all immunoglobulin subclasses. IVIG is used to treat autoimmune and inflammatory diseases and is known to induce regulatory T cell expansion and enhance their suppressive capabilities (Ballow, 2011; Maddur, et al., 2010). This may explain the reduction in proliferation to all stimuli observed in the cultures with IgG added. This may also mean that antibodies may need to be further purified to exclude all but those specific to MOG35-55 and MOG61-85 to see the expected results in cell culture.

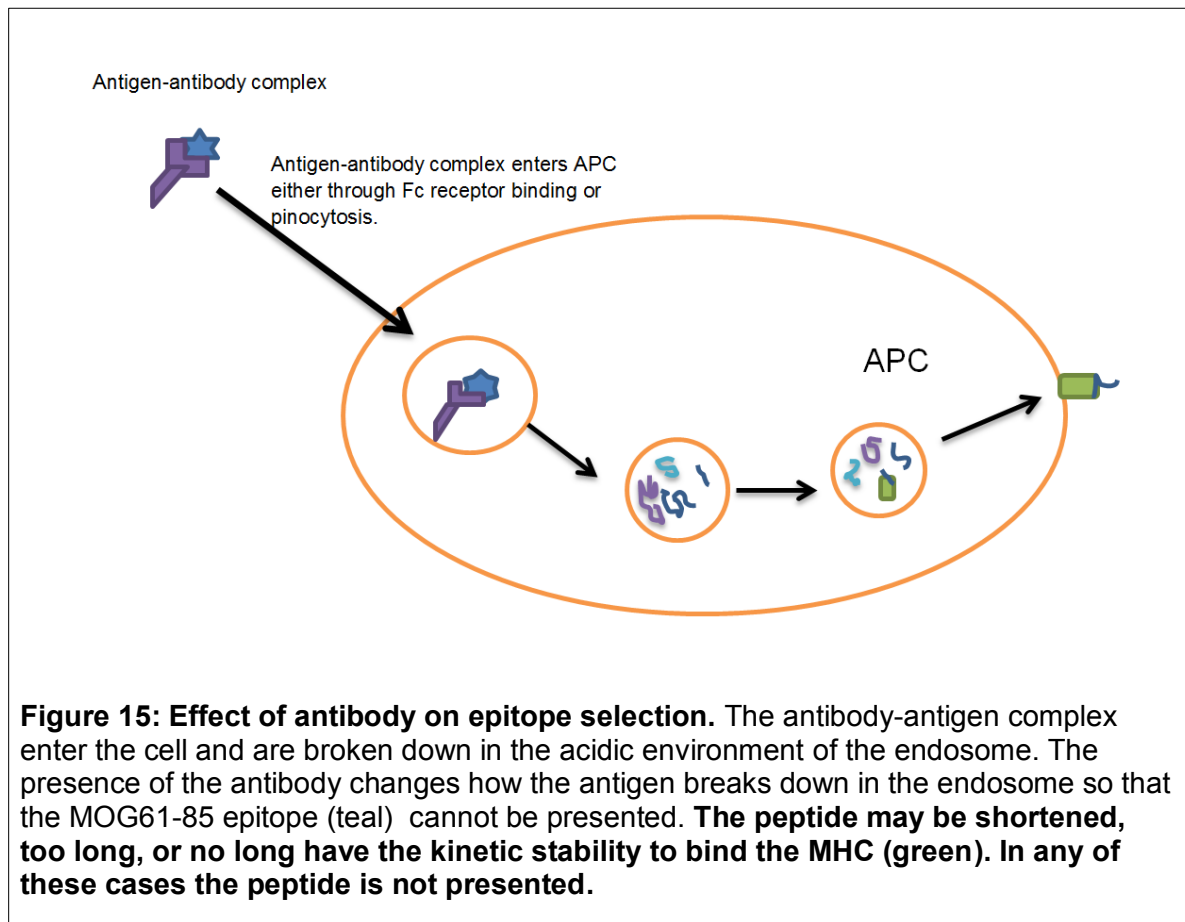
To determine if this effect would be observed *in vivo* mice were coimmunized and injected with purified IgG from rMOG primed serum or naïve IgG from WT mice. The mice given the immune IgG showed more severe

disease than the naïve IgG group. Furthermore, the naïve IgG group recovered faster than the immune IgG group. This may have been due to the same IVIG effect observed in the cell cultures. The disease course of the mice also indicated that antibody is important for disease initiation but also for disease progression in this model of EAE. The antibody used in this experiment was of the IgG isotype but based on the results of the ELISA analysis is most likely has more of some subclasses of IgG than others due to the incomplete purification of the serum sample. According to the Promega protocol the magnetic beads bind most strongly to the IgG1 and IgG2A subclasses but also bind IgG2B and IgG3. The subclasses present in both the sample and the eluted antibody should be determined to give a better indication of the mechanism of action of the IgG, since different subclasses are known to promote or inhibit epitope selection (Vidarsson, Dekkers, & Rispen, 2014). The role of IgM in the initiation of disease should also be ruled out in future studies.

Conclusion

Together these studies indicate that IgG is altering the processing and presentation of MOG protein in B6 mice. Alfonso et al. demonstrated that the addition of specific immunoglobulin to cultures inhibited the presentation of epitopes known to be presented in the absence of immunoglobulin (2003). I hypothesize that this occurs when APCs take in antigen bound by antibodies specific to MOG61-85 epitope through pinocytosis or Fc receptor binding. The suppression of epitopes in other models has not been able to be blocked by any available knock-out (Heyman, 2014). This gives credence to pinocytosis as the

entry method of the antigen-antibody complex over the Fc receptor. Though it does not rule out that more than one entry route is used. Once the antigen-antibody complex is taken in to the APC the complex moves to the endocytic compartment. Here the complex is exposed to a low pH environment. It is possible that the bound antibody affects the breakdown of MOG protein into epitopes in the endosome. Another possibility is that the antibody affects the peptides, produced in the endosome, ability to bind to the MHC. I think the alteration of peptide production is more likely since antibody is not stable in acidic environments. The altered MOG61-85 peptide may be too short, too long or not have side chains that allow it to create a stable binding with the MHC molecule. So that when the epitope tries to bind to the MHC the regulator protein MHC-DM removes MOG61-85 from the MHC because the binding is not stable enough. Meaning that MOG61-85 is not presented to T cell only MOG35-55 is presented leading to a proinflammatory response.



Chapter V: References

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