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A Novel Murine Myelin Oligodendrocyte Glycoprotein Fusion Protein, MOGtag, Induces Appropriate Autoimmune B Cell Germinal Center Responses And Central Nervous System Autoimmune Disease

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A NOVEL MURINE MYELIN OLIGODENDROCYTE GLYCOPROTEIN FUSION PROTEIN, MOG$_{TAG}$, INDUCES APPROPRIATE AUTOIMMUNE B CELL GERMINAL CENTER RESPONSES AND CENTRAL NERVOUS SYSTEM AUTOIMMUNE DISEASE

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by

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Abstract

The clinical success of B cell-depleting therapies in multiple sclerosis (MS) has identified an important, yet poorly understood pathogenic role for B cells in disease. An animal model of MS, experimental autoimmune encephalomyelitis (EAE), is typically induced through immunization with short myelin-derived peptides. B cells recognize whole proteins and not peptides, therefore their activation and involvement in peptide models of EAE is largely excluded. The goal of this study was to develop a novel fusion myelin protein reagent (MOG_{tag}) to induce autoimmune responses in mice that incorporate T and B cell recognition of antigen. Characterization of the autoimmune response revealed the formation of a T cell-dependent germinal center B cell response. Further, immunization with MOG_{tag} resulted in a chronic disease with evidence of an ongoing immune response, and central nervous system pathology featuring T cell infiltration of white and gray matter as well as formation of meningeal B cell clusters.

Keywords

Co-Authorship Statement

The text and images presented in this thesis are adapted from Dang, Amy K., R. W. Jain, H. C. Craig, and S. M. Kerfoot. (2015). B cell recognition of myelin oligodendrocyte glycoprotein autoantigen depends on immunization with protein rather than short peptide, while B cell invasion of the CNS in autoimmunity does not. Journal of Neuroimmunology, 278, 73-84. All experimental work was performed by Amy Dang, with the exception of Figures 6, 7, 8. Immunizations were performed with the assistance of Heather Craig. Immune cell quantification and scoring of histology images in Figures 12 and 13 were performed by Heather Craig.

Acknowledgments

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List of Abbreviations

Ab: Antibody

Ag: Antigen

A647: Alexa Fluor 647

APC: Antigen-presenting Cell

BCR: B Cell Receptor

BME: β-Mercaptoethanol

CFA: Complete Freund's Adjuvant

CNS: Central Nervous System

CSF: Cerebrospinal Fluid

DNA: Deoxyribonucleic Acid

EAE: Experimental Autoimmune Encephalomyelitis

EDTA: Ethylenediaminetetraacetic Acid

EBV: Epstein-Barr Virus

EDSS: Expanded Disability Status Scale

FACS: Fluorescent-activated Cell Sorting

FBS: Fetal Bovine Serum

GC: Germinal Center (s)

GFP: Green Fluorescent Protein

HLA: Human Leukocyte Antigen

HPLC: High-Performance Liquid Chromatography

Ig: Immunoglobulin

i.p.: Intraperitoneal

IPTG: Isopropyl β–D-1-Thiogalactopyranoside

L: Liter

LN: Lymph Node (s)

M: Molar

MBP: Myelin Basic Protein

mg: Milligram

MHC: Major Histocompatibility Complex

mL: Milliliter

mM: Millimolar

μM: Micrometer

MOG: Myelin Oligodendrocyte Glycoprotein

MRI: Magnetic Resonance Imaging

MS: Multiple Sclerosis

ng: Nanogram

NHS: N-Hydroxysuccinimide

nmol: Nanomole

NP: Nitrophenyl

OCT: Optimal Cutting Temperature

PBS: Phosphate-buffered Saline

PC: Plasma Cell (s)

PEG: Poly(ethylene) glycol

PLP: Proteolipid Protein

PTX: Pertussis Toxin

RFP: Red Fluorescent Protein

s.c.: Subcutaneous

SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SLO: Secondary Lymphoid Organ

TEV: Tobacco Etch Virus

TCR: T Cell Receptor

Chapter 1

1 Introduction and literature review

1.1 Multiple sclerosis

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) where host immune cells target myelin for destruction (1). Myelin is a fatty sheath produced by oligodendrocytes that surround and protect axons of nerve cells, and is essential for proper and rapid signal transduction (2-4). As the myelin is damaged, communication signals within the CNS, and consequently the rest of the body, are delayed and disrupted. This can lead to a wide range of symptoms and signs including fatigue, visual impairment, and motor and cognitive disabilities that may accumulate over time as the disease progresses (5).

MS is one of the most common autoimmune diseases of the CNS (6, 7). It affects 2.5 million people worldwide, with varied rates across different regions and populations (8). MS is particularly prevalent in Canada – affecting up to 350 in every 100, 000 Canadians (9). Separating by gender, MS is approximately two times more prevalent in females than males (10). MS is often referred to as a young adults' disease because it generally develops in early adulthood between 20-40 years of age, although cases of earlier and later development have been reported (8, 11). It is reported that most MS patients will be unemployed within 15 years of its first diagnosis due to the steady decline in motor and cognitive functions (8). Further, the overall life expectancy within the MS population is 5-10 years lower compared to the healthy, unaffected population (5).

The disease course and prognosis of MS following its initial diagnosis is highly variable and unpredictable (1). Instead, MS is broadly categorized into four subtypes based on differences in clinical presentation and progression over time (12). The most common form, relapsing-remitting MS, is characterized by episodes of inflammatory attacks on the CNS followed by periods of remission. Most patients with relapsing-remitting MS eventually fall into the secondary progressive MS category, where there is little to no recovery and there is a gradual decline in cognitive and physical abilities (13). Primary

progressive and primary relapsing MS display steady accumulation of neurological disability over time (14). It is unclear whether the clinical variability within these categories actually reflect different pathogenic mechanisms. However, the characteristic CNS inflammation and demyelinating features present in all four MS subtypes suggests that there is a common etiology.

At the moment, there are no cures or preventative measures for MS. Instead, available clinical therapies are intended only for individuals that have already been diagnosed with MS (further discussed in section *1.5.3 Available clinical therapies* below*)*. Taken together, MS is a chronic and debilitating disease in which we have little knowledge and control over its initial development and progression. As it stands, it remains a priority to identify the underlying cellular pathogenic mechanisms involved in the initiation and maintenance of disease, in hopes of developing more effective and targeted therapies for MS patients.

1.2 Genetics in Multiple Sclerosis

It is unclear how MS is first initiated in individuals. The etiology of MS remains complex, and it can be argued that there is not one single defining cause or factor that results in its initiation. Instead, it is now widely accepted that disease development is influenced through a combination of environmental and genetic factors (7).

1.2.1 Familial genetic predisposition

The idea that susceptibility to MS was in part influenced by genetics, and therefore could be inherited, was first proposed in the 1890s following the discovery of familial aggregation (15). Compared to the general population, families with a history of MS have an increased likelihood of developing the disease. Individuals with a first degree relative who has MS have a 15-25 fold greater risk in developing MS than the general population (15). Furthermore, the risk for offspring of parents with MS is significantly increased when both parents have MS, compared to only one parent (15-17). Adoption studies indicated that adopted relatives and non-related individuals raised in an MS household were at no greater risk in developing MS than the general population (15). Taken

together, the familial susceptibility in MS is due to inherited and shared genes within families with MS, and is likely less influenced by shared family environment.

1.2.2   Immune-mediated genetic predisposition

There has been an increase in effort over the past few decades to identify common genetic traits and alleles that confer susceptibility risk to MS. The presence of immune cells in MS pathology heavily suggested that immune-associated genes were involved. Indeed, genomic studies have confirmed that major histocompatibility complex (MHC) class II human leukocyte antigen (HLA) molecules are associated with MS, and that disease-associated variations of these molecules confer the greatest genetic risk to MS (15, 18, 19). MHC class II molecules are protein receptors expressed on the surface of antigen-presenting cells (APCs) and are essential for the activation of CD4$^+$ T cells. In an autoimmune context, it may confer risk by facilitating self-antigen presentation to pathogenic CD4$^+$ T cells (further discussed in section *1.4.Immunology of Multiple Sclerosis* below). Amongst the MHC class II locus, the allelic variations associated with disease have so far been restricted to the HLA-DR and HLA-DQ loci, including the HLA-DRB1*1501 or DQB1*0602 alleles (20, 21). Specific MHC class I HLA molecules, which are involved in presenting intracellular antigens (Ags) to CD8$^+$ T cells, have also been linked to confer risk or protection from MS, independently of MHC class II susceptibility (22-24).

More recent genome-wide association studies of MS have identified additional groups of immune-related genes in close association with disease susceptibility. These include, but are not limited to, genes involved in cytokine (*CXCR5, IL21B*) and co-stimulation (*CD40, CD80, CD86*) signaling pathways (18, 25). Taken together, the majority of disease-associated alleles that have been identified thus far are centered on the immune system, therefore providing strong evidence that MS is largely a disorder of the immune system.

1.3   Environmental Factors in Multiple Sclerosis

Despite a strong genetic basis in MS, not all individuals with genetic risk go on to develop MS. Instead, a complex interplay of non-genetic related factors are implicated in

triggering disease manifestation. To date, a multitude of other factors have been suggested, including: environmental (infectious agents, sunlight) and lifestyle (diet, smoking) factors (26-28). In this section I will focus on two common environmental factors implicated in MS.

1.3.1   Viral infections

Infectious agents such as viruses are common environmental candidates for MS, in part due to their involvement with other autoimmune diseases (29, 30). In MS, potential viruses that are speculated to initiate disease have been narrowed down to those within the *Herpesviridae* family, including the Human Herpesvirus 6 and Epstein-Barr virus (EBV) (7, 26). This is based on reported findings of 1) presence of EBV deoxyribonucleic acid (DNA) in the CNS of some MS patients and 2) MS development is positively correlated with anti-EBV antibody (Ab) titer levels in the serum (31, 32). Several theories have been suggested to explain how viruses play a role in initiating MS.

The first proposed mechanism is based on the concept of molecular mimicry, in which structural and sequence similarities shared between foreign- and self-antigens unintentionally direct immune cells to target the host (33-37). In support of this, T cells of some MS patients have been shown to cross-react with both EBV Ags and myelin-derived Ags found in the CNS (32, 38).

The second theory suggests an autoreactive B cell hypothesis. Under normal circumstances, the immune system has regulatory mechanisms to prevent immune cells from mounting an immune response against self-antigens (refer to *1.4.Immunology of Multiple Sclerosis* section). In MS, it is believed that virally-infected autoreactive B cells are able to escape apoptosis through expression of viral genes that mimic survival signals (31, 39). This can lead to an accumulation of autoreactive B cells that can subsequently activate nearby autoreactive T cells in the CNS to propagate an autoimmune response.

1.3.2   Sunlight exposure

The lack of sunlight has also been implicated in influencing the development of MS (40). Individuals located further from the equator are at a significantly greater risk for

developing MS than those in close proximity to the equator (7, 27, 41). More interestingly, geographical distribution specifically during early adolescence (0-15 years of age) is reported to influence the risk of disease (7). Migration studies reported that individuals who migrate from either low-to-high or high-to-low risk environments are likely to acquire the environmental risk of the new region. In contrast, individuals that migrate post-adolescence retain the environmental risk of their former geographic area (7). The reduced risk of MS in regions of greater sun exposure and with the use of vitamin D supplements suggests a protective role for vitamin D (42). Vitamin D linkage to factors associated with immune regulation is currently being investigated in animal models of MS.

1.4   Immunology of Multiple Sclerosis

The immune system plays an essential role in targeting invading foreign pathogens for destruction. To prevent the immune system from targeting its own cells, there are regulatory checkpoints to limit the generation of potential autoreactive cells (central tolerance) and to prevent their activation in the periphery (peripheral tolerance). Autoimmune diseases reflect a failure in immune regulation at these checkpoints.

1.4.1   Immune response against foreign antigens

Immune responses are initiated in secondary lymphoid organs (SLOs) such as lymph nodes (LNs) (Figure 1). LNs are immune structures consisting mainly of B and T lymphocytes in organized compartments referred to as B cell follicles and T cell zones within the lymphoid cortex, respectively (43). Tissue-derived Ags and foreign Ags present in the lymph enter LNs via afferent vessels (44). Immature dendritic cells that sample local tissue Ags also migrate via lymph to the LN and function as APCs upon maturation (45-47). APCs, such as dendritic cells, macrophages and B cells, are a group of cells with a specialized ability to internalize Ag for processing and loading of processed peptides onto their MHC II molecules for the activation of T cells (48, 49). Successful activation of T cells generally requires two signaling events. The first signal, as described above, involves T cell receptor (TCR) recognition and binding of an appropriate epitope or "peptide" Ag that is presented on surface MHC molecules of
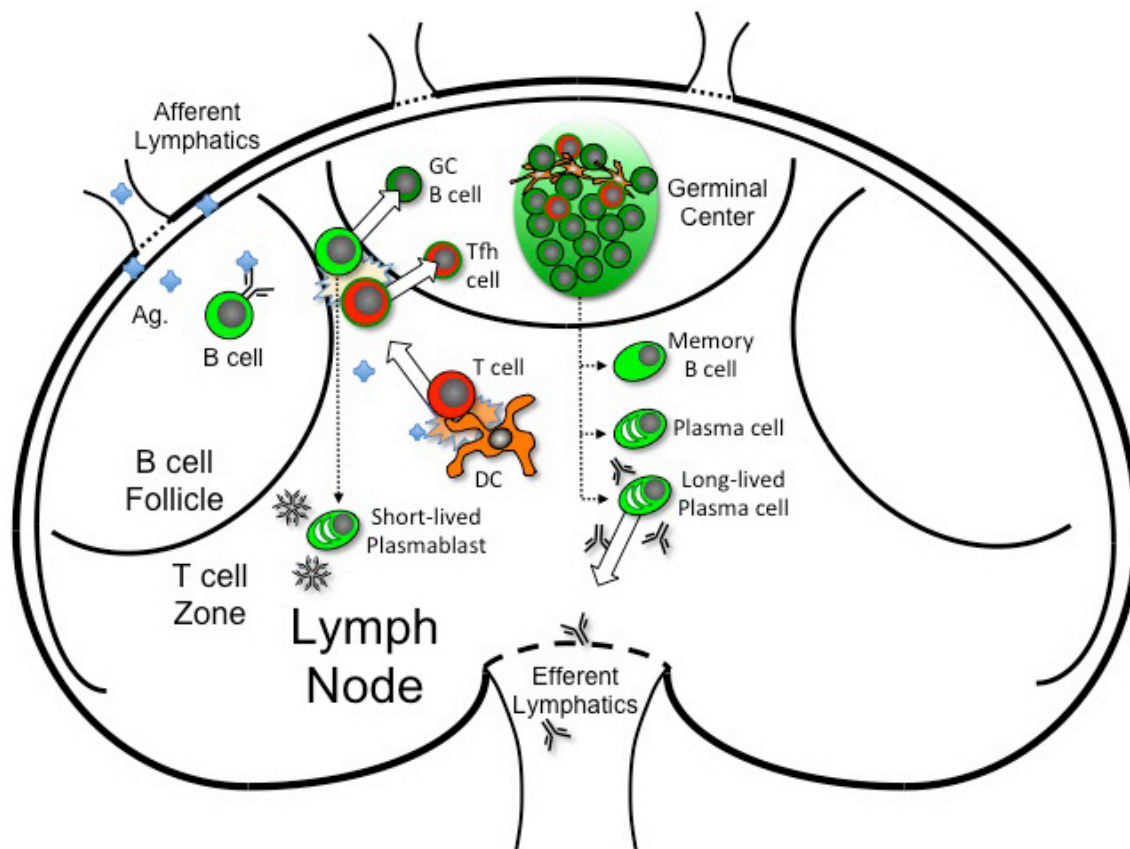
Figure 1: Immune responses are initiated in secondary lymphoid organs. Tissue-derived and soluble antigens found in lymph circulation enter lymph nodes via afferent lymphatics. Dendritic cells (DC) take up, process, and present antigens on their MHC II molecules to activate nearby T cells in the T cell zone. Activation of B cells occurs in the B cell zone upon binding of antigen to the B cell receptor. Newly activated B and T cells can relocate to the interfollicular zone and form interactions that will determine their fate commitment. B cells can either commit to the extrafollicular pathway to differentiate into short-lived plasmablasts, or commit to the germinal center pathway and enter the follicle. In case of the latter, T cells in the interfollicular zone commit to Tfh differentiation to participate in the germinal center response. B cells participating in the germinal center response undergo affinity maturation, somatic hypermutation and isotype switching. Long-lived, high-affinity memory B cells and antibody-secreting plasma cells are generated from the germinal response.

APCs (50). The second signal involves binding of costimulatory ligands and receptors expressed on APCs and T cells, respectively.

Unlike T cells, B cells are not dependent on MHC II Ag presentation and can bind directly to the Ag. The type of B cell response that is elicited depends on the Ag and its requirement for T cell help in generating an Ab response. Furthermore, B cells can also be potent APCs similar to dendritic cells, with the exception that Ag internalization is mediated through binding of the antigen-specific B cell receptor (BCR) (51). Ags taken up by the BCR are processed into short peptides and loaded onto MHC II molecules for presentation to T cells. Consequently, this results in B and T cell interactions where both cells are specific for the same Ag. Immune responses that incorporate B and T cell recognition of the same Ag results in the initiation of a germinal center (GC) response (52-54). GCs are highly specialized microenvironments within B cell follicles of SLOs. During this response, B cells undergo extensive clonal expansion, somatic hypermutation and isotype-switching (55). The end product is the generation of high-affinity plasma cells (PCs) or resting memory B cells that contribute to the long-lived humoral immune repertoire (56). Of particular interest are Ag-experienced memory B cells, which are capable of differentiating into PCs or initiating a germinal center response upon re-exposure to the Ag (57). Additionally, memory B cells can be highly effective APCs and can stimulate or reactivate autoreactive T cells to facilitate an autoimmune response (57). Indeed, these T-cell dependent GC responses are thought to produce pathogenic B cell subsets that may participate in the MS immune response.

Upon activation, antigen-experienced immune cells exit the SLO and home specifically to the site of inflammation. This process of leukocyte extravasation from the original site of activation to the inflamed region proceeds in a step-wise fashion involving a combination of adhesion molecules and chemokines. First, circulating leukocytes can tether to the endothelial membrane through the binding of selectins (58, 59). This interaction serves as the initial membrane attachment and tethering, and mediates leukocyte rolling in the direction of flow (60). Under normal non-inflammatory circumstances, rolling leukocytes form weak adhesion interactions through low-affinity integrins. However under inflammatory conditions, inflammatory chemokines help to

stabilize firm leukocyte:endothelium adhesion by increasing the receptor affinity of integrins. Consequently, the firmly adhered leukocyte becomes arrested at a single location and then proceeds with diapedesis into the tissue.

1.4.2   Immune response in Multiple Sclerosis

Similar to the immune response described above, the local CNS immune response in MS is speculated to be a reactivation of infiltrating antigen-experienced cells that were likely primed earlier in the periphery (61). While it has not been explicitly confirmed, previous and more recent studies suggest that CNS-draining cervical lymph nodes are the initial site of activation and maturation of immune cells involved in MS (61-64). Activation of T cells is an essential event in the early development of the autoimmune response in MS (65). Peripheral activation of autoreactive T cells may occur in the cervical LNs or in the circulation through direct encounter of degraded myelin fragments (62). Upon activation, autoreactive myelin-specific T cells home to the CNS where there is an abundance of processed myelin Ags being presented by local APCs (66, 67). Consequently, this leads to a cascade of inflammatory signaling events involving local activation and further recruitment of peripheral immune cells, thereby driving chronic progression of the disease response. Under non-inflammatory conditions, T cell entry into the healthy CNS is dependent on α_4-integrin (68). In MS, enhanced T cell recruitment to the inflamed CNS is reported to involve an additional adhesion molecule known as P-selectin (69). Identification of specific adhesion molecules involved in immune cell recruitment during inflammation has led to the development of successful clinical therapies used today. Natalizumab, a monoclonal Ab targeted against α_4-integrin, has been demonstrated to reduce clinical relapses in both animal models of MS and human MS by blocking T cell recruitment (70-72). In contrast, little is known regarding the recruitment mechanisms of B cells into the healthy and inflamed CNS.

1.5   Pathology of Multiple Sclerosis

1.5.1   Diagnosis and clinical monitoring

The heterogeneous and complex nature of MS makes it difficult for an immediate diagnosis. According to McDonald's criteria, the clinical definition of MS is defined by

episodes of neurological symptoms and disability caused by inflammatory attacks on the CNS that is disseminated over space and time (73-76). Magnetic resonance imaging (MRI) has become a central component in the clinical diagnosis and monitoring of MS owing to its sensitive ability in detecting white matter lesions (77, 78). Conventional MRI techniques can distinguish between older and "active" inflammatory lesions, and also quantify total lesion burden (79). Disease severity in MS is measured with the Expanded Disability Status Scale (EDSS), a 10-point rating scale that assesses clinical signs of neurocognitive and physical disability (80). The EDSS provides a general indication of the affected CNS areas and accumulation of disability over time. Together, MRIs and the EDSS are used to identify the clinical MS subtype (discussed above in section *1.1 Multiple Sclerosis*). Active lesions in conjunction with worsened clinical presentation reflect a "relapse", whereas clinical worsening in the absence of new or currently active lesions often reflects "progression".

1.5.2   White and gray matter pathology

Pathologically, MS is characterized by inflammatory immune cell infiltrates in close association with demyelinated regions throughout the brain and spinal cord (5). Areas of demyelination, more commonly referred to as lesions, or "plaques", can vary drastically in the size, the extent of myelin damage and repair in the surrounding area, and the cellular infiltrate composition (81). MS plaques likely reflect repeated inflammatory attacks, resulting in substantial axonal damage, loss of oligodendrocytes and eventually a scar-like appearance (5). Lesions commonly affect CNS white matter, however more recent histopathological analyses found evidence of gray matter pathology (82). By comparison, gray matter lesions had fewer immune cell infiltrates, suggestive of less inflammation (83). While gray matter pathology is most often seen in chronic MS, further studies are required to understand its implication in disease.

The composition of inflammatory cell infiltrates in MS is diverse between patients, mainly comprising activated microglia (a type of resident cell in the CNS), macrophages and B and T lymphocytes in surrounding areas of demyelination (84, 85). T lymphocytes, predominantly CD8 and to a lesser extent CD4 T cells, are found in human MS lesions (86-88). Aggregates or clusters of B cells are found in the CNS meninges of MS patients

(89, 90). B cell clusters vary in size, ranging from diffuse numbers of B cells to more densely organized structures resembling ectopic follicle-like structures. Memory B cells, PCs, and actively proliferating B cells, suggestive of GC B cells, have been identified in B cell clusters (90, 91). Further, PCs and their secreted Ab products are also detected in the cerebrospinal fluid (CSF) of MS patients. Compared to healthy individuals, almost all MS patients have similar IgG Ab patterns in the CSF, referred to as "oligoclonal bands" (92, 93). At the moment, Ag specificity of the autoimmune response in MS remains largely unresolved. Based on demyelinating hallmarks in MS, myelin-derived Ags have been implicated and investigated, but with conflicting results. In support of this, both myelin-reactive B and T cells, and Abs have been detected in lesions and CSF in a subset of MS patients (94-97). This finding was not universal across all MS patients, and in some cases was also detected in healthy controls (98, 99). Moreover, anti-myelin Abs have been mostly reported in pediatric MS patients (100), whereas these Abs were either rare or no longer detectable in adult MS. Further investigation is necessary to determine the pathogenic contribution of myelin-reactive cells in MS.

1.5.3   Available clinical therapies

Currently available disease-modifying therapies, classified as first- or second-line, have been demonstrated to reduce the number of clinical relapses and delay accumulation of neurological disabilities in relapsing MS (101). At the moment, there are few clinically effective treatments for patients with progressive MS (101). First-line therapies are general immune modifiers intended to suppress the pro-inflammatory immune response (101). Second-line therapies such as Natalizumab are cell-targeted and function to inhibit immune cell entry into the inflamed CNS. More recently, a B cell-depleting second-line therapy known as Rituximab showed surprising therapeutic success, considering our understanding of B cell involvement in an animal model of CNS autoimmunity (further discussed in section *1.6 Animal models of Multiple Sclerosis* below) (102). Rituximab is a monoclonal Ab directed against the CD20 transmembrane protein expressed on B cells. CD20 expression is present on pre-B cells and mature B cells, but lost upon differentiation into antibody-secreting cells (103, 104). Taken together, CD20^{+} non-

antibody-secreting B cells play an important, yet poorly understood role in the pathogenesis and progression of disease.

1.6   Animal models of Multiple Sclerosis

Our current understanding of human MS is mainly attributed to the collaboration of multidisciplinary studies ranging from genome-wide analyses of individual genes to more broad-scale population-based studies. While providing excellent qualitative data, the nature of these studies provides little to no insight on the actual cellular mechanisms underlying MS pathology. Instead, we are restricted to spinal taps and blood samples, and in rare cases biopsies or post-mortem autopsies to uncover the cellular intricacies behind its pathology. These do not provide an adequate nor thorough examination of the underlying pathology over the course of the disease. As such, experimental animal models have become an essential and integral component of human disease research by allowing researchers to tackle the disease from various different aspects and at different disease stages.

1.6.1   Experimental Autoimmune Encephalomyelitis

The most widely used animal model in MS research is experimental autoimmune encephalomyelitis (EAE). EAE is an experimental model with CNS inflammation and demyelination hallmarks characteristic of human CNS autoimmunity (61). EAE has now been adapted for use in a wide range of species and can be induced through various means, including viral and chemical agents (105). One of the most common induction method of EAE is through direct immunization with a myelin-derived Ag (106). In mice, EAE is typically induced through immunization with short myelin peptides mimicking dominant CD4$^+$ T cell epitopes. Peptide-induced models of EAE reflect a simplified version of a normally complex immune response, where activation of myelin-specific T cells represents the minimal requirement necessary for initiating disease (further described below). Alternatively, EAE could also be initiated passively through the adoptive transfer of previously activated myelin-specific CD4$^+$ T cells (107). Immunized animals typically develop clinical signs of disease around 10-14 days post-immunization, and are scored on a standard EAE scoring system beginning with muscle loss in the tail

and continuing with ascending paralysis in the hind and front limbs. Inflammation and disease pathology of most rodent models of EAE are predominantly located within the spinal cord, which is in contrast to human MS where inflammatory lesions are commonly associated with the brain (61).

EAE first started as an accidental discovery when a proportion of rabies vaccination patients receiving crude CNS extracts from infected rabbits developed acute episodes of paralysis (108). Eventually, it was confirmed that the muscle paralysis and encephalitis was actually a stereotypic immune response against the myelin components within the CNS (108). More systematic approaches of extraction and purification of individual myelin components identified myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) among the several encephalitogenic targets in this CNS-restricted immune response. In light of T cell biology advancements at the time, the EAE field shifted its focus to identifying the immunodominant T cell epitopes on myelin proteins that appropriately bound the MHC II/TCR complex. Myelin-derived peptides such as MOG_{35-55} that are analogous to the immunodominant epitopes were demonstrated to elicit an encephalitogenic immune response (109, 110). Combined with the relatively inexpensive cost and ease of synthesis, these short myelin peptides are now the most common form of Ag used today in EAE studies.

By comparison, EAE models induced with whole protein Ags generate a more complex immune response by incorporating responses from other immune cells. MOG has emerged as a leading myelin autoantigen due to the presence of anti-MOG Abs and MOG-specific T cells in MS patients (111, 112). Further, immunization with whole MOG, but not MBP or PLP, has been demonstrated to initiate both a demyelinating autoantibody response and an encephalitogenic T cell response in rodents and marmosets, thereby producing an inflammatory CNS disease with demyelinating lesions characteristic of human MS (109, 113-117). MOG is expressed on the outermost lamellae of the myelin sheath and has been characterized as a type I integral membrane protein with a single extracellular Ig variable domain at the N-terminus (118). In particular, the extracellular domain of MOG, analogous to MOG_{1-125}, has been identified as the encephalitogenic portion that is recognized by immune cells, and also contains the

immunodominant MOG_{35-55} peptide sequence recognized by $CD4^+$ T cells (113). At the moment, rat, mouse and human-based versions of the MOG_{1-125} protein immunogen have been produced (discussed further).

More recent to the EAE field are "spontaneous" models where genetically susceptible mice, in combination with environmental factors, can develop disease spontaneously – a scenario that better reflects human MS. In the majority of these spontaneous models, lymphocyte receptors are genetically modified to be myelin reactive, resulting in enhanced proportion of myelin-reactive cells that is sufficient to overcome immune tolerance. Other models of spontaneous EAE based on a modified cytokine profile have also been reported (119). To date, two spontaneous EAE models with known B cell involvement have been described (120-122). In one model, 2D2 mice (123) bearing TCRs specific for MOG_{35-55} are crossbred with IgH^{MOG} mice (124) expressing BCRs specific for MOG_{1-125} to generate a double mutant mouse with genetically modified B and T cell receptors that can recognize the MOG autoantigen (120, 121). The single transgenic 2D2 mouse strain develops EAE at very low incidences (120, 121). However disease incidence increases greatly when combined with genetically modified MOG-specific B cells (120, 121). Therefore the development of EAE in this model is largely B cell-dependent. The difficulty and unpredictability in disease manifestation in spontaneous EAE models remains one of the greatest challenges in preventing its wider adoption in the EAE field. Taken together, spontaneous models, while recapitulating many aspects of human MS, are a far more difficult and expensive alternative compared to induced models of EAE.

1.6.2 Lessons learned from EAE: good and bad

Since its first discovery, EAE has contributed greatly to our understanding of CNS autoimmunity, and has led to the development of clinical therapies used today. Recent clinical success of B cell-depleting therapies in MS revealed an important, but poorly understood pathogenic role of B cells in disease. This finding was unexpected based on experimental findings from earlier studies on the role of B cells in EAE. Induction of EAE is mainly dependent on $CD4^+$ T cells, with little to no requirement for B cell involvement. Indeed, several groups have demonstrated that EAE can be induced in B

cell-deficient mice (125, 126). With the exception of mice immunized with a protein Ag based on human MOG (127-130), neither short-peptide nor protein-induced EAE is absolutely dependent on the presence of B cells as measured by the development of typical signs of disease (125, 130, 131). While protein-induced models of EAE that incorporate B cell involvement have been examined, these studies almost exclusively looked at the Ab response which, based on the therapeutic mechanism of Rituximab is not the main contributors to disease. Instead, B cells derived from the GC response have been implicated (62, 132). Collectively, these findings indicate a need to reevaluate the appropriateness of peptide-induced models of EAE to investigate the role of B cells in pathology.

There are two conceptual reasons why peptide-induced EAE models cannot model a more complex pathogenic immune response incorporating both B and T cell recognition of an autoantigen. As discussed earlier (refer to section *1.4 Immunology of Multiple Sclerosis*), B cells often rely on conformational epitopes of whole proteins, and therefore short linear myelin peptides mimicking dominant $CD4^+$ T cell epitopes are not accessible. Further, peptides can be loaded directly onto the MHC II/TCR Ag complex, thus bypassing the BCR-mediated Ag uptake and processing for presentation to cognate T cells. This short-circuits the Ag specificity of the interactions with T cells and affects the competition for Ag binding between somatically mutated B cells that is inherent to the maturation of the GC response (133).

The primary factors limiting the use of larger protein Ags to induce EAE that incorporates more than just $CD4^+$ T cell recognition of the autoantigen is the relative cost and difficulty of obtaining or producing them. Few commercial sources exist, and available expression vectors for in-house production are based on inefficient and out-of-date expression systems. Further, purification protocols are complex, laborious, and often depend on specialized equipment (109), complicated in part by the insolubility of the MOG protein itself. It is also important to note that all expression systems that we have identified produce fusion proteins consisting of the MOG extracellular domain, various purification tags, and often additional sequences that we cannot identify. Most incorporate either human or rat MOG_{1-125} (134), and therefore when used in mice

represent a cross-reactive response not ideal for some investigations of autoimmune-specific cell interactions.

1.7 Thesis hypothesis and objectives

MS is a complex autoimmune disease characterized by heterogeneous CNS pathology involving many immune cells. Although the EAE model has contributed significantly to advancing our knowledge of CNS inflammation, peptide Ag models of EAE have inevitably shortsighted our understanding of the complex immune response in MS. There is an urgent need to revisit the experimental model to address these limitations. Ideally, short myelin peptides commonly used for EAE should be replaced with whole myelin protein Ags that incorporate other immune cells known to contribute to disease. The few available myelin protein Ags are expensive and difficult to produce, and are often based on a foreign species thereby generating a cross-reactive autoimmune response. Therefore the goal of this thesis is to develop a mouse-based myelin protein Ag using simple and inexpensive laboratory equipment to facilitate its wider availability and usage in the EAE field.

Hypothesis:

We hypothesize that EAE induced through immunization with a myelin protein Ag will initiate a more complex autoimmune CNS disease that incorporates B cell responses.

Research Objectives:

1) To develop a mouse-based myelin protein Ag for the induction of an autoimmune response in mice that incorporates B and T cell recognition of the Ag

2) To characterize the GC response and autoimmune CNS pathology induced by the mouse-based myelin protein

Chapter 2

2   Materials and Methods

2.1 Mice

C57Bl/6 and 2D2 TCR transgenic (123) mice were purchased from Jackson Laboratories. B1-8 mice (135) with a homozygous deletion of the Jκ locus (136) were a generous gift from Dr. Ann Haberman. IgH^{MOG} MOG-specific BCR knockin mice (124) were received as a gift from Hartmut Wekerle. Mice were housed in a specific pathogen-free barrier at West Valley Barrier. Animal protocols (#2011-047) were approved by the Western University Animal Use Subcommittee.

2.2 Antibodies for flow cytometry and histology

The following antibodies were purchased from BD Biosciences: anti-CD4-V450 (RM4-5), anti-CD45R-V450 (RA3-6B2), anti-CD138-BV421 (281-2), anti-CD19-BV711 (1D3), anti-CD95-PE-Cy7 (Jo2), anti-Bcl6-A647 (K112-91), anti-CD4-A647 (RM4-5), anti-IgG1-APC (A85-1), and anti-CD62L-A700 (MEL-14). The following antibodies were purchased from BioLegend: anti-IgKappa-Biotin (RMK-12), anti-IgLambda-Biotin (RML-42), and anti-His Tag-purified (J099B12). The following antibodies were purchased from eBioscience: anti-IgD-eF450 (11-26), anti-CD3-FITC (145-SC11), anti-CD38-PE (90), anti-CD4-PE-Cy5 (RM4-5), anti-IgM-PE-Cy5 (II/41), anti-CD279-Biotin (RMP1-30), and Streptavidin-eF780. FluoroMyelin Red for myelin staining was purchased from Invitrogen.

2.3 Recombinant mouse MOG$_{tag}$

2.3.1 Vector design

The MOG$_{tag}$ insert was designed using SnapGene software to include the following: a tobacco etch virus (TEV) cleavage sequence site (ENLYFQ/G), the extracellular immunoglobulin domain of mouse MOG (residues 1-125), a TAA stop codon and *Bgl*II and *Eco*RI restriction sites added to the 5' and 3' ends, respectively. MOG$_{tag}$ insert sequence was codon optimized for expression in *Escherichia coli* and synthesis in the

pQE-12 vector by Celtek Genes. The insert was then cloned into the pET-32a(+) vector containing the gene for thioredoxin (Novagen) and then transformed into BL21 *E. coli* using standard transformation procedures.

2.3.2   Protein expression and purification

A protocol for purification of MOG$_{tag}$ protein was adapted from previous systems (109). pET-32a(+) MOG$_{tag}$ BL21 *E. coli* was cultured in LB medium at 37°C to an O.D. of 0.6, when protein expression was induced overnight with 1 mM Isopropyl β–D-1-Thiogalactopyranoside (IPTG). Bacterial cells were pelleted and resuspended in lysis buffer (0.1 mg/mL hen egg lysozyme, 0.1% Triton-X (v/v) in PBS) then lysed to collect inclusion bodies. The inclusion body pellet was resuspended in 500 mM NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9 (Buffer A) and incubated at 4°C, then denatured with the addition of 6 M guanidine (Buffer B). The protein suspension was centrifuged at 4°C to collect the supernatant containing the solubilized proteins. Before protein absorption, His-Bind nickel resin (Novagen) was prepared according to the manufacturer's instructions. Briefly, the nickel resin was washed and treated with distilled water and 200 mM NiSO$_4$, then equilibrated with Buffer B. The solubilized proteins containing MOG$_{tag}$ was incubated with the charged nickel resin in a standard 50 mL centrifuge tube at 4°C. Following centrifugation, the supernatant was kept for further rounds of purification and MOG$_{tag}$ was eluted from the pelleted resin with 500 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, and 6 M guanidine. Elutions were pooled and exchanged by overnight dialysis at 4°C into the storage buffer containing 0.115% glacial acetic acid, 3 mM sodium acetate and concentrated with PEG 3350 and PEG 8000 (BioShop) to 5 mg/mL before storage at -20°C. Protein expression and quantification were confirmed using standard SDS-PAGE (Bio-Rad) and Bradford Assay kits (Bio-Rad) according to the manufacturer's instructions.

2.3.3   TEV protease cleavage of MOG$_{tag}$ protein

Purified MOG$_{tag}$ was dialyzed into 50 mM Tris-HCl, 0.5 mM EDTA and 5 mM BME. TEV protease enzyme (137) (a generous gift from Dr. John McCormick (138)) was added at a 1:20 (mg/mg) ratio TEV to MOG$_{tag}$ protein and left at 4°C for two days before

removal of the vector His_{tag} from the cut MOG_{1-125} using nickel resin purification as described above. Purified MOG_{1-125} was dialyzed into the storage buffer as described above and concentrated to 2.24 mg/mL before storage at -20°C.

2.3.4 MOG_{tag} Alexa Fluor 647 fluorophore conjugation

Direct conjugation of MOG_{tag} to Alexa Fluor 647 was performed using the NHS Ester and C_2 Maleimide fluorophore labeling kits (Life Technologies) according to the manufacturer's instructions.

2.4   Adoptive transfer and immunization

Naïve antigen-specific T and B cells were isolated from red fluorescent (RFP^+) 2D2 and green fluorescent (GFP^+) IgH^{MOG} mice, respectively, as previously described (139), and transferred into wild type C57Bl/6 recipients. Briefly, LNs and spleens of RFP^+ 2D2 and GFP^+ IgH^{MOG} mice were dissociated and T and B cells were isolated using EasySep Negative selection Mouse T and B cell Enrichment Kits (StemCell Technologies). Unless otherwise stated, 5×10^5 T cells and 5×10^6 B cells per mouse were transferred 2 days prior to immunization. To induce a GC response, mice were immunized in the footpad with 125 μg MOG_{tag} in Complete Freund's Adjuvant (CFA) (Sigma-Aldrich). Draining popliteal LNs were harvested at the indicated timepoints for analysis.

2.5   Induction of experimental autoimmune encephalomyelitis

6-8 week old C57Bl/6 mice were immunized s.c. on day 0 at two sites at the base of the tail with either 0.5 mg of MOG_{tag}, 0.224 mg of MOG_{1-125}, or 0.04 mg of commercially-synthesized MOG_{35-55} (Tocris) in CFA. At the same time mice were also administered 250 ng of pertussis toxin (PTX) (List Biological Laboratories) i.p. and, when indicated, again on day 2. Clinical disease was monitored daily and was scored as follows: 0) no clinical signs; 1) tail paralysis; 2) tail paralysis and hind limb weakness; 3) hind limb paralysis; and 4) complete hind limb paralysis and front limb weakness. Half points were given for intermediate scores.

2.6   Flow cytometry

Draining inguinal LNs were harvested from mice at the study endpoint and were prepared as previously described (139). Briefly, LN cell suspensions were blocked with an anti-Fc-γ receptor (CD16/32 2.4G2) in PBS containing 1% FBS before further incubation with a combination of the Abs mentioned above in section *2.2 Antibodies for flow cytometry and histology*). Dead cells were excluded by staining with the Fixable Viability Dye eFluor506 (eBioscience). Flow cytometry was performed on a LSRII cytometer (BD Immunocytometry Systems) and analyzed with FlowJo software (Treestar).

2.7   Immunofluorescent histology

At the end of the experiment or earlier, if the mouse reached a predetermined endpoint, spinal cords were extracted from mice and prepared as previously described (139). Briefly, 5-9 evenly spaced spinal cord tissues spanning the lumbar to cervical regions were cut and frozen in OCT (TissueTek) media. Serial cryostat sections (7 μm) were blocked in PBS containing 1% Bovine Serum Albumin, 0.1% Tween-20 and 10% rat serum before proceeding with staining. Sections were mounted with ProLong Gold Antifade Reagent (Invitrogen) and stored at -20°C. Tiled images of whole spinal cord sections (20x) were imaged using DM5500B fluorescence microscope (Leica).

2.8   Image and statistical analyses

Histology images were analyzed by a blinded reviewer based on the number of B cell clusters and for the presence and localization of CD4$^+$ T cell infiltration.

PRISM software was used to analyze FACs and histology data. Unless otherwise stated, T-tests were used for single comparisons, and ANOVA followed by either a Bonferroni or Tukey's post-hoc test were used for comparison between multiple groups. For all experiments: *p<0.05, **p<0.01 and ***p<0.001 were considered significant.

Chapter 3

3   Results

3.1   Design of the mouse MOG$_{1-125}$ thioredoxin tag expression vector

To develop an improved expression system for the production of large quantities of murine MOG extracellular domain, we began with the mouse sequence for MOG$_{1-125}$ (GenBank NM_010814.2), and linked the 5'end to a sequence generating a TEV protease cleavage site (Figure 2). An alternate form of the consensus cleavage site (ENLYFQ/G) (140) results in cleavage between the final glutamine and glycine, glycine being the first amino acid of MOG$_{1-125}$. Therefore, TEV protease cleavage results in the removal of additional tag sequences, leaving pure MOG$_{1-125}$ extracellular domain without any residual amino acids. This sequence was then codon optimized for efficient expression in bacteria resulting in changes in the DNA but not translated protein (Figure 2). The synthesized sequence was cloned into the pET-32a(+) expression vector and transformed into BL21 *E. coli* cells for efficient generation of a fusion protein consisting of the mouse MOG extracellular domain, purification tags including a 6xHis tag and an S-Tag, and finally thioredoxin to counteract the known insolubility of MOG itself (141).

3.2   Generation and purification of the mouse MOG$_{1-125}$ thioredoxin tag expression vector

While some purification protocols for current MOG expression systems require specialized equipment, such as HPLC (109), we developed an isolation protocol using only standard equipment available to the majority of immunology groups (see *Material and Methods* for detailed protocol). MOG$_{tag}$ protein expression in BL21 *E. coli* was induced by overnight culture with IPTG (Figure 3A). Inclusion bodies containing MOG$_{tag}$ were isolated from lysed bacterial cells via centrifugation and protein was denatured in 6 M guanidine prior to absorption onto nickel resin in a standard 50 mL centrifuge tube. Following centrifugation, the supernatant was kept for further rounds of purification, while pure MOG$_{tag}$ was eluted from the pelleted resin with imidazole. Due to the very
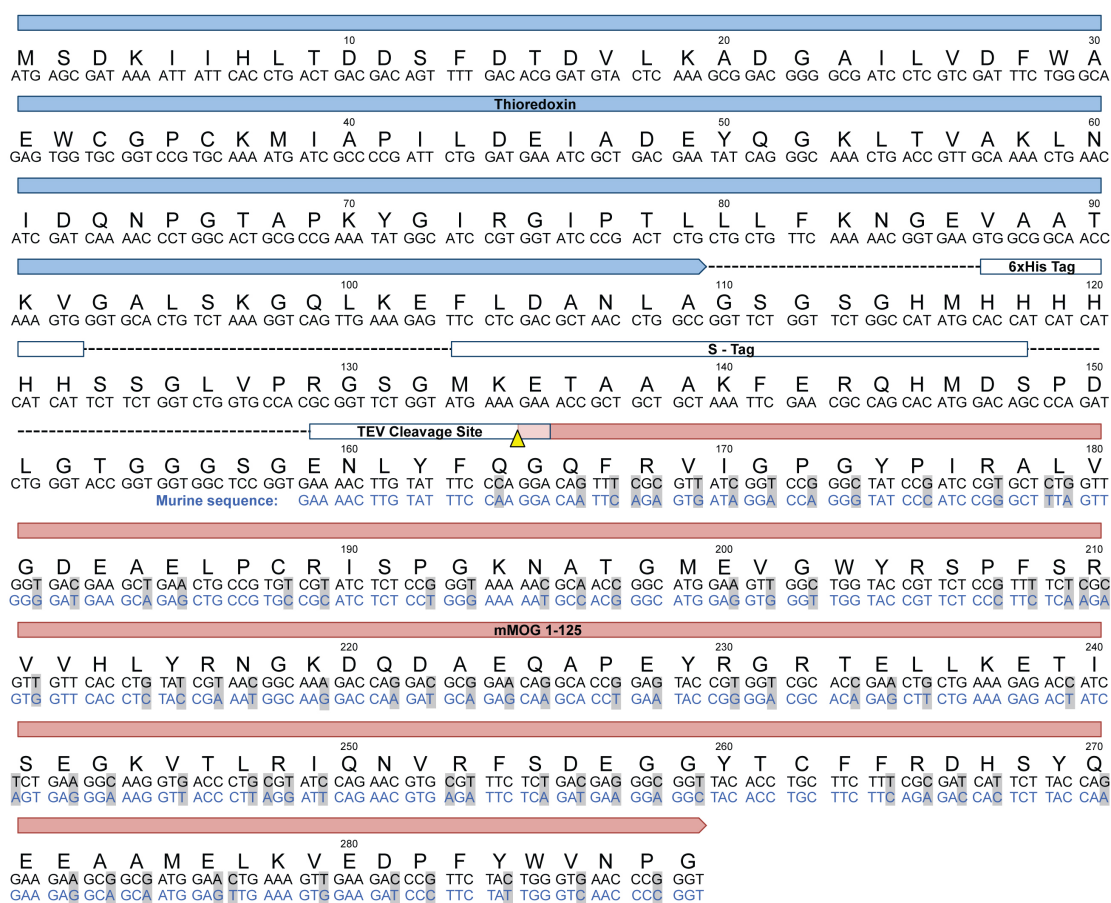
Figure 2: Generation of the MOG_{tag} fusion protein. Linear structure depicting the MOG_{tag} fusion protein, along with the amino acid and DNA sequences. The mouse DNA sequence for MOG$_{1-125}$ (in blue) and codon-optimized sequences (in black) are shown.
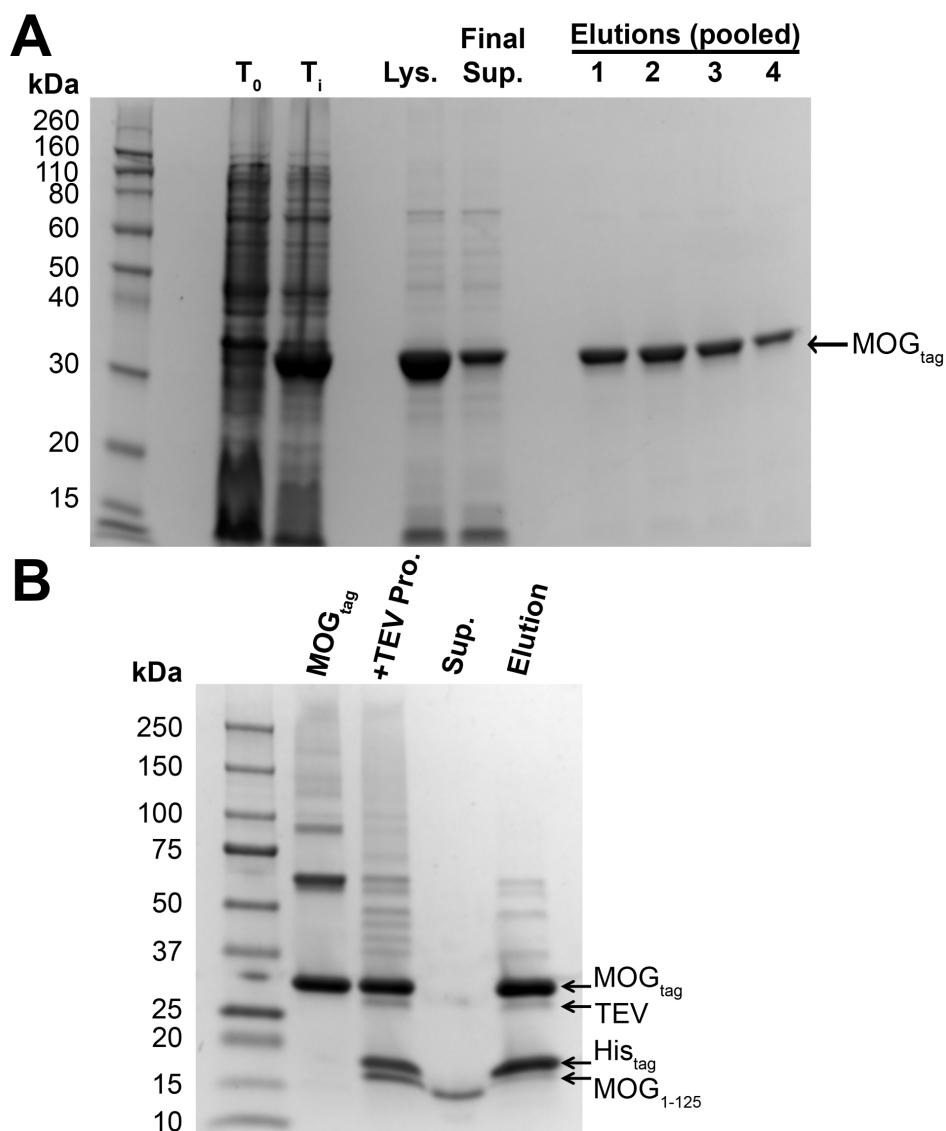
Figure 3: Production and purification of MOGtag fusion protein. (**A**) MOG$_{tag}$ purification from bacterial culture. Expression was induced in BL21 *E. coli.* by overnight culture with IPTG (*T$_0$ vs. T$_i$* (BL21 *E. coli.* induced with IPTG)). MOG$_{tag}$ protein was then purified from bacterial lysates (*Lys.*) through repeated absorption on nickel resin and elution of pure protein (*Elutions 1-4*). Some MOG$_{tag}$ protein remained following four rounds of absorption (*Final Sup.*), demonstrating that additional purification rounds could have yielded more protein. (**B**) TEV protease cleavage to remove the tag containing thioredoxin and purification sequences was performed after protein refolding. MOG$_{tag}$ was incubated with TEV protease *(+ TEV Pro.)*. Absorption on nickel resin was used to remove uncut MOG$_{tag}$ and cut tag (*Elution*), leaving pure MOG$_{1-125}$ (*Sup.*).

large amount of protein generated from the T7 expression system, 4-6 rounds of absorption were required. Resulting elutions were subsequently pooled. Greater than 250 mg of protein was obtained from an initial 2 L of bacterial culture. Protein was refolded through dialyzation to acetate buffer and concentrated to 5 mg/mL. Pure MOG_{1-125} was generated from MOG_{tag} by cleavage with TEV protease and removal of cut tag (Figure 3B). Taken together, MOG_{tag} is easily purified in large quantities and, to our knowledge is the only system that allows for the production of pure MOG protein without additional foreign tag sequences.

3.3   MOG-specific B cells recognize MOG_{tag}

An important precondition for the use of MOG_{tag} to induce an appropriate anti-MOG protein B cell response is that it must be recognized by MOG-specific B cells. IgH^{MOG} mice possess a BCR variable region derived from a MOG-specific hybridoma inserted into the endogenous locus. This greatly increases the number of MOG-specific B cells (124), presumably dependent on heavy chain pairing with an appropriate light chain. Further, IgH^{MOG} cells have been confirmed to be pathogenic as spontaneous CNS autoimmune disease develops with high incidence in the progeny of these mice crossed with mice bearing a transgenic TCR for MOG_{35-55} (120, 121).

In preliminary experiments, LN cells from wild type C57Bl/6 mice and IgH^{MOG} mice were incubated with MOG_{tag} followed by anti-His tag secondary and fluorescently-labeled anti-IgG1 tertiary Abs. FACS analysis revealed little MOG_{tag} binding by C57Bl/6 cells as expected, due to the extremely low numbers of antigen-specific cells in naïve mice. In contrast, ~15% of B cells from IgH^{MOG} mice, but not other lymphocytes, bound MOG_{tag} (Figure 4A). This confirms that B cells with known specificity for MOG protein appropriately recognize MOG_{tag}. It also demonstrates the utility of MOG_{tag} to identify MOG-specific B cells by FACS. To facilitate this, we directly conjugated MOG_{tag} to the fluorophore Alexa Fluor 647. Both amine- and thiol-reactive approaches were tested and the amine-reactive NHS ester was found to produce the best cell labeling (Figure 4B). MOG_{tag}-A647 effectively identified significant numbers of B cells in IgH^{MOG} mice but only rare cells in wild type C57Bl/6 (Figure 5A). Significantly more MOG-binding B
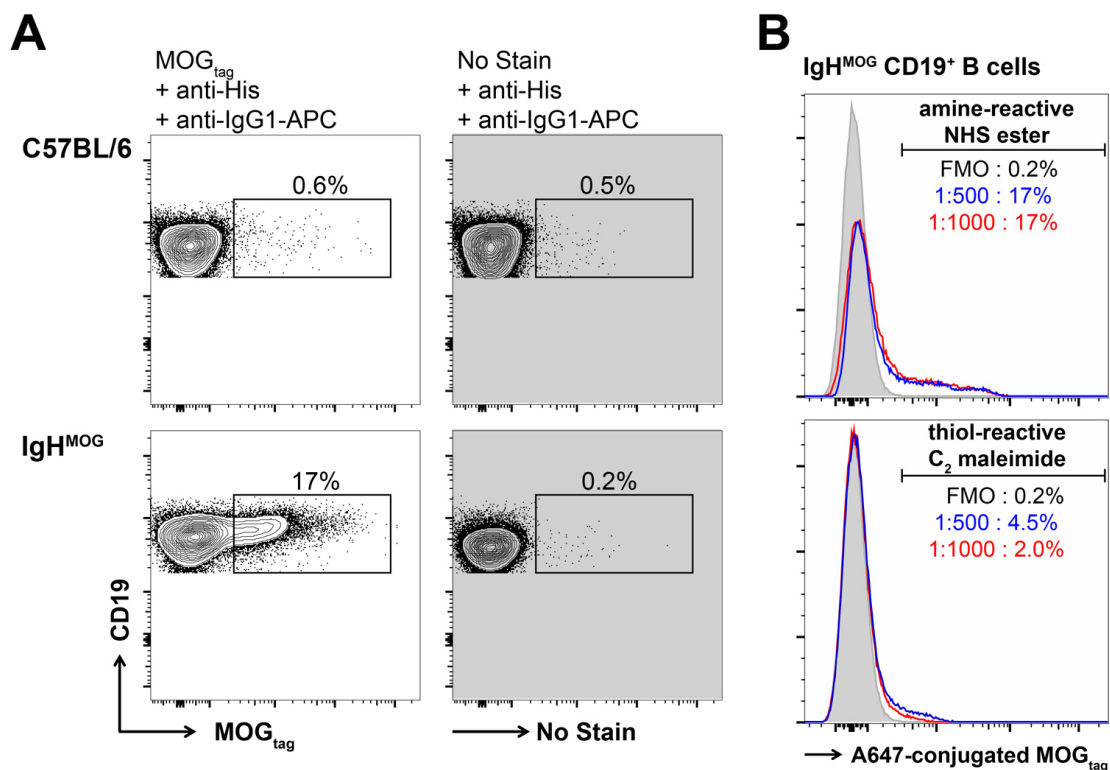
Figure 4: Binding of unlabeled MOG$_{tag}$ protein by MOG-specific B cells. (**A**) Lymph node cells from wild type C57Bl/6 and IgH^{MOG} mice were harvested and stained for FACS analysis. B cells (CD19$^+$ CD4$^-$) were analyzed for binding MOG$_{tag}$ using anti-His and anti-IgG1-APC secondary and tertiary antibodies, respectively. Representative stains are shown. n = 3 mice/group. (**B**) MOG$_{tag}$ was directly conjugated with Alexa Fluor 647 using amine-reactive (top) or thiol-reactive reagents. Binding of different titers of labeled MOG$_{tag}$ to IgH^{MOG} B cells was assessed by FACS. FMO – Fluorophore minus one (no MOG$_{tag}$).
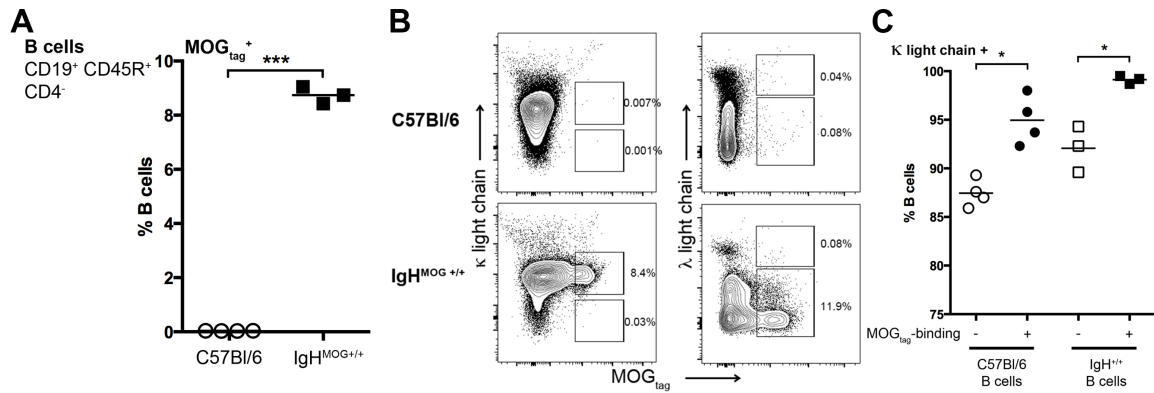
Figure 5: Identification of MOG-specific B cells with MOG$_{tag}$-A647. (**A**) Lymph node cells from wild type C57Bl/6 and IgH^{MOG} mice were harvested and analyzed for B cells (CD19$^+$, CD45R$^+$, CD4$^-$) binding to MOG$_{tag}$ conjugated to A647 using FACS. Symbols represent individual mice. ***p<0.001. (**B**) MOG$_{tag}$-binding B cell light chain usage. Lymph node B cells were analyzed for binding of MOG$_{tag}$ and κ or λ light chain antibodies. Representative plots from wild type and IgH^{MOG} mice are shown. n = 3-4. (**C**) Quantification demonstrating enrichment of κ usage in MOG$_{tag}$-specific B cells from wild type and IgH^{MOG} mice. Each symbol represents an individual mouse. *p<0.05.

cells expressed κ-light chain compared to the non-specific pool, suggesting that light chain usage is an important factor in specificity to MOG_{tag} in both wild type and IgH^{MOG} B cells (Figure 5B, C).

3.4 Effective generation of a T cell-dependent B cell germinal center response with MOG_{tag}

In order to determine if MOG_{tag} is able to activate B cells to initiate a GC, GFP^+ IgH^{MOG} B cells and RFP^+ 2D2 (MOG_{35-55} TCR transgenic (123)) T cells were isolated and transferred to wild type C57Bl/6 recipients. These mice were immunized with MOG_{tag} in CFA and draining popliteal LNs were harvested 10 days later. Development of the GC response was analyzed by FACS. Significantly greater numbers of $CD95^{hi}$ $CD38^{lo}$ GC B cells and $CD138^+$ $CD19^{int}$ PCs were detected in immunized mice compared to unimmunized controls (Figure 6A and B). As expected, transferred GFP^+ IgH^{MOG} B cells contributed to the GC response but, interestingly, remained a minor component as compared to recipient-derived GFP^- cells. This demonstrates that wild type and not just mutant IgH^{MOG} cells are activated by MOG_{tag}. Similarly, endogenous GFP^- cells made up the majority of PCs. Tfh differentiation in immunized mice was confirmed by PD-1 and Bcl-6 expression. Again, both transferred MOG_{35-55}-specific RFP^+ 2D2 Tfh cells and endogenous wild type RFP^- Tfh cells were induced by immunization with MOG_{tag} (Figure 6A and B, bottom).

In a separate experiment, draining LNs were harvested for histological analysis. Consistent with FACS findings, 10 days post immunization with MOG_{tag}, IgD^- GCs were apparent in follicles (Figure 7). Serial sections confirmed the expression of Bcl-6 on B cells, a transcriptional repressor that is required for GC B cell differentiation (142). Also consistent with FACS findings, transferred GFP^+ cells were present in the GC but the majority of $Bcl-6^+$ cells were GFP^-. RFP^+ transferred T cells were also clearly evident in the follicle and GC, many of which were also $PD-1^+$, representing Tfh cells. Therefore, immunization with MOG_{tag} effectively induced the development of a GC response with the participation of both endogenous and transferred antigen-specific T and B cells.
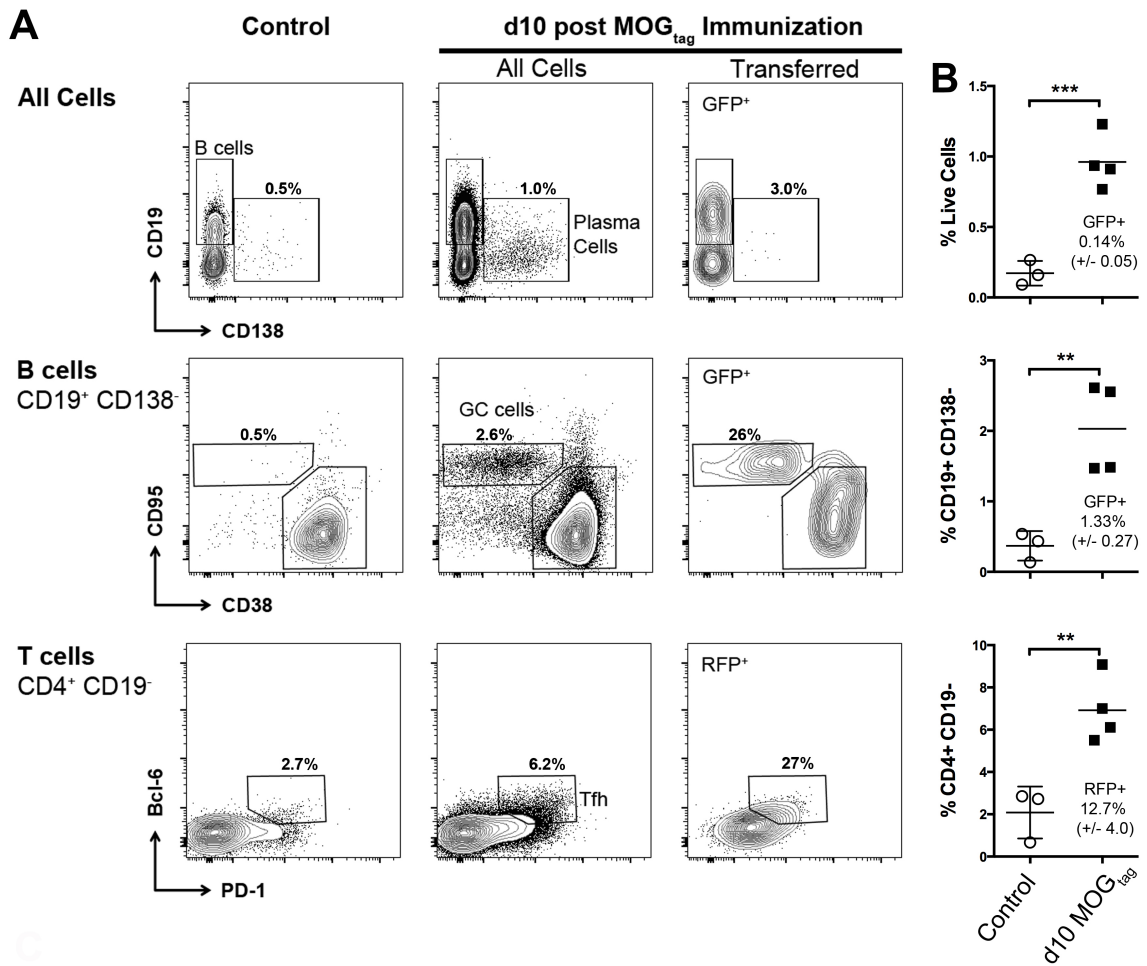
Figure 6: FACS analysis of the immune response initiated by MOG$_{tag}$. GFP$^+$ IgH^{MOG} B cells and RFP$^+$ 2D2 (MOG$_{35-55}$-specific) T cells were transferred to non-fluorescent wild type C57Bl/6 recipients. 10 days post immunization with MOG$_{tag}$ in CFA, draining popliteal lymph nodes were harvested for analysis. (**A**) FACS was performed to determine the frequency of CD138$^+$ CD19^{int} plasma cells (top row), CD95^{hi} CD38^{lo} GC B cells (middle row), and Bcl-6^{hi} PD-1^{hi} Tfh cells (bottom row) in unimmunized (Control) or immunized mice. Upstream gating is shown to the left of each row. (**B**) To specifically investigate the development of these cell types in transferred MOG-specific cells, cells were first gated on GFP (CD19$^+$ CD138$^-$ B cells) or RFP (CD4$^+$ T cells), right column. Each symbol represents an individual mouse. **p<0.01, ***p<0.001.
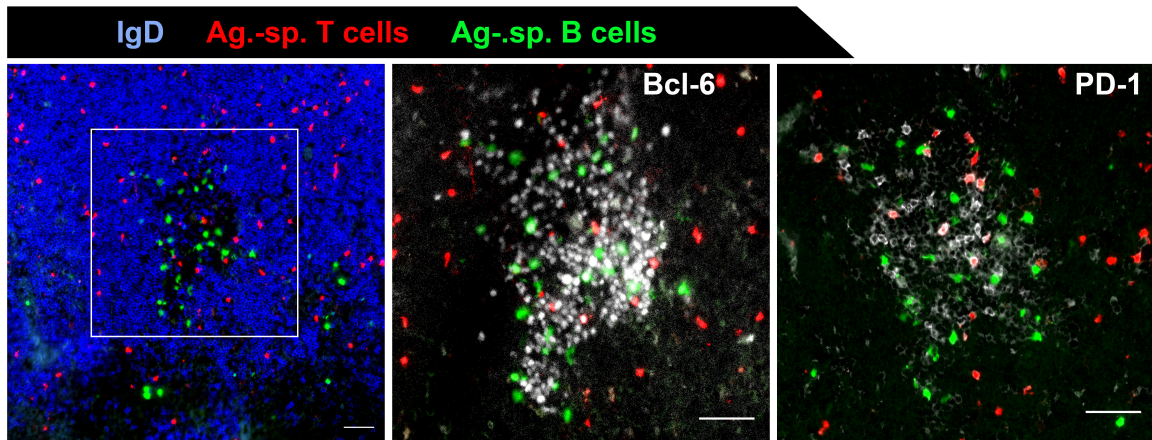
Figure 7: Histological analysis of the immune response initiated by MOG_tag. GFP$^+$ IgH^{MOG} B cells and RFP$^+$ 2D2 (MOG$_{35\text{-}55}$-specific) T cells were transferred to wild type C57Bl/6 recipients. 10 days post immunization with MOG$_{tag}$ in CFA, draining popliteal lymph nodes were harvested for analysis. Histological staining of lymph nodes from immunized mice. A magnified view of the area in the square (center) shows Bcl-6 expression in the same section, and PD-1 expression by RFP$^+$ MOG-specific T cells in the GC in an adjacent section. Representative stain of n = 3 mice. Scale bars represent 100 μm.

3.5   MOG$_{tag}$ and MOG$_{35-55}$ peptide activate different B cells to induce a germinal center response

To determine if MOG$_{35-55}$ peptide can also activate B cells to initiate a GC response, we transferred fluorescent MOG-specific B and T cells to wild type C57Bl/6 recipients and immunized them with equimolar (15 nmol) amounts of MOG$_{tag}$ (500 μg), pure MOG$_{1-125}$ (225 μg) generated by TEV protease cleavage (Figure 3B), or commercially-synthesized MOG$_{35-55}$ (40 μg). It should be noted that pure MOG$_{1-125}$ without the thioredoxin tag was highly insoluble, making it difficult to determine the precise amount of soluble and precipitated protein. Nevertheless, GC cells were evident after immunization with each of the three Ags (Figure 8A, top), with MOG$_{tag}$ being the most potent immunogen. Importantly, both MOG$_{tag}$ and pure MOG$_{1-125}$ induced transferred GFP$^+$ IgH^{MOG} B cells to activate and differentiate into GC B cells, while immunization with MOG$_{35-55}$ had no ability to do so (Figure 8A, middle). Further, a significantly greater proportion of GC B cells bound MOG$_{tag}$ in MOG$_{1-125}$-immunized mice, while binding was virtually non-existent in mice immunized with MOG$_{35-55}$ (Figure 8A, bottom). Note that GC B cells downregulate BCR surface expression, and therefore many antigen-specific cells will not be labeled with MOG$_{tag}$. Further, it should be noted that endogenous MOG on the surface of oligodendrocyte is glycosylated, and that it remains possible that the MOG$_{35-55}$-induced B cell response targets glycosylated but not unglycosylated MOG. As expected, there was no defect in T cell activation as measured by CD62L down-regulation, including transferred pathogenic RFP$^+$ 2D2 MOG$_{35-55}$-specific T cells (Figure 8B). Together, these experiments demonstrate that while MOG$_{35-55}$ can induce a GC response, pathogenic IgH^{MOG} B cells specific for MOG protein do not participate, nor do wild type endogenous cells capable of binding MOG$_{tag}$.

3.6   Characterization of MOG$_{tag}$-induced EAE

The above experiments confirm that immunization with MOG$_{tag}$ results in the initiation of an immune response that incorporates appropriate recognition of mouse MOG protein by both T and B cells. To determine if immunization also induces disease, wild type C57Bl/6 mice were immunized once and administered PTX i.p. at the time of immunization and again 2 days later. Due to a past report of relatively low disease
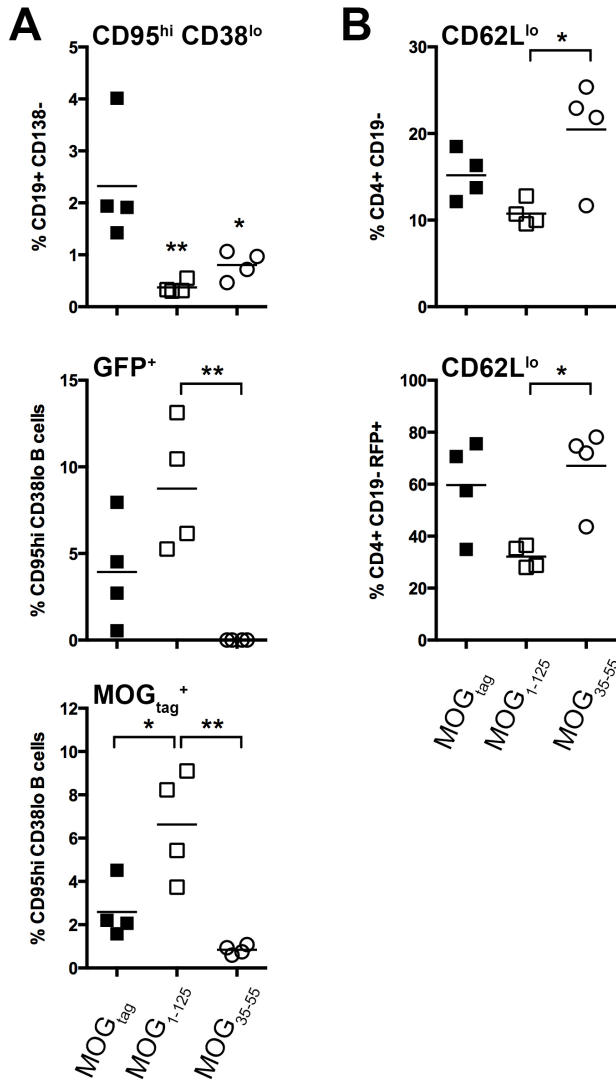
Figure 8: MOG$_{tag}$, MOG$_{1-125}$, and MOG$_{35-55}$ induce GC responses, but MOG$_{35-55}$ does not activate pathogenic B cells that bind MOG protein. GFP$^+$ IgH^{MOG} B cells and RFP$^+$ 2D2 T cells were transferred to wild type C57Bl/6 recipients that were then immunized with equimolar amounts of MOG$_{tag}$, pure MOG$_{1-125}$, or MOG$_{35-55}$ in CFA. Draining popliteal lymph nodes were analyzed by FACS 10 days later for (**A**) GC B cell (CD95^{hi} CD38^{lo}) development and (**B**) activation of T cells, as defined by downregulation of CD62L (shown as CD62L^{lo}). Percent of the indicated population for the relevant parent (**A**: B cells – top, GC B cells – middle and bottom, **B**: all T cells – top, transferred RFP$^+$ T cells – bottom) is shown. Each symbol represents an individual mouse. Multiple groups were compared using one-way ANOVA followed by a post-hoc Tukey's test.   *p<0.05, **p<0.01.

incidence following immunization with a different fusion protein containing mouse MOG_{1-125} (143), we also included mice with enriched numbers of MOG-specific immune cells, including wild type recipients of transferred IgH^{MOG} B cells and 2D2 T cells, as well as mice with enhanced endogenous MOG reactivity in the B cell compartment ($IgH^{MOG\ +/+}$) or in both the B and T compartments ($IgH^{MOG\ +/-}\ 2D2^{+/-}$). Surprisingly, all groups developed severe disease with high incidence (Figure 9). There was no difference in disease severity between wild type and groups with endogenously enhanced MOG reactivity, although disease emerged earlier and was more severe in mice that had received transferred MOG-reactive T and B cells. No evidence of recovery was observed in any group.

In a separate experiment, wild type mice were immunized with MOG_{tag} as described above with the exception that only one dose of PTX was administered to limit disease severity. Similar to our initial experiment, 10 of the 11 mice developed disease within 10 days of immunization (Figure 10A). Mice were sacrificed 23 days post-immunization (~2 weeks post onset of signs of disease). FACS analysis of inguinal draining LNs revealed an ongoing GC response at this late timepoint (Figure 10B), demonstrating an ongoing source for autoreactive T and B cells to drive chronic disease. Interestingly, the single mouse that did not develop overt signs of disease nevertheless had a GC response similar to that of mice that did develop disease.

Spinal cords were collected from the above mice and prepared for immunofluorescence analysis of cellular infiltrates and demyelination. Sections were taken from 7 to 9 evenly spaced sites spanning the cervical to lumbar regions of the spinal cord. Meningeal infiltration by $CD4^+$ and $CD45R^+$ cells (T cells and B cells, respectively) and $CD4^+$ infiltration of the white matter was evident in all mice that developed overt signs of disease (10/11 immunized mice) (Figure 11). No evidence of immune cells was observed in the single mouse that did not develop disease or in the three unimmunized mice. Clusters of B cells in the meninges were evident in all diseased mice, but only very rarely deeper in the spinal cord tissue. Clusters varied considerably in size from 5 to 20 cells (Figure 11i, iii) to much larger (Figure 11ii, vi). Similar to observations in human MS patients (144), these clusters were sometimes closely associated with white matter
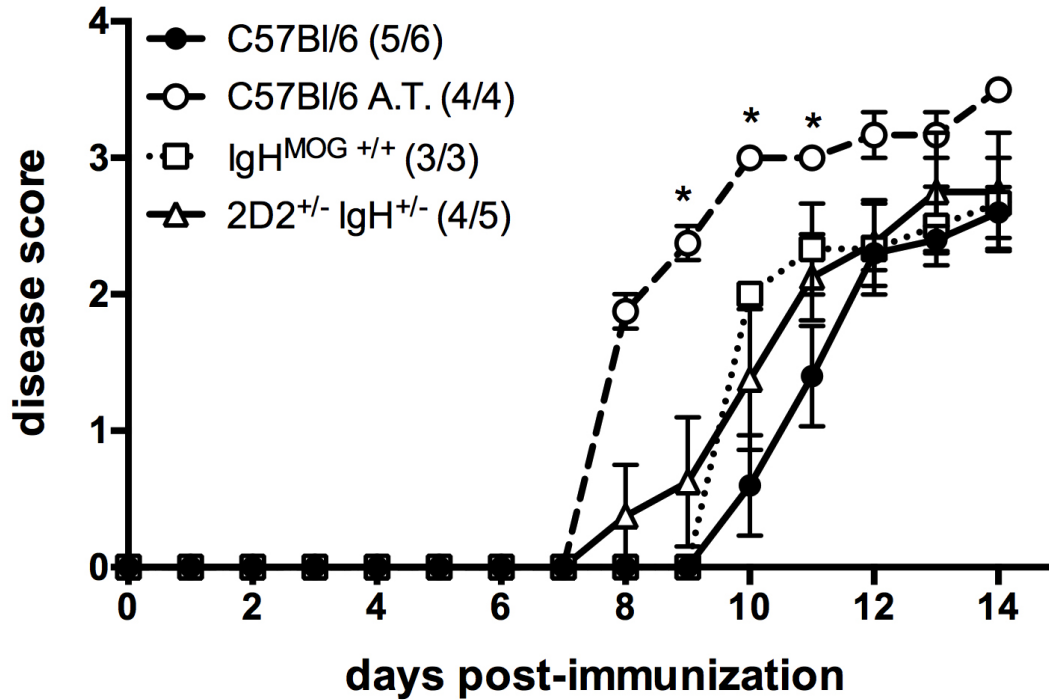
Figure 9: Characterization of MOG$_{tag}$-induced EAE. EAE development in wild type mice and in mice with enhanced immune responsiveness to MOG. Different strains of mice, including wild type C57Bl/6 mice, wild type recipients of MOG-specific T and B cells (A.T.), IgH^{MOG}, and 2D2$^{+/-}$ IgH^{MOG} mice, were immunized with MOG$_{tag}$ in CFA. PTX was administered at the time of immunization and again 2 days later. Mice were monitored and disease severity was scored. Scores are shown only for mice that developed signs of disease. Incidence for each strain is shown in the legend. Graph shows mean +/- SEM. n = 3-6. *p<0.05 vs C57Bl/6.
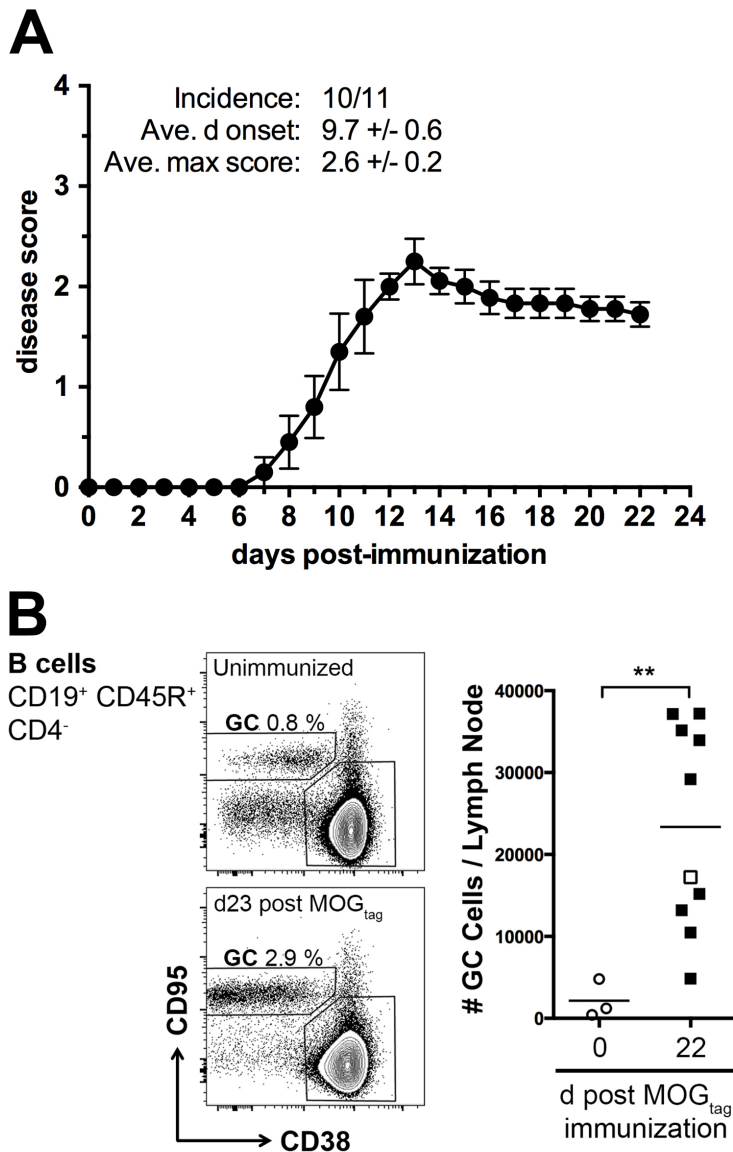
Figure 10: FACS analysis of MOG$_{tag}$-induced EAE. (A) EAE was induced with immunization with MOG$_{tag}$ in CFA and administered one dose of PTX at the time of immunization. Mice were monitored for signs of disease. Scores are shown only for mice that developed signs of disease. Incidence is shown in the legend. Graph shows mean +/- SEM. **(B)** Mice were sacrificed 23 days post immunization and the presence of GC B cells in draining inguinal lymph nodes was analyzed by FACs. (Left) Representative plots from unimmunized and MOG$_{tag}$-immunized C57Bl/6 mice and (right) quantification of GC (CD95^{hi} CD38^{lo}) B cells in mice are shown. Each symbol represents an individual mouse. Note that the open square represents the individual mouse that did not develop signs of disease. **p<0.01.
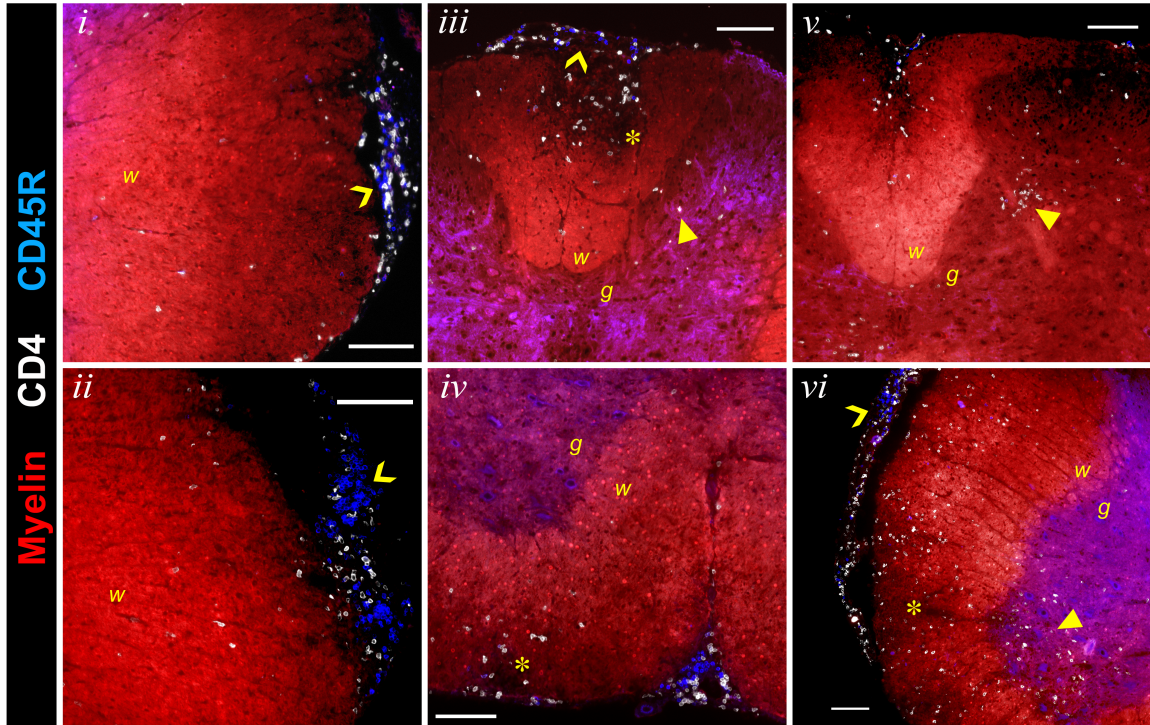
Figure 11: Histological analysis of MOG_{tag}-induced EAE. Spinal cords were collected and prepared for histology. 7-9 evenly spaced segments were prepared for each spinal cord, spanning the cervical to lumbar regions. Representative images from MOG$_{tag}$ immunized mice showing common patterns of demyelination and T cell (CD4$^+$) and B cell (CD45R$^+$) infiltration. Representative of n = 10 diseased mice. * - region of white matter demyelination, open arrow – B cell meningeal clusters, closed arrow – T cell grey matter infiltrates, *g* – grey matter, *w* – white matter. Scale bars represent 200 μm.

infiltration by T cells (Figure 11iii, vi). While loss of myelin was typically associated with extensive T cell invasion and was observed in 7/10 diseased mice (Figure 11iii, iv, vi), it was not always associated with B cell clusters (Figure 11iv). Importantly, recent reports from human MS patients have noted inflammatory lesions in the gray matter (145). $CD4^+$ T cells were apparent in the gray matter of 8/10 diseased mice, often only as diffuse cells within the tissue (Figure 11iii), but sometimes as distinct clusters of invading cells (Figure 11v) or as an apparent continuation of extensive white matter infiltration (Figure 11vi). Blinded evaluation of the tissue sections revealed that T and B cell infiltration was evenly distributed throughout the spinal cord (Figure 12). The average number of clusters per section correlated with disease severity, while infiltration by $CD4^+$ T cells did not (Figure 13). Taken together, MOG_{tag} is a potent immunogen capable of inducing EAE characterized by ongoing GC responses and autoimmune CNS pathology features similar to human MS.

3.7 Comparison of EAE induced by MOG_{tag}, MOG_{1-125}, and MOG_{35-55}

A separate experiment was performed comparing EAE induced by MOG_{tag}, pure MOG_{1-125}, and MOG_{35-55}. Separate groups of wild type C57Bl/6 mice were immunized with equimolar (15 nmol) amounts of the given Ag in CFA and administered one dose of PTX at the time of immunization. Although signs of disease appeared most rapidly in MOG_{tag}-immunized mice, all mice eventually showed signs of disease (Figure 14A). There was no difference in disease severity between the groups (Figure 15A). Consistent with above, at the end of the experiment (22 days post-immunization) ongoing GC responses were observed in mice immunized with MOG_{tag} (Figure 15B). Interestingly, the GC response was significantly greater in these mice compared to mice immunized with either pure MOG_{1-125} or MOG_{35-55}. Further, MOG_{tag} binding by GC B cells was significantly greater in MOG_{tag}-immunized mice and almost non-existent in MOG_{35-55}-immunized mice (Figure 14B), consistent with our findings of MOG_{tag} binding by B cells activated by MOG protein and peptide (Figure 8).

Histological analysis of spinal cord sections again revealed extensive $CD4^+$ T cell infiltration in the meninges and white matter of the majority of MOG_{tag}-immunized mice.
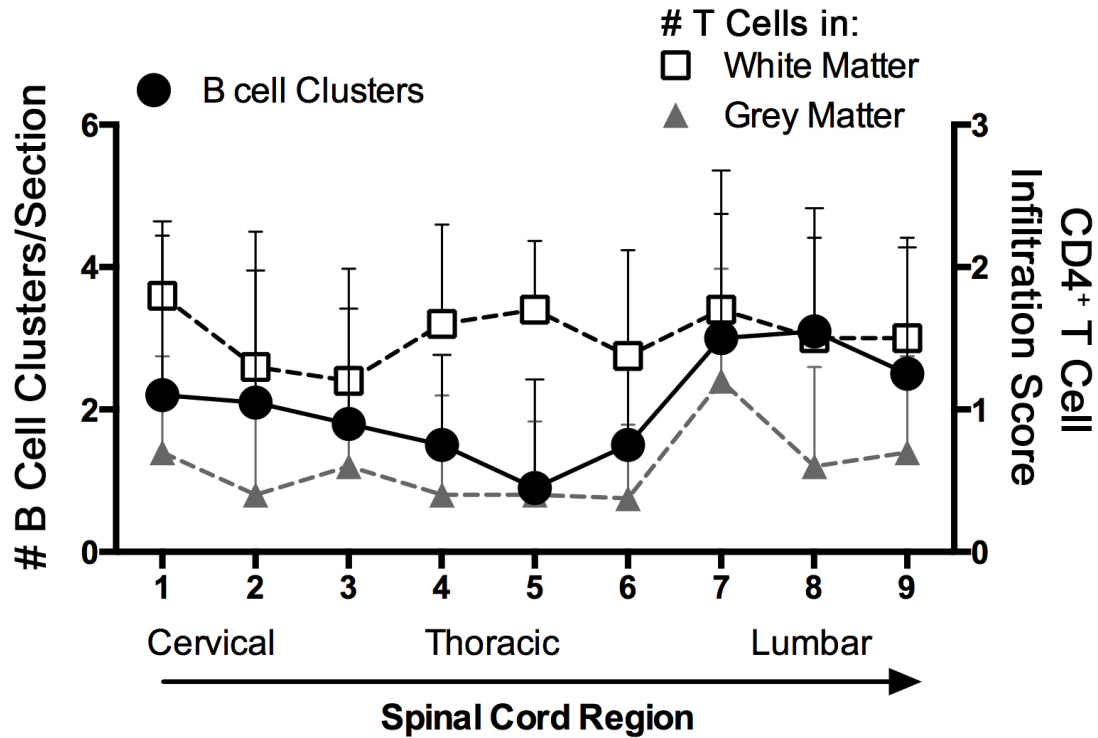
Figure 12: Quantification of B and T cell infiltration in MOG$_{tag}$-induced CNS pathology. Spinal cords were collected and prepared for histology. 7-9 evenly spaced segments were prepared for each spinal cord, spanning the cervical to lumbar regions. CD4$^+$ T cell infiltration of the white and grey matter was scored separately (0 – no cells, 1 – diffuse infiltration, 2 – severe infiltration) and CD45R$^+$ B cell clusters were counted for each section for each mouse by a blinded reviewer.
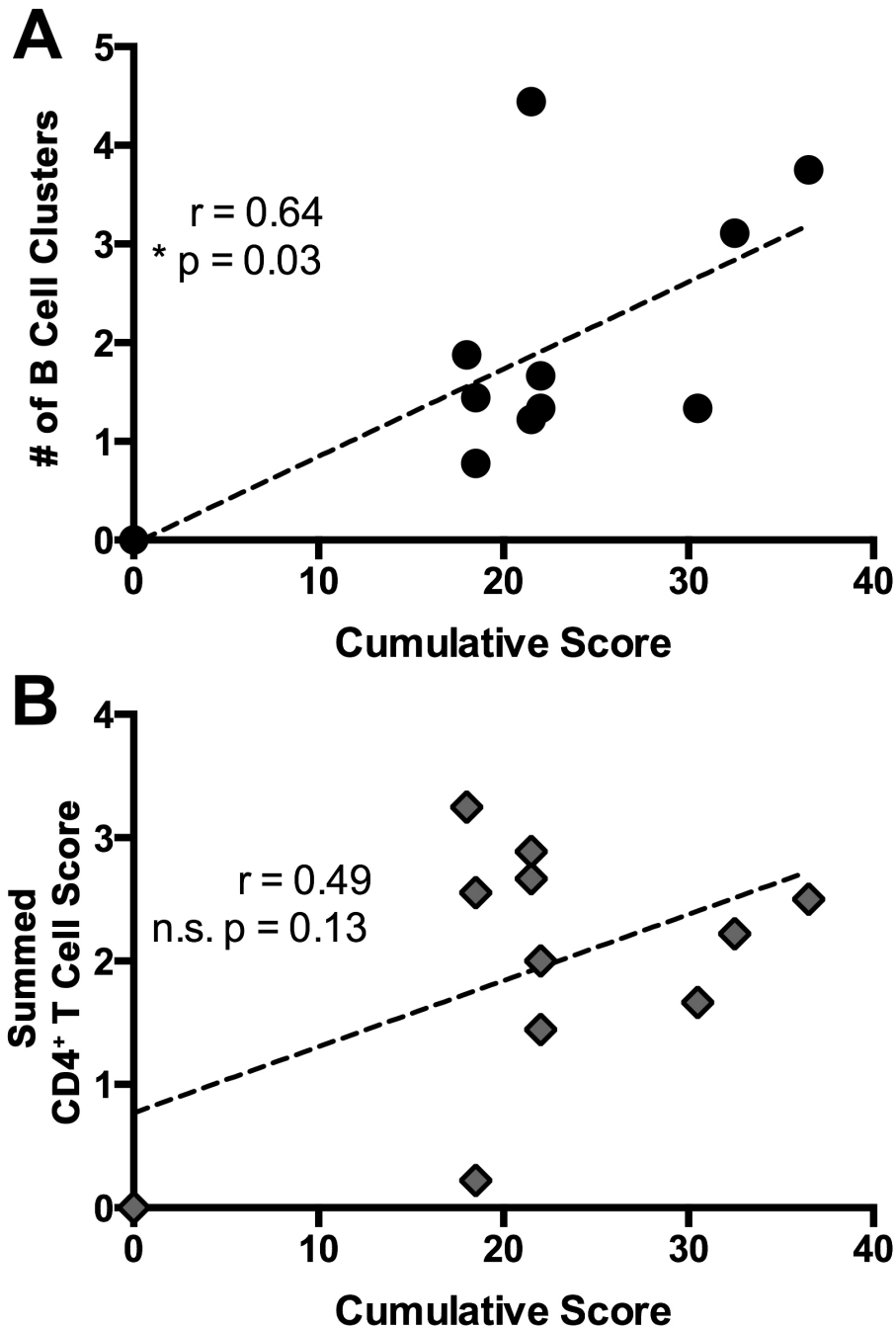
Figure 13: Correlation of immune cell infiltration in the CNS and MOG$_{tag}$-induced EAE disease score. (**A**) The average number of B cell clusters counted per spinal cord section analyzed compared to the cumulative disease score for each mouse. (**B**) The sum CD4$^+$ cell infiltration score of the white and grey matter (0 – no cells, 1 – diffuse infiltration, 2 – severe infiltration) of all spinal cord sections compared to the cumulative disease score for each mouse. Each point represents an individual mouse.
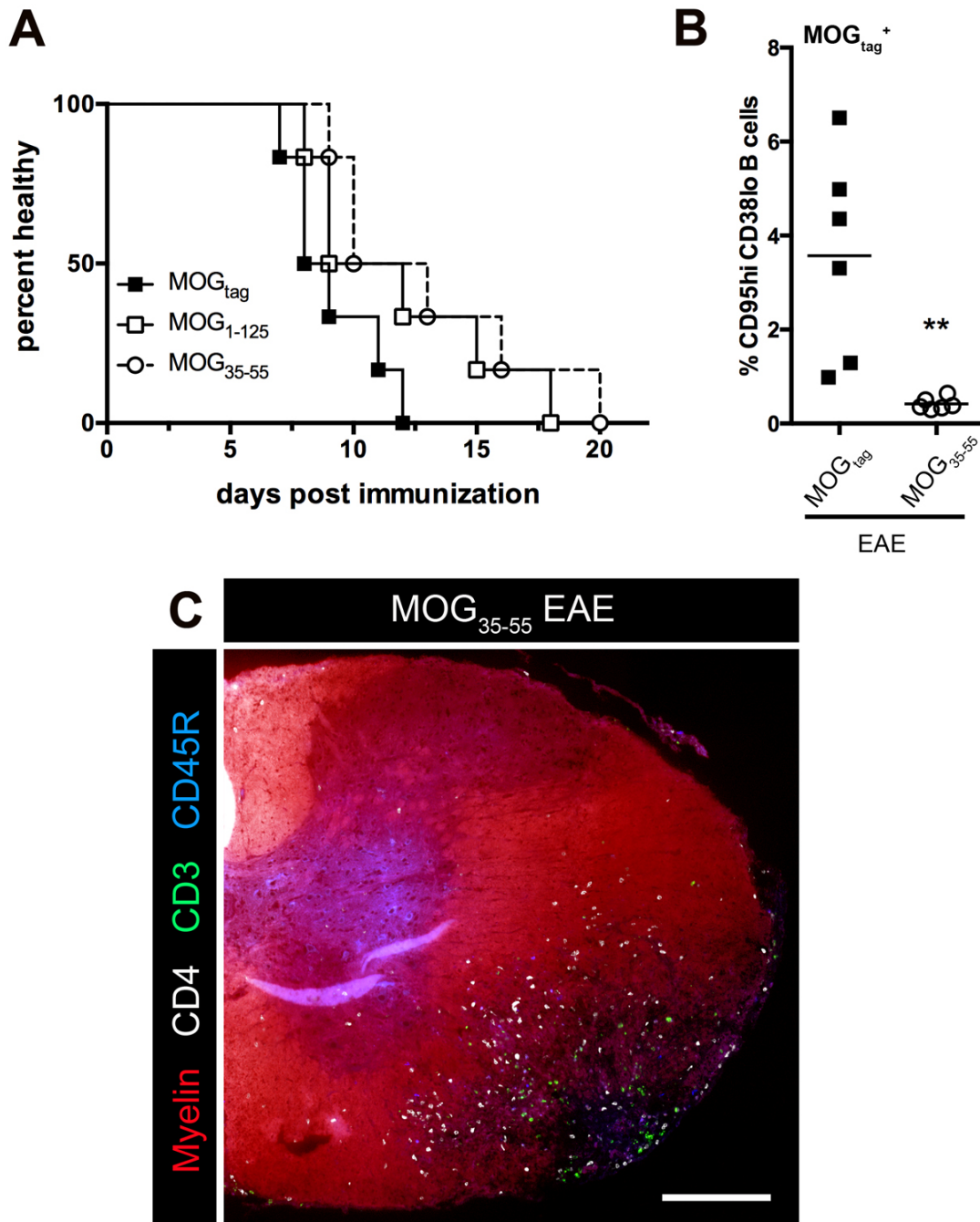
Figure 14: EAE induction by MOG$_{tag}$, pure MOG$_{1-125}$, and MOG$_{35-55}$ peptide. (A) Disease incidence and onset in C57Bl/6 mice immunized with equimolar amounts of MOG$_{tag}$, pure MOG$_{1-125}$, or MOG$_{35-55}$ peptide. **(B)** MOG$_{tag}$ binding by GC B cells from draining lymph nodes of MOG$_{tag}$ and MOG$_{35-55}$ EAE mice. Each symbol represents an individual mouse. **p<0.01. **(C)** Uncommon severe CD4$^+$ T cell infiltration into white matter of spinal cord, as observed in one MOG$_{35-55}$-induced EAE mouse.
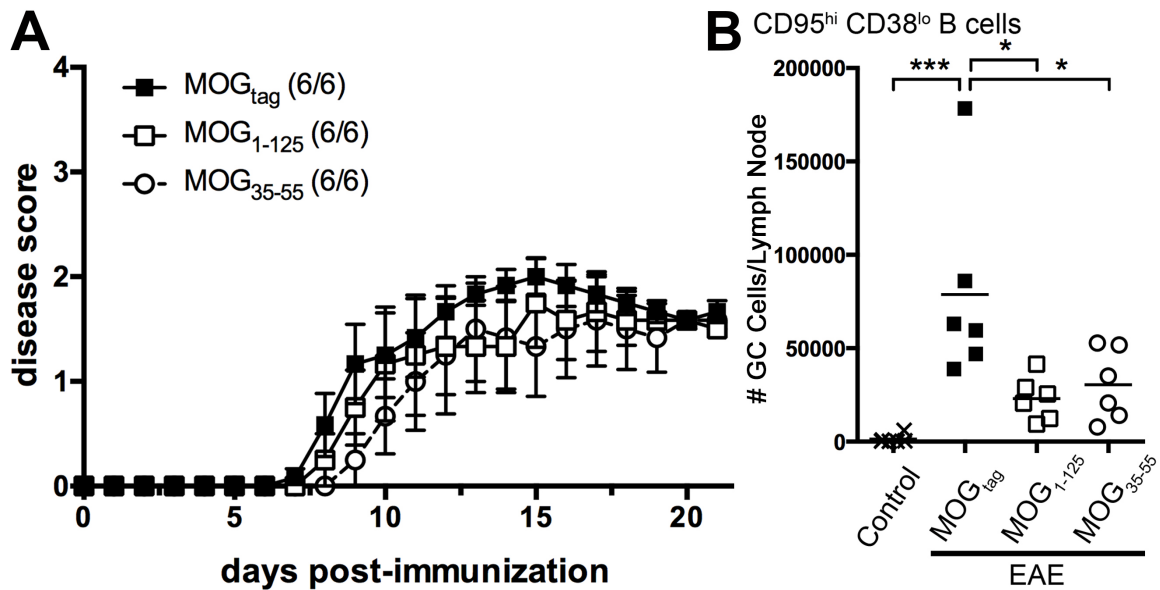
Figure 15: FACs analysis of EAE induced by MOG$_{tag}$, pure MOG$_{1-125}$, and MOG$_{35-55}$ peptide. (**A**) EAE was induced in wild type C57Bl/6 mice via immunization with equimolar amounts of MOG$_{tag}$, pure MOG$_{1-125}$, or MOG$_{35-55}$ peptide in CFA and administered one dose of PTX at the time of immunization. Disease incidence is indicated in the legend. Graph shows mean +/- SEM. (**B**) Mice were sacrificed 22 days post-immunization and draining inguinal lymph nodes were analyzed by FACS for the ongoing presence of CD95^{hi} CD38^{lo} GC B cells in unimmunized (Control) and immunized mice. Each symbol represents an individual mouse. Multiple groups were compared using one-way ANOVA followed by a post-hoc Tukey's test. *p<0.05, ***p<0.001.

B cell clusters in the meninges were evident in 4/6 mice (Figure 16). More variable patterns of inflammation, ranging from diffuse to more severe, were observed in MOG_{35-55}-immunized mice, and B cell cluster formation was only prevalent in 2/6 mice in the group (Figure 16). Demyelination was also less extensive, with the exception of one mouse that had massive white matter infiltration by both $CD4^+$ T cells and $CD45R^+$ B cells (Figure 14C). Surprisingly, this mouse was the last to show overt signs of disease at 20 days post-immunization and had by far the lowest cumulative score of all mice in the experiment (3, as compared to the group average of 16.6). Therefore, as with human MS patients, outward physical disability is not always a reliable measure of underlying pathology. Further, these findings demonstrate that B cell clusters can form independently of B cell binding of endogenous autoantigen.

3.8 MOG$_{tag}$-induced EAE in the absence of B cell recognition of antigen

Finally, to determine the contribution of anti-myelin B cells to MOG_{tag}-induced EAE pathology, we immunized wild type C57Bl/6 and B1-8 $J\kappa^{-/-}$ mice with MOG_{tag} in CFA followed by one dose of PTX, and followed disease development. B1-8 mice possess a heavy chain knockin that, when paired with an appropriate lambda light chain, confers specificity for the hapten nitrophenol (NP) (135), an irrelevant foreign Ag. The deletion of the kappa light chain ($J\kappa^{-/-}$) results in >95% B cell specificity for NP in these mice (136), eliminating the endogenous B cell response to MOG while maintaining otherwise relatively normal B cell populations and lymphoid tissue development.

Consistent with previous studies showing that EAE induced by rodent MOG proteins is B cell independent (125, 130, 131), most (4/5) B1-8 $J\kappa^{-/-}$ mice developed disease that was initially indistinguishable from that in wild type mice (Figure 17A). Following the acute phase, however, disease score was significantly lower in B1-8 $J\kappa^{-/-}$ mice, perhaps indicating that B cells contribute to processes later in disease. FACS analysis of LNs at 22 days post-immunization revealed that while ongoing GC responses were again evident in wild type mice, they were essentially absent in B1-8 $J\kappa^{-/-}$ mice (Figure 17B). B cell binding of MOG_{tag} was also completely absent in B1-8 $J\kappa^{-/-}$ mice compared to wild type
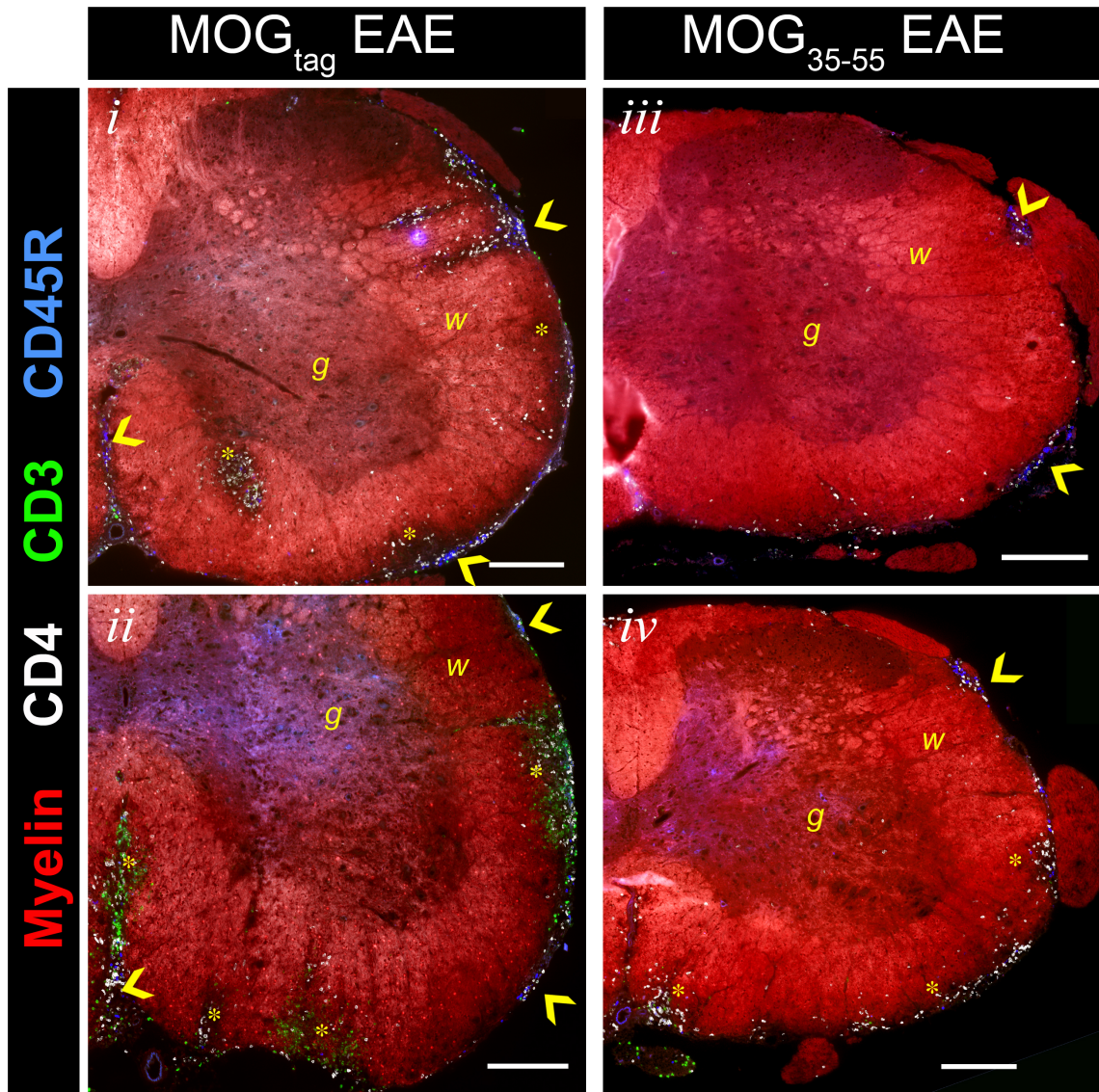
Figure 16: Histological analysis of EAE induced by MOG$_{tag}$, pure MOG$_{1-125}$, and MOG$_{35-55}$ peptide. EAE was induced in C57Bl/6 mice via immunization with equimolar amounts of MOG$_{tag}$, pure MOG$_{1-125}$, and MOG$_{35-55}$ peptide. Representative images of spinal cords from EAE mice induced with MOG$_{tag}$ (i, ii) (n = 6) or MOG$_{35-55}$ (iii, iv) (n = 6) showing common patterns of pathology. * - region of white matter demyelination, open arrow – B cell meningeal clusters, g – grey matter, w – white matter. Scale bars represent 200 µm.
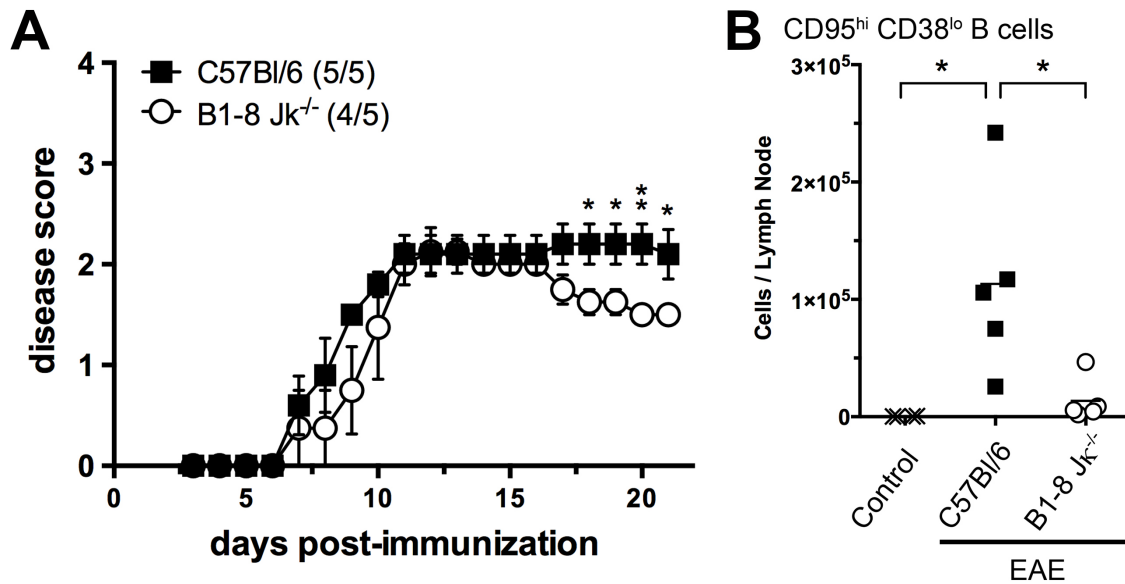
Figure 17: MOG$_{tag}$-induced EAE in C57Bl/6 and B1-8 Jκ$^{-/-}$ mice. (**A**) EAE was induced in C57Bl/6 or B1-8 Jκ$^{-/-}$ mice via immunization with MOG$_{tag}$. Disease incidence is indicated in the legend. Scores are shown only for mice that developed signs of disease. Graph shows mean +/- SEM. (**B**) Mice were sacrificed 22 days post immunization and draining inguinal lymph nodes were analyzed by FACS for the ongoing presence of CD95^{hi}CD38^{lo} GC B cells in unimmunized (Control) and immunized mice. Each symbol represents an individual mouse. Multiple groups were compared using one-way ANOVA followed by a post-hoc Tukey's test. *p<0.05.

mice, as expected (Figure 18A). In contrast, there was no reduction in CD4$^+$ T cell activation, consistent with the elimination of the anti-MOG B cell response but a largely normal T cell response in these mice (Figure 18B).

Histological analysis of spinal cord sections revealed that CD4$^+$ T cell infiltration in the meninges and white matter was often closely associated with regions of demyelination in both wild type and B1-8 Jκ$^{-/-}$ groups (Figure 19). However, demyelination was less extensive in B1-8 Jκ$^{-/-}$ mice. Similarly, while meningeal B cell clusters were consistently observed in diseased wild type mice, they were less common and tended to be considerably smaller in B1-8 Jκ$^{-/-}$ mice. Altogether, these findings suggest that B cells can infiltrate the CNS and form clusters independently of their ability to recognize the endogenous autoantigen.
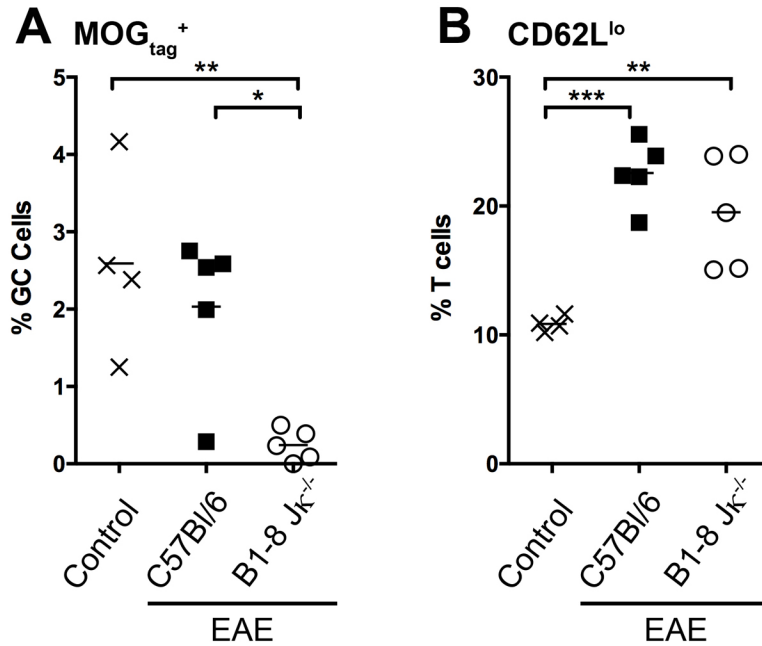
Figure 18: MOG$_{tag}$-induced EAE in C57Bl/6 and B1-8 Jκ$^{-/-}$ mice. EAE was induced in C57Bl/6 or B1-8 Jκ$^{-/-}$ mice via immunization with MOG$_{tag}$ in CFA and administered one dose of PTX. Mice were sacrificed 22 days post immunization and draining inguinal lymph nodes of unimmunized (Control) and immunized mice were analyzed by FACS. (**A**) CD95^{hi} CD38^{lo} GC B were analyzed for binding of MOG$_{tag}$. (**B**) Activation of CD3^{+} CD4^{+} T cells was determined by their downregulation of CD62L. Each symbol represents an individual mouse. Multiple groups were compared using one-way ANOVA followed by a post-hoc Tukey's test. *p<0.05, **p<0.01, ***p<0.001.
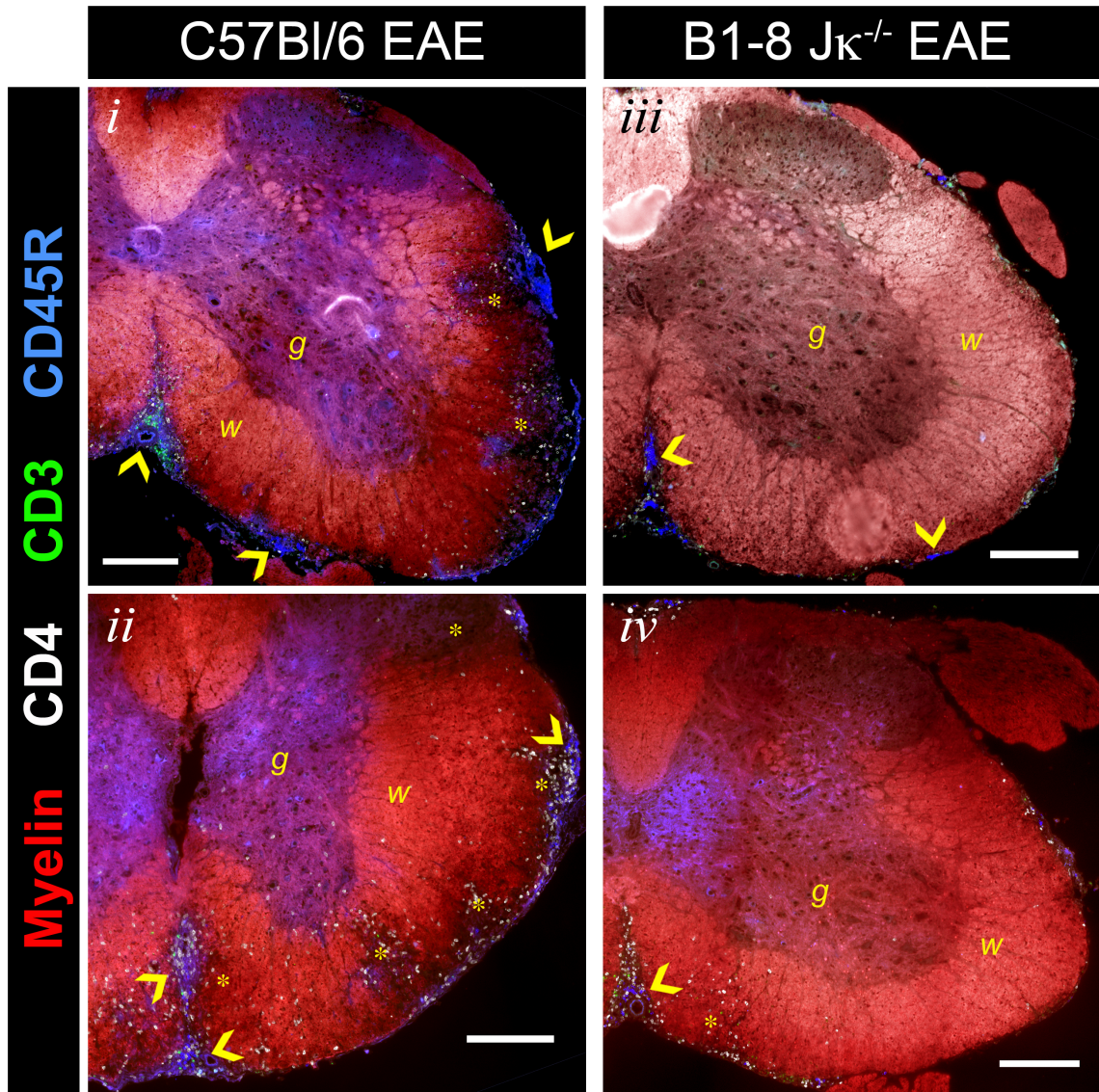
Figure 19: Histological analysis of MOG$_{tag}$-induced EAE in C57Bl/6 and B1-8 Jκ$^{-/-}$ mice. EAE was induced in C57Bl/6 or B1-8 Jκ$^{-/-}$ mice via immunization with MOG$_{tag}$. Representative images of spinal cords from C57Bl/6 (i, ii) (n=5) or B1-8 Jκ$^{-/-}$ (iii, iv) (n=4) EAE mice showing common patterns of pathology. * - region of white matter demyelination, open arrow – B cell meningeal clusters, *g* – grey matter, *w* – white matter. Scale bars represent 200 μm.

Chapter 4

4   Discussion

In this study we developed a new fusion protein (MOG$_{tag}$) containing the extracellular domain of mouse MOG for the purpose of inducing a CNS autoimmune response. Our system allows for the simple purification of large quantities of protein using common laboratory equipment. We characterized the T cell and B cell immune response that MOG$_{tag}$ initiates and confirm the contribution of B cells to pathology in this model of CNS autoimmunity. Interestingly, we report that B cell recognition of the endogenous Ag was not a requirement for infiltration and retention in the CNS.

4.1   Advantages of the MOG$_{tag}$ system

While other similar protein Ags are available, our protein system has several advantages that we hope will facilitate its wider adoption in the EAE field. The hydrophobic properties of MOG makes it highly insoluble and difficult to purify in large quantities. To improve the solubility and facilitate purification of MOG, we fused mouse MOG$_{1-125}$ to a tag containing thioredoxin (141). Indeed, while some MOG fusion proteins have adapted similar approaches to counteract this issue, these systems are based on human MOG (134). To our knowledge, we have not identified a fusion protein based on mouse MOG using a similar system to ours. Additionally, other MOG fusion proteins that we inspected contain tags for purification and, in some cases, additional sequences that we were not able to identify. Unique to MOG$_{tag}$, the entire extraneous tag can be removed by cleavage with TEV protease, leaving only pure MOG$_{1-125}$. As expected, this reduces solubility and this may in part explain its lower immunogenicity compared to MOG$_{tag}$. Alternatively, it is possible that the presence of the foreign thioredoxin domain in MOG$_{tag}$ may help to overcome tolerance mechanisms. Nevertheless, pure MOG$_{1-125}$ remained capable of inducing an anti-MOG response and EAE. To further facilitate its production, we codon optimized and used a more efficient T7 (146) expression system that resulted in the production of large amounts of protein from relatively small culture volumes. Here we describe a simple centrifugation based method of purification that can be performed

in virtually any laboratory with basic equipment. Indeed, while protein purification was the primary factor limiting the amount of protein that we could produce, we acknowledge that HPLC or other specialized protein handling equipment may improve yield.

4.2 Protein versus peptide antigens in modeling B and T cell germinal center interactions

The initiation of a GC response is dependent on B and T cell interactions for the same cognate Ag – a phenomenon that is evident in immune responses against whole proteins. Here we confirm appropriate recognition of MOG_{tag} fusion protein by MOG-specific IgH^{MOG} B cells, and the induction of a GC response upon immunization with MOG_{tag} in CFA. Activation of MOG-specific IgH^{MOG} B cells is of particular relevance to this model as these cells have been confirmed to contribute to autoimmune pathology (120, 121). In similar studies using a model foreign Ag system (NP-haptinated ovalbumin), transferred pre-rearranged antigen-specific B cells dominated the GC response (139). Interestingly, the contribution of transferred MOG-specific B cells to the GC was much more limited. Since MOG_{1-125} was fused to a thioredoxin tag, it was possible that endogenous B cells recognizing the foreign tag were outcompeting autoreactive MOG-specific B cells. However this was not the case as mice immunized with pure MOG_{1-125} also had a similarly low autoimmune GC response. It is not clear why endogenous B cells would dominate the anti-MOG GC response in the presence of elevated numbers of transferred cells with predetermined Ag specificity. A study by Dal Porto et al. demonstrated that higher affinity B cells can exclude cells with fixed lower affinity from the GC (147). This does not seem to be the case as the IgH^{MOG} mutation is derived from a hybridoma selected for its ability to bind MOG and has the mutant variable gene inserted at the endogenous locus (124), thereby allowing for appropriate somatic hypermutation and affinity maturation to occur. Further studies are required to investigate the differences in GC development following immunization with foreign Ag and autoantigen.

Immunization with MOG_{35-55} peptide was also capable of inducing a GC response, although not as well as immunization with MOG_{tag}. This was interesting as Ag presentation by B cells is normally limited to conformational Ags that bind the Ag-specific BCR. Nevertheless, short linear peptide Ags can also bind the BCR.

Furthermore, peptide Ags can also load directly onto MHC II, bypassing BCR recognition and intracellular processing (148). Therefore activated B cells may present peptides independent of their BCR specificity. More importantly, the GC response initiated by MOG_{35-55} did not activate nor incorporate participation of MOG-binding or pathogenic IgH^{MOG} B cells, consistent with the idea that short peptides cannot model a response incorporating both T and B cell recognition of protein.

The majority of EAE studies have analyzed B cell responses primarily in the context of Ab production (111, 112). However human studies of CD20 B cell-depleting therapies indicate that the primary pathogenic mechanism of B cells in MS is not through the production of these Abs (102). There is a growing body of evidence suggesting that GC-derived autoreactive B cells contribute to pathology. We are unaware of a prior description of the GC response, and therefore are the first group to characterize such a response in EAE induced with mouse MOG_{35-55} peptide and MOG_{1-125} protein. Therefore we believe EAE models induced with protein Ags like MOG_{tag} may be more appropriate to investigate these pathological mechanisms.

4.3 MOG_{tag}-induced EAE features autoimmune CNS pathology similar to human MS

Immunization with MOG_{tag} resulted in a monophasic EAE disease with little evidence of recovery. The ongoing GC response and clear evidence of active inflammation in the spinal cords of EAE mice confirms the chronic nature of disease. The CNS pathology in EAE induced with MOG_{tag}, as we describe here, has important features that are otherwise absent, or overlooked in some peptide EAE models. The observation of immune cell infiltrates in gray matter of some mice is of particular relevance to increasing reports of gray matter pathology in human MS patients (83). In contrast, inflammatory infiltrates are usually restricted to the white matter in other EAE models. Also of importance are the meningeal B cell clusters that were often found in association with demyelinating lesions in MOG_{tag}-immunized mice. These structures are reminiscent of so-called tertiary lymphoid tissues that have been reported in human MS and are often associated with more severe disease (89, 149).

An additional advantage of the use of larger protein Ag such as MOG_{tag} to induce EAE is that its utility is not limited to specific strains, unlike short peptides mimicking MHC-restricted T cell epitopes. These highly reduced models were developed shortly after the mechanisms of antigen-recognition by T cells were discovered and great effort was expended to map T cell epitopes. Very few contemporary EAE studies benefit from such highly reduced models. Therefore MOG_{tag}-induced EAE may be a useful model to investigate the role of B cells and these clusters in the pathology of autoimmune-driven inflammation of the CNS.

4.4   B cells contribute to ongoing CNS pathology in MOG_{tag}-induced EAE

While we did not observe a significant difference in disease score between MOG_{tag}- and MOG_{35-55}-induced EAE, this in part could be attributed to the lack of sensitivity of the EAE grading scale. The overall histopathology in the spinal cord tended to be more severe in MOG_{tag}-immunized mice, with evidence of more immune cell infiltration and demyelination. While this could simply be a function of a larger immune response induced by MOG_{tag}, it seems unlikely as equimolar amounts of protein and peptide Ag were used for inducing EAE. Regardless, it is possible that multiple immunizations with MOG_{35-55} or increasing its concentration could result in equal pathology. Alternatively, the differences may be the result of a more complex immune response that incorporates B cell targeting of Ag, as we demonstrate here, or potentially $CD8^+$ T cells as well.

The role of B cells in EAE has long been controversial with conflicting views within the field. It is well established that $CD4^+$ T cells alone are sufficient to mediate the CNS autoimmune response. B cells are not required for EAE induced with either short peptides or protein Ags based on rodent MOG. However as demonstrated in our studies, the reduced CNS pathology in B1-8 $J\kappa^{-/-}$ mice suggests that B cell mechanisms do contribute to ongoing pathology in this model of MOG_{tag}-induced EAE. Additionally, we were surprised to find similar meningeal B cell clusters in some mice immunized with MOG_{35-55} peptide, suggesting that B cell recognition of autoantigen may not be a prerequisite for cluster formation. Further investigation will be required to rule out a contribution from B cells that recognize the glycosylated form of endogenous MOG, as lack of binding to

unglycosylated MOG$_{tag}$ does not exclude this possibility. Nevertheless, small meningeal B cell clusters were also evident in some B1-8 Jκ$^{-/-}$ mice that, because of the absence of kappa light chain and expression of a heavy chain directed towards an irrelevant Ag, almost certainly has no capacity to recognize any form of MOG.

Ab contribution to pathology was not analyzed in this particular study, however their role in disease has been described in the field of EAE. Transfer of autoantibodies against MOG was reported to contribute to demyelination and exacerbate disease in rats, mice and primates with EAE (127, 150, 151). Of particular importance is that the pathogenic ability of Abs is dependent on the type of MOG used. In a comparative study performed by Marta et al., mice immunized with rodent-based protein Ags produced Abs that did not bind glycosylated endogenous MOG nor contribute to pathology (152). Interestingly, the Ab response induced by human MOG in mice was demonstrated to cross-react with the glycosylated endogenous Ag on the surface of oligodendrocytes, despite the fact that the bacterially-produced Ag is not glycosylated itself (152). We do not consider MOG$_{tag}$ protein to be drastically different from currently available protein Ags. Therefore it is possible that the Abs generated in MOG$_{tag}$-induced EAE contribute limited pathology due to an inability to recognize the glycosylated endogenous Ag. Further investigation is necessary to determine the role and Ag-specificity of autoantibodies in this model of CNS autoimmunity.

4.5 Significance

MS is a heterogeneous and complex autoimmune disease in part complicated by the multitude of immune cell players. While MS was originally thought to be T cell-mediated, recent clinical studies have shed light on the importance of B cells in contributing to ongoing pivotal pathogenic processes in this disease. Prior to this discovery the role of B cells in MS were largely overlooked, and consequently poorly understood. This was in part due to highly reduced peptide models of EAE which, as we demonstrate here in our study, are not appropriate models for studying the role of B cells in the ongoing immune response and CNS pathology.

Although EAE studies have improved our knowledge of CNS inflammation, these highly reduced peptide models have inevitably shortsighted our understanding of the complex immune response in MS. There is a clear need for alternatives to short-peptide induced models of EAE that better reflect the complexity and other features of human MS. We show here that MOG_{tag} induces a more complex immune response and consistent CNS autoimmunity. Importantly, the involvement of pathogenic B cells in this model of EAE provides the critical foundation for which to study the development, recruitment and pathogenic processes of B cells to ongoing disease. Further combined with its expression efficiency and relatively simple purification protocol, MOG_{tag} becomes an attractive candidate for more general use in the EAE field. By making a MOG protein autoantigen more accessible, we hope that this model of CNS autoimmunity can begin to regain dominance from minimalist peptide-induced models.

4.6   Future directions

The goal of this study was to develop a myelin protein Ag appropriate for studying pathogenic B cell responses in a model of CNS autoimmunity. Of particular importance to the emerging B cell field in EAE is the presence of meningeal B cell clusters, indicating that B cells are recruited and retained in the inflamed CNS in this model. Thus future studies are required to characterize the B cell phenotypes within the meningeal clusters and to elucidate their potential role in CNS autoimmune disease pathogenesis. FACS and histological studies will be performed on spinal cord tissues to identify if B cells found within the inflamed CNS represent PCs (CD138^{+}), Ag-experienced memory B cells (PD-L2, CD80, CD73) or naïve-like follicular B cells (IgD^{+}, IgM^{+}). More importantly, these studies will determine if meningeal B cell clusters represent tertiary lymphoid structures capable of initiating germinal centers (as determined by Bcl6^{+} expression on B and T cells and the presence of CD35^{+} follicular dendritic cells) as suggested by some studies of human MS. Furthermore, additional ELISPOT and ELISA assays will be performed and compared to disease in order to examine the contribution of Abs to CNS pathology in the MOG_{tag}-induced EAE model.

In this study we demonstrate that B cells are recruited to the CNS independent of their Ag-specificity. Thus it is of interest for future studies to elucidate the Ag-specificity of

the B cells in order to determine if disease progression is facilitated through direct targeting of the autoAg or non-specifically via other mechanisms. Based on the methods used in this thesis, we did not completely rule out the contribution from B cells that recognize the glycosylated form of endogenous MOG. Therefore future studies will be performed to adapt our MOG_{tag} vector for expression in a eukaryotic insect cell line for the purpose of producing glycosylated mouse MOG protein.

At the moment, the mechanisms of B cell recruitment into the inflamed CNS are not well understood. While the therapeutic effects of Natalizumab (anti-α_4-integrin) has been mainly attributed to the blocking of T cells, it is possible that it also functions by preventing the entry of B cells into the CNS. Therefore future studies involving an adoptive transfer of activated B cells, followed by the administration of anti-α_4-integrin and/or anti-P-selectin blocking Abs will be performed to identify the adhesion molecules involved in B cell trafficking into the inflamed CNS.

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