Published in final edited form as:

J Neuroimmunol. 2016 November 15; 300: 66–73. doi:10.1016/j.jneuroim.2016.06.006.

Amelioration of EAE by a Cryptic Epitope of Myelin Oligodendrocyte Glycoprotein

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Abstract

Previous work demonstrated that EAE induced by recombinant human MOG was B cell-dependent. Data presented here reveal a T cell response to MOG61-85 in human rMOG-immunized B cell^{-/-} mice not observed in WT mice. Further study revealed this peptide to be a cryptic epitope in WT mice. Co-immunization of B cell^{-/-} mice with MOG35-55 and MOG61-85 peptides led to less severe disease compared to mice immunized with MOG35-55 alone. Disease amelioration was associated with decreased production of Interferon- γ by lymph node cells. Thus, MOG61-85 represents a protective epitope to human rMOG induced EAE in B cell^{-/-} mice.

Graphical Abstract

Keywords

Experimental Autoimmune Encephalomyelitis; Myelin Oligodendrocyte Glycoprotein; B cells; Antibody; T cell epitopes; Immune regulation

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1.0 Introduction

Multiple sclerosis (MS) is considered an autoimmune inflammatory demyelinating disease of the central nervous system (Furlan et al., 2009). The instrumental role of CD4⁺ Th1 and Th17 myelin-reactive T cells in mediating disease pathology is well accepted. A role of B cells and antibody in the disease process is also acknowledged. A pathologic role for antibody is suggested by the correlation of oligoclonal bands in the CSF of MS patients with disease severity (Avasarala et al., 2001). Furthermore, the importance of antibodies specific for conformational epitopes of myelin in the destruction of the myelin sheath is recognized (Brehm et al., 1999, Linington et al., 1988). Clinical benefits upon B cell-depletion support a role for B cells in the disease process (Hauser et al., 2008, Kappos et al., 2011, Naismith et al., 2010, Sorensen et al., 2014), although the exact role of B cells and their products in MS pathogenesis is still unclear.

Previous work from this group in the Experimental Autoimmune Encephalomyelitis (EAE) model of MS demonstrated that B cells and/or antibody were critical for the development of EAE when C57BL/6 (B6) mice were immunized with a recombinant form of human myelin oligodendrocyte glycoprotein (hrMOG) but not when immunized with the encephalitogenic peptide MOG35-55 (pMOG35-55) epitope (Lyons et al., 1999). Our subsequent studies revealed that adding back either B cells or serum isolated from hrMOG-primed wild-type (WT) B6 mice was able to reconstitute disease in B cell-deficient (B cell^{-/-}) mice. MOG-specific serum was as effective, if not more effective, in reconstituting disease in B cell deficient mice than was adding back B cells. This suggested a role for an antigen-specific soluble factor in promoting hrMOG-induced disease in WT mice (Lyons et al., 2002). However, the mechanism(s) by which B cells/antibody contribute to hrMOG-induced EAE remain under investigation.

Although our previous data demonstrated proliferative and cytokine responses to the encephalitogenic MOG35-55 epitope in hrMOG-immunized WT and B cell^{-/-} mice, it is possible that additional epitopes are recognized in either the presence or absence of B cells or antibody that contribute to disease. We addressed this hypothesis using a panel of overlapping peptides spanning the extracellular portion of MOG, and discovered that T cells from hrMOG-immunized B cell^{-/-} mice, but not WT mice, proliferated in response to MOG61-85. Subsequent experiments demonstrated that the MOG61-85 epitope inhibited disease initiation by the pMOG35-55 epitope.

2.0 Materials and Methods

2.1 Mice

Female WT and B cell^{-/-} B6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and immunized at 6-8 weeks of age. Mice were housed in specific pathogen free conditions according to NIH and University guidelines.

2.2 Antigens

MOG aa35-55 (pMOG35-55; M-E-V-G-W-Y-R-S-S-F-S-R-V-V-H-L-Y-RN-G-K); MOG aa 61-85 (pMOG61-85; Q-A-P-E-Y-R-G-R-T-E-L-L-K-D-A-I-G-E-G-K-V-TL-R-I); and Hen's

Egg Lysozyme (HEL) aa 46-61 (pHEL46-61; N-T-D-G-S-T-D-Y-G-I-LQ-I-N-S-R). Initially, peptides were synthesized by Sigma-Genosys (The Woodlands, TX). More recently, MOG35-55 and MOG61-85 were synthesized by Genscript (Piscataway, NJ). Proteolipid Protein (PLP) aa 180-199 (pPLP280-199; W-T-T-C-Q-S-IA-F-P-S-K-T-S-A-S-I-G-S-L) was synthesized by Genscript (Piscataway, NJ). The purity of all peptides was confirmed by HPLC. Human rMOG (hrMOG) consisting of the 120-amino acid extracellular domain of MOG, was isolated from the culture supernatant of High-5 insect cells infected with a recombinant baculovirus expressing the hrMOG protein, as previously described (Devaux et al., 1997).

2.3 Immunizations

Mice were immunized (s.c.) with pMOG35-55 (50 µg/mouse when peptides were synthesized by Sigma-Genosys; 100 µg/mouse when peptides synthesized by Genscript) and/or pMOG61-85 (50 µg/mouse [Sigma-Genosys]; 100 µg/ mouse [Genscript]), or pHEL46-61 (50 µg/mouse) emulsified (1:1) in IFA containing 0.3 mg/mouse Mycobacterium tuberculosis, strain H37RA, 0.1ml emulsion/mouse. Co-immunization experiments with pMOG35-55 and pPLP180-199 were performed with 100 µg of each peptide emulsified together in a single emulsion with CFA. Emulsions were prepared using an Omni-Mixer (Omni International, Warrenton, VA). Mice also received 300ng pertussis toxin (List Biological Laboratories, Campbell, CA) intravenously at the time of immunization and 72h later. As indicated, co-immunization was performed with both peptides in the same emulsion or in separate emulsions. When prepared in separated emulsions, peptides were administered at separate sites. The development of EAE was followed and graded on a scale of 0-5 by a blinded observer as previously described (Cross et al., 1993).

2.4 Serum injection

Polyclonal serum specific for MOG35-55 (mouse sequence) and MOG61-85 was raised in rabbits (Sigma Genosys, The Woodlands Texas), resulting in high titer serum (1:500,000) specific for each peptide. Serum was heated overnight at 56°C to inactivate complement. Mice received 100 µl (i.p.) of a mixture of pMOG35-55 and pMOG61-85 serum or preimmune rabbit serum diluted 1:100 in normal mouse serum at the time of immunization and once daily for the following three days,

2.5 Proliferation assay

Lymph node and spleen cells were isolated from mice and cultured in quadruplicate at 2.5×10^6 cells/ml with antigen (pMOG35-55 or pMOG61-85) or mitogen (ConA, 1µg/ml) and 5% FBS in complete RPMI-1640. Culture with irrelevant antigen (pHEL46-61) was included as a negative control. ^3H -thymidine (0.5 µCi/well) was added during the final 18h, and its incorporation counted (Betaplate 1205; Wallace, Gaithersburg, MD). Results are reported as the Stimulation Index (SI; cpm with antigen/cpm with medium alone). $\text{SI} > 2.0$ was considered significant.

2.6 Epitope Mapping Experiments using hrMOG-Primed LNC from WT vs. B cell^{-/-} Mice

T cells were isolated by negative selection from draining lymph nodes of hrMOG-primed mice immunized 10 days prior with hrMOG. T cells were cultured with the indicated peptides at 5-30 µg/ml, as indicated, and naïve spleen cells as antigen presenting cells. Proliferation in response to a panel of overlapping 20-25 mer peptides spanning the length of the hrMOG protein was assessed by tritiated thymidine incorporation. A stimulation index (cpm with antigen/cpm with medium alone) greater than 2.0 was considered significant.

2.7 Cytokine ELISAs

Lymph node and spleen cells were isolated from immunized mice and cultured at 2.5×10^6 cells/ml with the indicated peptide antigen at 10 µg/ml and 5% FBS in complete RPMI-1640. Cell culture supernatants were collected at the indicated times and frozen at -80°C until assayed. Supernatant fluids were analyzed in duplicate for IFN γ (detection limit: 2pg/ml), IL-10 (detection limit: 4pg/ml), and IL-13 (detection limit: 1.5 pg/ml) by QuantikineM ELISA (R&D Systems, Minneapolis, MN) per manufacturer's instructions.

3.0 Results

3.1 Lymph node cells from B cell^{-/-} mice, but not WT mice, reveal a second epitope within human MOG

To determine if epitopes other than the immunodominant 35-55 epitope within hrMOG elicited a proliferation response, mapping experiments were performed on lymph node cells isolated from hrMOG-primed B cell^{-/-} and WT mice at 10 dpi. Lymph node cells were assayed for antigen-specific proliferation to 12 overlapping 20-25-mers of hrMOG (Figure 1A). As expected, LNC from both strains responded to the immunogen hrMOG. Similarly, as we had shown previously, both strains responded to the known encephalitogenic epitope within hrMOG, MOG31-55. WT LNC did not proliferate to any additional epitopes. However, hrMOG-primed LNC from B cell^{-/-} mice proliferated to an additional epitope, pMOG61-85. WT mice failed to proliferate to this pMOG61-85 at concentrations ranging from 5-30 µg/ml (Figure 1B).

3.2 MOG61-85 is not an encephalitogenic epitope

pMOG61-85 was used to immunize mice to induce EAE, following the same regimen as used with pMOG35-55. Mice (n=20) were followed for 30 days. However, immunization with pMOG61-85 alone did not result in clinical signs of EAE in either WT or B cell^{-/-} mice (Table 1).

3.3 Co-immunization with pMOG35-55 and pMOG61-85 abrogates EAE in WT and B cell^{-/-} mice

Previous data demonstrated that B cell^{-/-} mice were resistant to hrMOG-induced EAE, despite similar proliferative and cytokine responses to the encephalitogenic pMOG35-55 epitope compared to susceptible WT mice (Lyons, San, 1999). This suggested that the difference in the EAE response to hrMOG in susceptible WT and resistant B cell^{-/-} mice might be due to factors other than differences in antigen processing to the major

encephalitogenic epitope, MOG35-55. Thus, to investigate whether the response to MOG61-85 seen in B cell^{-/-} mice might play a role in the resistance of these mice to hrMOG-induced EAE, the disease course was compared in WT and B cell^{-/-} mice immunized with pMOG35-55 in the presence or absence of pMOG61-85. Mice were immunized with pMOG35-55 alone, pMOG61-85 alone, or coimmunized with both peptides. Initially, WT and B cell^{-/-} mice were immunized at the same site, using a single emulsion containing both peptides, and were followed clinically for the development of EAE (Figure 2 & Table 1). As previously described, WT and B cell^{-/-} mice presented with similar disease in response to the pMOG35-55 epitope alone (Hjelmström et al., 1998, Lyons, San, 1999). EAE was significantly ameliorated in B cell^{-/-} and WT mice upon co-immunization with pMOG61-85 and pMOG35-55 (Figure 2). The amelioration was near complete in the B cell^{-/-} mice, whereas it was partial in the WT mice (Figure 2B, D).

Next, mice of each strain were immunized with the two peptides at different sites. When immunized at different sites, pMOG35-55 was injected on the left side at each flank, and MOG61-85 was administered on the right side at each flank. Again, amelioration of disease was observed upon immunization with the two peptides compared to only a single pMOG35-55 peptide immunization (Figure 2A, C). That results were similar whether both peptides were emulsified together and injected at the same sites (Figure 2B, D) or when separate emulsions for each peptide were prepared and injected at sites draining into different lymph nodes (Figure 2A, C) suggested that reduction of clinical signs by immunization with pMOG61-85 was not due to local competition for MHC binding. Moreover, co-immunization of B cell^{-/-} mice with another myelin antigen (pPLP180-199) or an irrelevant antigen, pHEL46-61, together with pMOG35-55 did not alter the clinical outcome when compared to mice immunized with pMOG35-55 alone (Table 1). These data suggest that the response to MOG61-85 noted in B cell^{-/-} mice immunized with hrMOG may be involved in the amelioration of disease in B cell^{-/-} mice.

3.4 Co-immunization with pMOG35-55 and pMOG61-85 inhibits antigen-specific proliferation in C57BL/6 mice

To investigate the EAE ameliorating effect of co-immunization with pMOG61-85, antigen specific proliferation and cytokine secretion by cells from co-immunized mice in response to hrMOG and the pMOG35-55 epitope were examined. Mice were immunized with separate emulsions of pMOG35-55 and pMOG61-85 at different sites, and antigen-specific proliferation by LNC and spleen cells was assayed at 31 days post-immunization. Although co-immunization with pMOG35-55 and pMOG61-85 had less effect on disease progression in WT mice, WT mice did respond to pMOG61-85 when immunized with this peptide alone, but not when co-immunized along with the pMOG35-55 epitope (Figure 3A, C). Concurrent immunization with pMOG35-55 and pMOG61-85 at different sites depressed antigen-specific proliferation to pMOG35-55 in WT mice (Figure 3A, C), and to either peptide alone in B cell^{-/-} mice (Figure 3B, D). The effect was more pronounced using cells isolated from B cell^{-/-} mice (Figure 3), in agreement with a more pronounced effect on disease course in B cell^{-/-} mice upon concurrent immunization with pMOG35-55 and pMOG61-85 (Figure 2, Table 1).

3.5 Co-immunization with pMOG35-55 and pMOG61-85 inhibits cytokine secretion in C57BL/6 mice

In both WT and B cell^{-/-} mice, concurrent immunization with pMOG35-55 and pMOG61-85 at different sites depressed antigen-specific secretion of IFN γ by LNC compared with immunization with pMOG35-55 alone (Figure 4 A, B).

To determine if the MOG61-85 epitope induced the production of cytokines hypothesized to be protective in the EAE/MS disease process, we assessed the expression of the anti-inflammatory cytokines, IL-10 and IL-13, in supernatant fluids from the cell assays. We failed to detect measureable quantities of IL-10 in any of the cell culture supernatant fluids tested (4 separate experiments; data not shown). IL-13 was readily detectable, particularly in the B cell^{-/-} mouse cell cultures. IL-13 production was reduced in supernatant fluids from LNC derived from co-immunized mice, whether WT or B cell^{-/-}. (Figure 4C, D).

3.6 Antigen-specific serum regulates EAE amelioration by MOG61-85

Our previous data demonstrated that administration of hrMOG-primed serum allowed hrMOG-induced EAE to develop in B cell^{-/-} mice. To determine if peptide-specific serum was important to amelioration of EAE by MOG61-85, polyclonal serum specific for pMOG35-55 and pMOG61-85 was generated in rabbits. Heat-inactivated serum was diluted 1:100 in normal mouse serum before injection (i.p.) in to B cell^{-/-} mice at the time of immunization with pMOG35-55 and pMOG61-85 and for 3 consecutive days following immunization. A group of mice similarly immunized received heat-inactivated preimmune rabbit serum as a negative control. All mice were followed for 27 days to observe the clinical severity of EAE. Injection of a mixture of serum specific for pMOG35-55 and pMOG61-85 resulted in ablation of the amelioration of EAE by pMOG61-85 (Figure 5; Table 2).

4.0 Discussion

Multiple sclerosis is widely accepted as an autoimmune disease involving myelin-reactive CD4⁺ Th1 and /or Th17 T cells. Although a role for B cells and antibody in MS pathogenesis seems clear, the mechanism(s) by which these immune factors contribute to disease pathogenesis are still being elucidated. Our and other investigators' work in the EAE model has demonstrated resistance to EAE in C57BL/6 mice lacking B cells when it is induced with a recombinant form of the human MOG protein (Lyons, San, 1999, Monson et al., 2011). Here, we present data that the failure of B cell^{-/-} mice to develop EAE in response to hrMOG immunization is due in part to the immune response to a cryptic epitope (MOG61-85). While not naturally processed and recognized in hrMOG-immunized WT mice, MOG61-85 is targeted for processing and presentation in B cell deficient mice that were immunized with the longer protein. Moreover, pMOG61-85 ameliorated EAE induction in B cell^{-/-} mice when mice were co-immunized with the encephalitogenic pMOG35-55 and the pMOG61-85 peptide.

The role of B cells as antigen presenting cells in the initiation of EAE has received considerable attention (Archambault et al., 2013, Molnarfi et al., 2013, Parker Harp et al., 2015). Through multiple complementary approaches, Molnarfi et al. clearly demonstrated

that antigen-specific B cells serve as antigen-presenting cells for the initiation of hrMOG-induced EAE in C57BL/6J mice. Furthermore, these authors demonstrated that MOG-specific antibody secretion was not critical for disease induction in the presence of MOG-specific B cells (Molnarfi, Schulze-Topphoff, 2013). Recent data by Parker Harp et al. further supported a role for B cells in antigen presentation to T cells in hrMOG-induced disease and demonstrated that a sufficient precursor frequency of MOG-specific B cells is a critical factor in determining the APC function of neuroantigen specific B cells (Parker Harp, Archambault, 2015).

Complementing the role of antigen-specific B cells as APCs in the activation of myelin-reactive T cells, a recent report clearly demonstrates that opsonization of myelin antigen by MOG-specific antibody and subsequent antigen processing and presentation by an Fc γ R-dependent mechanism is also capable of activating MOG-reactive T cells *in vivo* (Kinzel et al., 2016). High titer antibody facilitated the processing and presentation of limiting amounts of neuroantigen to myelin-reactive T cells by antigen nonspecific myeloid cells. Activation of neuroantigen-specific T cells via antibody-mediated opsonization and Fc γ R-mediated uptake by APCs was evident in the EAE model and in patients with neuromyelitis optica.

Others have investigated the presence of additional MOG epitopes in the EAE model (Delarasse et al., 2003, Shetty et al., 2014). Neither group found a response to pMOG61-85. Furthermore, both groups observed a response to pMOG111-130 not observed in the current work. This could be explained by the fact that both groups utilized MOG of the mouse/rat sequence, whereas the current work utilized MOG of the human sequence. Previous data demonstrated that human rMOG and mouse/rat rMOG caused disease by different mechanisms, and only disease induced with human rMOG was B cell dependent (Hjelmström, Juedes, 1998, Lyons, San, 1999, Oliver et al., 2003, Weber et al., 2010). There are sequence differences between rodent and human MOG, including the MOG61-85 peptide, most of which are conservative substitutions (Johns and Bernard, 1999). However, it is interesting to note that one non-conservative substitution between the human and rodent sequence occurs at position 41: in the human sequence, position 41 is occupied by proline, whereas in the rodent sequence, position 41 is occupied by serine. The consequences of this substitution on the conformation of the mature protein could explain the differences in the processing and presentation of the different epitopes observed in this study compared to the previous studies.

The mechanism leading to the selection of the MOG61-85 epitope remains under investigation. We recently demonstrated antibody formation to an island of epitopes within hrMOG spanning amino acids 41-85 (Liu et al., 2012). Potentially relevant to the B cell dependence of hrMOG-induced EAE and to the selection of the MOG61-85 epitope, it is noteworthy that this region is overlapping with both the pathogenic MOG35-55 epitope and the protective MOG61-85 epitope. Previous studies in other models demonstrated the role of surface Ig and soluble Ab in directing antigen processing (Amigorena and Bonnerot, 1998, Watts et al., 1998, Watts and Lanzavecchia, 1993). In particular, Ig- or Fc-mediated internalization of whole antigens or Ab-antigen complexes, respectively, can suppress the processing and presentation of some epitopes while selecting others (Simitsek et al., 1995, Watts and Lanzavecchia, 1993). Thus, it is possible that antibody recognizing an epitope in

the aa41-85 region of MOG is able to direct the selection of the encephalitogenic MOG35-55 epitope and/or prevent the processing of the protective MOG61-85 epitope in WT mice. Conversely, the absence of antibody in B cell^{-/-} mice may allow for the presentation of the MOG61-85 epitope and the prevention of disease induction in these animals.

Sercarz et al. first promulgated the idea of “suppressor epitopes” of antigens (Adorini et al., 1979). Various suppressive epitopes have been identified, collectively acting in *trans* to dampen pathologic T cell responses. Commonly, suppressive epitopes result from the generation of altered peptide ligands (APL), through mutation of T cell receptor (TCR) or major histocompatibility complex (MHC) contact residues, affecting the affinity or avidity of peptide/MHC/TCR complex interactions and the resulting T cell responses (De Palma et al., 2000). More recently, regulatory T cells specific for suppressive epitopes derived from the Fab and Fc portion of immunoglobulin have been proposed as the mechanism of protection following injection of IVIg (Cousens et al., 2013). In addition, regulatory T cell responses to stress-induced proteins, such as HSP70, provide long-lived suppressor function in autoimmune arthritis (van Herwijnen et al., 2012). To the best of our knowledge, this is the first report of a *cis*-acting suppressive epitope, derived from the same antigen as the pathologic epitope.

While our data demonstrate the suppressive potential of the MOG61-85 epitope, the mechanism of suppression remains unknown. Our data indicated the down-regulation of antigen-specific production of IFN γ when mice are co-immunized with pMOG35-55 and pMOG61-85 compared to mice immunized with pMOG35-55 alone. We did not detect up-regulation of anti-inflammatory IL-10 production, indicating that protection may not simply be due to shifting of a pathogenic Th1 phenotype to a protective Th2 phenotype. The higher expression of IL-13 in the absence of MOG61-85 may provide insight into the mechanism of suppression by MOG61-85, as well as to potential biologic functions of IL-13. IL-13 has been considered a Th2 cytokine, and thus might be expected to down-regulate Th1 responses and protect against EAE/MS (Ochoa-Reparaz et al., 2008, Wynn, 2003, Young et al., 2000). However, previous data demonstrated gender-specific effects of IL-13 in promoting the MOG 35-55 EAE model (Sinha et al., 2008). In the latter report, the authors demonstrated that the presence of IL-13 contributed to the increased expression of MHC class II by macrophages and increased pro-inflammatory cytokines in female B6 mice. Thus, it is possible that a response to MOG61-85 in some manner reduces IL13 production, e.g., by the production of TGF β (preliminary data; (Li et al., 2006), thus limiting MHC class II presentation by macrophages that would be expected to be critically important APCs in the absence of B cells (Figure 6).

Our data demonstrate that the generation of a response to MOG61-85 following hrMOG immunization occurs only in the absence of B cells and antibodies. A role for B cells and/or antibodies in the induction of EAE is recently recognized (Kinzel, Lehmann-Horn, 2016, Molnarfi, Schulze-Topphoff, 2013, Parker Harp, Archambault, 2015). Thus, hrMOG immunization of WT mice, in the presence of B cells/antibody, leads to the activation of the encephalitogenic MOG35-55 T cell response and the onset of clinical EAE. In the absence of B cells/antibody to focus the immune response to the immunodominant epitope, hrMOG

immunization of B cell^{-/-} mice results in reduced response to the encephalitogenic MOG35-55 and enhanced response to the protective MOG61-85, leading to the amelioration of clinical EAE. Conversely, both WT and B cell^{-/-} mice respond when immunized with pMOG61-85, demonstrating the cryptic nature of this epitope. Co-immunization of in both WT and B cell^{-/-} mice with pMOG35-55 and pMOG61-85 results in the amelioration of clinical disease compared to mice immunized with pMOG35-55 alone. Given the generation of this response in B cell^{-/-} mice, it is likely that the pMOG61-85 response is primed following direct binding of pMOG61-85 to MHC for presentation to the responding T cell population (Figure 6).

Data presented here support the hypothesis that the lack of hrMOG-induced EAE in B cell^{-/-} mice is due at least in part to the generation of a response to a protective epitope, a response that does not occur in WT mice immunized with hrMOG. This observation supports the idea that autoimmunity may be due in some cases to the lack of protective autoimmunity rather than directly due to the generation of pathogenic autoimmunity. This hypothesis does not contradict the notion that B cells are critical as antigen presenting cells; the response to the protective region of hrMOG may serve to re-focus processing and presentation away from the pathogenic region. That the MOG61-85 epitope is a cryptic epitope that does not induce autoimmunity itself opens the possibility to generate protective immune responses in MS patients by direct immunization with protective epitopes, either as a monotherapy or in combination with other disease modifying agents, such as Rituximab. If this could be accomplished, such a therapy would be very appealing in humans as a means to subvert autoimmunity without requiring long-term treatment and with few expected side effects.

Acknowledgments

The authors dedicate this work to the memory of our colleague, John L. Trotter MD. We thank Dr. Neville Rapp, Bob Mikesell and Robert Lopez for technical assistance, and Dr. John Russell for discussion. Funded in part by the National Institutes of Health (NS34947) and the Multiple Sclerosis Society (RG-3138). Dr. Lyons was a Fellow of the National MS Society USA during part of these studies (FG-1259). Funding also provided by the UWM College of Health Sciences SEED grant (JAL); Graduate Student Research Award (MMR; AMA).

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Highlights

- MOG61-85 represents a cryptic epitope of rMOG in wild-type C57BL/6 mice.
- MOG61-85 is naturally processed in B cell-deficient C57BL/6 mice.
- Immunization with MOG35-55 + MOG61-85 ameliorates clinical EAE in B cell^{-/-} mice
- Processing of MOG61-85 is regulated by an antibody-dependent mechanism

A. **B.**

Figure 1. B cell^{-/-} mice, but not WT B6 mice, respond to MOG61-85, when immunized with hrMOG

WT and B cell^{-/-} were immunized with hrMOG in CFA. (A). T cells were isolated by negative selection from draining lymph nodes 10 days post immunization, and proliferation to a panel of overlapping 20-25mer peptides (10 µg/ml) spanning the hrMOG protein was assessed. Analysis revealed an additional response to MOG61-85 by cells isolated from B cell^{-/-} mice that was not noted in WT mice. Stimulation index: (cpm with antigen)/(cpm with medium alone); dotted line: SI=2; Error bars: S.D. of data from 3 separate experiments. (B). The failure of hrMOG-immunized WT B6 mice to generate a response to pMOG61-85 was confirmed using peptide concentrations ranging from 5-30 µg/ml. Data representative of 4 separate experiments.

Figure 2. Co-immunization of B6 mice with pMOG35-55 and pMOG61-85 at different sites (Fig 2A, 2C) or at the same site (Fig 2B, 2D) ameliorates EAE

WT (A, B) or B cell^{-/-} (C, D) C57BL/6 mice were immunized with the encephalitogenic pMOG35-55 alone (closed circles) or with pMOG61-85 (open circles), and the clinical course of EAE was followed. EAE clinical course was significantly less severe in B cell^{-/-} mice co-immunized with both peptides compared to animals immunized with pMOG35-55 alone (human or mouse MOG35-55 sequence; data shown for mice immunized with mouse sequence). Immunization with pMOG61-85 alone (closed squares) failed to induce clinical EAE. $P = <0.007$ (WT mice, different sites) and $P = <0.0001$ (B cell^{-/-} mice different sites/same sites; WT mice, same sites) by 2-way ANOVA, pMOG35-55 alone vs. co-immunization with pMOG61-85. Representative of 4 experiments

Figure 3. Co-immunization with pMOG35-55 and pMOG61-85 decreases cell proliferation to encephalitogenic antigen

Lymph nodes (A, B) and spleens (C, D) were isolated from WT (A, C) or B cell^{-/-} mice (B, D) immunized with pMOG35-55, pMOG61-85, or both antigens at separate sites. Cells were cultured *in vitro* with the indicated antigens, and antigen-specific proliferation was determined. Stimulation Index = (cpm + antigen)/ (cpm - antigen); N.D.: not detected.

Figure 4. Co-immunization with pMOG35-55 and pMOG61-85 decreases cytokine secretion to encephalitogenic antigen

Lymph nodes were isolated from WT (A, C) or B cell^{-/-} mice (B, D) immunized with pMOG35-55, pMOG61-85, or both antigens at separate sites. Cells were cultured *in vitro* with the indicated antigens, and (A, B) IFN γ secretion and (C, D) IL-13 secretion was determined. P values calculated by unpaired t-test

Figure 5. Antigen-specific serum regulates EAE amelioration by MOG61-85 in B cell^{-/-} mice
Mice were immunized with pMOG35-55 + MOG61-85 in a single emulsion. On the day of immunization and the three following days, mice received pre-immune rabbit serum (□; N=10) or serum from rabbits immunized with pMOG35-55 and pMOG61-85 (■; N=8). Rabbit serum was heat-inactivated and diluted 1:100 in naïve mouse serum prior to I.P. injection. Clinical data presented as the average clinical score of all mice on each day. Error bars represent SEM of clinical scores. P=0.0013 by Mann-Whitney U-test of AUC. Representative of 2 separate experiments.

Figure 6. Generation of the protective MOG61-85 epitope

(Solid lines): In the presence of surface immunoglobulin (on B cells) or soluble Ab (interacting through Fc receptors), B cell epitopes are bound and focus antigen processing and presentation to the encephalitogenic MOG35-55 epitope but not the protective MOG61-85 epitope. This leads to activation of MOG35-55-specific T cells and the initiation of clinical disease. (Dotted lines): In the absence of MOG-specific surface Ig or soluble Ab reactive to the relevant B cell epitopes, or with immunization with the pMOG61-85 epitope, both MOG5-55 T cells and MOG61-85 T cells are primed. The MOG61-85-reactive T cells, through an as of yet uncharacterized mechanism, inhibit the full activation of the MOG35-55-specific T cells and prevent the onset, or ameliorates the severity, of clinical disease. One possible mechanism by which this may occur is via production of TGFβ by the MOG61-85-specific T cells, leading to the down-regulation of IL13 production and the subsequent down-regulation of MHCII by antigen presenting cells priming the encephalitogenic T cell population.

Table 1

Co-immunization with pMOG35-55 and pMOG61-85 abrogates clinical EAE

Antigens used in Immunization	Incidence	Onset	Median Max Score (range) ^a
pMOG35-55			
WT	8/8	14.4±2.5	4.0(1.0-5.0)
B cell ^{-/-}	8/8	13.1±1.0	3.2 (2.0-4.0)
pMOG61-85			
WT	0/5	N/A	
B cell ^{-/-}	0/5	N/A	
pMOG35-55 + pMOG61-85			
<u>same site</u>			
WT	6/10 ^b	13.3±1.2 ⁱ	2.5(0-5.0) ⁱ
B cell ^{-/-}	0/5 ^d	N/A	0(0) ^g
<u>different sites</u>			
WT	5/10 ^c	12.6±1.9 ⁱ	2.0(0-5.0) ^h
B cell ^{-/-}	4/9 ^d	18.5±6.2 ^e	0 (0-2.0) ^f
<u>B cell^{-/-}</u>	5/5	15.6±3.0	
pMOG35-55 alone			
+ pHEL46-61	5/5	14±0	
B cell ^{-/-}			
pMOG35-55	5/5	10.0±0.7	4.0 (3.0-4.0)
+ pPLP180-199	5/5	10.4±1.1	4.0 (4.0-5.0)

^a of all mice^b p=0.09 (Fisher's exact test; compared to WT mice immunized with pMOG35-55)^c p=0.0359 (Fisher's exact test; compared to WT mice immunized with pMOG35-55)^d p<0.005 (Fisher's exact test; compared to B cell^{-/-} mice immunized with pMOG35-55)^e p=0.03 (unpaired t-test; compared to B cell^{-/-} mice immunized with pMOG35-55)^f p=0.001 (Mann-Whitney test; compared to B cell^{-/-} mice immunized with pMOG35-55)^g p=0.0078 (Wilcoxon signed rank test; compared to B cell^{-/-} mice immunized with pMOG35-55)^h p=0.083 (Mann-Whitney test; compared to WT mice immunized with pMOG35-55)ⁱ not significant (unpaired t-test; compared to WT mice immunized with pMOG35-55). All Data derived from a single experiment; Co-immunization with pMOG35-55 and pMOG61-85 representative of 4 experiments. Co-immunization with pMOG35-55 and pHEL46-61 and pMOG35-55 and pPLP180-199 each representative of a single experiment.

Table 2

Passive Transfer of pMOG35-55 + pMOG61-85 specific serum abrogates protection by MOG61-85

Serum source	Incidence ^a	Onset ^b	Median Max Score (range) ^{c,d}
Preimmune	5/10	12.8 ± 1.3	2.8 ± 1.2
Immune	7/8	10.3 ± 2.4	4.1 ± 0.8

^a not significant by Chi-square

^b p=0.03 by Mann-Whitney test

^c of mice presenting with clinical disease

^d p<0.001 by Wilcoxon signed ranked test