1-12-2021 11:00 AM

Meningeal B cells in central nervous system autoimmunity: Their phenotype and susceptibility to therapeutic depletion

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Microbiology and Immunology

Abstract

B cell depleting therapies have been effective in the treatment of Multiple Sclerosis (MS), yet to date little is known about how B cells promote disease pathogenesis. B cells can be found invading the meninges around the brain and spinal cord in MS, where they cluster in association with T cells. These meningeal B cells clusters are often adjacent to demyelinating lesions suggesting this may be a site where B cells are exerting their pathogenic effects. *The purpose of this thesis was to understand the contribution of meningeal B cells to central nervous system (CNS) autoimmunity, by characterizing their phenotype and determining their susceptibility to B cell depletion*. Using an animal model of MS, immunofluorescence analysis, flow cytometry, or single-cell sequencing analysis was used to analyze B cells within the inflamed CNS. Interestingly, while anti-myelin T cells were readily identified in the inflamed CNS, anti-myelin B cells were excluded from this site. Non-specific B cells within the CNS were phenotypically unique from naïve B cells, exhibiting a non-classical activation status. Following treatment with anti-CD20, B cells were rapidly depleted in peripheral tissues such as the blood, lymph node and spleen, while depletion in the CNS was delayed. Following treatment there was minimal evidence that anti-CD20 accessed meningeal B cells directly, but rather that depletion was indirect and the result of ongoing turnover of the meningeal population and elimination of the peripheral pool from which it is sustained. Depleting B cells from the CNS resulted in significantly less demyelination and T cell accumulation within meningeal clusters. This suggests that B cells may be required to help with T cell reactivation within the meninges, and that depletion of B cells over time prevents ongoing local pathology. Collectively, this thesis elucidates the phenotype of B cells within the inflamed CNS of anti-myelin autoimmunity, supporting a role for their involvement in disease pathogenesis. By selectively targeting pathogenic populations of B cells this will help minimize the adverse effects that exist with current complete B cell depletion therapies.

Keywords

Multiple Sclerosis, EAE, autoimmunity, inflammation, B cells, T cells, CNS, CD20

Summary for Lay Audience

B cells, an important cell of the immune system, are the targets of some of the most effective therapies currently used to treat multiple sclerosis (MS). Despite the success these therapies have in treating patients with MS, the removal of B cells may have negative effects over time such as a weakened immune response to fighting infections. These therapies confirm that B cells are involved in MS, yet we still do not understand how B cells are driving disease. Interestingly, B cells can be found gathering into large clusters in the meninges, a protective covering of the brain and spinal cord, next to areas where damage has occurred. In this thesis I used animal models of MS to determine how B cells found within the spinal cord contribute to disease, and whether they can be targeted for removal by current B cell targeting therapies. I found that B cells located next to damaged parts of the spinal cord are unable to recognize myelin antigens, the main target in MS. Our findings suggest these B cells are interacting with other immune cells in the damaged spinal cord to further intensify the immune response. Finally, using a mouse version of B cell targeting therapy used in humans, I revealed that B cells in the spinal cord are not being targeted for removal by treatment, since the drug was not capable of crossing into the brain and spinal cord. However, by removing B cells in the periphery, B cells in the spinal cord were able to slowly be reduced as their replacement over time was stopped. This removal of B cells in the spinal cord reduced the damage that occurred over the course of disease. My thesis provides a new understanding of the damaging role of B cells in MS. Future work to characterize these B cells will allow us to identify specific targets for new MS therapies.

Co-Authorship Statement

The investigations reported in this thesis were predominantly performed by Yodit Tesfagiorgis under the supervision and guidance of Dr. Steven M. Kerfoot. The contributions of others for each section are described below:

Sections of chapter 3 are adapted from:

Tesfagiorgis Y, Zhu SL, Jain R and Kerfoot SM (2017) Activated B cells participating in the anti-myelin response are excluded from the inflamed central nervous system in a model of autoimmunity that allows for B cell recognition of autoantigen. *J. Immunol.* 199: 449–457.

Text and images were reproduced with permission from the Journal of Immunology (Appendix A). S Zhu helped analyze histological sections contributing to Figure 3.2C. R Jain helped generally with flow cytometry experiments. SM Kerfoot conceived the project, secured funding and provided guidance, supervising all aspects of the study. The manuscript was written by Y Tesfagiorgis with guidance from S Kerfoot. The amended pilot single-cell RNA-sequencing experiment was done through the London Regional Genomics Center under the supervision of D Carter using their newly acquired 10x Genomics Chromium Controller. ER Christensen analyzed this data set under the supervision of Dr. P. Shooshtari.

Chapter 4 is prepared in a manuscript for submission:

Tesfagiorgis Y and Kerfoot SM (2020) Systemic administration of anti-CD20 indirectly reduces B cells in the inflamed meninges in a chronic model of central nervous system autoimmunity.

Y Tesfagiorgis helped to conceive the project, designed and executed experimental work, analyzed and interpreted data and wrote the manuscript with guidance from S Kerfoot. K Parham assisted with preparation for flow cytometry experiments. SM Kerfoot conceived the project, secured funding, and provided guidance, supervising all aspects of the study and manuscript preparation.

Acknowledgments

This PhD could not have been completed without the constant support and guidance from my supervisor Dr. Steven Kerfoot. Steve, thank you for teaching me everything I know, challenging me relentlessly, and being more than just a supervisor. You not only taught me how to be the scientist I am today but gave me the confidence, resilience and critical thinking skills that will allow me to succeed wherever life takes me.

To my advisory committee mentors, Dr. Lisa Cameron and Dr. Rodney Dekoter, you have also played an instrumental role in my success throughout this PhD. You have encouraged my love for science and provided me perspectives and outside the box thinking that is required in any good scientist. Dr. Cameron, thank you for reading and editing this thesis prior to submission, your insightful comments were among the reasons I was able to confidently defend my work.

To my examiners, Dr. Javier Di Noia, Dr. Arthur Brown, Dr. Lillian Barra, and Dr. Dekoter, thank you for giving me one last big picture look at what I have accomplished these past five years. I appreciated the feedback and scientific discussion we had.

To my parents, Okbai and Rishan, thank you for being such a positive influence in my life. You fostered an environment where I was allowed to be curious, ensured I realized my full potential, and instilled in me that nothing in life should be taken for granted. Without the sacrifices you made for me, I would not have been able to be the person I am today.

Helen, this PhD started in hopes of being able to make a difference in your life. Despite the constant challenges you face, you never cease to amaze me with your everlasting faith and strength. Sophia, you have been my biggest supporter and have taught me through example the value of a good work ethic. I will forever love you both and am grateful for the lessons you have given me.

To the current and previous KerfootLab members, thank you for making these past few years a memorable experience. Animal work is no easy feat and often requires all hands-on deck, so thank you for those early morning starts and those many late nights at lab. Thank you for debating the nuances of immunology and constantly supporting me during the hardships of science.

To the MNI department, both past and present, thank you for providing me a nurturing environment to grow. The endless nights spent at the grad club talking science helped make this whole experience a lot more enjoyable. To my friends who saw me through these past five and a half years, I appreciate your continual support and for motivating me to do my best. There are many people who have made this PhD experience one I will never forget – I could not have done it without you!

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List of Abbreviations

5D2 – anti-mouse-CD20

Anti-mCD20 – Anti-mouse-CD20

APC – Antigen presenting cell

APRIL – A proliferation-inducing ligand

AQP4 – Aquaporin-4

ASC – Antibody secreting cell

BAFF – B cell activating factor

BBB – Blood brain barrier

BCR – B cell receptor

BCSFB – Blood-cerebrospinal fluid barrier

BLMB – Blood-leptomeningeal barrier

BTK – Bruton's tyrosine kinase

CIS – Clinically isolated syndrome

CNS – Central nervous system

CSF – Cerebral spinal fluid

DC – Dendritic cell

EAE – Experimental autoimmune encephalomyelitis

EBV – Epstein-Barr virus

FDC – Follicular dendritic cell

GC – Germinal center

GFAP – Glial fibrillary acidic protein

GM-CSF – granulocyte macrophage colony stimulating factor

HEV – high endothelial venules

HLA – Human leukocyte antigen

hMOG – Human myelin oligodendrocyte glycoprotein 1-120

i.p. – Intraperitoneally

i.v. – Intravenously

Ig – Immunoglobulin

MBP – Myelin basic protein

MFI – Mean fluorescence intensity

MHC – Major histocompatibility complex

$mMOG_{tag}$ – mouse myelin oligodendrocyte glycoprotein 1-125

MOG – myelin oligodendrocyte glycoprotein

MP4 – fusion protein of myelin basic protein and proteolipid protein

MRI – Magnetic resonance imaging

MS – Multiple sclerosis

NMO – Neuromyelitis optica

NP – Nitrophenyl

OCB – Oligoclonal bands

PLP – Proteolipid protein

PPMS – Primary progressive multiple sclerosis

PSGL-1 – P-selectin glycoprotein ligand

PTX – Pertussis toxin

rMOG – Rat myelin oligodendrocyte glycoprotein 1-125

RRMS – Relapsing remitting multiple sclerosis

sc-RNA-seq – Single cell RNA sequencing

sEAE – spontaneous experimental autoimmune encephalomyelitis

SEM – Standard error of the mean

SLO – secondary lymphoid organs

SPMS – Secondary progressive multiple sclerosis

TCR – T cell receptor

Tfh – T follicular helper cells

TNF – tumor necrosis factor

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Chapter 1

1 Introduction

Our immune system protects us from foreign invaders, such as viruses, bacteria, and parasites, which can infect us and cause harm. Unfortunately, sometimes our immune system makes a mistake and instead of protecting us from foreign invaders it mounts an immune response against ourselves. When this aberrant immune response occurs, the individual can be left with a chronic autoimmune disease. Multiple sclerosis (MS) is a chronic autoimmune disease of the brain and spinal cord (the central nervous system – CNS), that is characterized by demyelination and inflammation resulting in neurological deficits over time. T cells have long been thought to be the primary drivers of the autoimmune response; however, the recent success of B cell depleting therapies (1–6) confirms the role of B cells as critical players in this disease. Relative to T cells, much less is known about basic B cell biology and even less is known about how these cells contribute to MS. It is likely that depending on their antigen specificity, location within the body, and effector phenotype (regulatory vs pathogenic), different subsets of B cells may contribute to pathogenesis utilizing distinct effector mechanisms. This highlights how essential it is to understand exactly how these mechanisms work and where they occur. Furthermore, it is unknown whether B cell depleting therapies universally effect all compartments of B cells, or if only specific populations are targeted. By addressing these uncertainties, we may then be able to move beyond trying to treat the disease with broadly depleting therapies and instead create more targeted and effective treatments with the advantage of having fewer adverse effects.

1.1 Multiple sclerosis overview

Multiple sclerosis is a complex disease that, despite being first described in 1868 by French neurologist Charcot (7), still has no known etiology. Over the years, researchers have identified genetic, environmental and infectious agents that are all involved one way or another in causing MS. In this section, I will briefly describe the mechanisms thought to contribute to susceptibility to MS, as well as the clinical manifestation of this disease.

1.1.1 MS susceptibility: genetics

The current view is that MS develops in genetically susceptible individuals in response to the right environmental triggers. Studies looking at the familial recurrence rate of MS show the odds of developing the disease are higher in closely related family members, with no evidence of genetic transmissibility (8, 9). Genome wide association studies have been used to identify allelic risk variants among individuals with MS. Consistent with the idea that this disease is the result of an autoimmune response, the strongest association linked to MS susceptibility comes from human leukocyte antigen (HLA) class II alleles, located within the major histocompatibility complex (MHC) region (10–12). MHC class II is expressed on the surface of antigen presenting cells (APCs) and is essential for the activation of $CD4^+$ T cells. When looking at non-MHC alleles, the most recent international genome wide association study found 200 risk variants associated to MS susceptibility (13). This included genes involved in both the adaptive and innate immune system, implicating cytokine and chemokine pathways, co-stimulatory molecules, adhesion molecules, signaling pathways and transcription factors. Nevertheless, these risk variants only account for a modest association, suggesting additional factors must be accounting for disease susceptibility.

1.1.2 MS susceptibility: environmental factors

Several environmental factors have also been implicated in MS susceptibility. First, the odds of developing the disease increase the further away you move from the equator, suggesting that vitamin D deficiency is associated with MS (14). Indeed, when looking at patients who have MS, those with lower levels of vitamin D were more likely to develop new brain lesions observed through magnetic resonance imaging [MRI (15)]. Second, studies looking at environmental exposure to passive tobacco smoke have also shown an association with increased risks of developing MS (16, 17). Third, infections as a young adult with Epstein-Barr virus (EBV) increases the risk of developing MS (18), with incidence being reduced in EBV seronegative individuals (19–21). This finding is attributed to various factors including molecular mimicry of an EBV DNA polymerase peptide, $EBV_{627\text{-}647}$ to a self-protein with four DRB1 T cell receptor (TCR) peptide contacts

identical for the immunodominant epitope of myelin basic protein (MBP) amino acids 85-99 (22–24), which is a major myelin antigen. This is further supported by the finding that B cells infected with EBV accumulate in brain lesions (25, 26). Finally, diet and the gut microbiota have also been proposed as a risk factor contributing to MS (27, 28), as an increased body mass index (29, 30) and numerous microbial infections have been associated with this disease (31, 32).

1.1.3 Diagnosis and clinical manifestation

The complexity of MS is heightened by the heterogeneity of the disease in terms of the onset, clinical manifestations, and severity experienced by individuals. The key to determining if a patient is presenting with features of MS is to be able to demonstrate a dissemination in time and space, whereby new lesions are observed both over time and in distinct anatomical locations within the CNS (33). To do this, MRI techniques are used to identify lesions in the brain and spinal cord (34), lumbar punctures identify the presence of intrathecal immunoglobulin (Ig) synthesis [predominately IgG, although IgM, IgD and IgA have also been observed (35)] within the cerebral spinal fluid (CSF) referred to as oligoclonal bands (OCB) (36), and evoked potential tests are used to measure nerve responses to stimulation (37). The most frequently experienced symptoms of MS include motor impairment, vision loss, sensory deficiencies (tingling, numbness), spasticity, cognitive dysfunction, depression and fatigue (38).

Clinically definite MS is broadly characterized into three distinct subtypes based on initial clinical presentations and disease progression over time. Typically, before being diagnosed with MS, patients may exhibit a clinically isolated syndrome (CIS), whereby inflammation and demyelination in the CNS leads to neurological deficits that last for a minimum of 24 hours, followed by a complete or partial recovery (33). CIS patients may go on to develop clinically definite MS if an MRI identifies new or existing brain lesions, or CSF OCB are observed. Relapsing-remitting MS (RRMS) is the most common form of MS that occurs in 85% of cases (39). It is characterized by clinical episodes of disease worsening with active inflammatory demyelinating lesions forming in the CNS, followed by partial or total recovery. Over time, a majority of RRMS patients develop a secondary progressive form

of the disease (SPMS), where it is thought that ongoing local inflammation occurs within the CNS leading to neurodegeneration, oligodendrocyte loss and progressive disability (40, 41). Alternatively, 15% of individuals will develop primary progressive MS (PPMS), characterized by continuous disease progression from the onset (33). PPMS, like SPMS, results in fewer newly active inflammatory demyelinating lesions, and instead is characterized by having chronic active or slowly expanding lesions with occasional lymphoid neogenesis observed (41–43). PPMS is typically diagnosed in older populations, with frequent lesion formation in the spinal cord (44).

Various additional demyelinating disorders exist that should not be confused with MS. Devic's disease, otherwise known as neuromyelitis optica (NMO), is a relapsing disease that affects the optic nerve and spinal cord. This disease is differentiated from MS by minimal brain involvement or OCB and instead results in continuous spinal cord lesions and the presence of serum aquaporin-4 (AQP4) antibodies (45, 46). In contrast to NMO, myelin oligodendrocyte glycoprotein (MOG) antibody disease is a monophasic or relapsing neurological disorder with a better prognosis for patients. Individuals affected with this disease frequently present with serum MOG-autoantibodies while the presence of OCB is uncommon (47).

1.2 CNS pathology in MS

The brain and spinal cord historically were considered to be immune privileged sites that are highly restrictive in the migration of cells and molecules (48). The CNS is surrounded by the meninges, a highly vascularized tissue, made up of three layers being the outermost dura, arachnoid, and inner pia mater. The arachnoid and pia mater form the leptomeninges, and the subarachnoid space between them is where CSF circulates (49). The inner pia mater continuously envelops the brain and spinal cord parenchyma, following all the gyri and sulci (50). The pia mater also lines the endothelium of smaller arteries and veins that can penetrate into the parenchyma (51, 52), thereby separating the perivascular space from the subarachnoid space (49).

Within the CNS there are three major barriers that regulate the transport of molecules from the peripheral blood: the blood brain barrier (BBB), blood-leptomeningeal barrier (BLMB) and the blood-CSF barrier (BCSFB). The BBB is best characterized, having multicellular components; endothelial cells that line the BBB are sealed by tight junctions and covered by pericytes which together secrete the extracellular matrix of the inner vascular basement membrane (53). The CSF draining perivascular space encompasses the vascular basement membrane with perivascular macrophage, an APC, surveying the area. Finally, the border ends at the glia limitans, comprised of the parenchymal basement membrane (created by astrocytes) and astrocyte endfeet (54). In contrast to this multicellular BBB, the BLMB is unicellular with endothelial cells only sealed by tight junctions. Leptomeningeal macrophages survey the subarachnoid space for any infiltrating cells. Finally, the BCSFB is the tight junction sealed epithelial layer of the choroid plexuses (produces CSF) that separates fenestrated capillaries within the choroid plexus from the CSF (55). Epiplexus cells, a type of APC, line the endothelial cells, patrolling for any migration through this area. Analysis of adhesion molecules of these various endothelial surfaces revealed the BLMB as the easiest barrier to breach as endothelial cells constitutively express P-selectin (56). Together these various barriers highly regulate the migration of molecules and immune cells in and out of the CNS.

Since MS is thought to be the result of an autoimmune response initiated against the myelin sheath surrounding the axons in the brain and spinal cord, leukocyte infiltration must occur in order to initiate the disease. Until recently, the most common CNS pathology in MS was white matter lesions. Active focal white matter lesions characterized by an overall inflammatory phenotype with significant BBB disruption, lymphocyte infiltration, and complement deposition (57), have frequently been observed in early phases of the disease (CIS and RRMS), while largely being absent in progressive forms. Instead, in progressive MS it is more common to observe slowly expanding, chronic lesions of the white matter, with a low degree of demyelination and no BBB disruption (58). With the advent of more sensitive MRI methodologies it became clear that grey matter lesions, also known as cortical lesions, are also a recognized feature in MS pathology (59, 60). Typically, grey matter lesions are associated with more progressive forms of the disease (61), with lesions that are broadly characterized as cortical, intracortical, or subpial (62). Subpial lesions are

often associated with meningeal inflammation that can be either diffuse or densely packed with B and T cells that can occasionally be observed forming into ectopic B cell follicles (discussed in more detail in section 1.2.2) (63). This meningeal inflammation is primarily observed in SPMS (63–68) and PPMS (42, 43, 69), and is correlated with substantial cortical pathology (42, 65–68). However, it is becoming increasingly accepted that cortical demyelination and meningeal inflammation can also be observed in early MS (70, 71). Overall, infiltrating cells of MS lesions include CD4 and CD8 T cells, macrophage, microglia (CNS resident macrophage), dendritic cells (63, 72, 73), and B cells (74). Invading immune cells are able to mediate demyelination via $CD8^+$ cytotoxic T cell responses, complement dependent cell cytotoxicity (CDC), antibody dependent cell cytotoxicity (ADCC), and the release of toxic mediators such as reactive oxygen species.

1.2.1 T cells in MS

It is widely proposed that MS is initiated by the activation of autoreactive T cells in the periphery in response to myelin antigens (1) or through molecular mimicry (20, 22). CNS-draining cervical lymph nodes have been implicated as the site where immune cells are activated due to their abundance of myelin antigens (75, 76). The recruitment mechanisms T cells use to gain access to the CNS have been best characterized using rodent models of MS (more details in section 1.3). Under healthy conditions, low levels of activated (but not naïve) $CD4^+$ T cells, regardless of their specificity, cross the various BBBs using the adhesion molecules α4-integrin and P-selectin glycoprotein ligand (PSGL-1) (77–79). Because of the constitutive expression of P-selectin on the BLMB, T cell migration across this barrier is more efficient and frequently observed (80). Once in the CNS, $CD4^+$ T cells must then be reactivated by local APC, such as perivascular macrophages or dendritic cells (DC), in order to be retained within the perivascular space and parenchyma below (81–86). This reactivation subsequently leads to increased BBB permeability as T cells proliferate and secrete proinflammatory cytokines and chemokines, further driving immune cell recruitment to the CNS resulting in an inflammatory lesion (79, 87). Activated $CD8^+$ T cells like $CD4^+$ T cells have also been shown to utilize α4-integrin to cross the BBB (88); however, it is unknown whether reactivation is required for their retention. Once CNS

inflammation occurs, α4-integrin, endothelial P- and E-selectins and a wide array of inflammatory chemokines are responsible for T cell access to the CNS (89–93).

Despite genome wide association studies frequently linking susceptibility of MS to MHC class II (10, 13), histological evaluation of MS lesions reveals $CD4^+$ T cells as a minor population of immune cell infiltrates (69). Nevertheless, $CD4^+$ T cells infiltrating the CNS are observed more frequently in the perivascular spaces and meninges (68, 94–96). It has been proposed that $CD4^+$ T cells – in addition to contributing to the initiation of disease in the periphery (87) – contribute to ongoing disease pathogenesis through numerous mechanisms, including cytokine-mediated toxicity, ligand-mediated apoptosis, reactive oxygen species, and matrix metalloproteinases (97). Furthermore, CSF and peripheral blood Th1 T cells from SPMS patients have been shown to secrete granzyme and perforin in response to anti-CD3 and anti-CD28 T cell activation (98). In contrast to $CD4^+$ T cells, $CD8^+$ T cells are predominately observed in the lesions of MS patients (68, 69, 94, 99), phenotypically characterized containing granzyme B cytoplasmic granules indicating cytotoxic differentiation (69, 88, 100, 101). HLA class I molecules are constitutively expressed on BBB endothelial cells and perivascular macrophage, and its expression on oligodendrocytes, astrocytes and neurons can be found in active inflammatory lesions within the CNS (102), thereby creating a means for antigen specific $CD8^+$ T cell mediated cytotoxicity. In fact, CD8 T cell infiltration in lesions has been shown to correlate with axonal injury (103), supporting the immunopathogenic capabilities of this cell. $CD8^+$ T cells have also been observed inducing cytokine mediated toxicity (104, 105) and FasL-mediated cell death (106).

1.2.2 B cells in MS

For years, the contribution of B cells to MS has been solely attributed to the identification of antibodies in the CSF seen as OCB (33, 107). Indeed, deposition of antibodies can be seen within some MS lesions (108–111) and the presence of OCB has been shown to correlate to more severe disease (112–114), suggesting antibodies are involved in disease pathogenesis. Interestingly, OCB are unique to the CSF and not found in peripheral blood, with further investigation revealing that plasma cells in the CSF are the main contributor

to this polyclonal B cell humoral response (115). Nevertheless, when characterizing the target of Igs produced in the CSF, consistent results are rarely observed, with some MS patients purified antibodies from OCB targeting cellular debris (116). When analyzing the target antigen of CSF B cells as a means to identify OCB Ig specificity, such specificity towards lipids (117–119), myelin proteins (120–124), bacterial antigens such as *Chlamydia pneumoniae* (125), and viral antigens (126–129) could all be identified at varying frequencies. A study by O'Connor et al., found IgG purified from CNS parenchyma had strong specificity for MOG in 50% (7/14) of cases while CSF and serum IgG did not (130). Interestingly, recent developments have even been made to further delineate distinct demyelinating diseases based on antibody specificity within peripheral blood, with AQP4 being a strong indicator of NMO (46) and anti-MOG antibodies suggestive of MOG antibody disease (47). Nevertheless, this heterogeneity of Ig specificities and inability to frequently recapitulate the presence of myelin-autoantibody reflects the complexity of MS as a disease and the varying role autoantibodies may play.

Clusters of B cells have also been observed in the meninges of patients with SPMS (63–68) and PPMS (42, 43, 69). These clusters of B cells are often found next to demyelinating lesions with severe cortical pathology and often result in an aggressive clinical course (42, 65–68), suggesting that they may be important anatomical sites from which B cells are exerting their pathogenic effects. Certain studies investigating these B cell clusters sometimes term them as ectopic B cell follicles (63), meaning they become organized similar to secondary lymphoid tissue. Based on this preconception, most attention has been focused on looking for events as they occur in secondary lymphoid tissue like germinal centers (GCs). Indeed, when analyzing sections of post-mortem human brain and spinal cords, some groups are able to demonstrate that $CD20^+$ B cells, $CD138^+$ plasma cells, $CD35^+$ follicular dendritic cells (FDC), and $Ki67^+$ proliferating $CD20^+$ B cells can be found within meningeal B cell clusters (64–67). Occasionally they can also be seen containing activation-induced cytidine deaminase, the enzyme responsible for somatic hypermutation, activated caspase-3, which induces apoptosis of low affinity B cell receptors (BCRs), and Bcl-2, which promotes survival of GC B cells (63). Another group was also able to identify $CD38^{hi}CD77^+Bcl2^-$ centroblasts and $CD38^{hi}CD77^-Bcl2^-$ centrocytes in the CSF of patients with MS, indicative of a GC secondary lymphoid follicle (131). The human memory B cell

marker CD27 has also been identified in these meningeal B cell aggregates, representing a majority of the B cells present in this location (80% – 98%) (25, 66). Studies looking at the clonal relationship of B cells within the CSF and CNS compartments reveal shared Ig-heavy (132–136) and light chain variable sequences (36, 137), with evidence of clonal expansion, somatic hypermutation, and variable heavy gene segment bias (Vh4) (138–141), which these studies suggest is representative of a localized antigen-driven B cell response. However, longitudinal studies (142), as well as paired tissue Ig receptor sequencing (135, 143–145) have also demonstrated frequent input from peripheral B cells. Nevertheless, the antigen(s) that drive this immune response have yet to be identified, as antibodies generated from clonally expanded B cells from MS brain tissues did not bind to autoantigens that were unique to MS, while non-MS-derived antibodies had similar binding specificity (146). Based on these human post-mortem CNS findings it has been suggested that meningeal B cell clusters may be where B cells are being locally amplified in the CNS, differentiating and contributing to the inflammatory response found in surrounding tissues (74, 147). Nevertheless, on a closer analysis of the literature it is quite clear that these events are rare and instead diffuse meningeal inflammation or disorganized meningeal B cell clusters are a more frequent phenomenon (42, 43, 65–69). Although this provides important information on aspects of disease pathologies from time-points much further in disease progression, human experiments fall short in their ability to understand how the initiation of disease transpires. This draws attention to the many important questions that still remain unclear, such as what these meningeal B cells are and whether they are involved in disease pathogenesis.

1.2.3 B cell targeting therapies in MS

Although the availability of treatment options have increased over the years, there is still no cure and most effective therapies are broadly immunomodulatory, targeting a wide range of undefined mechanisms (148). First generation therapies including the cytokine β interferon and synthetic protein glatiramer acetate were described as functioning by modulating the T cell response of the disease (149, 150). The mechanistic understanding of T cell recruitment to the inflamed CNS was key in establishing one of the first and most potent second-generation therapies in RRMS, natalizumab (anti-α4-integrin) (151).

Natalizumab was effective in reducing relapses in RRMS patients, as well as reducing $CD4^+$ and $CD8^+$ T cell and B cell migration from the peripheral blood to the CSF (152); however, its use is restricted due to the increased risk of developing progressive multifocal leukoencephalopathy, an opportunistic brain infection resulting from reemergence of John Cunningham virus (153). In contrast, therapeutic strategies that directly target $CD4^+$ T cells, have been ineffective in treating patients with MS despite effective depletion of peripheral $CD4^+$ T cells (154).

More current therapies tend to be more aggressive, depleting entire branches of the immune response. This includes anti-CD20 monoclonal antibody therapies such as rituximab (chimeric human/mouse IgG1), ocrelizumab (humanized IgG1), and ofatumumab (fully human IgG1), which have been effective in treating patients with RRMS (1–3) and more importantly progressive forms of MS (5, 155) which currently have no other FDA approved therapeutic options. CD20 is a transmembrane calcium channel implicated in B cell activation, proliferation and differentiation (156), and is expressed by pre-B, immature, mature, and memory B cells, but not pro-B or antibody secreting cells (ASC). Indeed, following treatment with anti-CD20, clinical trials have confirmed that while CSF B cells are reduced, there is no impact on the level of antibodies present (2, 157, 158). Therefore, while autoantibodies may be key in disease pathogenesis in some MS patients, NMO, and MOG-antibody disorders, it must not be the only mechanism B cells are using to contribute to disease pathogenesis in MS.

Besides the ability to mature into antibody producing plasma cells, B cells are also capable of producing pro-inflammatory (159, 160) or regulatory cytokines (161, 162), as well as being able to act as APCs (64, 163–167). These in turn could be alternative mechanism(s) B cells are employing that further aid in disease pathogenesis. Studies looking at the peripheral blood following anti-CD20 treatment highlight some of these roles B cells play in disease pathogenesis as peripheral blood B cells stimulated *ex vivo* have been shown to produce decreased levels of IL-6 (159) and granulocyte macrophage colony stimulating factor (GM-CSF) (160) following B cell depletion. While anti-CD20 B cell depleting therapies have been effective in limiting new and existing brain lesions, clinical relapses and disability progression (1–6, 168–170), they still, however, are broadly

immunosuppressive as they non-specifically target immune cell populations and have been shown to increase the risk of opportunistic infections, autoimmune disorders and cancer (148, 171). Furthermore, it is unclear whether the reduction of B cells in the CSF is due to the direct entry of anti-CD20 into the CNS or due to the indirect depletion of the trafficking pool of B cells that enter from the periphery into the CNS.

Despite the clinical success anti-CD20 therapies have on MS, attempts to deplete B cells via alternative mechanisms have also been investigated with varying results. Atacicept, a fully humanized recombinant fusion protein containing the extracellular ligand-binding portion of the human TACI receptor linked to a recombinant Fc domain of human IgG (TACI-Fc), was terminated early due to exacerbations in disease (172). TACI-Fc is a soluble recombinant cytokine receptor similar to TACI that results in B cell depletion by blocking the effect of B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL), two key cytokines from the tumor necrosis factor (TNF) family that result in B cell proliferation, maturation and survival (173, 174). The conflicting results of TACI-Fc to anti-CD20 therapies may be attributed to the broader effects of blocking BAFF and APRIL in comparison to depleting CD20 expressing cells, as monocytes, dendritic cells, neutrophils and T cells have also been shown to express BAFF receptors (175, 176). Importantly, TACI-Fc depletes antibody producing plasma cells in addition to B cells, suggesting a protective function B cells may be providing in CNS autoimmunity. In contrast to atacicept, inebilizumab, an anti-CD19 B cell depleting therapy which targets a broader range of B cells, including pro-B cells and Ig-producing plasmablasts, was successful in reducing new gadolinium-enhancing lesions in RRMS in a recent phase I clinical trial (177). Interestingly, a clinical trial of inebilizumab treatment in NMO, which is known to result in autoantibodies, was halted early due to a clear therapeutic benefit (178); however, whether this was due to a reduction in anti-AQP4 antibodies remains to be addressed. Finally, Evobrutinib, a Bruton's tyrosine kinase (BTK) inhibitor which inhibits BCR signaling (179) is being tested in RRMS patients with promising results (180). Overall, the therapeutic success of anti-CD20 B cell depleting therapies confirms B cells contribute to the pathogenesis of MS, although further research is required to understand the various B cell populations involved in disease pathogenesis in order to allow for more selective targeting of pathogenic populations and maintenance of regulatory B cell pools.

1.3 Animal models of MS: EAE and lessons we have learned

Animal models are essential and integral components to human disease research enabling the ability to understand the disease from various different stages, genetic modifications, and manipulations (181). Serendipitously discovered, experimental autoimmune encephalomyelitis (EAE), was first observed in humans immunized with rabies vaccines that contained crude rabbit spinal cord extracts (182), resulting in an anti-myelin inflammatory response with symptoms of paralysis and CNS inflammation (183–185). Adapted for research purposes, the most commonly used animal model for human MS is usually represented by the use of mouse models of EAE, as the reagents and targeted gene-deletion models are abundant in this species. EAE can be induced by various different mechanisms, and depending on how it is induced and which strain of mouse is used – C57BL/6 mice are most common, followed by SJL/J and Biozzi antibody high mice – can have major implications on the role B cells play in disease pathogenesis. Nonetheless, EAE recapitulates several symptoms of MS including limb weakness/paralysis, leukocyte infiltration and inflammation in the CNS typically restricted to the spinal cord in most models of EAE (186). Less common symptoms of EAE include altered gait and ataxia, which result when brain inflammation occurs (187, 188). Furthermore, EAE is influenced by the sex, age, season and housing facilities (189, 190). Here, I will briefly describe the initiation of immune responses and various models of EAE that taught us about the role B cells play in anti-myelin autoimmunity.

1.3.1 Initiation of immune responses

Before being able to understand the various models of EAE, a basic understanding of how antigen-specific immune responses are initiated is required. Typically, immune responses occur in secondary lymphoid organs (SLO) such as the lymph nodes and spleen. Cells of the adaptive immune system, including T and B cells, reside in these tissues and are constantly surveying for antigen that they recognize and can mount an immune response against. Naïve CD4 T cells are incapable of recognizing antigen on their own and instead require help from a mature DC acting as an APC to present epitopes from antigens to the TCR via MHC class II complexes. Similarly, naïve CD8 T cells require the aid of APCs,

though in the context of TCR-MHC class I receptor signaling. In contrast, B cells are able to bind directly to protein antigens via their BCR; however, additional help from $CD4^+$ T follicular helper cells (Tfh) is required. B cells, like DCs and macrophage, are a type of APC that expresses MHC class II. Using their BCR they can endocytose antigen, processing it into short peptides that can then be loaded onto their MHC class II for presentation to T cells. This epitope loaded MHC class II interaction with Tfh cells, with the addition of co-stimulatory receptors, allows for a cognate interaction between B and T cells that recognize the same antigen leading to a GC response. GCs allow for B cells to undergo extensive clonal expansion, somatic hypermutation and class switch recombination, with an end product of high-affinity memory B cells and plasma cells.

1.3.2 Peptide and passive transfer EAE models

For the last two decades, peptide models of EAE have dominated the field as they can be synthesized efficiently at a relatively low cost. The most common peptide model of EAE is based on the extracellular domain of MOG, MOG_{35-55} (191). This allowed for effective characterization of the $CD4^+$ T cell response that occurs in MS as this peptide represented an immunodominant $CD4^+$ T cell epitope that could be presented to T cells by APCs (192, 193). Using this model of EAE, $CD4^+$ T cells are cast as the star of immunopathogenesis, as immunization bypasses antigen processing and presentation by B cells, diminishing the role B cells play in immune responses. Moreover, if B cells are depleted prior to MOG_{35-55} disease induction, or disease is induced in B cell-deficient mice, this results in similar and in some cases more severe disease (161, 162, 194, 195), which is associated with a reduction in IL-10 producing regulatory B cells (161, 162). Overall, this model sheds light on the regulatory roles of B cells in MS but diminished their contributions to disease pathogenesis.

Passive EAE, which results from the adoptive transfer of pre-primed myelin reactive T cells into naïve recipient mice, has demonstrated that both Th1 and Th17 T cells play an important role in EAE pathogenesis (196, 197). An integral study done by Jager and colleagues revealed that different effector T cell subsets can alter pathologies and patterns of immune cell infiltration (198). Indeed, it has been shown that IL-17 and IL-22 cytokine

production, in addition to lymphotoxin αβ expression and podoplanin on the cell surface of Th17 cells, is important for the formation of ectopic B cells follicles in EAE (199, 200). Passive transfer of myelin reactive CD8 T cells into naïve recipient mice is also capable of inducing EAE (188, 201); however, how B cells play a role in this model of EAE is not well characterized.

1.3.3 Protein models of EAE

The role of B cells in EAE was deemed dispensable with animal models frequently utilizing the classical MOG_{35-55} induced EAE in C57Bl/6 mice. However, immunization with larger protein antigens, which allow for the incorporation of tertiary structure, permit the incorporation of B cells and additional lymphocytes creating a more complex interplay of immune cell involvement. Our lab has shown that B cells activated by MOG_{35-55} induced EAE do not recognize MOG protein (202), limiting the use of this model to characterize B cell involvement in CNS autoimmunity. Protein induced models of EAE have therefore become more common with the best characterized model being based on rat or human MOG. Rat MOG_{1-125} (rMOG), which contains the encephalitogenic $CD4^+$ T cell epitope MOG_{35-55}, induces EAE that is not dependent on B cells (203). In contrast, an important distinction was made when immunization of C57Bl/6 mice with human MOG_{1-120} (hMOG) protein induced B cell dependent disease (194, 203). This protein differs from rMOG at several sites, however a key substitution from serine to proline at position 42, results in a weak encephalitogenic $CD4^+$ T cell epitope (204). In B cell deficient mice, hMOG immunized mice were resistant to EAE, although this was not due to B cells being required to act as APCs (203), but was dependent on antibody production (205–207). Alternative protein models of EAE also exist, such as MP4, a fusion protein of MBP (21.5-kDa isoform of human MBP) and proteolipid protein (PLP [three hydrophilic loops of PLP]), that induces antibody dependent EAE (208), that also involves CD8 T cells (209), and the formation of meningeal B cell clusters (210).

Nevertheless, at its core MS is an autoimmune response to self-antigen and while rMOG, hMOG and MP4 allow for a more complex immune response than peptide induced or passive EAE, they are not naturally occurring proteins in the mouse CNS. Therefore, our

lab generated a new fusion protein ($mMOG_{tag}$) based on the mouse MOG_{1-125} extracellular domain (202, 211), that contains the immunodominant $CD4^+$ T cell epitope (MOG_{35-55}) like rMOG. Overall, our lab has demonstrated that immunization with $mMOG_{tag}$ results in induction of a GC response that incorporates B cell recognition of MOG, unlike the response initiated by MOG_{35-55} peptide (202). In addition, the disease featured extensive white matter infiltration by $CD4^+$ T cells, demyelination, and regular formation of B cell clusters within the meninges making it an attractive model to investigate how these structures are involved in CNS autoimmunity.

1.3.4 Spontaneous models of EAE

While inducible models of EAE allow for consistent and reliable experimental conditions, it is an artificial autoimmune response that manipulates the initiation of the disease towards large quantities of antigen and potent adjuvants. In contrast to these models, spontaneous models of EAE (sEAE) have been developed that are reflective of the spontaneous onset in MS. Typically, sEAE occurs in mice that have genetically modified lymphocyte receptors with specificity towards myelin, allowing for enhanced recognition of myelin antigens. For example, TCR transgenic mice, such as SJL mice expressing a transgenic TCR specific for MOG_{92-106} (TCR^{1640}), develop sEAE that results in the expansion of endogenous MOG-reactive B cells and development of autoantibodies specific for native MOG protein (212). This model of sEAE results in inflammation in the brain and spinal cord. Alternatively, C57Bl/6 mice expressing a transgenic TCR specific for MOG_{35-55} (2D2) develop sEAE that occurs at a much lower incidence (4%) (213). Interestingly, if you cross 2D2 mice with IgH^{MOG} mice (214), expressing a MOG_{1-125} specific BCR knockin, the double mutant offspring develop sEAE at a much higher incidence (215, 216), emphasizing the pathogenic role B cells play in the initiation of this model. In contrast to TCR^{1640} sEAE, inflammation in 2D2 IgH^{MOG} mice is confined to the spinal cord and optic nerve. Nevertheless, both models replicate the formation of meningeal B cell clusters and are therefore an alternative model that can be used in investigating the role of meningeal B cell clusters in MS pathology (212, 215–217).

Most of our understanding of T and B cell biology in MS has come from what we have learned from animal models of CNS autoimmunity. The various models of EAE are heterogeneous in their onset, course and inflammation, which is why each model continues to be used to account for the many complexities of MS. When trying to understand the role B cells play within meningeal B cell clusters, utilization of the right model of EAE is necessary.

1.4 B cell contributions to EAE

By using various animal models of MS, researchers have been able to recapitulate many aspects of the disease enabling the ability to better characterize potential contributions B cells may play in disease pathogenesis. In this section, I will describe the B cell mechanisms that are thought to contribute to anti-myelin CNS autoimmunity.

1.4.1 Meningeal B cell clusters in animal models

Meningeal B cell clusters are a common occurrence in all stages of MS, with better characterization being performed in progressive phases of the disease (42, 43, 63–70). This phenomenon can also be recapitulated in certain animal models of CNS autoimmunity, making them an attractive model to study how these structures are involved in disease pathogenesis. Nevertheless, just like meningeal B cell clusters in MS patients, these clusters of B cells in EAE often take on the form of unorganized aggregates of B and T cells, lacking the phenotypic characteristics of tertiary lymphoid tissue. Indeed, an analysis by Magliozzi and colleagues revealed the formation of meningeal B cell clusters occurring in the brain stem and spinal cord of two models of EAE: SJL mice immunized with peptide $PLP_{139-151}$, and Biozzi antibody high mice immunized with spinal cord homogenate (218). These clusters occasionally expressed markers of GC phenotypes such as CXCL13, a chemokine that drives B cell zone organization, FDCs, and Ki67, a proliferative cell marker; however, these features were only identified in a small percentage of the clusters analyzed, and often only one of these phenotypes were observed. In contrast, MP4 induced

EAE was shown to generate tertiary lymphoid tissue in the meninges and the parenchyma of C57Bl/6 mice 61% of the time during chronic disease (d30-57 after disease onset) (210). These clusters were only classified as tertiary lymphoid tissue if there was B and T cell compartmentalization, high endothelial venules (HEV), and at least one additional characteristic phenotype such as FDCs, plasma cells, $Ki67^+$ B or T cells, reticulin fibers and activation-induced cytidine deaminase expression, highlighting that with time, these clusters can go on to form tertiary lymphoid tissue.

Preliminary analysis characterizing the immune response in 2D2 IgH^{MOG} sEAE mice revealed profound infiltration in the spinal cord white and grey matter by $CD4^+$ T cells, demyelination, and formation of large B cell clusters (217). These clusters often lacked evidence of tertiary lymphoid tissue as there was no evidence of $CD138^+$ plasma cells, little proliferation in comparison to $CD4^+$ infiltrating T cells, and no evidence of class switching. Interestingly, when analyzed via flow cytometry, spinal cord B cells did possess an elevated level of CD80 and a reduced level of CD62L in comparison to lymph node B cells, suggesting some level of activation had occurred. Moreover, we were able to demonstrate that disease severity correlated with cluster size, suggesting that while follicular differentiation may occur with time it is not the minimum requirement for pathogenic effects to be observed (217), consistent with some human reports (42, 70). While characterized to a lesser extent, my lab has also shown that $mMOG_{tag}$ induced EAE results in the development of meningeal B cell clusters (202). However, these clusters were also apparent in mice immunized with MOG_{35-55}, albeit at a lower frequency, and in mice with a mutant BCR specific for an irrelevant foreign antigen, suggesting the ability of a B cell to recognize MOG protein is not a requirement for meningeal cluster formation to occur. It is important to note that MS is a highly variable disease and that with time these follicle-like structures may result. Still, the models we have combined allow us to address critical questions regarding the role of meningeal B cell clusters in CNS autoimmunity and their susceptibility to anti-CD20 B cell depleting therapies.

1.4.2 Potential pathogenic B cell mechanisms in EAE and MS: antibody production

In many models of EAE, like MOG_{35-55}, MBP and PLP induced EAE models, production of autoantibodies is not a major contributor to disease pathogenesis. Nevertheless, some models of EAE do generate pathogenic autoantibodies, such as hMOG protein induced EAE in C57Bl/6 mice (203, 205, 206, 219, 220), that can restore disease in B cell deficient mice (207). In contrast, while pathogenic autoantibodies are produced in MP4 protein induced EAE in C57BL/6 mice, these antibodies are not pathogenic on their own (208). It has also been shown that antibody production by IgH^{MOG} B cells allows for concentration of target myelin antigens allowing for more efficient T cell activation with either MOG_{35-55} or rMOG, resulting in increased disease incidence and severity, as well as an earlier onset of clinical symptoms (221). This is supported by work from Kinzel et al., that show that CNS antigen opsonization by myelin reactive Ab produced by IgH^{MOG} B cells allows for efficient uptake by myeloid cells via the Fc receptor, allowing for an increased incidence of sEAE in 2D2 mice (36%, 5/14 mice) supporting the pathogenic capabilities of antibodies in EAE (222). Indeed, as mentioned in previous sections, high levels of antibodies in CSF, in the form of OCB (107, 223), is a hallmark feature in MS, and the presence of plasma cells have been reported within meningeal B cell clusters (43, 64, 65, 69). Although these features may contribute to pathology in a subpopulation of MS patients and in some animal models (224), CD20-targeting therapies complicated this notion as plasma cells are not depleted, nor are antibody levels impacted within a therapeutically relevant timeframe (225). Therefore, while antibodies may still influence the immune response or contribute to disease severity, they must not be the primary pathogenic contribution of B cells to disease.

1.4.3 Potential pathogenic B cell mechanisms in EAE and MS: cytokine production and toxic factors

Independent of antibody production, B cells can modulate T cell responses through the production of proinflammatory and regulatory cytokines. Indeed, it has been shown that the B cells isolated from the peripheral blood of MS patients secrete elevated levels of

proinflammatory cytokines TNF-α, lymphotoxin-α, IL-6, and GM-CSF (159, 160, 226, 227), while anti-inflammatory IL-10 producing B cells are reduced in the peripheral blood of MS patients (228, 229). Furthermore, peripheral blood B cells from RRMS patients, but not control patients, have been shown to secrete non-Ig, non-cytokine, and non-complement mediated molecules that are toxic to oligodendrocyte *in vitro* and results in apoptosis (230, 231), suggesting B cells could be influencing CNS pathology through this as of yet unidentified mechanism. Consistent with human studies of MS, animal models of EAE have also shown that B cells can produce proinflammatory cytokines. B cells from MOG_{35-55} induced EAE secrete elevated levels of IL-6 compared to control mice (159), and selective knock out of IL-6 from B cells results in reduced disease severity and decreased Th17 responses (165). An important finding in animal models was the identification that B cells that lack the expression of IL-10, result in more severe disease in C57Bl/6 mice immunized with MOG_{35-55} (161). Indeed, since then it has been shown that ASC are a key source of anti-inflammatory cytokines such as IL-10 and IL-35 (162, 232, 233). This led to the observation that while pathogenic B cells exist in MS, anti-inflammatory B cells can also be playing a role in regulating disease pathogenesis. Supporting this notion was the identification of a rare population of IL-10 producing B10 like B cells in the peripheral blood of MS patients (234). Overall, it is likely that different populations of B cells contribute differently to CNS autoimmunity, similar to what is seen with T cells.

1.4.4 Potential pathogenic B cell mechanisms in EAE and MS: antigen presentation

B cells constitutively express MHC II which allows them to act as APCs, influencing T cell activation through direct interactions (163, 235). Since B cells specifically take up antigen that bind to their BCR they, unlike other APCs, present antigen to T cells through "cognate" interactions, where T and B cells are specific for the same antigen (236). Cognate interactions are foundational to a GC response (237); therefore, it is almost certain that B cells must participate in this way during the initiation of the original anti-myelin response leading to MS. Furthermore, T cells that are strongly reactivated in the CNS are reportedly capable of increasing the degree of parenchymal inflammation (83), which is dependent on

the affinity of the TCR for its cognate ligand and the number of myelin-epitope-MHC complexes available on the surface of APCs, making B cells a probable candidate for reactivation within meningeal clusters. Indeed, animal models looking at the importance of B cell APC function in CNS autoimmunity have reported that the presence of MHC II on B cells is required for disease initiation by hMOG (165). B cells are also capable of serving as the sole MHC class II expressing APC in passive EAE (166), and when controlled temporally for their expression of MHC class II can result in rapid disease onset in comparison to recipient mice with unaltered MHC class II expression (167). As mentioned previously, sEAE also occurs in a greater proportion of 2D2 mice when crossed with myelin specific IgH^{MOG} mice (215, 216), highlighting the importance of myelin-specific B and T cells cooperating to induce disease. Furthermore, it has been shown that 2D2 IgH^{MOG} mice that are genetically altered to be unable to secrete antibodies are still capable of developing sEAE with meningeal B cell clusters (165), emphasizing the critical role B cells play as APCs in the initiation of EAE independent of antibody production. Consistent with these animal models of CNS autoimmunity, a recent report by Jelcic et al., revealed that memory B cells isolated from the peripheral blood of RRMS patients are potent inducers of myelin-specific T cells responses (238). Coupled with the observation that T cell accumulation is reduced in the CSF of patients following Rituximab treatment (158), this supports a role for B cells in T cell activity within the inflamed CNS.

While B cells have clearly been identified as capable APCs in the peripheral EAE immune response, their role as APCs within meningeal B cell clusters have been far less characterized. B cells have been proposed to function as local APCs within the naive CNS, reactivating newly recruited myelin-specific T cells in passive transfer EAE, as B cells make up the predominant MHC class II population in the naïve CNS (239). However, the function of meningeal B cell clusters during chronic EAE remains to be addressed.

1.5 Rationale and objectives

This thesis aims to characterize B cells within the inflamed CNS in animal models of anti-myelin autoimmunity. Based on observations of B cell accumulation in the meninges observed in human and animal models of MS, I initially hypothesized that myelin-specific autoimmune B cells infiltrate the CNS and drive local pathology, particularly through cognate interactions with T cells. Secondly, I hypothesized that anti-CD20 treatment after chronic EAE is established would reduce meningeal B cells and CNS pathology. To this end, I designed a series of studies to identify pathogenic populations of B cells, their anatomical location, and determine their susceptibility to targeted depletion by anti-CD20 B cell depleting therapies.

1.5.1 Rationale for Chapter 3: *Activated B cells participating in the anti-myelin response are excluded from the inflamed central nervous system in an inducible and spontaneous model of experimental autoimmune encephalomyelitis*

Unlike T cells, B cells have not been well characterized within the context of CNS autoimmunity. We know B cells can produce proinflammatory or regulatory cytokines and act as APCs; however, we have yet to identify exactly what these B cells are doing within the CNS to promote or potentially even mitigate inflammation. To begin to understand how meningeal B cells contribute to disease, I characterized their specificity and activation status using two animal models of EAE: $mMOG_{tag}$ induced EAE and 2D2 IgH^{MOG} sEAE. I hypothesized that B cells would behave like T cells in their recruitment to the inflamed CNS in that they would have to be activated, to gain entry and be specific for CNS antigen to be retained (Fig 1.1). To determine whether antigen specific B cells accumulate in the CNS, I transferred fluorescent MOG-specific B and T cells into non-fluorescent recipient mice to track antigen-specific cells throughout disease. Histology and flow cytometry were completed to elucidate the phenotype of these B cells, comparing them to various B cell populations (naïve or GC B cells). Further analysis on the role of B cells in CNS

pathogenies will be explored with initial analysis focused on their role in antigen presentation and interactions with T cells (e.g. CD80, MHC class II, ICOSL, and SLAM family receptors), although if other proinflammatory mechanisms are uncovered (e.g. cytokine production) these will also be pursued.

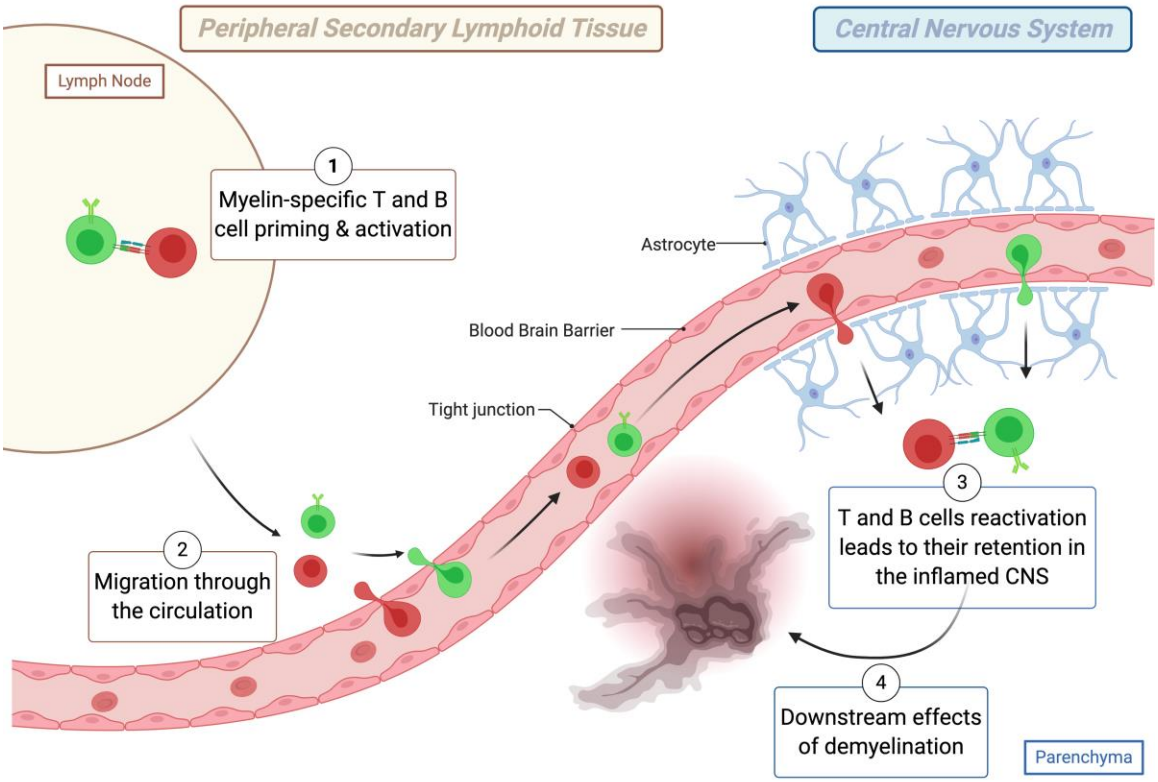


Figure 1.1 | Proposed Model of B cell recruitment to the inflamed CNS.

During CNS autoimmunity we propose that autoreactive myelin specific $CD4^+$ T cells (red) and myelin-specific B cells (green) are primed and activated in the periphery. This allows them to cross the BBB and make it into the inflamed CNS (2). Once there, they must find their respective antigen and be reactivated (3) in order to be retained which leads to the downstream effects of demyelination (4). This figure was adapted from Goverman, 2009 (87) and was created with BioRender.com.

1.5.2 Rationale for Chapter 4: *Systemic administration of anti-CD20 indirectly reduces B cells in the inflamed meninges in a chronic model of central nervous system autoimmunity*

While B cell depleting therapies have been effective in the treatment of MS (3, 5, 6, 168), little is known about how these therapies result in their beneficial effects, and whether meningeal B cell clusters are susceptible to depletion. Previously published data from our lab has shown that meningeal B cell cluster size and number correlates to disease severity (217). Since B cell deleting therapies have been highly effective in treating progressive multiple sclerosis, a course that has been correlated to the presence of B cell follicles in the meninges (42, 64, 65), I hypothesized this drug has a role in targeting CNS B cells. To understand the contribution of meningeal B cells to CNS autoimmunity, I characterized their susceptibility to B cell depletion during the chronic phase of disease using the 2D2 IgH^{MOG} sEAE model. B cell depletion in the CNS was compared to B cell depletion in the peripheral blood and secondary lymphoid tissue using flow cytometry and histology. The degree of CNS pathology was measured by histology, counting the number of infiltrating cells and the extent of demyelination.

Chapter 2

2 Materials and Methods

2.1 Mice

Wild-type C57BL/6, 2D2 TCR transgenic (213), and OTII TCR-transgenic (OTII) mice (4194;Tg(TcraTcrb)425Cbn/J) were purchased from the Jackson Laboratory. IgH^{MOG} MOG-specific BCR knockin mice (214) were received as a gift from Dr. H. Wekerle. B1-8 mice (240) with a homozygous deletion of the Jκ locus (B1-8 $J\kappa^{-/-}$) (241) were a gift from Dr. A. Haberman. Mice expressing fluorescent proteins within all nucleated cells, either dsRed (6051; Tg(CAG-DsRed∗MST)1Nagy/J) under control of the β-Actin promoter or eGFP via the ubiquitin promoter [4353; Tg(UBC-GFP)30Scha/J] were obtained from the Jackson Laboratory. All mice were housed under specific pathogen-free conditions at the West Valley Barrier Facility at Western University Canada. Animal protocols (number 2019-123) were approved by the Western University Animal Use Subcommittee (Appendix B).

2.2 Adoptive transfer

Naive Ag-specific T and B cells were isolated from either RFP^+ 2D2 and GFP^+ IgH^{MOG} or RFP^+ IgH^{MOG} mice, respectively, as previously described (242). Hapten-specific GFP^+ B cells were similarly isolated from GFP^+ B1-8 Jκ–deficient mice representing foreign specific B cells, and nonspecific T and B cells were isolated from RFP^+ and GFP^+ C57BL/6 mice respectively, when noted. Briefly, lymph nodes and spleens of donor mice (as indicated) were dissociated, and T or B cells were isolated using EasySep Negative selection Mouse T and B cell Enrichment Kits (StemCell Technologies). Cells were transferred intravenously (i.v.) into wild-type C57BL/6 recipients, or in some experiments, OTII (foreign T cell specific mice) or B1-8 $J\kappa^{-/-}$ mice (foreign B cell specific mice). Unless otherwise stated, 5×10^5 T cells and 5×10^6 B cells per mouse were transferred 2 d prior to immunization.

2.3 Induction of EAE

To induce a T and B cell autoimmune response targeting MOG-self–antigen I used a novel fusion protein Ag based on the extracellular domain of mouse MOG [$mMOG_{tag}$ (202)]. $mMOG_{tag}$ protein was isolated and purified as previously described (211). Mice 6–8 wk old were immunized subcutaneously at two sites on each flank with a total of 0.5 mg of $mMOG_{tag}$ (and 0.5 mg nitrophenol [NP]-$mMOG_{tag}$, when indicated) in CFA (Sigma-Aldrich). On days 0 and 2 following immunization, mice were administered 250ng of pertussis toxin (PTX) intraperitoneally (i.p.) (List Biological Laboratories). Clinical disease was monitored daily and 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.

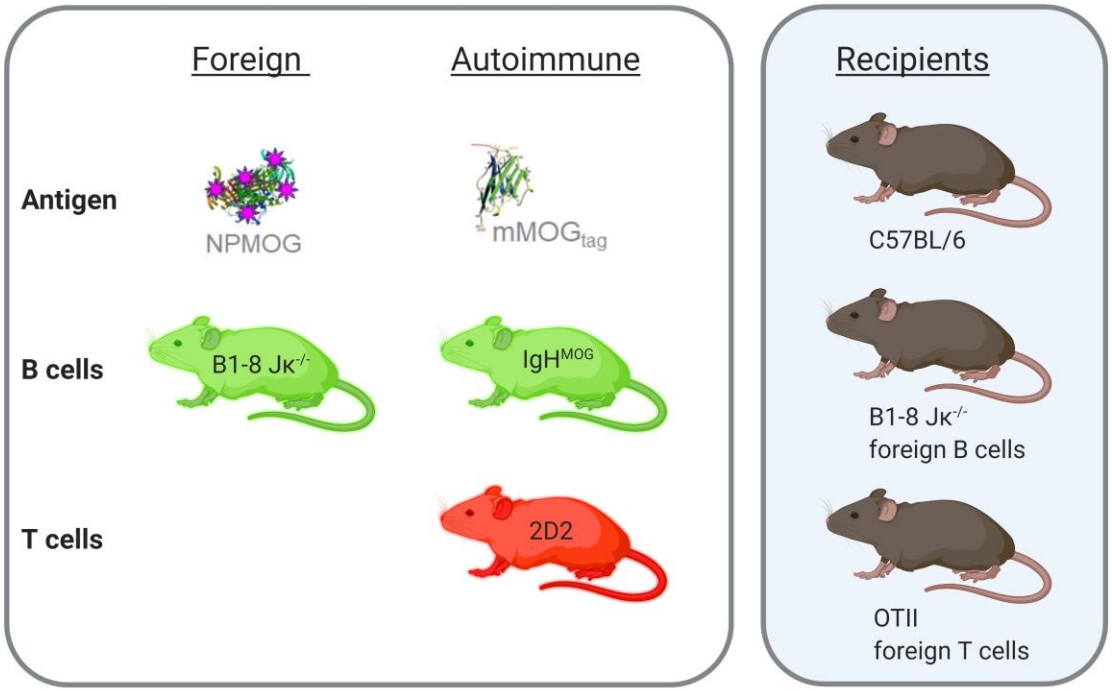


Figure 2.1 | Model of adoptive transfers for tracking myelin specific B and T cells during EAE induction.

To track myelin specific B and T cells during EAE, IgH^{MOG} mice bearing B cells with a MOG-specific BCR knockin (214) were bred onto a green fluorescent background, while 2D2 transgenic mice bearing T cells with a MOG$_{35-55}$ TCR (213) were bred onto a red fluorescent background. These cells were then transferred into wild type C57BL/6 mice in order to track where myelin specific cells home. Mice were then immunized with mMOG$_{tag}$ antigen 2d post transfer to induce EAE. When comparing autoimmune myelin specific B cells to foreign specific B cells, B1-8 Jκ$^{-/-}$ mice were used where >95% of B cells are specific for the irrelevant NP hapten Ag (241). Finally, to exclude the endogenous autoimmune response, cells were transferred into B1-8 Jκ$^{-/-}$ mice to exclude the B cells endogenous response or OTII transgenic mice to exclude the T cell endogenous response. This figure was created with BioRender.com.

2.4 Spontaneous 2D2 IgH^{MOG} EAE model

IgH$^{MOG+/+}$ mice were crossed with 2D2$^{+/-}$ mice. Approximately 80% of IgH$^{MOG+/-}$ 2D2$^{+/-}$ double mutant offspring spontaneously result in EAE (sEAE) at 31 – 42 days of age. Only mice that developed signs of disease and met the conditions of chronic disease were included in experiments. As both sexes develop chronic disease, both male and female mice were used in experiments. Clinical disease was monitored daily with a modified 0-20 clinical scoring system to better evaluate tail paralysis, weakness and paralysis for each individual limb, and righting reflex, with more sensitivity then the typical 5-point scale. Scores were determined as follows, tails were scored as: 0, asymptomatic; 2, partial tail paralysis; 4, complete tail paralysis. Each hind limb was scored as: 0, asymptomatic; 1, hind limb weakness with a wobbly gait; 2, weight bearing but knuckling; 3, not weight bearing; 4, complete hind limb paralysis. Each forelimb was scored as: 0, asymptomatic; 1, weak grasp yet weight bearing; 2, not weight bearing; 3, complete forelimb paralysis. Finally, the righting reflex was assessed as: 0, asymptomatic; 1, delayed righting reflex; 2, no righting reflex.

2.5 Abs for flow cytometry and histology

The following Abs 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-CD62L-A700 (MEL-14), anti-IgG1-APC (A85-1), anti-IgG2a-biotin (R19-15), anti-CD45R-A647 (RA3-6B2), and anti-CD49d-PE-CF594 (R1-2). The following Abs were purchased from BioLegend: anti-IgKappa-Biotin (RMK-12), anti-IgLambda-Biotin (RML-42), anti-CD80-PECy7 (16-10A1), anti-His Tag-purified (J099B12), anti-CD4-A647 (RM4-5), anti-CD3-A488 (17A2), anti-CD45R-APC-Cy7 (RA3-6B2), anti-Ly6G-A647 (1A8), anti-rabbit-DyLight555 (Poly4064), anti-CD19-A488 (6D5), anti-GFAP-A488 (2E1.E9), anti-IL-17A-PE-Cy7 (TC11-18H10.1), anti-IL-10-A647 (JES5-16E3), and anti-IFNγ-A700 (XMG1.2). The following Abs 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-APC, Streptavidin-eF570. FluoroMyelin Red for myelin staining was purchased from Invitrogen. The following antibodies were purchased from Abcam: Anti-Myelin Basic Protein (MBP)-rabbit polyclonal antibody and anti-Ki67 (SP6) unconjugated. The following antibodies were purchased from Life Technologies: anti-ICOSL-PE (HK5.3), and anti-Ly108-APC (13G3-19D). $mMOG_{tag}$ was conjugated to Alexa Fluor 647 ($mMOG_{tag}$-A647) using the NHS Ester and C2 Maleimide fluorophore labeling kits (Life Technologies) according to the manufacturer instructions.

2.6 Anti-mCD20 B cell depletion

Anti-mouse-CD20 (5D2), the murine surrogate of rituximab was received as a generous gift from Genentech, South San Francisco, USA. 150μg of the drug was administered i.v. (unless otherwise stated) during the chronic phase of disease, ~2 weeks post disease onset. Anti-IgG2a (MG2a-53) acquired from BioLegend was used as an isotype control and injected at the same concentration i.v. Mice were monitored for EAE disease severity for either 2- or 7-days following treatment before collecting the blood, lymph node, spleen and spinal cord for flow cytometry or histology.

2.7 Flow cytometry

The blood, lymph nodes (inguinal, axillary, and cervical), spleen, liver, intestines, and the spinal cord were harvested from mice for flow cytometry analysis as previously described (202, 243). Briefly, blood was isolated through a cardiac puncture with needles pre-washed with 0.5M EDTA. All other tissues were harvested after perfusion with ice-cold PBS. Individual spinal cords, livers, and intestines were additionally dissociated through a wire mesh after which leukocytes were isolated using a Percoll (GE Healthcare Life Sciences) gradient. Leukocytes were collected at the 37/90% Percoll interface. The spleen, blood, and liver were then lysed for 2 min at 37°C to remove RBCs. Dead cells were identified by staining with a Fixable Viability Dye eFluor506 (eBioscience) according to manufacturer's protocol. All cells were then blocked with an anti-Fcγ receptor, CD16/32

2.4G2 (BD biosciences), in PBS containing 2% FBS for 30 min on ice. Cells were then stained on ice for 30 mins with the listed combination of staining Abs, followed by a secondary stain with streptavidin for 15 mins on ice where necessary. Spleen cells were fixed in 2% PFA in PBS prior to running cells to prevent cell clumping. Flow cytometry was performed on a BD Immunocytometry Systems LSRII cytometer. Analysis was then completed using FlowJo software (TreeStar).

For cytokine staining, 2 x 10^6 lymph node cells and 4 x 10^5 spinal cord cells were stimulated with cell activation cocktail with brefeldin A (Biolegend) for 2 hrs at 37°C then stained as listed above. Intracellular staining of IL-10, IFNγ and IL-17 was completed as previously described (244). Briefly, cells were fixed and permeabilized with Cytofix / Cytoperm solution (BD Bioscience) after cell surface staining. Fixed cells were then intracellularly stained for IL-10, IFNγ and IL-17 at 4°C overnight. The next day fixed cells were washed, and flow cytometry was performed as stated above.

2.8 10x Genomics single cell library preparation and Illumina NextSeq Sequencing

All samples were sequenced at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada; http://www.lrgc.ca) using the Illumina NextSeq 500 (Illumina Inc., San Diego, CA). A fully dissociated cell suspension was quantified using the CellDrop Automated Cell Counter (DeNovix, Wilmington, DE, USA) to measure total cell counts and cell viability using Acridine Orange / Propidium Iodide (AO/PI) dyes. Two thousand lymph node and six thousand spinal cord cells were selected for GEM generation and library construction, as per the user guide (Chromium Next GEM Single Cell 3' Reagent Kits v3.1 Rev D). Library quantity was determined using the Qubit 2.0 Fluorimeter (Thermo Fisher Scientific, Waltham, MA) and size determined using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA) and the DNA High Sensitivity kit (Caliper Life Sciences, Mountain View, CA). The library was sequenced on an Illumina NextSeq 500 as a paired end run, 28 x 91 bp, using a High Output v2 kit (150 cycles).

Fastq data files were analyzed using Partek Flow (St. Louis, MO). After importation, tags were trimmed, data was aligned to the *Mus musculus* mm10 using STAR 2.5.3a and annotated using Ensembl v98. Unique Molecular Identifiers (UMIs) were de-multiplexed and quantified to create single cell counts. Guided by violin plots, the cells were filtered based on total reads > 512, expressed genes >11 and mitochondrial reads percent < 5.50%. The raw counts data was normalised and clustered using Adobo, and cell types within each cluster were identified using the Adobo marker-based cell type prediction method (245). B cells identified in both the spinal cord (129 B cells) and lymph node (661 B cells) samples were then isolated from the rest of the clusters and fed to Seurat for integration and differential expression analysis (246).

2.9 Immunofluorescent histology

Spinal cord and lymph node tissue were prepared for histology as previously described (217). Briefly, at the end of the experiment or earlier, if mice reached a predetermined endpoint lymph node sections and spinal cord tissue spanning the cervical to lumbar regions were isolated and fixed in PLP. Spinal cords were then cut into five to nine evenly spaced sections and frozen in OCT (TissueTek) media. Tissue was then cut in serial cryostat sections at 7μm. Prior to staining, all slide-mounted tissue sections were blocked with PBS containing 1% BSA, 0.1% Tween-20, and 10% rat serum. After staining, sections were mounted with ProLong Gold Antifade Reagent (Invitrogen). When staining for MBP, tissue sections were additionally pre-treated to remove lipids in a series of ethanol gradients (0%, 50%, 70%, 90%, 95%, 100%, 95%, 90%, 70%, 50% and 0% ethanol in deionized water for one minute at each concentration) prior to the blocking step. Tiled images of whole spinal cord sections were collected using a DM5500B fluorescence microscope (Leica) at 20x.

2.10 Imaging and Statistical analysis

Microscopy images were analyzed using Fiji software. The number of infiltrating B and T cells were determined by cell counting the number of cells per spinal cord area. The total area of the spinal cord was determined by either FluoroMyelin staining or glial fibrillary

acidic protein (GFAP) staining, while regions of demyelination were determined by the absence of MBP. The percent of demyelination was calculated by dividing the area of demyelination to the total area of the spinal cord. To determine the size of the cluster area, I looked at regions of the meninges (determined by the lack of GFAP staining) that contained B and T cell infiltration.

PRISM software (GraphPad, La Jolla, California) was used for all statistical analysis. A Student t test was used for single comparisons, and ANOVA followed by a Student t test with Bonferroni correction was used for multiple comparisons.

Chapter 3

3 Activated B cells participating in the anti-myelin response are excluded from the inflamed central nervous system in various models of autoimmunity that allows for B cell recognition of autoantigen

Texts and figures within this section were adapted with permission (Appendix A) from the previously published manuscript:

Tesfagiorgis, Y., S. L. Zhu, R. Jain, and S. M. Kerfoot. 2017. Activated B Cells Participating in the Anti-Myelin Response Are Excluded from the Inflamed Central Nervous System in a Model of Autoimmunity that Allows for B Cell Recognition of Autoantigen. *J. Immunol.* 199: 449–457.

3.1 Introduction

Multiple sclerosis (MS) is a chronic disease characterized by inflammation of the central nervous system (CNS) and demyelination of axons, thought to be driven by an autoimmune response targeting myelin antigens (247, 248). The importance of B cells to ongoing disease was demonstrated by clinical studies using anti-CD20 Abs to deplete B cells in MS patients (225). This finding is complicated by subsequent studies using a different approach to deplete B cells through the blockade of BAFF and APRIL, key cytokines that drive B cell proliferation, maturation, and survival. In contrast to CD20-targeting therapies, there was no evidence of benefit and, instead, disease may have been accelerated (172). Therefore, although B cells as a whole are major players in disease, there is an urgent need to identify the pathogenic and protective subsets and the mechanisms by which they influence disease.

The B cell lineage is best known for production of Abs, via plasmablasts and plasma cells, which target immune effector mechanisms to specific antigens. However, plasma cells are not depleted by anti-CD20, nor are Ab levels effected in the therapeutic time frame (225, 249). Therefore, although Abs may contribute to MS, they are not the primary mechanism by which B cells drive disease. B cells may also contribute to MS by influencing inflammation and the autoimmune T cell response through production of cytokines or by acting as APC (250, 251). Particular attention has recently been paid to B cells that infiltrate the CNS in MS (252). Indeed, several studies of human tissue (42, 43, 63–68) and in animal models (218, 210, 217, 202, 199, 253, 254) describe B cell accumulation specifically within the meninges, often forming clusters immediately adjacent to demyelinating lesions. Consensus is forming around the hypothesis that these are sites where activated autoimmune B cells promote pathology from within the CNS in MS (251, 252, 255).

Some studies of human MS brain tissue report that meningeal B cells assemble into highly organized follicles separated from T cell zones (64, 66, 67, 218). Like the structures in lymph nodes that they resemble, these so-called tertiary lymphatic tissues may be capable of sustaining GC responses in which antigen specific B cell clones proliferate and differentiate into memory and effector subsets (256). Similarly, antibody producing plasma

cells have been described in MS brain tissue (43, 64, 65, 69). In more recent studies, evidence of class switch and accumulation of somatic mutations was found in BCR genes isolated from MS brain tissue (143, 251), both of which are strong indicators that B cells in the CNS derive from a GC response. The apparent accumulation of antigen experienced B cell clones indicates that they may be responding to an autoantigen present in the tissue.

Nevertheless, much less is known about B cell pathological mechanisms and B cell invasion of the inflamed CNS in autoimmune disease compared with what is known about T cells. It is well established that, as with other tissues, antigen activated T cells are able to gain access to the CNS and that those T cells that encounter their specific antigen within the tissue are retained and reactivated to exert their effector function (87). The evidence presented above suggests that the same may also be true of B cells, but this has not yet been demonstrated.

I set out to define the B cell relationship with the inflamed CNS in anti-myelin autoimmunity using both an inducible and spontaneous mouse model of experimental autoimmune encephalomyelitis (EAE). The inducible model results from immunization of C57BL/6 mice with a fusion-protein based on the extracellular domain of mouse myelin oligodendrocyte glycoprotein ($mMOG_{tag}$) (211). Our lab has previously demonstrated that, unlike most models used currently, the inducible model incorporates both T and B cell recognition of the autoantigen (202). By tracking transferred T and B cells with known specificity for MOG, I was surprised to find that, although activated autoimmune T cells are enriched in the diseased CNS as expected, the opposite was true of myelin-specific B cells. This finding was further corroborated by the utilization of a different B-cell dependent spontaneous model of EAE that develops in mice expressing both 2D2 TCRs specific for MOG_{35-55} (213) crossed with IgH^{MOG} BCRs specific for MOG_{1-125} (214). This model resulted in mice with $mMOG_{tag}$ specificity excluded from the inflamed CNS. This finding is a significant challenge to our assumptions about how B cells contribute to autoimmune disease pathology from within the CNS.

3.2 Results

3.2.1 Activated, autoimmune T cells are enriched in the inflamed spinal cords of $mMOG_{tag}$-induced EAE mice

Our lab recently demonstrated that immunization of C57BL/6 mice with a novel protein Ag based on the extracellular domain of $mMOG_{tag}$ results in chronic CNS autoimmunity driven by both anti-myelin T and B cells (202). To identify and track the location and differentiation of pathogenic autoimmune cells over the course of the disease, I transferred fluorescent anti-MOG T and B cells to nonfluorescent recipient mice prior to disease induction. Red fluorescent T cells were isolated from 2D2 TCR transgenic mice that also ubiquitously express RFP, and green fluorescent B cells were isolated from IgH^{MOG} BCR H chain knockin mice that also ubiquitously express GFP. These cells were transferred to wild-type C57BL/6 mice 2 d prior to immunization with $mMOG_{tag}$ in CFA and i.p. administration of PTX to induce disease. Our lab previously showed that immunization induces a large expansion of the transferred autoimmune T and B cells via a germinal center response (202).

Consistent with our previous observations (202), immunized mice developed physical signs of disease ~5 d post immunization (Fig. 3.1A). Diseased mice were sacrificed 11 d post immunization in the acute phase of disease, and spinal cords were harvested for immunofluorescence analysis of pathology and inflammation. Extensive pathology was evident throughout the spinal cord featuring large regions of white matter demyelination associated with $CD4^+$ T cell (Fig. 3.1B) and $CD8^+$ T cell (data not shown) infiltration. $CD45R^+$ B cells were largely confined to the meninges, often directly adjacent to underlying demyelinating lesions (Fig. 3.1B).

It is well established that activated but not naive T cells are able to gain access to the noninflamed CNS, and that encountering a specific Ag is required for retention of a T cell in the tissue (77). Although the literature typically generalizes this to include the inflamed CNS in EAE, the evidence for this is limited. Using intravital microscopy of the pial microvasculature in a rat model of EAE, Bartholomäus et al. (84) observed endothelial

transmigration of activated T cells regardless of specificity, but noted that only myelin-specific cells were able to migrate away from the immediate vicinity of the vessel. However, our analysis of T cell infiltration of the spinal cords of mice described above revealed that both RFP^+ 2D2 and RFP^- endogenous $CD4^+$ T cells were evident throughout the white matter and meninges (Fig. 3.1C). The proportion of infiltrating $CD4^+$ T cells that were RFP^+ was variable between individual mice (Fig. 3.1D), yet in most cases exceeded the proportion of RFP^+ cells observed in the inguinal lymph nodes in which the anti-MOG response itself was initiated (never exceeding 2–44% as determined by flow cytometry; data not shown). The relative enrichment for MOG-specific RFP^+ T cells in the inflamed CNS was confirmed in a separate experiment that used flow cytometry to quantify the accumulation of RFP^+ T cells in the spinal cord, circulation, and lymphoid tissues of EAE mice. Indeed, RFP^+ cells made up a greater percentage of the total $CD4^+$ T cell population in the spinal cord compared with circulating blood, spleen, lymph nodes, or draining inguinal lymph nodes (Fig. 3.1E).

In the above experiments it is likely that recipient-derived nonfluorescent MOG-specific T cells also responded to $mMOG_{tag}$ immunization. Therefore, at least some of the infiltrating RFP^- T cells observed in the spinal cord may have also been MOG specific, potentially resulting in an underestimation of enrichment for MOG specificity. To largely limit the anti-MOG T cell response to transferred RFP^+ 2D2 T cells, OTII mice in which the great majority of T cells express an irrelevant TCR against the foreign Ag OVA were used as recipients. Following transfer of fluorescent MOG-specific T and B cells as described above, OTII recipients developed disease in response to immunization with $mMOG_{tag}$ (Fig. 3.1F). Again, RFP^+ cells made up a large proportion, but not all, of the infiltrating $CD4^+$ T cells (Fig. 3.1D). Therefore, although MOG-specific T cells are highly enriched in the inflamed spinal cord in EAE, Ag specificity is not likely to be an absolute requirement.

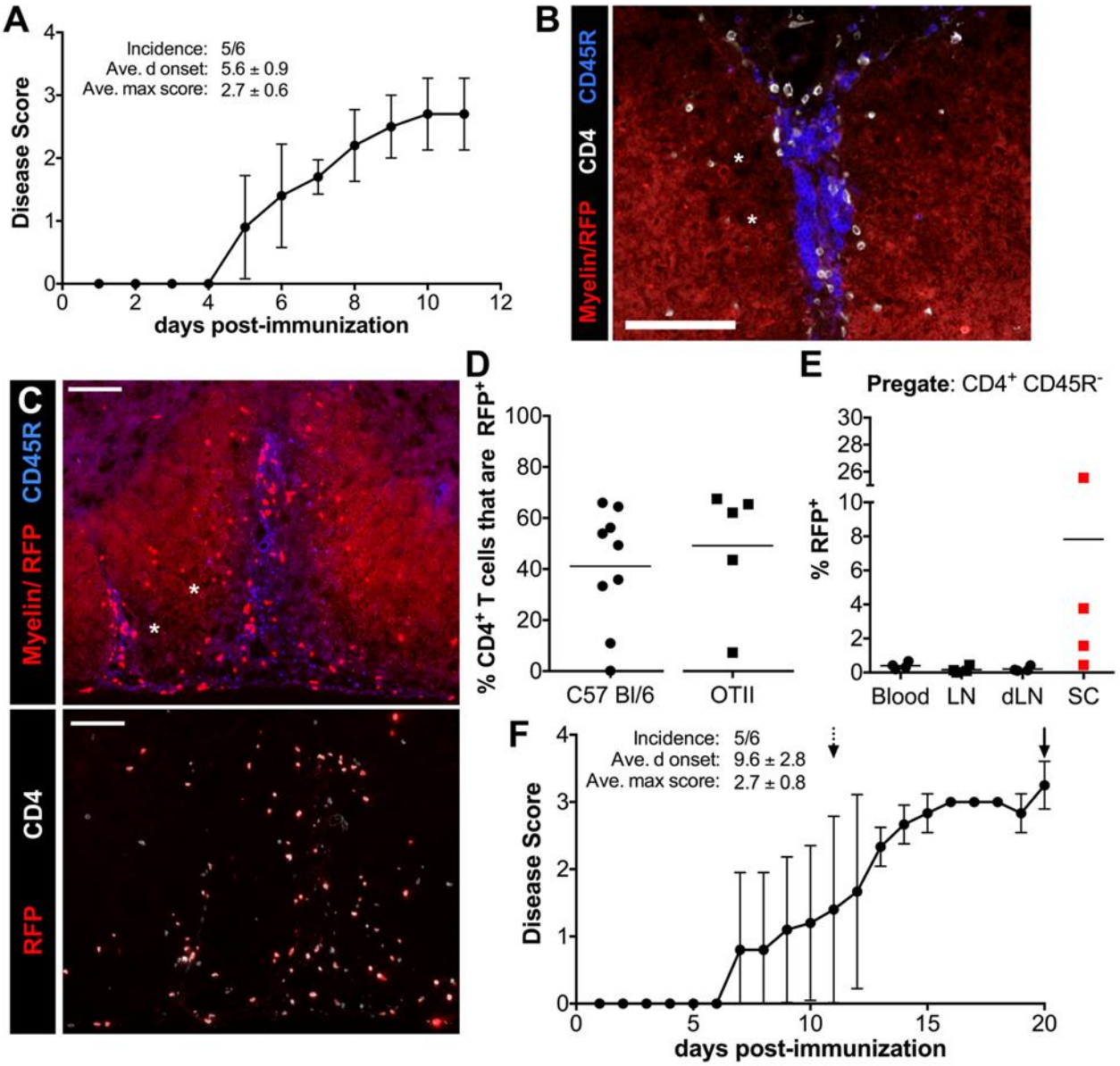


Figure 3.1 | CNS infiltrating T cells are enriched for autoimmune T cells.

GFP^+ IgH^{MOG} B cells and RFP^+ 2D2 T cells were transferred into wild type nonfluorescent C57BL/6 recipients 2 d prior to induction of EAE via immunization with $mMOG_{tag}$ in CFA and i.p. administration of PTX. Mice were monitored daily for signs of disease (**A**). Note that disease scores are shown only for mice that developed disease. (**B**) Representative immunofluorescence image of a spinal cord section collected 11 d post immunization from an EAE mouse demonstrating T and B cell infiltration and demyelination. Regions depicting myelin loss are starred. (**C**) Representative serial sections demonstrating a region

of extensive myelin loss in the white matter (*top*; myelin loss is starred) and extensive infiltration of $CD4^+$ cells, many of which were also RFP^+ indicating they derived from our transferred 2D2 RFP^+ T cells (*top and bottom*). (**D**) $CD4^+$ cells in spinal cord sections of C57BL/6 or OTII-recipient EAE mice (**F**) were quantified and the percent of RFP^+ cells is shown. Each symbol represents an individual mouse from three pooled independent experiments (C57BL/6) or a single OTII experiment. (**E**) Fluorescent MOG-specific T and B cells were transferred to wild-type C57BL/6 recipients prior to EAE induction as described above. Then 20 d post immunization, circulating blood, pooled lymph nodes (LN), draining inguinal lymph nodes (dLN), and spinal cord (SC) were analyzed by flow cytometry. The percent of $CD4^+$ cells that are also RFP^+ is shown for each tissue. Each symbol represents an individual mouse. (**F**) Disease scores for OTII recipient EAE mice used for analysis of RFP^+ T cell infiltration of the spinal cord shown in (**D**). Two mice reached disease severity endpoint and were sacrificed early (dotted arrow) and remaining mice were sacrificed at the solid arrow. Scale bars, 100 μm. Data is shown as mean +/- SEM.

Flow cytometry analysis revealed that spinal cord T cells express higher levels of the activation marker CD44 and lower levels of CD62L compared with lymph node T cells (Fig. 3.2A, 3.2B). By histology, a slight majority of both RFP^+ 2D2 T cells and endogenous RFP^- T cells co-stained with CD44, regardless of whether C57BL/6 or OTII recipient mice were used (Fig. 3.2C). Similarly, both RFP^+ and RFP^- T cells stained for intracellular Ki67 in approximately equal numbers (Fig. 3.2D), indicating that many were in cell cycle. However, I observed no evidence that T cells in either the meninges or infiltrating deeper into the CNS parenchyma expressed PD-1 (data not shown), excluding the possibility that they were either T follicular helper cells or exhausted T cells. Therefore, consistent with established rules for T cell surveillance of the healthy CNS (77), activated anti-myelin T cells are highly enriched in the inflamed CNS in autoimmunity.

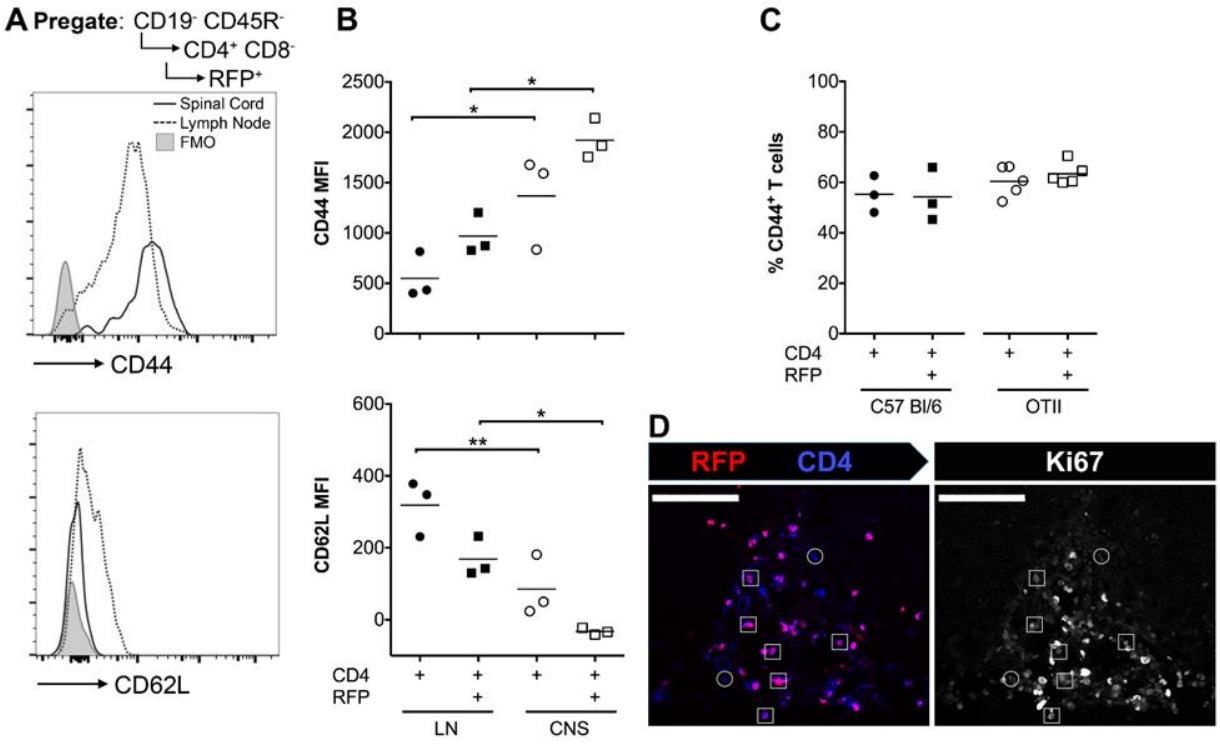


Figure 3.2 | Infiltrating T cells have an activated phenotype regardless of anti-myelin specificity.

GFP^+ IgH^{MOG} B cells and RFP^+ 2D2 T cells were transferred into C57BL/6 recipients, 2 d prior to induction of EAE. Lymph nodes and spinal cords were harvested from diseased mice 20 d post $mMOG_{tag}$ immunization and $CD4^+$ T cells were analyzed by flow cytometry. (**A**) Representative histograms of the mean fluorescence intensity of CD44 (*top*) and CD62L (*bottom*) staining of $CD4^+$ RFP^+ cells isolated from the inguinal lymph node or the CNS of the same mouse. (**B**) Mean fluorescent intensity (MFI) of CD44 (*top*) and CD62L (*bottom*) staining of endogenous RFP^- and transfer-derived RFP^+ $CD4^+$ T cells from lymph nodes or spinal cords. Each symbol represents an individual mouse. *$p < 0.05$, **$p < 0.01$. (**C**) In two separate similar experiments in which MOG-specific T and B cells were transferred into either C57BL/6- (*left*) or OTII- (*right*) recipient mice, spinal cords were harvested and prepared for immunofluorescence staining to characterize infiltrating immune cells (disease score shown in Fig. 1F). The percentage of RFP^- and RFP^+ $CD4^+$ T cells co-staining with CD44 was quantified. Each symbol represents an individual mouse. (**D**) Representative image from the C57BL/6-recpient experiment as described in Fig. 1A

showing Ki67 staining of both RFP^+ (squares) and RFP^- (circles) $CD4^+$ T cells in the spinal cord of an EAE mouse. Scale bars, 100 μm.

3.2.2 Autoimmune B cells are excluded from the inflamed spinal cords of $mMOG_{tag}$-induced EAE mice

The analysis above confirms that activated, myelin-specific T cells are highly enriched in the inflamed CNS in anti-myelin autoimmunity. To determine if the same is true for infiltrating B cells, spinal cord sections from the experiment described above (Fig. 3.1A) were separately analyzed for B cell infiltration and accumulation of GFP^+ IgH^{MOG} B cells. As noted above, $CD45R^+$ B cells were largely confined to the meninges (Fig. 3.1A) and were only very rarely observed in the parenchyma (data not shown; also see below). Surprisingly, no GFP^+ B cells were found in any of the sections I investigated (example shown in Fig. 3.3A). By flow cytometry, GFP^+ B cells were evident in the draining inguinal lymph nodes of the same mice (Fig. 3.3B), confirming that the transfer and activation of IgH^{MOG} B cells was successful. Further, both RFP^+ T cells and GFP^+ B cells were clearly evident by histology in lymph nodes harvested from separate mice at a similar timepoint (Fig. 3.3C), confirming both that immunization with $mMOG_{tag}$ induced a germinal center response incorporating the progeny of the transferred MOG-specific T and B cells, and that GFP^+ B cells can be detected by histology if they are present in the tissue. The exclusion of GFP^+ B cells from the CNS was further confirmed in a separate experiment using flow cytometry to analyze diseased spinal cord, circulating blood, and lymphatic tissue harvested from $mMOG_{tag}$-induced EAE mice. Although GFP^+ B cells were evident in peripheral tissues, they were completely absent from the CNS (Fig. 3.3D).

Finally, a similar experiment was performed to track GFP^+ MOG-specific B cells in preclinical mice to determine if autoimmune cells are present in the CNS at an earlier timepoint. Again, although RFP^+ MOG-specific T cells were readily apparent in the spinal cords of $mMOG_{tag}$-immunized mice (data not shown), GFP^+ cells were absent from the spinal cords (Fig. 3.3E), but detectable in the draining inguinal lymph nodes, other lymphatic tissues (other peripheral lymph nodes and spleen) and liver. Except for a single mouse, very few were observed in the intestines.

As for T cells, nonfluorescent, C57BL/6 recipient-derived B cells would also be able to respond to immunization with $mMOG_{tag}$. Therefore, it is possible that GFP^- B cells

infiltrating the spinal cord were myelin specific and somehow outcompeted the transferred GFP^+ IgH^{MOG} cells for access to the tissue. To exclude this possibility, fluorescent IgH^{MOG} B cells were transferred to B1-8 $J\kappa^{-/-}$ recipient mice in which >95% of B cells are specific for the irrelevant NP hapten Ag (241), effectively preventing an endogenous anti-MOG response. Disease was induced via immunization with $mMOG_{tag}$, as described above (Fig. 3.3F). Again, meningeal B cell clusters were clearly evident in the spinal cords of diseased mice (Fig. 3.3G, left). $CD45R^+$ cells were only very rarely observed in the white matter parenchyma (Fig. 3.3G, right). No GFP^+ MOG-specific B cells were observed in any section imaged. Therefore, anti-MOG B cells are excluded from the inflamed tissue in a model of CNS autoimmunity that incorporates B cell activation and recognition of myelin Ag (202).

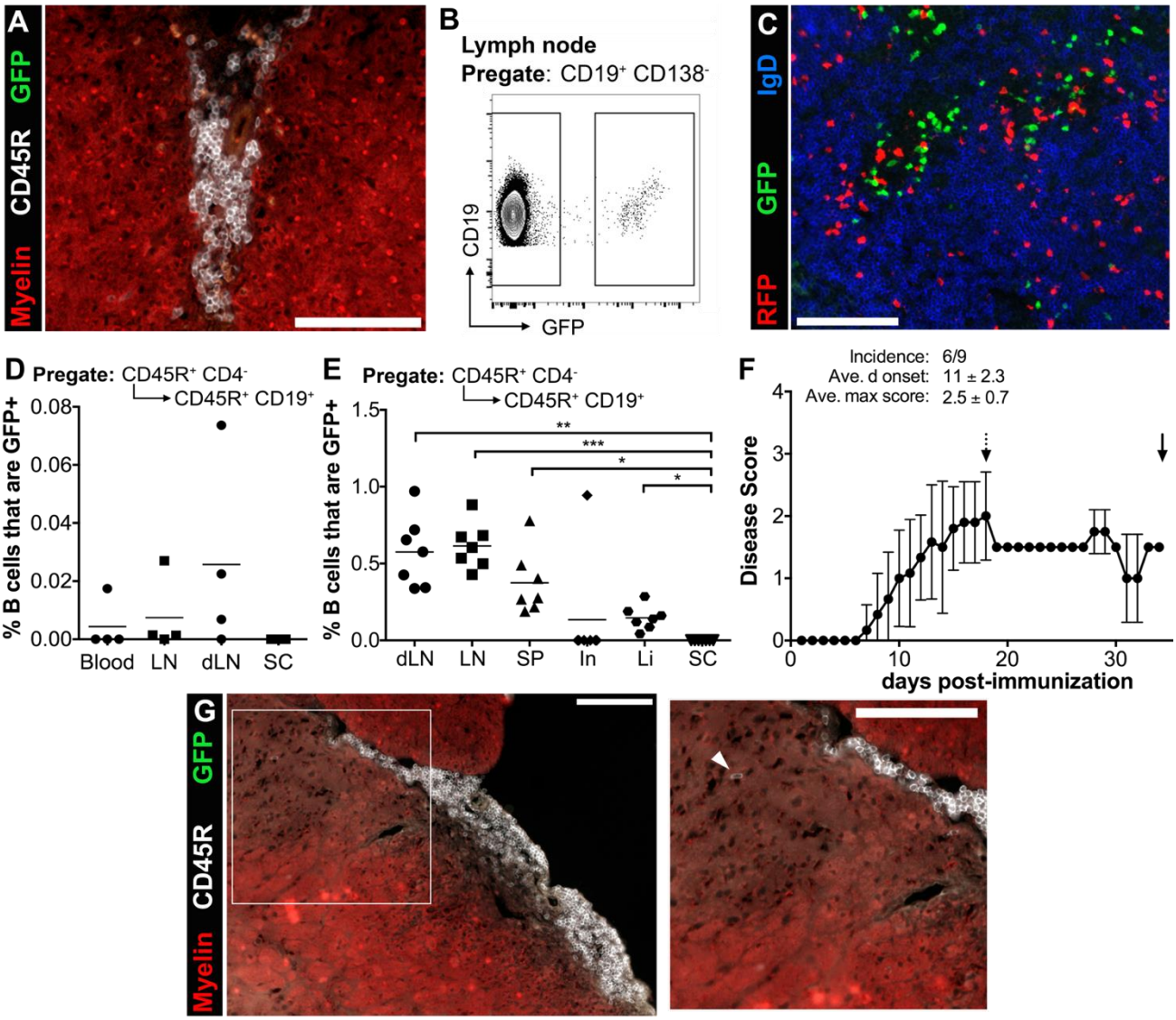


Figure 3.3 | Autoimmune B cells are excluded from the inflamed spinal cords of $mMOG_{tag}$-induced EAE.

GFP^+ IgH^{MOG} B cells and RFP^+ 2D2 T cells were transferred into wild-type nonfluorescent C57BL/6 recipients 2 d prior to induction of EAE (disease score shown in Fig. 1A) (**A**) Representative immunofluorescence image of a spinal cord section showing a meningeal B cell cluster devoid of transfer-derived GFP^+ B cells. (**B**) Flow cytometry analysis of the inguinal lymph nodes of the same mouse demonstrating the presence of GFP^+ B cells. (**C**) In a separate experiment, RFP^+ and GFP^+ MOG-specific T and B cells were transferred into C57BL/6 recipient mice and 2 d later $mMOG_{tag}$ was administered via footpad

injection. Immunofluorescence analysis of a draining inguinal lymph node harvested 10 d post immunization reveals the presence of MOG-specific RFP^+ T cells and GFP^+ B cells. (**D**) As described in Fig. 1E, fluorescent MOG-specific T and B cells were transferred to wild-type C57BL/6 recipients prior to EAE induction. 20 d post immunization, circulating blood (Blood), pooled lymph nodes (LN), draining inguinal lymph nodes (dLN), and spinal cord (SC) were analyzed by flow cytometry. The percent of $CD45R^+$ $CD19^+$ cells that are also GFP^+ is shown for each tissue. (**E**) In a separate experiment, MOG-specific RFP^+ T cells and GFP^+ B cells were transferred into nonfluorescent C57BL/6 recipient mice 2 d prior to induction of EAE. Then 8 d post immunization, cells isolated from the dLN, LN, spleen (SP), intestine (In), liver (Li), and SC were analyzed by flow cytometry. Each symbol represents an individual mouse. Comparisons between multiple groups were done using a one-way ANOVA with Bonferroni correction, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (**F**) In two separate but repeat experiments, fluorescent MOG-specific T and B cells were transferred to B1-8 $J\kappa^{-/-}$ recipient mice bearing a mutant BCR specific for an irrelevant Ag. One experiment with n = 4 ended 18 d post immunization (dotted arrow), whereas the other went out 34 d (n = 2). Errors bars represent SEM. (**G**) Immunofluorescence was performed on spinal cords harvested 34 d post $mMOG_{tag}$ immunization. One representative image is shown of a typical meningeal B cell cluster devoid of GFP^+ cells. One infiltrating parenchymal B cell (closed triangle) was evident in the white matter of a diseased mouse (see inset box shown at higher magnification, right). Scale bars, 100 μm.

3.2.3 Activated B cells are excluded from the inflamed spinal cords of $mMOG_{tag}$-induced EAE mice

In the above experiments transferred GFP^+ B cells may have been excluded due to their specificity for a CNS Ag or alternatively due to their activation status. To differentiate between these possibilities, I transferred both RFP^+ IgH^{MOG} MOG-specific B cells and GFP^+ NP-specific B1-8 $J\kappa^{-/-}$ B cells into nonfluorescent wild-type C57BL/6 mice. Recipients were immunized with both $mMOG_{tag}$ and NP-haptenated $mMOG_{tag}$ in CFA. Disease developed normally in these mice (Fig. 3.4A) and draining inguinal lymph nodes and spinal cords were harvested for analysis 21 d post immunization. Flow cytometry analysis of the lymph nodes confirmed that both transferred MOG-specific RFP^+ and NP-specific GFP^+ B cells were activated and proliferated in response to immunization with the combined antigen (Fig. 3.4B, left). Despite this, neither RFP^+ nor GFP^+ B cells were present in the spinal cords of diseased mice (Fig. 3.4B, right; 3.4C). This suggests that activated B cells, regardless of specificity, are excluded from the inflamed CNS.

Phenotypic analysis of CNS-infiltrating B cells supports the contention that they are not activated in a conventional way, as occurs in response to specific antigen in lymphatic tissue. By flow cytometry, spinal cord B cells were exclusively $CD38^{hi}$ $CD95^{lo}$, a phenotype shared by naive and memory B cells (Fig. 3.4D). However, compared with lymph node B cells of the same $CD38^{hi}$ $CD95^{lo}$ phenotype, spinal cord B cells expressed elevated levels of surface CD80 (Fig. 3.4E), indicating that they are activated to some degree. Importantly, this phenotype is identical to that of spinal cord B cells, which our lab recently described in a different B cell–dependent EAE model that develops spontaneously in mice expressing both the 2D2 TCR and IgH^{MOG} BCR (217), therefore it is not an artifact unique to the $mMOG_{tag}$-induced model.

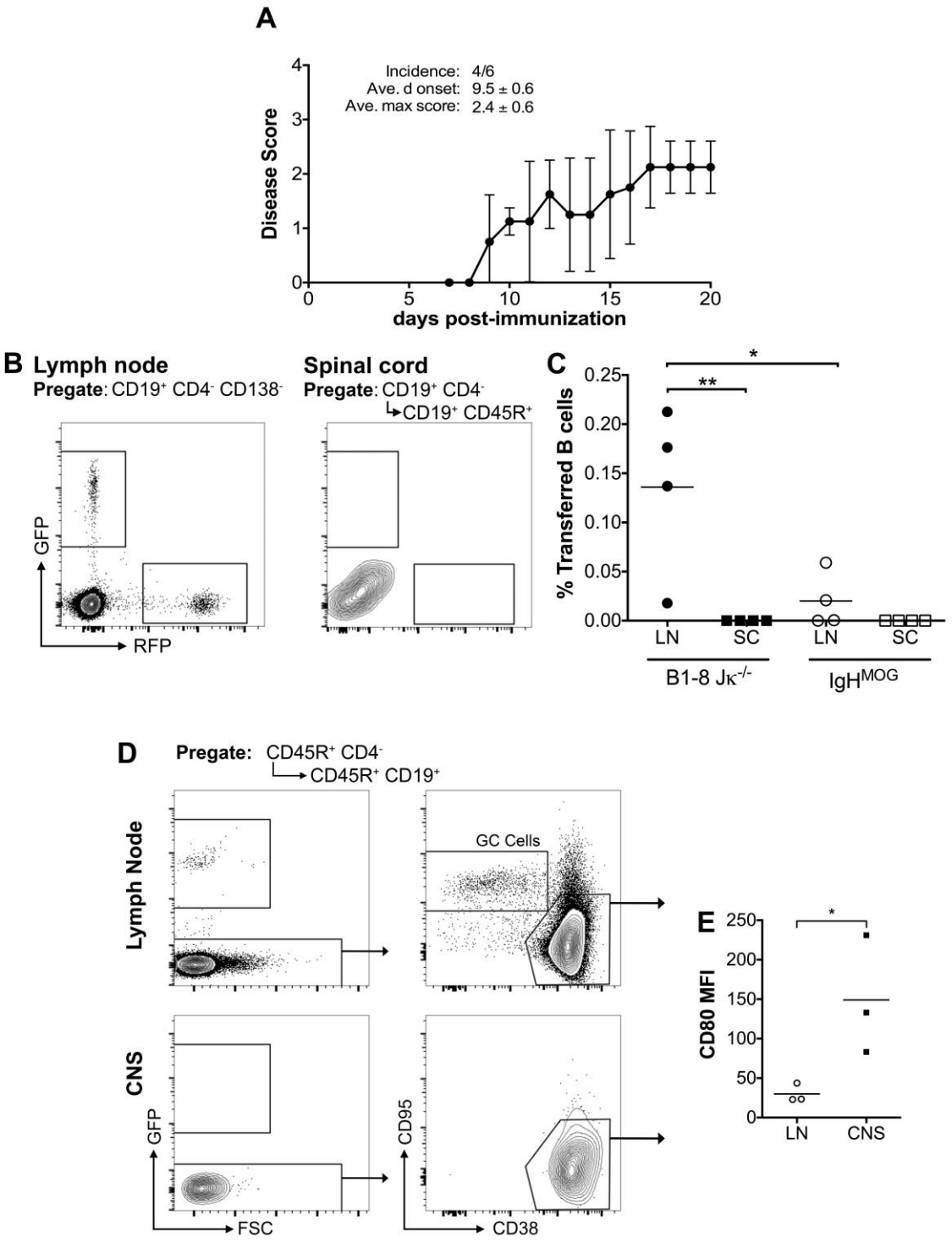

A
Incidence: 4/6
Ave. d onset: 9.5 ± 0.6
Ave. max score: 2.4 ± 0.6
Disease Score
days post-immunization
B Lymph node
Pregate: CD19+ CD4- CD138-
Spinal cord
Pregate: CD19+ CD4-
CD19+ CD45R+
GFP
RFP
C
% Transferred B cells
LN
SC
LN
SC
B1-8 Jκ-/-
IgHMOG
D
Pregate: CD45R+ CD4-
CD45R+ CD19+
Lymph Node
GC Cells
CNS
GFP
FSC
CD95
CD38
E
CD80 MFI
LN
CNS

Figure 3.4 | Activated B cells are excluded from the inflamed CNS in MOGtag-induced EAE.

RFP^+ IgH^{MOG} and GFP^+ B1-8 $J\kappa^{-/-}$ B cells were transferred into C57BL/6 mice 2 d prior to immunization with both $mMOG_{tag}$ and NP-MOG_{tag} in CFA, accompanied by PTX i.p. Mice were monitored daily for signs of disease (**A**). Then 21 d post immunization, lymph nodes and spinal cords were harvested from sick mice and analyzed by flow cytometry for the presence of RFP^+ and GFP^+ B cells (**B and C**). One representative set of plots (n = 4) for a lymph node (*left*) and spinal cord (right) from the same mouse is shown in (**B**). Each data point represents an individual mouse in (**C**). *p < 0.05, **p < 0.01. (**D**) In a separate experiment, GFP^+ IgH^{MOG} B cells and RFP^+ 2D2 T cells were transferred to C57BL/6 recipients 2 d prior to EAE induction. Lymph nodes and spinal cords were harvested from sick mice 20 d post immunization and analyzed by flow cytometry. Representative plots gated on B cells (left) show the presence of GFP^+ B cells in lymph nodes (*top*) but not the spinal cord (*bottom*). The CD38/CD95 expression profile of GFP^- B cells was further analyzed to determine activation status (*right*). (**E**) CD80 expression by naive/memory phenotype B cells ($CD38^{hi}$ $CD95^{lo}$) from the lymph node and spinal cord (*arrow*) was quantified. Each symbol represents an individual mouse. Data is shown as mean +/- SEM.

In a repeat experiment with no MOG-specific B and T cell transfer (disease scores shown in Fig 3.5A), surface receptor expression of ICOSL, Ly108, and CD49d on B cells in the inflamed CNS compared to peripheral lymph node B cells was analyzed. Interestingly, a bimodal relationship was observed for B cells expressing ICOSL (Fig 3.5B), with some B cells upregulating the expression of ICOSL relative to LN B cells, while other B cells do not. This represents the first observation of two distinct B cell population in the inflamed CNS in $mMOG_{tag}$ induced EAE. B cell expression of Ly108 was significantly upregulated in the inflamed spinal cord (Figure 3.5C, left), while despite all B cells expressing CD49d (α4-integrin), they had a slight downregulation (Figure 3.5C, right) in comparison to lymph node B cells.

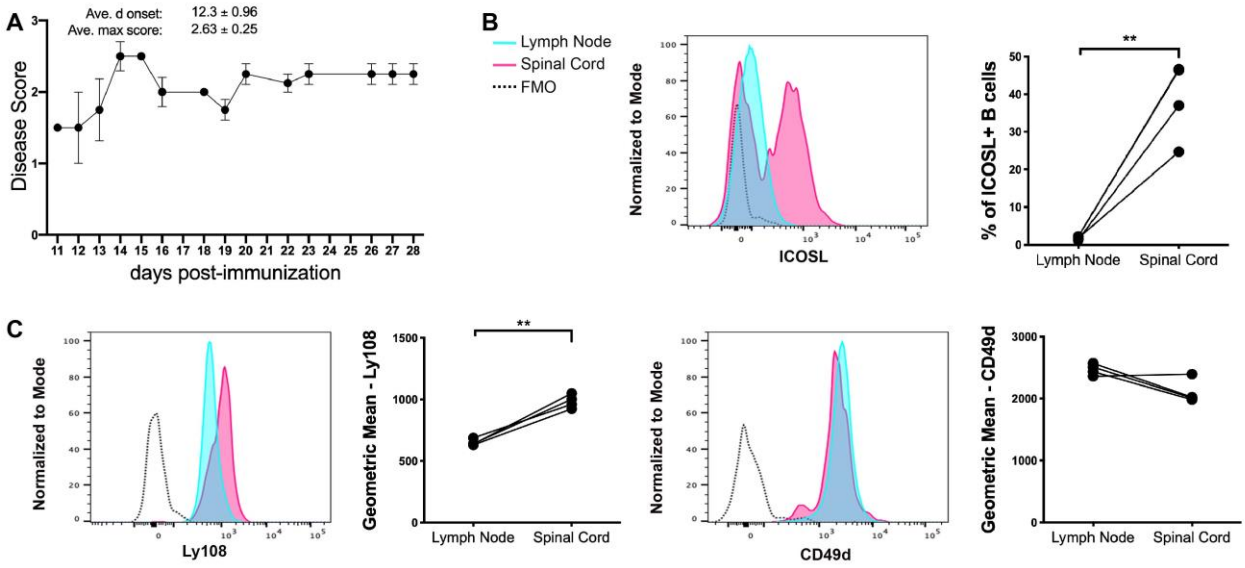


Figure 3.5 | B cells in the inflamed CNS upregulate the expression of ICOSL and Ly108.

C57Bl/6 recipient mice were immunized with $mMOG_{tag}$ and during the chronic phase of disease (d16 post disease onset, [**A**]), lymph nodes and spinal cords were harvested from mice and analyzed by flow cytometry (n=3). B cell surface expression of ICOSL (**B**), Ly108 and CD49d (**C**) were quantified in the lymph node and spinal cord. Each symbol represents an individual mouse. Comparisons were done using a paired two-tailed Student's T-test, **p < 0.01. Data is shown as mean +/- SEM.

Follow-up analysis by histology did not find any evidence of class switch in meningeal B cells, as they were exclusively IgD^+ and no $IgG1^+$ cells were observed (Fig. 3.6A). Parenchymal B cells were too rare to analyze reliably. Further, I did not find any evidence of plasma cells in spinal cord tissue as indicated by the absence of cells with the intracellular L chain (data not shown), an indicator of large-scale Ab production, which is a fundamental property of plasma cells. In contrast to T cells (see above), very few B cells in the meninges or rare parenchymal cells stained with Ki67 (Fig. 3.6B), indicating that the large majority were not proliferating.

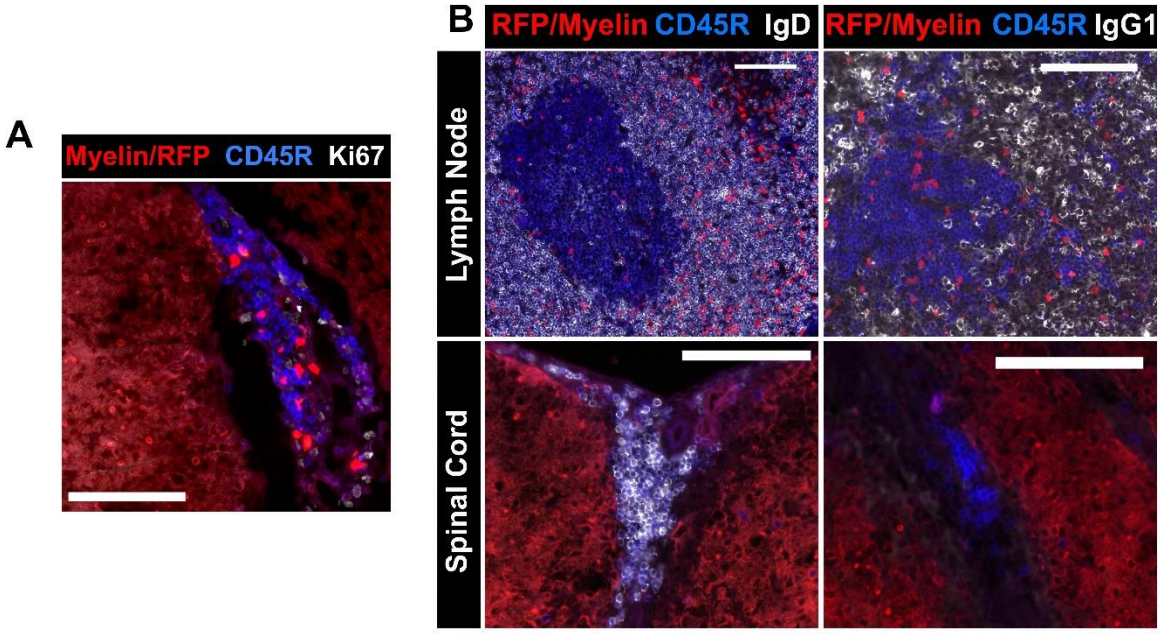


Figure 3.6 | B cells in the inflamed CNS are not actively proliferating and are IgD^+.

(**A**) Serial sections of spinal cord tissue from the experiment described in Fig 1A were stained by immunofluorescence showing no Ki67 staining of meningeal B cells. (**B**) IgD (*bottom left*) but no IgG (*bottom right*) staining, was observed in the spinal cord of diseased mice. Lymph nodes were stained as a positive control (*top*). Scale bars, 100 μm.

To determine if unactivated, naive B cells are able to access the CNS in EAE and to confirm that B cell exclusion from the CNS was not due to their expression of GFP or due to the method of cell transfer, B cells were isolated from GFP-expressing but otherwise wild-type mice and transferred to already immunized mice at the preclinical stage of disease (day 6 post immunization). Two days post transfer, draining inguinal lymph nodes, pooled lymph nodes, and spinal cords were harvested for analysis by flow cytometry. As expected, GFP^+ B cells were recovered from lymph nodes (Fig. 3.7A), consistent with normal homing of naive cells to lymphatic tissue. Small numbers of GFP^+ cells were also recovered from spinal cords and, although very rare in absolute terms (data not shown), the proportion of total B cells isolated from spinal cords that were GFP^+ was not significantly different from lymphatic tissue. Similarly, in a separate experiment RFP^+ IgH^{MOG} and GFP^+ B1-8 $J\kappa^{-/-}$ B cells were isolated from unimmunized mice and transferred into recipients with already established $mMOG_{tag}$-induced EAE (Fig. 3.7B). Two days post transfer, draining inguinal lymph nodes, pooled lymph nodes, and spinal cords were harvested for analysis by flow cytometry. Both GFP^+ and RFP^+ B cells were recovered from lymph nodes, (Fig. 3.7C) and, consistent with the previous experiment, even though in absolute terms the number of transferred B cells recovered from the spinal cord was very small (Fig. 3.7C, left), they made up a similar proportion of total B cells recovered from both tissues (Fig. 3.7C, right). No significant bias between NP- or MOG-specific B cells was observed, suggesting that the activation state rather than specificity is the primary factor limiting initial access to the tissue. Together, these experiments suggest that, unlike activated B cells, small numbers of unactivated cells are recruited to the inflamed spinal cord in EAE. However, because transferred B cells make up a similar proportion of the total B cell pool, the relative rate of recruitment is similar in the spinal cord and lymphatic tissue. The complete absence of MOG-specific B cells observed in previous experiments could be due to the subsequent removal or loss of cells that encounter Ag once in the CNS, or because all Ag-specific cells were activated by immunization.

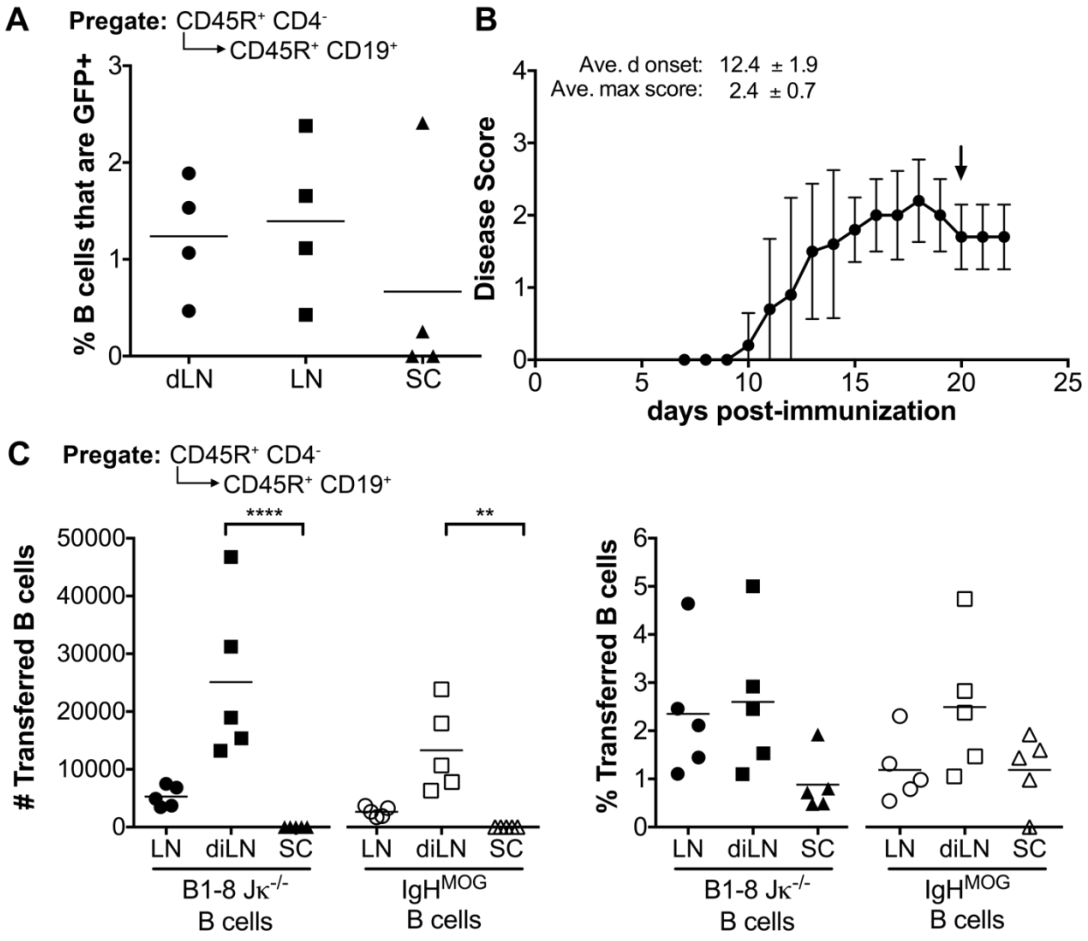


Figure 3.7 | Naive B cells can access the inflamed CNS.

EAE was induced in C57BL/6 mice and 6 d post immunization wild-type RFP^+ T cell and GFP^+ B cells were transferred i.v. into recipient mice (**A**). Then 2 d later mice were sacrificed and inguinal lymph nodes that drain the site of immunization (dLN), axial and brachial lymph nodes (LN), and spinal cords (SC) were analyzed by flow cytometry for the presence of nonspecific GFP^+ B cells. (**B**) In a separate experiment EAE was induced in C57BL/6 mice. Once disease was established, RFP^+ IgH^{MOG} B cells and GFP^+ B1-8 $J\kappa^{-/-}$ B cells were isolated from healthy donor mice and transferred i.v. into the already sick EAE recipient mice (timepoint of transfer indicated by the arrow). Errors bars represent SEM. (**C**) After 2 d mice were sacrificed and LN, dLN, and the SC were analyzed by flow cytometry for the presence of both the absolute number (*left*) and percent of (*right*) RFP^+ and GFP^+ B cells. Each symbol represents an individual mouse. ** $p < 0.01$, **** $p < 0.0001$.

3.2.4 B cell phenotype in the inflamed spinal cord of sEAE is similar to $mMOG_{tag}$ induced EAE

Previous work from our lab identified a positive correlation between B cell infiltration in the spinal cord and disease severity (217), using the sEAE model (Fig 3.8A). To confirm whether the phenotype of activated, autoimmune B cells being excluded from the inflamed CNS occurs in this model of EAE, I characterized the antigen specificity of B cells within the inflamed CNS of chronic sEAE mice. To do this I conjugated $mMOG_{tag}$ to Alexa Fluor 647 ($mMOG_{tag}$-A647) to identify B cells specific for $mMOG_{tag}$ by flow cytometry. Consistent with the inducible $mMOG_{tag}$ model, B cells isolated from the inflamed CNS of chronic sEAE (~2wks post disease onset [Fig 3.8B]) had significantly reduced specificity for $mMOG_{tag}$ compared to B cells isolated from peripheral LNs (Fig 3.8C). Follow-up analysis by immunofluorescence microscopy in a separate repeat experiment revealed little to no evidence of class switch in meningeal B cells with the large majority being IgD^+ (Fig 3.8D, left) and only rare $IgG1^+$ B cells observed (Fig 3.8D, right). MHC class II expression was also observed on all CNS infiltrating B cells (Fig 3.8E).

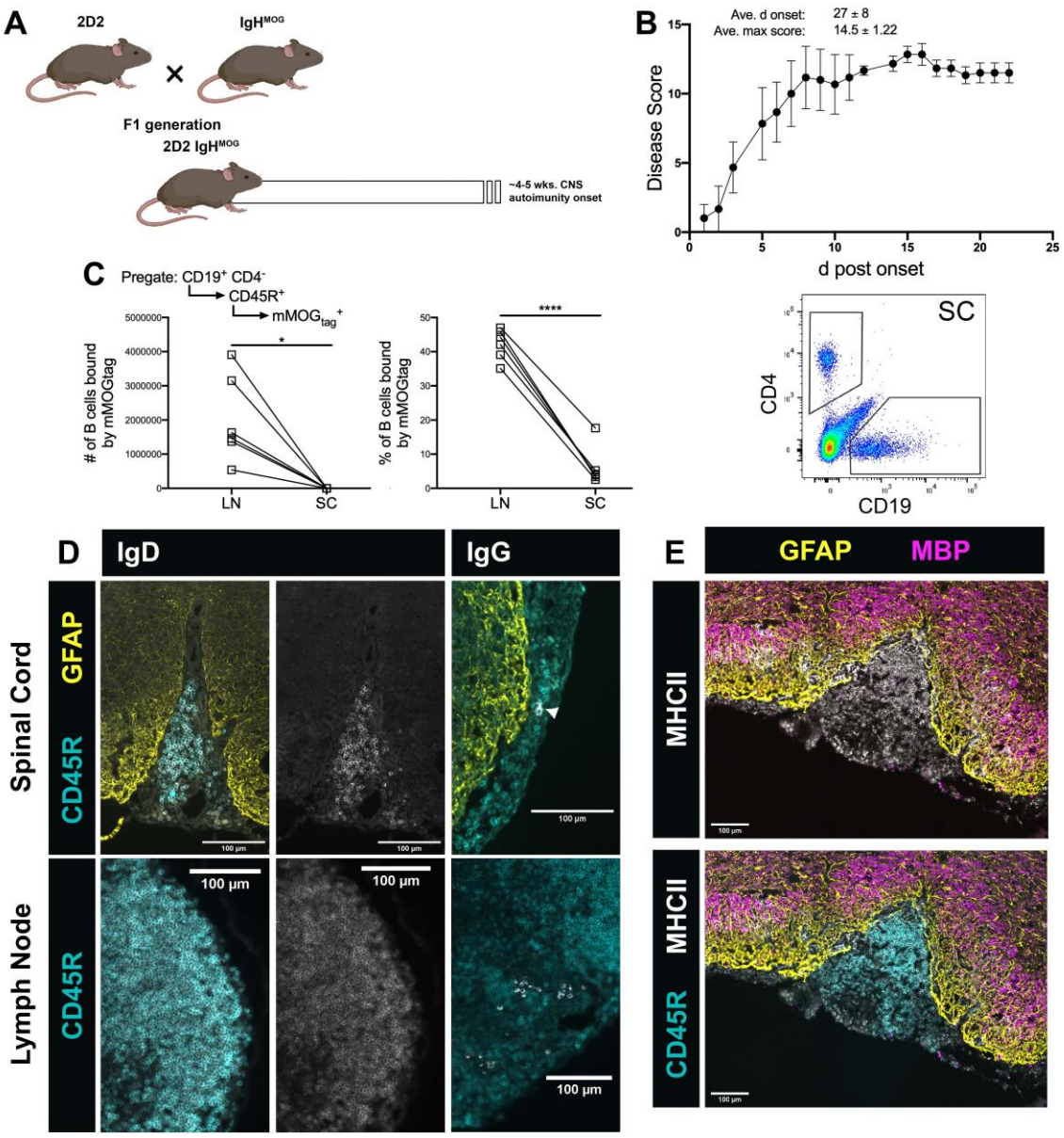


Figure 3.8 | B cells in the inflamed CNS of sEAE are not specific for MOG antigen and are predominately not class switched.

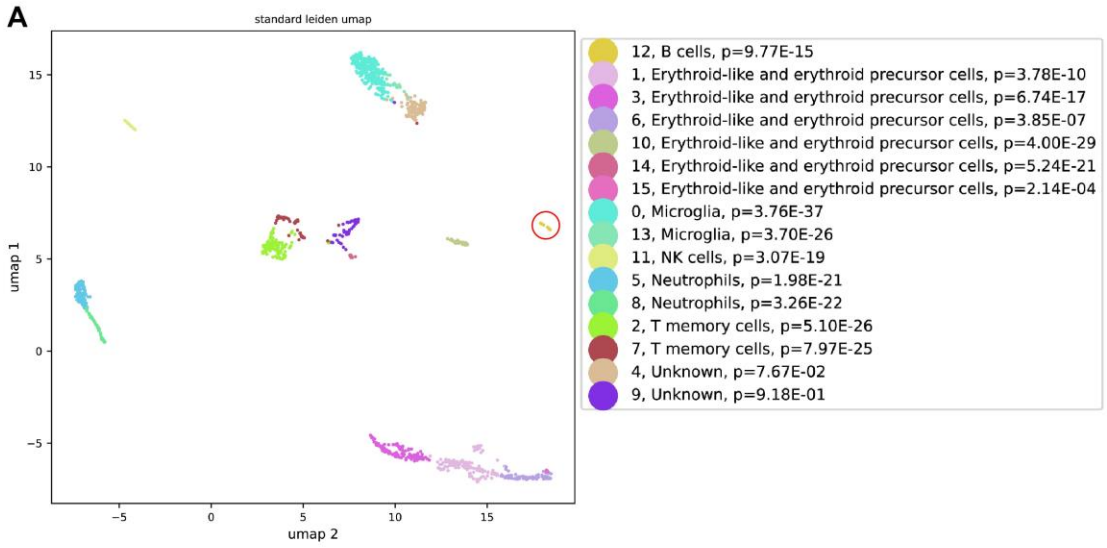
Double mutant offspring from the F1 generation of 2D2 mice crossed with IgH^{MOG} mice (**A**) were monitored and scored daily for signs of sEAE (**B**). Data is shown as mean +/- SEM. The peripheral lymph nodes (axial, brachial and inguinal [LN]) and spinal cord (SC) were harvested from chronic sEAE mice (sick 22 days post onset) and cells were prepared and analyzed by flow cytometry. (**C**) $CD19^+$ $CD45R^+$ B cells were analyzed for their

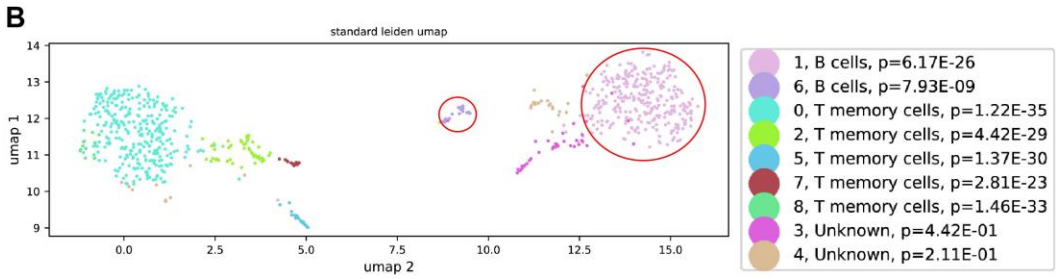
specificity for $mMOG_{tag}$ using $mMOG_{tag}$ conjugated to Alexa Fluor 647. A representative flow plot of B cells in the spinal cord is shown on the *right*. Each symbol represents an individual mouse. Comparisons were done using a paired two-tailed Student's T-test, *p<0.05., ****p<0.0001. In a repeat experiment once, disease was established (sick 20 days post onset), serial sections of spinal cord tissue were stained by immunofluorescence to characterize infiltrating B cells. (**D**, *left*) Nearly all $CD45R^+$ B cells in the CNS co-stained with IgD while few IgG positive B cells observed (**D**, *right*, closed triangle). Lymph nodes were stained as a positive control (**D**, *bottom*). All $CD45R^+$ B cells also co-stained with MHC class II (**E**). Demyelination can be observed by loss of myelin (MBP) within the total spinal cord area delineated by GFAP staining. Representative images from 2 mice are shown (n = 6).

In the same experiment from above (Fig. 3.8B), cells were taken from the spinal cords and peripheral lymph nodes of three male mice for single-cell RNA sequencing (sc-RNA-seq) analysis using a 10x genomics chromium controller. Information on the severity and days post onset (duration of disease) of these mice can be found in Table 1. Preliminary analysis comparing B cells in the spinal cord (Fig 3.9A) to B cells in peripheral lymph nodes (Fig 3.9B) for differentially expressed genes using Seurat revealed 4108 differentially expressed genes with a $p < 0.001$, with the top 25 differentially expressed genes expressed in Fig 3.9B. Overall, this preliminary analysis supports the unique phenotype of B cells within the inflamed CNS. Future studies comparing B cells in the spinal cord to a more targeted B cell populations in the lymph node (GC B cells, naïve B cells, memory B cells) or peritoneal cavity B1 B cells will need to be completed to better characterize the phenotype of B cells within the inflamed CNS.

Table 1 | Phenotype of mice used in single-cell RNA sequencing experiment.

	Average	**STDEV**	**n**
days post onset	17.0	5.29	
max score	14.7	1.15	3
end score	11.3	2.31	


A
standard leiden umap
umap 1
umap 2
12, B cells, p=9.77E-15
1, Erythroid-like and erythroid precursor cells, p=3.78E-10
3, Erythroid-like and erythroid precursor cells, p=6.74E-17
6, Erythroid-like and erythroid precursor cells, p=3.85E-07
10, Erythroid-like and erythroid precursor cells, p=4.00E-29
14, Erythroid-like and erythroid precursor cells, p=5.24E-21
15, Erythroid-like and erythroid precursor cells, p=2.14E-04
0, Microglia, p=3.76E-37
13, Microglia, p=3.70E-26
11, NK cells, p=3.07E-19
5, Neutrophils, p=1.98E-21
8, Neutrophils, p=3.26E-22
2, T memory cells, p=5.10E-26
7, T memory cells, p=7.97E-25
4, Unknown, p=7.67E-02
9, Unknown, p=9.18E-01


B
standard leiden umap
umap 1
umap 2
1, B cells, p=6.17E-26
6, B cells, p=7.93E-09
0, T memory cells, p=1.22E-35
2, T memory cells, p=4.42E-29
5, T memory cells, p=1.37E-30
7, T memory cells, p=2.81E-23
8, T memory cells, p=1.46E-33
3, Unknown, p=4.42E-01
4, Unknown, p=2.11E-01

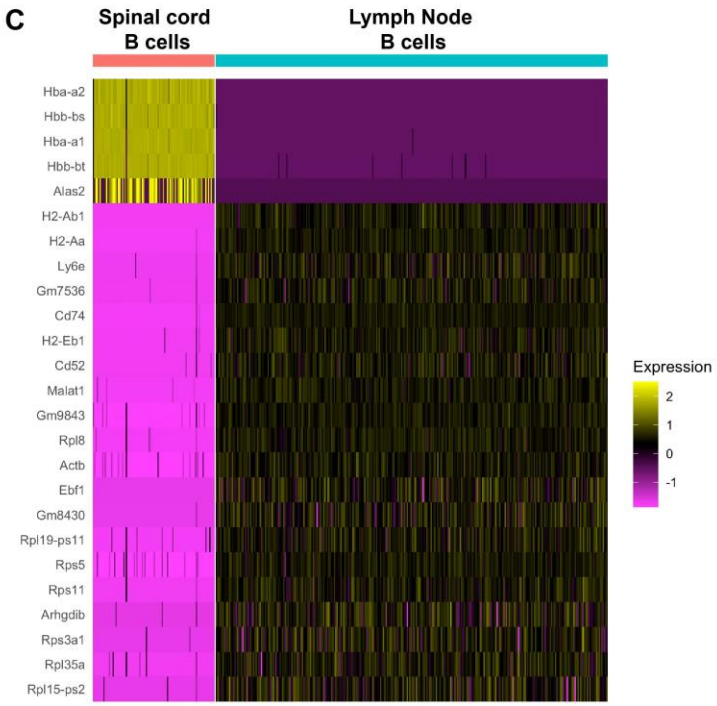

C
Spinal cord B cells
Lymph Node B cells
Hba-a2
Hbb-bs
Hba-a1
Hbb-bt
Alas2
H2-Ab1
H2-Aa
Ly6e
Gm7536
Cd74
H2-Eb1
Cd52
Malat1
Gm9843
Rpl8
Actb
Ebf1
Gm8430
Rpl19-ps11
Rps5
Rps11
Arhgdib
Rps3a1
Rpl35a
Rpl15-ps2
Expression
2
1
0
-1

Figure 3.9 | Single cell-RNA-sequencing analysis reveals a unique B cell population in the spinal cord of sEAE mice in comparison to lymph node B cells.

Following cell isolation from the lymph node and spinal cord of sEAE mice, two thousand lymph node cells and six thousand spinal cord cells were selected for generation of single cell library construction using 10x Genomics and the library was sequenced using an Illumina NextSeq 500 as a paired end run, 28 x 91 bp, using a High Output v2 kit (150 cycles). Fastq files were analyzed using Partek Flow and data was aligned to the mouse genome (*Mus musculus* mm10). Guided by violin plots, the cells were filtered based on total reads > 512, expressed genes >11 and mitochondrial reads percent < 5.50%. Raw counts data was normalized and clustered using Adobo, and cell types within each cluster were identified using Adobo's marker-based cell type prediction method with the default parameters for all steps. Labelled cell clusters from the spinal cord (**A**) and lymph node (**B**) are shown. B cell clusters identified using Adobo are labelled with a red circle. (**C**) Total B cells from the spinal cord (129 cells) were then compared to B cells in the peripheral lymph nodes (661 cells) for differentially expressed genes using Seurat.

3.3 Discussion

Together, our findings suggest that B cells have a very different relationship with the inflamed CNS in autoimmunity than T cells do, in that antigen specific cells actively participating in the anti-myelin response are excluded from the tissue rather than enriched. The absence of activated, autoimmune B cells in the inflamed CNS in two mouse models of anti-myelin autoimmune disease [in this study and (217)] may be interpreted as being at odds with findings from studies of human MS. However, close reading of this literature suggests that this may not be the case (221). Descriptions of B cell infiltration in MS brain tissues have largely focused on the most highly organized structures resembling lymphoid tissue that may be able to sustain a germinal center, however, it is clear that most clusters of B cells in the meninges are much less organized. Not all studies of human MS brain tissue found evidence of organized tertiary tissues (257), and even those that did report that most B cells were in less organized clusters not containing germinal centers (42, 43, 64–69), perhaps more reminiscent of the clusters we have described in our models [in this study and (202, 217)]. Similarly, although plasma cells have been reported to be in MS brain tissue (42, 43, 64, 65, 257), this too is not a universal finding (64, 143).

Nevertheless, studies analyzing BCR genes cloned from MS brains report that they are often class switched and show evidence of accumulating somatic mutations (137, 143, 251), both of which are very strong indications that they come from B cells derived from a germinal center response. Indeed, a recent important study from Stern et al. (143) traced the clonal lineage of brain B cells and showed that some were related to B cells found in deep cervical lymph nodes, building on their previous study demonstrating that B cells in the meninges are also clonally related to those deeper parenchyma (137). Importantly, they found no evidence of germinal center responses within the CNS tissues they analyzed, nor did they observe plasma cells. Therefore, although they provide clear evidence that the B cells found in MS brains had previously been activated and likely derived from germinal center responses in cervical lymph nodes, the cells in the tissue were not themselves in an activated state, at least not in a conventional sense as we understand antigen specific B cell activation in lymph nodes. Most critically, in a recent follow-up study from the same group, no evidence was found that BCRs isolated from MS brains recognized CNS or MS-specific

antigens (146). Therefore, instead of contradicting the human literature, I believe that our experimental findings in mouse models are entirely consistent with observations of human MS brain tissue.

This study employs a unique experimental system, adapted from our studies of B cell responses to foreign model antigens (242), which allows us to identify and track myelin-specific cells throughout the developing autoimmune response and associated disease pathology. This system depends on the transfer of small numbers of fluorescent T and B cells with known anti-MOG specificity to nonfluorescent recipients, which are then activated and expanded to large numbers in lymph nodes via immunization with Ag. The success of this transfer is demonstrated by the presence of GFP^+ cells in draining popliteal lymph nodes as, in our experience, without immunization transferred naïve B cells are lost and not detectable in any tissue after a small number of days. Further, as appreciable numbers of GFP^+ B cells were also found in other tissues including noninvolved lymphoid tissue and the liver, the egress and subsequent recirculation of the progeny of transferred GFP^+ B cells is also not defective. Exclusion from the inflamed CNS may be determined by recruitment mechanisms limiting that access of newly activated B cells. Alternatively, myelin-reactive cells may be induced to leave the tissue or die after encountering specific antigen, although this would likely also be tied to activation status as naive MOG-specific B cells were detectable in the inflamed CNS for at least 2 d post transfer.

In the work presented in this study I was not able to differentiate between naive and IgM memory B cells. By definition, memory B cells derive from previously activated cells but are themselves quiescent (258). Unless they have undergone class switch, memory B cells are very difficult to differentiate from naive B cells phenotypically. In humans, CD27 is often used as a surface marker to identify memory B cells and $CD27^+$ B cells have been reported to be present or even enriched in meningeal clusters in MS (25). Unfortunately, no equivalent memory marker exists in the mouse model system. Nevertheless, because I observed virtually no class switch in the meningeal B cells in our model, I suspect that most were naive, rather than memory. This does contrast with human MS studies that, in addition to IgM^+ B cells, also note IgG and IgA switched cells (111, 143). However, although this does represent a discrepancy between human MS and our model, it should be

noted that humans accumulate large numbers of memory B cells over decades of exposure to numerous pathogens. Like central memory T cells, memory B cells are thought to have homing properties similar to naive B cells (259) and therefore would accumulate in the same tissues. Our short-lived mouse models housed under very clean conditions will not accumulate B cell memory to nearly the same degree and therefore do not recapitulate this aspect of human disease. This could explain why I only observe apparently naive B cells in our mouse models, whereas memory cells are reported to be common in human MS tissue.

The distribution of B cells in the inflamed CNS may represent another discrepancy between our mouse model and human MS. In our models, B cells in the spinal cord are largely restricted to the meninges with only very rare cells found deeper in the parenchyma. Some histological studies of human MS brain tissue do report B cells in the parenchyma and white matter lesions (137, 223, 260). However, as for descriptions of plasma cells (see above), parenchymal B cells are not always observed (111). This is further complicated by descriptions of parenchymal cells as perivascular (25, 137), as these can be considered to be associated with the pia mater. In the few cases where the distinction is made, parenchymal B cells not directly associated with a venule are very sparse, as I observe in our models. Regardless, analysis of BCR genes isolated from the peripheral meninges and from deeper parenchymal MS brain tissue demonstrated that they were clonally related (137). While ICOSL staining by flow cytometry revealed two unique populations of B cells in the spinal cord (which would also incorporate rare parenchymal cells), no evidence by histology indicated that the rare parenchymal B cells were activated any more than meningeal cells (by Ki67 staining for example, data not shown).

Regardless of whether they are naive or memory, it is not clear how antigen-nonspecific meningeal B cells could promote CNS pathology. B cells are capable of presenting antigen to T cells and the physical association between T and B cells in meningeal clusters in addition to the elevated expression of CD80 by B cells in the CNS [Fig. 3.4E and (217)] strongly suggests that this is a role they play within the CNS. The upregulation of ICOSL in meningeal B cells, as well as Ly108 (Fig 3.5B and C) and presence of MHC class II (Fig 3.8C) also suggest B and T cell interactions (261) and entanglement (262) are occurring.

However, B cell presentation of antigen is best understood in the context of the germinal center response where B cells internalize antigen that binds their own specific BCR for presentation to T cells specific for the same antigen. Little is known about nonspecific Ag presentation by B cells. Further research is required to determine how B cells acquire their semi-activated phenotype and if their APC function is indeed enhanced. Future work will also need to be completed to understand the mechanisms and outcomes of noncognate interactions with autoimmune T cells in the CNS, as these may differ considerably from those that occur in the GC response in lymphatic tissue. Although sc-RNA-seq analysis was presented within this thesis, this analysis was very preliminary and a more targeted comparison of B cells in the spinal cord to different populations of B cells will be required in order to better characterize this population of cells.

Finally, the work presented in this study has important implications for our understanding of the mechanistic contribution(s) of autoimmune B cells to CNS autoimmunity. Both our lab and others have demonstrated that anti-myelin B cells do indeed contribute to the incidence and/or severity of disease in models that accommodate B cell recognition of antigen (202, 203, 215–217). Nevertheless, contrary to our expectations, autoimmune B cells are excluded from the inflamed site. More studies will be required to determine if this is a general feature of B cell responses during tissue inflammation or if it is unique to autoimmunity or the CNS environment. Regardless, I propose a model where B cells contribute to disease through (at least) two different mechanisms exerted from different anatomical sites. First, anti-myelin B cells may drive disease from the periphery, perhaps by influencing T cell activation and in some cases Ab production (221). Second, antigen-nonspecific cells may contribute to local pathology from within the CNS, although I have not yet confirmed that this is the case. Potential pathogenic mechanisms include noncognate antigen presentation to T cells and production of inflammatory mediators. Investigations of the activation status of meningeal B cells will aid greatly in resolving this important issue.

Chapter 4

4 Systemic administration of anti-CD20 indirectly reduces B cells in the inflamed meninges in a chronic model of central nervous system autoimmunity

This chapter is based on work that is in preparation for submission:

Tesfagiorgis, Y., and S. M. Kerfoot. 2021. Systemic administration of anti-CD20 indirectly reduces B cells in the inflamed meninges in a chronic model of central nervous system autoimmunity. *In preparation*.

4.1 Introduction

Anti-CD20 B cell depleting therapies, which includes rituximab, ocrelizumab, and ofatumumab are effective in treating patients with relapsing(1–3), and more importantly progressive forms of multiple sclerosis (MS) (5, 6, 155). Clinical trials have demonstrated that anti-CD20 treatment reduces the number of B cells in the cerebral spinal fluid (CSF) (152, 155, 157, 158) and peripheral blood (3, 170). This also results in a reduction of important measures of disease activity including a reduction in new and existing brain lesions, clinical relapses, disability progression (1–6, 168, 170) and, importantly, T cell accumulation in the CSF (158) and peripheral blood (157). However, because anti-CD20 therapies do not target antibody producing plasma cells, nor do they reduce antibody levels within the therapeutic time frame (2, 157, 158), it is not clear how B cells are contributing to disease pathogenesis.

B cells are known to accumulate in the central nervous system (CNS) of MS patients, where they often form clusters within the meninges directly adjacent to demyelinating lesions (64, 65, 67, 68). Importantly, these clusters have been shown to correlate to early onset of disease and severe cortical pathology (65, 67). We and others have shown that this phenomenon is recapitulated in certain animal models of CNS autoimmunity known as experimental autoimmune encephalomyelitis (EAE) (199, 202, 210, 217, 263). These clusters can become more organized with separate T cell zones and B cell follicles reminiscent of lymphoid tissue (25, 64). However more frequently they are disorganized mixtures of B and T cells. It has been hypothesized that B cell clusters may be a local environment within the CNS where B cells can promote disease. Nevertheless, it remains unclear whether not these meningeal cluster B cells contribute to disease pathogenesis or if they are susceptible to therapeutic depletion.

B cells play multiple roles in MS and EAE, complicating the analysis of B cell depleting therapies. Work by ourselves and others suggests that, depending on the stage of disease, both autoimmune and non-specific B cells contribute different mechanisms. For instance, in a peptide induced model of EAE, depletion of all B cells, including non-specific cells, prior to disease induction results in more severe disease (162, 195). This was attributed to

IL-10 production, demonstrating that some B cells can play a regulatory role. In contrast, anti-myelin B cells contribute to disease initiation in models that incorporate B cells in the autoimmune response. This includes 2D2 IgH^{MOG} spontaneous EAE (sEAE) (215, 216) and models induced by larger protein (195, 264, 265). Nevertheless, when analyzing the anti-myelin response to mouse MOG_{1-125} our lab has shown that the B cell anti-myelin response is short-lived, and that anti-myelin B cells largely disappear (244), and do not accumulate in B cell clusters in the inflamed spinal cord (243). Therefore, the role of anti-myelin-specific B cells may be confined to the initiation of the disease, and B cell contributions from the chronic phase likely comes from another population; including those that accumulate in the CNS.

I set out to determine whether meningeal B cell aggregates are susceptible to depletion by anti-mCD20 treatment. I specifically investigated the chronic phase of disease, after the establishment of B and T cell inflammation in the CNS, to isolate the contribution of meningeal B cells from the role of anti-myelin B cells in disease initiation. Further, knowing that these cells have the potential to play both protective and pathogenic roles in disease, I wanted to determine if there is an effect on disease outcome. To address these questions, I employed the 2D2 IgH^{MOG} sEAE model which our lab has shown results in a chronic disease course with consistent and robust B cell accumulation in the spinal cord adjacent to demyelinating lesions that correlate to disease severity (217). I found that systemic administration of anti-mCD20 does not target B cells in meningeal clusters directly, but does reduce their numbers over time, likely due to their ongoing turnover and the elimination of peripheral B cells. Further, I found this corresponds to reduced demyelination in the adjacent lesion. Finally, and unexpectedly, B cell depletion also corresponded to an increase in T cells specifically in the meninges, but not white matter.

4.2 Results

4.2.1 CNS-infiltrating B cells are reduced 7d post i.v. treatment with anti-mCD20.

To determine the optimal dose of anti-mCD20 administration that effectively depletes B cells in our model, I administered either 150μg or 250μg of anti-mCD20 (5D2), based on previously published studies (264, 266, 267), into wild type mice i.v. and analyzed B cells numbers in the blood, peripheral lymph nodes and spleen by flow cytometry 1d post treatment. As expected, B cells were almost entirely depleted in the blood (Fig 4.1A), demonstrating that depletion of B cells in the circulation is rapid and effective. B cell numbers in the peripheral lymph nodes (axial, brachial and inguinal), and the spleen were also reduced, but not to the same degree as observed in the blood. This suggests that anti-mCD20 that is administered i.v. has limited access to B cells in tissues compared to cells in the circulation, consistent with previous analysis of i.p. administered anti-mCD20 (195) As no difference was observed between the two doses, all subsequent experiments used 150μg as the treatment dose.

To determine if systemic depletion of B cells also reduces the number of B cells in the inflamed spinal cord in sEAE, I administered anti-mCD20 i.v. during the chronic phase of disease (~2wks post onset, [Fig 4.1B]) then waited either 2-or 7-days post treatment to determine the extent of B cell depletion. Isotype-control treated mice were sacrificed 2-days post treatment to represent the average size of B cell clusters. Data on the disease duration, severity, and sex of experimental mice can be found in Table 2, with no significant difference between the days post onset for each group. By this timepoint, we have shown that B cell meningeal clusters and T cell infiltration of the parenchyma is well established, and that there is evidence of ongoing inflammation and demyelination (217). I then analyzed B cell numbers in the blood, peripheral lymph nodes, spleen and spinal cord by flow cytometry 2- or 7-days following anti-mCD20 administration. As expected, based on the above pilot studies, B cells in the circulating blood were almost undetectable 2d post administration and remained absent 7d post treatment with a 99% reduction by 7d post treatment, signifying that B cells were not replaced by hematopoiesis by this time

point (Fig 4.1C). No decrease in CD4 or CD8 T cells were observed, as expected (data not shown). B cells were also significantly reduced in lymphoid tissue (lymph nodes and spleen) 2d and 7d post anti-mCD20-treatment compared to isotype-treated mice, but to a lesser extent than the circulation (76% and 88% reduction respectively [Fig 4.1C]). In contrast, there was little indication of a reduction in B cells in the inflamed spinal cord by 2d post treatment and it was not until 7d post treatment that a significant reduction of B cells was observed (69% reduction), both as a percentage of live cells and absolute number of B cells. This delay in the depletion of B cells in the inflamed CNS could be because it takes longer for the Ab to cross the BBB, or it could be because there is ongoing turnover of the CNS B cell population, and the elimination of circulating cells prevents the recruitment of replacement B cells to the inflamed CNS.

Table 2 | Phenotype of mice treated in flow cytometry experiments.

		Average	**STDEV**	**n**
Isotype Control	age of onset	29.89	3.14	9
	days post onset	15.11	5.95	
	max score	15.67	0.71	
	end score	14.33	1.66	
	female (%)	55.56		
d2 5D2 Rx	age of onset	28.44	2.88	9
	days post onset	14.44	5.90	
	max score	14.89	1.27	
	end score	12.67	2.24	
	female (%)	55.56		
d7 5D2 Rx	age of onset	32.67	1.86	6
	days post onset	17.83	5.95	
	max score	14.83	1.33	
	end score	11.67	3.20	
	female (%)	33.33		

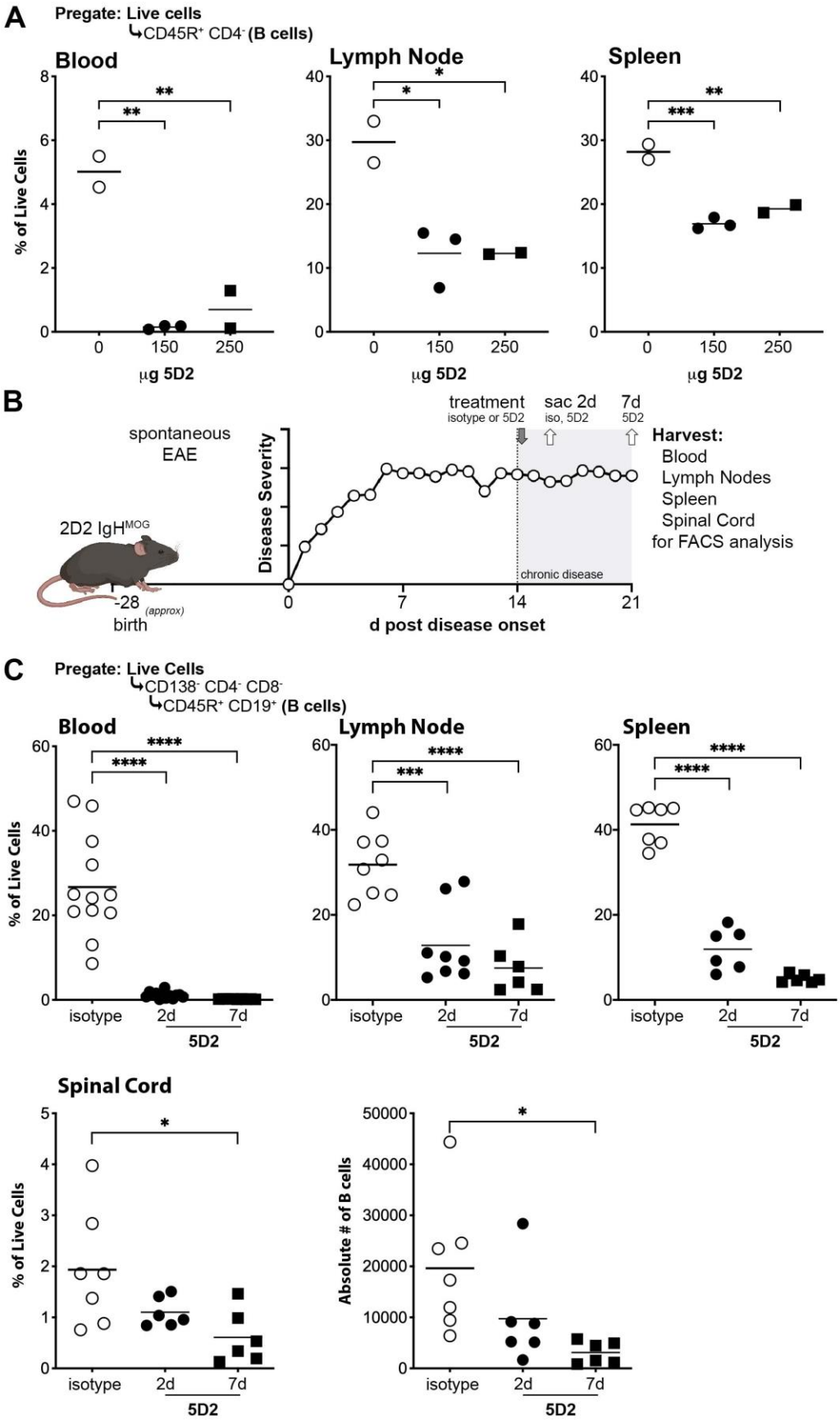

A
Pregate: Live cells
↳CD45R+ CD4- (B cells)
Blood
Lymph Node
Spleen
% of Live Cells
μg 5D2
B
spontaneous EAE
2D2 IgH^MOG
-28 (approx)
birth
Disease Severity
treatment
isotype or 5D2
sac 2d
iso, 5D2
7d
5D2
chronic disease
d post disease onset
Harvest:
Blood
Lymph Nodes
Spleen
Spinal Cord
for FACS analysis
C
Pregate: Live Cells
↳CD138- CD4- CD8-
↳CD45R+ CD19+ (B cells)
Blood
Lymph Node
Spleen
% of Live Cells
isotype
2d
7d
5D2
Spinal Cord
Absolute # of B cells

Figure 4.1 | Depletion of meningeal B cells by systemically administered anti-mCD20 is delayed compared to other tissues.

(**A**) Wild type mice were administered saline or either 150μg or 250μg of anti-mCD20 (5D2) i.v. 1d post injection, blood, lymph nodes (axial, brachial and inguinal), and the spleen were harvested, for flow cytometry analysis of B cell numbers. (**B**) 2D2 IgH^{MOG} sEAE mice in the chronic phase of disease were treated with anti-mCD20 antibodies or isotype control. Tissue was harvested 2d or 7d later for analysis by flow cytometry. (**C**) Data is represented as percentage of live cells or absolute number of cells, as indicated. Each symbol represents and individual mouse. Datasets were analyzed using one-way ANOVA followed by a t test with Bonferroni correction. *p <0.05 and **** p <0.0001. Mouse was created using BioRender.com.

4.2.2 Anti-mCD20 administration reduces the number of B cells in the meninges and this corresponds with less demyelination.

Because meningeal B cell clusters correlate with disease severity in MS (65, 67) and EAE (210, 217), I performed additional experiments to determine if systemic B cell depletion also reduces B cell accumulation in the meninges as well as spinal cord pathology in chronic sEAE. sEAE mice in the chronic phase of disease were administered anti-mCD20 i.v. and spinal cords were harvested 7d later for histological analysis (see Table 3 for disease parameters). Initial analysis revealed no differences in any measured parameters between isotype control treated mice and untreated mice, and therefore both were included in the control group.

As we observed previously (217), clusters of B cells were evident in the spinal cord meninges of sEAE mice, and these clusters typically formed adjacent to regions of T cell infiltration into the spinal cord white matter and regions of demyelination (Fig 4.2A). Consistent with the reduction in CNS B cells observed by flow cytometry (Fig 1C) above, the number of meningeal B cells appear to be reduced following anti-mCD20 treatment compared to untreated controls (Fig 4.2B). This was particularly evident when looking at the density of B cells within meningeal clusters (Fig 4.2C). Interestingly, the area of the clusters themselves did not change over this time period (Fig 4.2D). Importantly, the area of demyelination in the white matter was significantly reduced in treated mice (Fig 4.2E and F), however, this did not translate to a significant difference in disease score in this 7d timeframe (Fig 4.2G), consistent with recent observations by others (268). Nevertheless, these observations suggest that meningeal B cells contribute to local tissue pathology.

Table 3 | Phenotype of mice treated in immunofluorescence experiments.

		Average	STDEV	n
Control	age of onset	29.25	3.105295	8
	days post onset	17.5	4.14	
	max score	15.3	0.71	
	end score	13.8	1.75	
	female (%)	50		
d7 5D2 Rx	age of onset	27.8	3.63	5
	days post onset	22.2	1.30	
	max score	15.2	0.84	
	end score	10.8	2.28	
	female (%)	40		

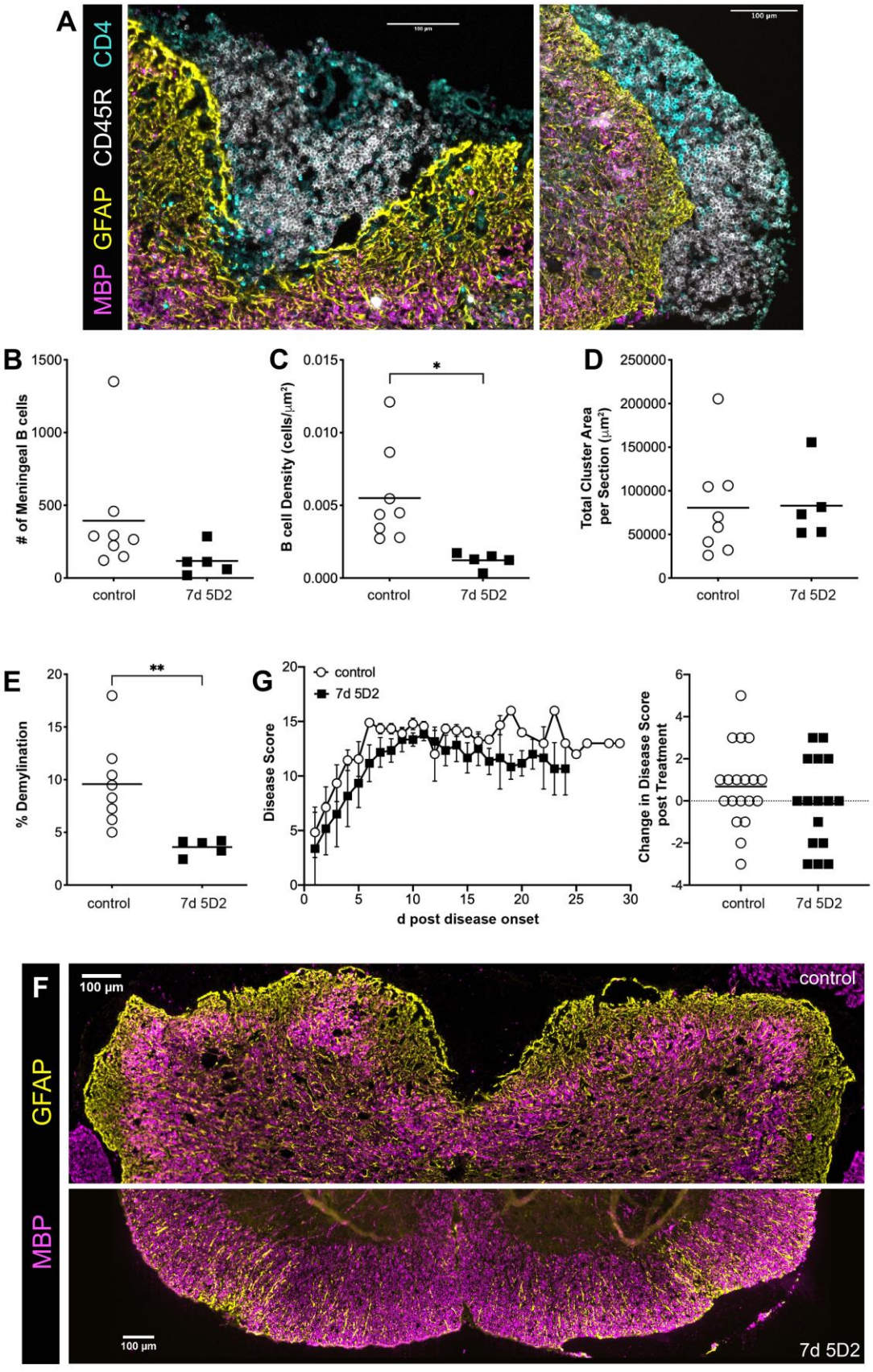

A
MBP GFAP CD45R CD4
100 µm
B
# of Meningeal B cells
1500
1000
500
0
control
7d 5D2
C
B cell Density (cells/µm²)
0.015
0.010
0.005
0.000
*
control
7d 5D2
D
Total Cluster Area per Section (µm²)
250000
200000
150000
100000
50000
0
control
7d 5D2
E
% Demylination
20
15
10
5
0
**
control
7d 5D2
G
control
7d 5D2
Disease Score
d post disease onset
Change in Disease Score post Treatment
control
7d 5D2
F
GFAP
MBP
100 µm
control
7d 5D2

Figure 4.2 | Anti-mCD20 treatment reduces meningeal B cell infiltration and demyelination by 7d post treatment.

(**A**) Representative immunofluorescence images of spinal cords from an isotype-control treated mouse (***left***) and d7 anti-mCD20 treated mouse (***right***) showing a large B cell cluster adjacent to a region of demyelination. Using ImageJ, (**B**) the number of meningeal B cell infiltrates and (**C**) density of B cells/μm^2 in meningeal clusters was determined from multiple sections from each mouse. (**D**) Total cluster area, defined by the presence of meningeal B and T cells, was determined in μm^2. (**E**) The extent of demyelination was evaluated by measuring regions identified as myelin basic protein (MBP) negative and glial fibrillary acidic protein (GFAP) positive expressed as a percentage of total spinal cord area. Each symbol represents an individual mouse. (**F**) Representative immunofluorescence images of spinal cord demyelination from control and d7 anti-mCD20 treated mice is shown. (**G**) Disease curves of control and anti-mCD20 treated mice used in the histological analysis above was tracked (***left***), along with the relative change in disease score over the final 7d prior to sacrificing (***right***). Data is shown as mean +/- SEM. Datasets were analyzed using a Student's t test, *$p < 0.05$ and **$p < 0.01$.

4.2.4 Reductions in meningeal B cells are not likely the result of direct anti-mCD20-mediated depletion.

In the above experiments I established that depletion of B cells in the spinal cord following systemic administration of anti-mCD20 is delayed relative to that observed in the circulation and lymphatic tissues (Fig 4.1C). To determine if i.v. administered anti-mCD20 has differential access to B cells in the inflamed spinal cord compared to B cells in the circulating blood and lymphatic tissue, I used flow cytometry to identify B cells bound by anti-mCD20 2 or 7d post treatment. Of the very few detectable $B220^+$ $CD19^+$ B cells remaining in the circulation 2d post treatment, approximately half were bound by anti-mCD20 (Fig 4.3A). This suggests that B cells bound by anti-mCD20 are rapidly eliminated in the blood, perhaps as they rejoin the circulation from peripheral tissues. In lymphoid tissue (spleen and lymph nodes), where measurable numbers of B cells were still evident, the large majority of B cells were bound by anti-mCD20 by d2 and remained so at d7. This may indicate that anti-mCD20-mediate depletion within tissues is slower than it is the blood. In contrast, almost none of the B cells in the spinal cord were bound by anti-mCD20 2d after i.v. administration. This increased to ~10% of CNS B cells 7d post administration, suggesting that the drug may not be able to cross the blood brain barrier to label cells already in the tissue, or at least that access to the CNS is much slower than other tissues.

In order to differentiate between these possibilities, I performed a separate experiment to look by histology for direct evidence of B cells bound by anti-mCD20 in the meninges, and also to determine the pattern of binding within meningeal clusters. If systemically administered anti-mCD20 does cross the blood brain barrier by 7d post administration to directly bind B cells in meningeal clusters, we would expect to see all or most B cells in a given cluster to be evenly bound by drug. This is not what we observed, however. Instead, anti-mCD20-bound B cells were most commonly observed in meningeal clusters as individual, brightly stained cells distributed among unlabeled cells (Fig 4.3B, left), and it was common to find anti-mCD20 bound B cells immediately adjacent to unlabeled cells (Fig 4.3B, right). This pattern is more consistent with B cells being heavily labelled in the circulation and then carrying the drug with them as they are recruited to the cluster, rather than the Ab crossing the BBB and labelling cells for depletion directly in the tissue.

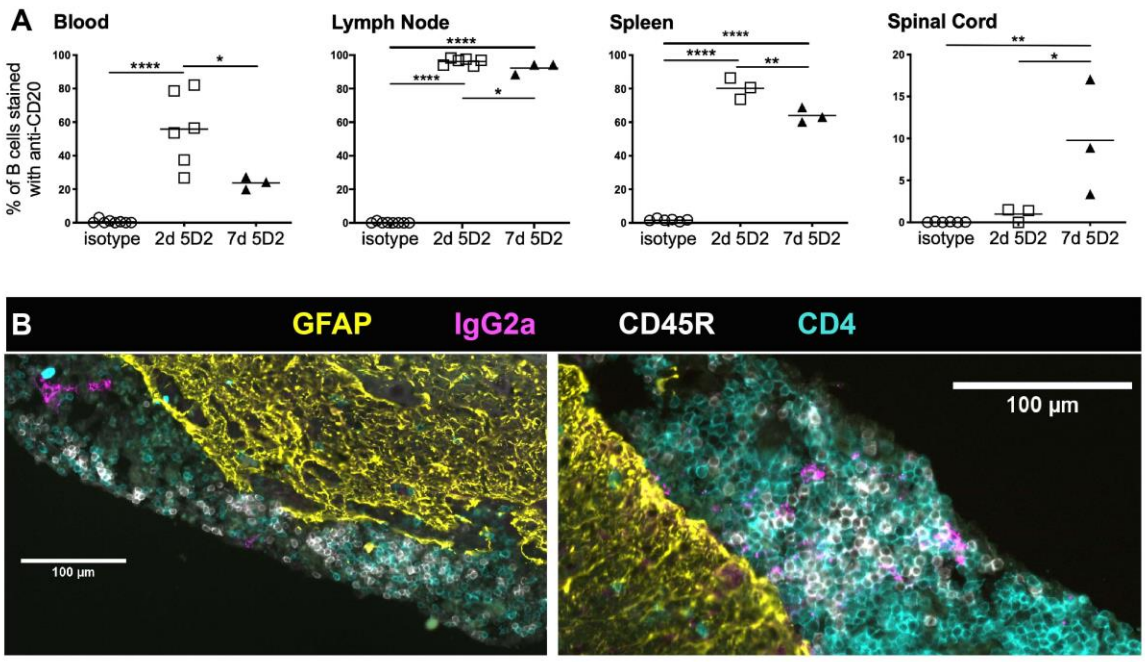


Figure 4.3 | Meningeal B cell depletion is indirect and is likely the result of eliminating circulating B cells.

The blood, peripheral lymph nodes (axial, brachial and inguinal), spleen and spinal cord were harvested from 2D2 IgH^{MOG} mice during the chronic phase of disease after isotype or anti-mCD20 (5D2) treatment. (**A**) Cells were prepared and analyzed by flow cytometry with anti-IgG2a, a secondary antibody, to determine if B cells ($CD45R^{+}$ $CD19^{+}$ $CD138^{-}$ $CD4^{-}$ $CD8^{-}$) were bound by i.v. injected anti-mCD20. Each symbol represents an individual mouse. Datasets were analyzed using one-way ANOVA followed by a t test with Bonferroni correction, *p <0.05, **p <0.01 and **** p <0.0001. (**B**) Representative immunofluorescence images of spinal cord meningeal B cell clusters from d7 anti-mCD20 treated mice reveal anti-mCD20 binding of meningeal B cells is not evenly distributed (*left*), and B cell bound by anti-mCD20 are adjacent to unlabeled cells (*right*).

4.2.5 T cells accumulate in the meninges following anti-mCD20 depletion of B cells.

Interestingly, despite a significant reduction in the number of B cells, meningeal cluster size remained comparable between control and anti-mCD20-treated mice (Fig 4.2B, right). Further analysis by histology revealed an accumulation of $CD4^+$ T cells in meningeal clusters in d7 anti-mCD20 treated mice (Fig 4.4A, bottom) compared to isotype control treated mice (top). Indeed, the absolute number of $CD4^+$ T cells in the meninges significantly increased, as did the corresponding density of $CD4^+$ T cells within clusters (Fig 4.4B and C). Overall, this resulted in a significant shift in the dominant cell type within clusters from B cells to T cells (Fig 4.4D). This increase in the number of $CD4^+$ T cells was not due to proliferation, as there was no difference in Ki67 expression as measured by microscopy (Fig 4.4E) or flow cytometry (data not shown). There was no difference in the number of parenchymal $CD4^+$ T cells by d7 post treatment, indicating that they are not likely to be leaving the parenchyma to populate the meninges (Fig 4.4F). These findings suggest that meningeal B cells may be reactivating newly recruited $CD4^+$ T cells to enter the lesion. However, preliminary analysis failed to show a difference in the expression of IL-10, IFNγ, or IL-17 by $CD4^+$ T cells in the inflamed spinal cord observed by flow cytometry (Fig 4.4G), indicating that at this time point the reduction in meningeal B cells may not appear to measurably alter T cell phenotype.

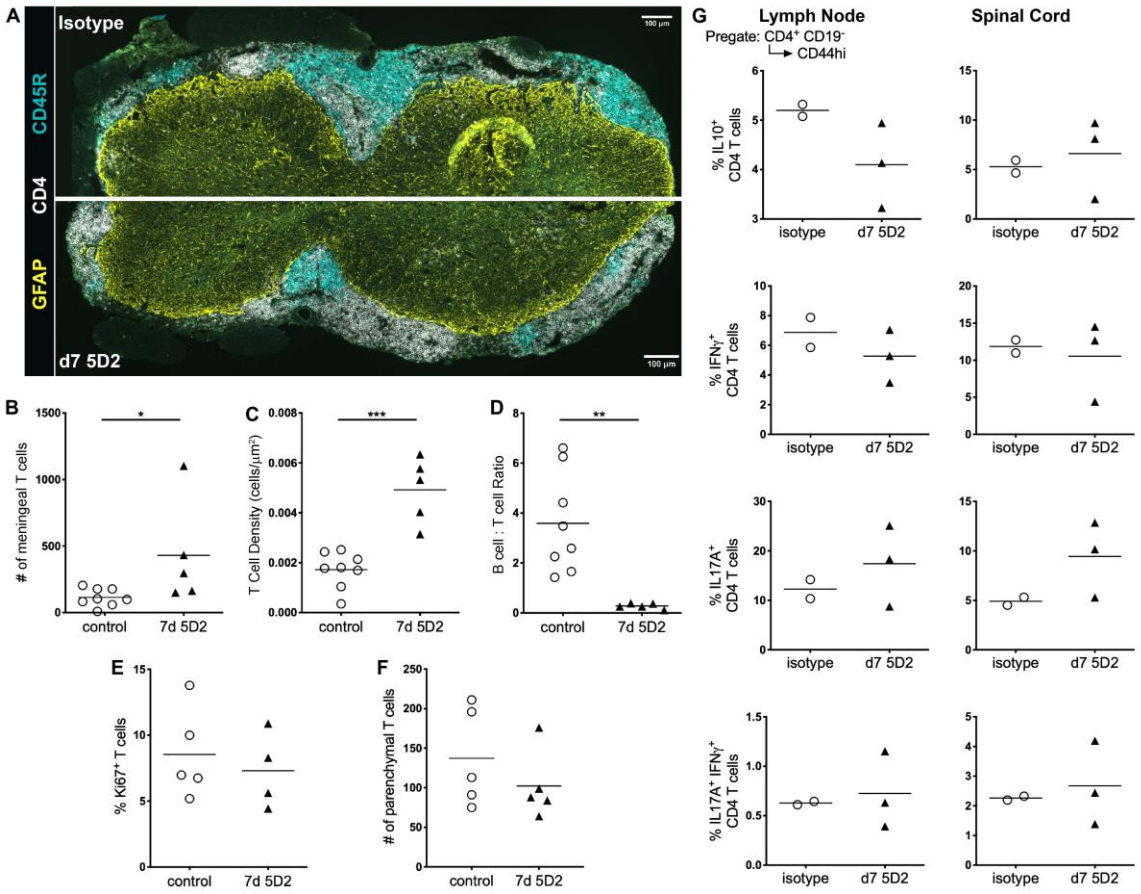


Figure 4.4 | T cell infiltration of the meninges increases following B cell depletion.

(**A**) A representative immunofluorescence image of a spinal cord from an isotype-control treated mouse (*top*) and a d7 anti-mCD20 (5D2) depleted mouse (*bottom*) showing a change in the dominant meningeal infiltrates from B cells to T cells. $CD4^+$ and $CD45R^+$ cells in the meninges were counted and the area of meningeal cluster was measured to determine the number (**B**) and density (**C**) of T cells in clusters, and to calculate the ratio between B cells and T cells in the meninges (**D**). Separate sections were stained with Ki67 and CD4 to determine the percentage of $CD4^+$ T cells in cell cycle (**E**). $CD4^+$ T cells in the spinal cord parenchyma were counted (**F**). In a separate repeat experiment, peripheral lymph nodes (*left*) and the spinal cord (*right*) were harvested for analysis of intracellular cytokine expression using flow cytometry (**G**). Each symbol represents an individual mouse. Datasets were analyzed using a Student's t test, *p <0.05, **p <0.01.

4.3 Discussion

Here, I characterized the effects of anti-mCD20 depletion during the chronic phase of sEAE in 2D2 IgH^{MOG} mice. I found that B cell depletion in the inflamed spinal cord was delayed compared to peripheral tissues, including circulating blood, lymph nodes, and the spleen. I attribute this delay to the mechanism of reduction of meningeal B cell numbers, which I believe results from the ongoing turnover of the population and the removal of the peripheral B cell pool from which it is replenished. Indeed, I did not observe evidence consistent with the anti-mCD20 Ab crossing the BBB on its own, but rather B cells appear to be bound by anti-CD20 in the periphery and carry the Ab with them as they are recruited into the inflamed CNS. Indeed, previous work from our lab shows that B cells are continuously recruited to meningeal clusters from the circulation at the chronic phase of EAE (243). I was surprised to find that B cell depletion did not reduce the size of meningeal clusters, which instead became dominated by T cells. I speculate that the increase in T cells within the meninges is the result of newly recruited cells that do not go on to infiltrate the white matter parenchyma due to the lack of B cell derived signals they normally would receive. Interestingly, even in this short 7d timeframe a reduction in demyelination was observed following B cell depletion.

Most studies investigating the effects of B cell depletion in models of CNS autoimmunity to date, have focused on the initiation and acute phase of disease. Indeed, it is clear that depending on the model, B cell depletion before or shortly after disease induction results in less or more severe disease. For example, depleting B cells prior to disease onset in MOG_{35-55} peptide induced EAE results in increased disease severity (162, 195, 267), with reduced regulatory B10 cells observed (162). In contrast, when B cells are depleted prior to protein induced EAE, disease severity is reduced (195, 264, 265, 267, 269). Consistent with this finding, when B cells are depleted during the acute phase of recombinant MOG_{1-117} EAE (4d post EAE onset), frequencies of Th1, Th17 and regulatory T cells in the peripheral blood and CNS were decreased 14d post treatment (195), supporting a role for B cells in the activation and differentiation of T cells. Nevertheless, it is unclear where these effects are occurring as the immune response is initiated in the periphery and depletion was targeted before established B cell infiltration in the CNS occurs. When

analyzing the long-term effects of anti-CD20 treatment in the peripheral blood of MS patients, proinflammatory Th1 and Th17 responses are reduced (227), indicating that T cell activation is altered by B cells.

The role of meningeal B cells in the chronic phase of EAE is not well characterized. In a very recent study investigating the chronic phase (30d post EAE onset) of MP4 protein induced EAE, B cell depletion similarly resulted in reduced demyelination, with the additional observation of reduced axonal damage. Interestingly, they also found a significant increase in remyelination when mice were treated with Obinutuzumab a novel humanized type II anti-CD20 monoclonal antibody (268). However, like us, over the short timeframe of B cell depletion no change in disease severity was observed. In people living with MS, anti-CD20 therapies led to a reduction in clinical relapses and disability progression (2–6), suggesting that with more time, changes in clinical disease severity in chronic EAE may become more apparent.

When characterizing the role B cells play in human and animal models of MS, both pathogenic and regulatory mechanisms can be identified. Peripheral B cells from MS patients have been shown to produce increased levels of proinflammatory cytokines such as IL-6 (159), GM-CSF (160), lymphotoxin and TNFα (226), which result in enhanced proinflammatory peripheral blood mononuclear cell responses. Nevertheless, B cells in the peripheral blood of MS have also been shown to play regulatory roles through their production of IL-10 by a rare B cell population (234). In fact, many animal models of CNS autoimmunity highlight this regulatory mechanism of B cells (161, 232, 233, 270). Interestingly, after the cessation of anti-CD20 treatment in MS patients, peripheral blood B cells have diminished proinflammatory cytokine production (159, 160). Interestingly, this phenomenon is only observed in peptide induced models of EAE, where B cells are shown to play more of a regulatory role in disease pathogenesis, but not in protein induced EAE where B cells are activated and involved in the proinflammatory responses (267). Indeed, Duddy et al., highlight the multifaceted role B cells can play depending on the signals they receive (271), and in turn, their activation milieus.

The identification of meningeal inflammatory foci in both secondary progressive (64, 65, 67) and primary progressive MS (42, 43) led to the hypothesis that local compartmentalized immune responses are propagating the pathogenic response. Meningeal lesions have been correlated to accelerated disease severity (67), and increased axonal atrophy in the brain and spinal cord (66, 68), suggesting they are a deleterious site of inflammation. Here, I aimed to characterize the effects of B cell depletion during the chronic phase of sEAE in an attempt to isolate the contribution of meningeal B cells to disease pathology. This sEAE model was strategically chosen for the reminiscent meningeal B cell clusters that form in addition to the chronic disease course that is experienced (217). Future studies will be required to determine the mechanistic action of meningeal B cells to chronic CNS pathology and to identify why T cells accumulate within the meninges following anti-CD20 treatment.

Chapter 5

5 Overall discussion and future directions

The findings in this thesis address the phenotype of meningeal B cells within the inflamed spinal cord of an animal model of CNS autoimmunity, and determined their susceptibility to therapeutic depletion with anti-mCD20, the murine surrogate of Rituximab. I began with the assumption that myelin specific B cells would be the predominant constituent of meningeal B cell clusters, contributing to the local pathogenic B cell mechanisms in the inflamed CNS. This was based on the observations that a majority of meningeal B cell clusters were comprised of $CD27^{+}$ memory B cells (25, 66), suggesting they were previously activated, and the description of ectopic B cell follicles, reflective of GCs (64). Surprisingly I challenged the hypothesis that B cells behave like T cells in their recruitment to the inflamed CNS. Instead, I discovered that unlike T cells, activated myelin-specific B cells are excluded from the inflamed CNS. The data in Chapter 3 suggested that clusters of meningeal B cells are activated through a non-antigen-specific pathway to participate in the local inflammatory immune response in both $mMOG_{tag}$ induced EAE and 2D2 IgH^{MOG} sEAE (217). B cells identified in the inflamed CNS of these models, were $CD38^{hi}CD95^{lo}$ indicative of a naïve/memory B cell, yet they upregulated the expression of CD80 and ICOSL in comparison to lymph node B cells, suggesting they are involved in antigen presentation to T cells. Nevertheless, because they are not specific for myelin antigen, I propose that B cells are presenting antigen to T cell via noncognate interactions with autoimmune T cells. In Chapter 4 I reveal that meningeal B cells can be targeted for depletion following systemic anti-mCD20 treatment, resulting in reduced CNS pathology, supporting the pathogenic nature of these meningeal B cells. Interestingly, this coincided with an influx of meningeal T cells, suggesting B cells may be required for the reactivation of T cells, allowing them to enter the parenchyma and promote further damage.

In MS, memory B cells have been identified in meningeal B cell aggregates as well as in white matter lesions of MS patients (25, 66). Typically, CD27 has been used to identify memory B cells in humans, though there is no equivalent phenotypic marker of memory B cells in mice. CD27 can be a marker for a cell that is activated or that has been activated in

the past, with ~40% of B cells in the human peripheral blood of adults expressing CD27 (272). However, with the heterogeneity of human memory B cells (273) functional contributions need to be better characterized in MS to determine if there is indeed an enrichment for memory B cells compared to other lymphoid structures. Further complicating the matter in mice, IgM^+ memory B cells are indistinguishable from naïve IgM^+ B cells. However, subsets of previously activated B cells have been identified based on the differential expression of CD80, CD73 and PD-L2 (274–276), therefore future studies should be aimed at addressing the potential memory phenotype of meningeal B cells in these models.

While the presence of meningeal B cell follicles, with GC formation in the meninges has been proposed as a phenomenon that can occur in SPMS (64), a thorough analysis of the literature reveals a different story. The overall majority are more frequently reminiscent of disorganized B cell clusters (42, 43, 67–70), similar to our findings in $mMOG_{tag}$ induced EAE and 2D2 IgH^{MOG} sEAE (217). In fact, reanalyzing SPMS post-mortem tissue revealed that these structures were characterized by having the presence of proliferating B cells ($Ki67^+$), a reticulum of $CD35^+$ FDCs producing $CXCL13^+$, as well as the presence of Ig^+ plasmablasts or plasma cells. While these appearances were observed in 2 of 3 individuals with SPMS, upon subsequent descriptions analyzing an additional 26 SPMS tissues (in addition to the previous 3), 41% of SPMS patients contained these meningeal B cell follicles (12/29 cases) (65). However, yet another reinvestigation using 28 of the previously analyzed 29 tissues, with an additional 9 more, contained 54% of SPMS tissues (20/37 cases) with features of follicle like structures (66). Yet unlike previous observations, they lacked the presences of a GC surrounded by a mantle zone, were predominantly $CD27^+$ memory B cells and contained CD4 and CD8 T cells, highlighting the predominate nature of these meningeal B cell clusters to, more often than not, represent disorganized aggregates of cells. Again, while some subsequent analyses characterize these meningeal B cell clusters to be reminiscent of lymphoid tissue, with additional phenotypic makers such as activation-induced cytidine deaminase, caspase-3, and Bcl-6 (63), this was an occasional phenomenon.

B cells in the meninges and parenchyma have been shown to be clonally related based on variable heavy chain BCR repertoire analysis (137). Furthermore, studies looking at B cells in the CNS show they are clonally related to B cells within cervical draining lymph nodes (143), suggesting B cell maturation occurs outside the CNS within these structures. Consistent with this idea is the observation that B cells from the CSF and peripheral blood of MS patients are also clonally related (136, 144), suggesting an ongoing migration of B cells out of the CNS, with frequent input of B cells from the periphery back into the CNS. Indeed, meningeal lymphatic vessels are known to drain into the deep- and superficial-cervical lymph nodes (277), supporting the idea that immune cells and macromolecules can drain from the CSF into the cervical lymph nodes. This also creates an opportunity for B cells to find relevant CNS antigens, as numerous myelin antigens have been identified within draining cervical lymph nodes (75, 76). Nevertheless, when analyzing the specificity of CNS resident B cells by producing recombinant human IgG from MS post-mortem tissue, no candidate antigens previously implicated in MS or high-throughput approaches were able to identify the target antigen of these B cells (146). This is consistent with our observations in Chapter 3 that meningeal B cells clusters are not specific for myelin antigen (243). This is supported by observations from another group that failed to detect RFP^+ MOG-specific B cells within inflammatory CNS lesions, while GFP^+ MOG-specific T cells were readily observed (221). While somatic hypermutation and affinity maturation in these clonal analyses had originally suggested B cells were undergoing an antigen-driven immune response, the observation that memory B cells are recruited, suggests this could just be derived from previous antigen-activation. Indeed, class-switched IgA gut derived plasma cells specific for microbial antigens can be found in the inflamed CNS of MOG_{35-55} induced EAE (233), which could explain the observation of somatically mutated B cells within the inflamed CNS.

While the results of Chapter 4 suggest that meningeal B cells clusters are contributing to CNS-autoimmunity, these studies do not exclude the possibility that B cells are promoting the disease from another anatomical location in chronic EAE. However, I hypothesize that by depleting B cells at the chronic phase of disease I was able to isolate the contribution of meningeal B cells from the initiation role of myelin-specific B cells. This is based on previous work from our lab that shows that the autoimmune $mMOG_{tag}$ GC response is

truncated in comparison to foreign antigen GC responses, which results in the generation of short-lived memory B cells that are not responsive to secondary stimulations (244). Furthermore, studies looking at the effects of B cell depletion in various models of EAE and at different time points, reveal the multifaceted role B cells play in CNS autoimmunity. Most of these studies looked at the effects of B cell depletion prior to disease induction, which differentiate the role of B cells during the initiation of disease (Table 4). For example, when looking at the effects of B cell depletion prior to EAE induction using MOG_{35-55}, B cells appear to play a regulatory role as disease severity is exacerbated in treated mice (162, 195, 266, 267). This finding was attributed to reduced $CD1d^{hi}CD5^{+}$ regulatory B cells (B10 cells) (162), and an influx of encephalitogenic T cells into the CNS (195), highlight the protective role B cells play in peptide induced EAE, when they are not activated through their BCR (194). Indeed, in peptide induced models of EAE B cells have been shown to regulate disease severity through their production of IL-10 (161, 232, 270). In contrast to peptide induced EAE, treatment of mice with anti-CD20 antibodies prior to the induction of EAE using larger protein antigens such as hMOG (264, 265, 269, 278) or MOG_{1-117} (195, 267) resulted in a reduction in disease severity, as well as reduced volumetric brain volume changes (265). Overall, these finding highlight that B cell depletion in protein induced EAE, is beneficial to clinical or pathological disease outcomes and can be attributed to the loss of pathogenic, BCR activated, MOG-specific B cells. While attempts were made to deplete B cells in both MOG_{35-55} peptide and various protein models during the acute phase of disease, I believe that meningeal B cell cluster formation is not fully established within the CNS by these early time points (day 1-4 post disease onset) and therefore the effects observed were solely due to the role of peripheral B cells.

Table 4 | The effects of B cell depletion in various models of EAE.

EAE Model	Strain and Sex	Rx type	Rx timepoint	Findings	Ref
MOG 35-55	C57BL/6 (F)	MB20-11 200μg i.v.	-7d pi	**Increased disease severity**. More demyelination, leukocyte infiltration, less B10 cells ($CD1d^{hi}$ $CD5^{+}$). IgM and IgG Ab response is abrogated. Increased T effector cells in the CNS (Th1 and 17).	(162)
			7d pi (no symptoms)	**No effect on clinical scores**	
			14d pi (symptomatic)	**Reduced disease severity.** Saw reduced demyelination, CD4 T cells, T effector cells, and Tregs in the CNS, with reduced T cell proliferation.	
			21d pi (recovery)	**No effect on clinical scores**	
	hCD20	m2h7 200μg i.p.	-21d pi	**Increased disease severity** $CD11b^{+}$ APC have higher TNF production, reduced IL10 production and stimulated Th1 T cell differentiation	(195)
			12d pi, ~d2 po	**Increased disease severity**, inflammation, demyelination, reduced CNS B cell numbers. No change in Th1 or Th17 T cells in the CNS, a reduction of Tregs in the CNS.	
	C57BL/6 (F)	200μg i.p	-21d pi Maintenance - wkly	**Increased disease severity.** Linked to splenic $CD11b^{+}$ monocyte TNF production. Removal or Tregs by anti-CD25 only modestly increased disease severity suggesting the reduction of Treg is not why disease is exacerbated in anti-CD20 treated mice	(266)
	C57BL/6 (F)	5D2 200μg i.p.	-14d, -7d, 0d pi	**Increased disease severity** 6wks pi. As B cells returned disease severity dampened. Saw enhanced cellular infiltration. B cell numbers in the CNS were restored	(267)
rMOG 1-117	hCD20	m2h7 200μg i.p.	-21d pi	**Reduced disease severity**	(195)
			13d pi, ~d4 po	**Reduced disease severity** – reduced B cells in the CNS (specifically meningeal B cells reduction). Reduced frequency of Th1, Th17 and Treg ($CD25^{+}$, $FoxP3^{+}$) 14d after Rx, and reduced anti-MOG antibody titers d30 after immunization and onwards.	
	C57BL/6 (F)	5D2 200μg i.p.	-14d, -7d, 0d pi	**Reduced disease severity** 14wks post disease induction. As B cells returned, severity worsened. Saw no difference in CNS infiltration or demyelination. B cell numbers restored with an increase in autoreactive MOG_{1-117} specific B cells.	(267)

EAE Model	Strain and Sex	Rx type	Rx timepoint	Findings	Ref
rhMOG $_{1-125}$	hCD20	100μg	3d pi Daily Rx for 3d 20d pi (8d po)	**Reduced disease severity** – B cells in the peripheral blood and spleen are reduced (13% reduction in the CNS), reduced CD4 T cells in the CNS. MOG-specific T cells unaltered however, T cell proliferation to MOG_{1-125} is reduced with a specific reduction in IL17 T cells but not IFNγ. **Reduced disease severity** – MOG specificity is not altered but in vitro T cell proliferation to MOG_{1-125} is reduced.	(264)
	hCD19 (M)	MB20-11 250μg i.p.	-7d pi	**Reduced disease severity –** Reduced anti-MOG IgG antibodies in the spinal cord but not the brain or serum	(269)
	C57BL/6 (F)	18B12 20mg s.c.	-1d and d3 pi,	**Reduced disease severity**	(278)
	C57BL/6 (F)	100μg s.c.	-21d pi Maintenance – wkly	**Reduced disease severity** 3-4 wks pi. Rotarod performance improved and reduced volumetric brain volume loss.	(265)
MP4	C57BL/6 (M)	18B12 5mg/kg i.v.	30, 33, and 36d after peak EAE,	**No effect on clinical scores**: **18B12**: significantly reduced axonal damage and lesion area **OBZ:** significantly reduced axonal damage, lesion area, and demyelinating axons, with a significant increase in remyelination.	(268)
	CD20dbtg	OBZ 5mg/kg i.v.	50, 53, 56 and 59 after peak EAE	D50 Rx mice saw a significant increase in remyelination, and a significant decrease in demyelinating axons.	
sEAE	TCR^{1640} SJL/J	18B12 20mg s.c.	3d after birth Maintenance – every 2 wks	**Reduced disease severity** and incidence	(212)
	2D2 IgH^{MOG}	200μg i.p.	3-4wks preventatively	**No effect on clinical scores** and incidence	(222)

Definitions: pi – post immunization, po – post onset, i.v. – intravenous, i.p. – intraperitoneal, s.c. - subcutaneous

Despite recent reports that subcutaneous administration of ^{89}Zr-labeled anti-CD20 therapy allowed for higher uptake in the CNS in comparison to i.v. administration (279), I opted to administer the drug i.v. consistent with current methods used to treat MS patients with ocrelizumab. Although B cell depletion in the CNS was delayed by this method, I was still able to observe a significant reduction in meningeal B cells over time. The BBB is selectively permeable through the presence of specialized endothelial cells with tight junction that prevent paracellular permeability (280). During neuroinflammation that occurs in MS, the BBB becomes leaky which I hypothesized would allow for anti-CD20 to gain access to the inflamed CNS. Immunofluorescence analysis revealed that while the drug was apparent in the inflamed CNS, it was not restricted to certain areas but rather observed in various meningeal clusters. This finding suggests that the drug was likely bound to peripheral B cells and made its way into the CNS with newly recruited B cells. Previous reports from our lab show that B cells are continuously recruited to the inflamed CNS during EAE (243) which supports this theory. While previous attempts to deplete B cells via intrathecal anti-CD20 treatment have been attempted in EAE, with a modest improvement compared to i.p. administration (281), this was not effective in clinical trials of progressive MS (282).

Meningeal B cell clusters could be contributing to CNS pathology through many effector mechanisms such as cytokine production, as B cells have been shown to produce proinflammatory cytokines IL-6, GM-CSF, lymphotoxin α, and TNFα (159, 160, 226, 227), which can influence the differentiation of T cells and myeloid cells. Furthermore, it has been shown that B cells isolated from the peripheral blood of RRMS patients secrete toxic factors that are not cytokines, or antibodies that induce apoptosis of neurons *in vitro* (230, 231), suggesting the B cells in the inflamed CNS could also be producing these as of yet uncharacterized toxic factors to promote tissue damage. Nevertheless, with our observation that B cells in the inflamed CNS up regulate the expression of CD80 and are in direct association of myelin-reactive T cells, it is likely that B cells are presenting antigen to T cells via non-BCR-uptake mechanisms. Indeed, the work in Chapter 4 suggests that meningeal B cells are involved in the reactivation of meningeal T cells in the inflamed CNS, as I observed no change in the number of parenchymal, white matter T cells

following anti-mCD20 treatment. In this study I was not able to determine why this influx of T cells results, however this avenue of research in the future could help solidify the role of B cells in meningeal B cell clusters. In particular, I would expect that transfer of activated myelin specific T cells following B cell depletion during the chronic phase of 2D2 IgH^{MOG} sEAE would result in the accumulation of myelin reactive T cells in the meninges, while in control mice myelin specific T cells would be observed throughout the inflamed CNS. Although no change in the cytokine profile of T cells was observed in preliminary analysis, this finding was restricted by the analysis of total CNS infiltrating T cells, and not just meningeal T cell phenotypes, as well as a small sample size (n=2 for control and n=3 for anti-mCD20 treated mice). Future experiments should be completed to characterize the cytokine profile of meningeal T cells following B cell depletion, with an ELISA to also determine total cytokine production.

Future experiments should also be aimed at addressing the mechanism by which B cells in the inflamed CNS may be presenting antigen to T cells. Since B cells are typically known for endocytosing antigen through their BCR, determining whether meningeal B cells have the capacity to take up antigen through BCR-independent pathways such as phagocytosis will be evaluated. Peritoneal cavity B-1 cells have previously been shown to differentiate into mononuclear phagocytes *in vitro* and *in vivo*, with the capability of phagocytosing various antigen targets (283–285). Further supporting the capability of B cells to become phagocytic was the observation that follicular B cells can be observed phagocytosing antigen (286), suggesting that B cells in certain contexts and with the right stimulation can go on to present antigen to T cells in a phagocytic-dependent manner. While I completed a pilot sc-RNA-seq experiment to better characterize meningeal B cells, preliminary analysis revealed that B cells within the inflamed spinal cord of 2D2 IgH^{MOG} sEAE mice are unique from lymph node B cells. Future experiments will be aimed at comparing meningeal B cells to additional B cell subsets such as naïve B cells, GC B cells, memory B cells, marginal zone B cells, and peritoneal cavity B-1 B cells in order to identify how meningeal B cells may be contributing to disease pathogenesis.

Animal models, at their core, have many limitations in addressing human diseases, such as the initiating events that trigger the onset of MS. Indeed, the use of $mMOG_{tag}$ as an

immunogen in EAE does not contain post-translational modifications that would be found on endogenous MOG protein in vivo making it difficult to understand the mechanisms by which post-translational modifications affect the autoimmune response. Indeed, extracellular MOG found on the surface of oligodendrocytes is post-translationally modified at arginine 31 to be glycosylated, resulting in altered B cell recognition and antibody binding (206, 287). Furthermore, inflammation in EAE tends to be localized to the spinal cord, with a more limited spectrum of clinical signs as opposed to MS, highlighting the limitations of these models in representing MS. Nevertheless, EAE has played a pivotal role in allowing us to determine mechanistic insights in MS. Furthermore, these findings are consistent with results observed by others in that myelin specific B cells appear to be excluded from the inflamed CNS (146, 221). While I have confirmed meningeal B cells are not specific for myelin antigen in two different models of EAE, confirming these results in additional models such as MP4 induced EAE, a model that has been cited to result in ectopic GC like follicles at a more frequent occurrence (210), could help solidify this finding. Furthermore, it is unknown whether these meningeal B cell aggregates are similar to those found in other sites of inflammation such as in spinal cord injury, colo-rectal cancer, Sjorgen's syndrome and systemic lupus erythematosus (288–290) to name a few.

While the contribution of antibodies was not investigated in this thesis, their role in EAE and MS like neurological variants of disease such as NMO and MOG-antibody disease have been reported. Characterizing the antibody response in various EAE models revealed that hMOG induced EAE generates pathogenic antibodies that cross-react with glycosylated MOG and oligodendrocytes (206). These antibodies have been shown to be pathogenic as transfer into B cell deficient mice immunized with hMOG restored susceptibility to EAE in these otherwise resistant mice. In contrast, while rMOG still generates a functioning antibody response, these antibodies are not able to bind glycosylated MOG or contribute to pathology. Future experiments analyzing the antibody response in $mMOG_{tag}$ induced EAE will be required to determine how the antibodies generated in this immune response contribute to disease. Clinical trials of anti-CD20 have confirmed that antibody levels are not affected within the therapeutic time frame (2, 157, 158), consistent with the idea that these therapies do not target antibody producing plasma

cells as they are $CD20^-$. Although I did not measure the antibody response following anti-mCD20 treatment in Chapter 4, I suspect that minimal alterations in the antibody response would be observed based on these clinical trials. 2D2 IgH^{MOG} mice are known to produce large amounts of MOG-specific IgM and IgG1 antibodies regardless of whether they have EAE onset or are asymptomatic (215), suggesting their role in disease is limited.

5.1 A model for B cell involvement in CNS autoimmunity

I hypothesize that at a minimum, two B cell populations are contributing to the pathogenesis of CNS autoimmunity (Fig 5.1). The first population is myelin-specific B cells that are involved in the induction of disease. Indeed, MHC class II-dependent B cell APC function is required for hMOG induced CNS autoimmunity (165). In this model of EAE, class switch recombination of the BCR is critical for the generation of pathogenic autoantibodies, which have also been shown to be sufficient to restore EAE susceptibility to hMOG in activation induced cytidine deaminase (AICD) knockout mice (207). Likewise, another group showed that when B cells are the only cell expressing MHC class II, that increasing the frequency of MOG-specific B cells restores susceptibility to rMOG induced EAE (166). Consistent with this role of B cells contributing to initial APC activation of T cells, when MHC class II is restricted to DCs alone, they are inefficient at inducing rMOG EAE (291). Furthermore, conditional and temporal regulation of MHC class II expression on B cells reveals MOG-specific B cells are capable of inducing an accelerated onset of EAE in comparison to a full compartment of MHC class II APC subsets (167). Again, while the APC function of myelin-specific B cells is clearly supported, MOG-specific autoantibodies have also been shown to enhance antigen presentation of resident CNS APCs, thereby enhancing the activation of incoming effector T cells (221). Finally, sEAE occurs at an increased incidence when both MOG-specific TCRs and BCRs are present (212, 215–217), and this has been shown to be independent of their ability to produce antibodies (165). However, it has been reported that even in this spontaneous model of EAE that the addition of serum from IgH^{MOG} mice into 2D2 recipients can increase the incidence of sEAE (6/14 compared to 0/12) (222). While autoantibodies have been shown to be involved in some variants of MS such as NMO and MOG-antibody disorders (46, 47), this is a less common occurrence in clinically definite

MS. Overall, these findings point towards an involvement of myelin-specific B cells in CNS autoimmunity, potentially allowing for the activation of autoreactive T cells that can go on to drive inflammation within the CNS.

The second population I propose is contributing to CNS autoimmunity is non-specific B cells involved in the local CNS inflammatory responses, where I have shown that myelin-specific B cells are largely excluded (243). These meningeal B cell clusters have been shown to correlate to disease severity in 2D2 IgH^{MOG} sEAE (217), and following B depletion during the chronic phase of sEAE results in a loss of meningeal B cells, and reduced tissue pathology (Chapter 4). Consistent with our findings that myelin-specific B cells are largely excluded from the inflamed CNS, was the identification that compared to foreign antigen GC responses, the MOG GC response is relatively short-lived and gives rise to fewer plasma cells relative to foreign-antigen responses (244). Instead, these anti-myelin GC responses predominantly produced short-lived memory B cells, that fail to respond to secondary challenge, suggesting that the role of myelin-specific B cells may not extend beyond disease induction.

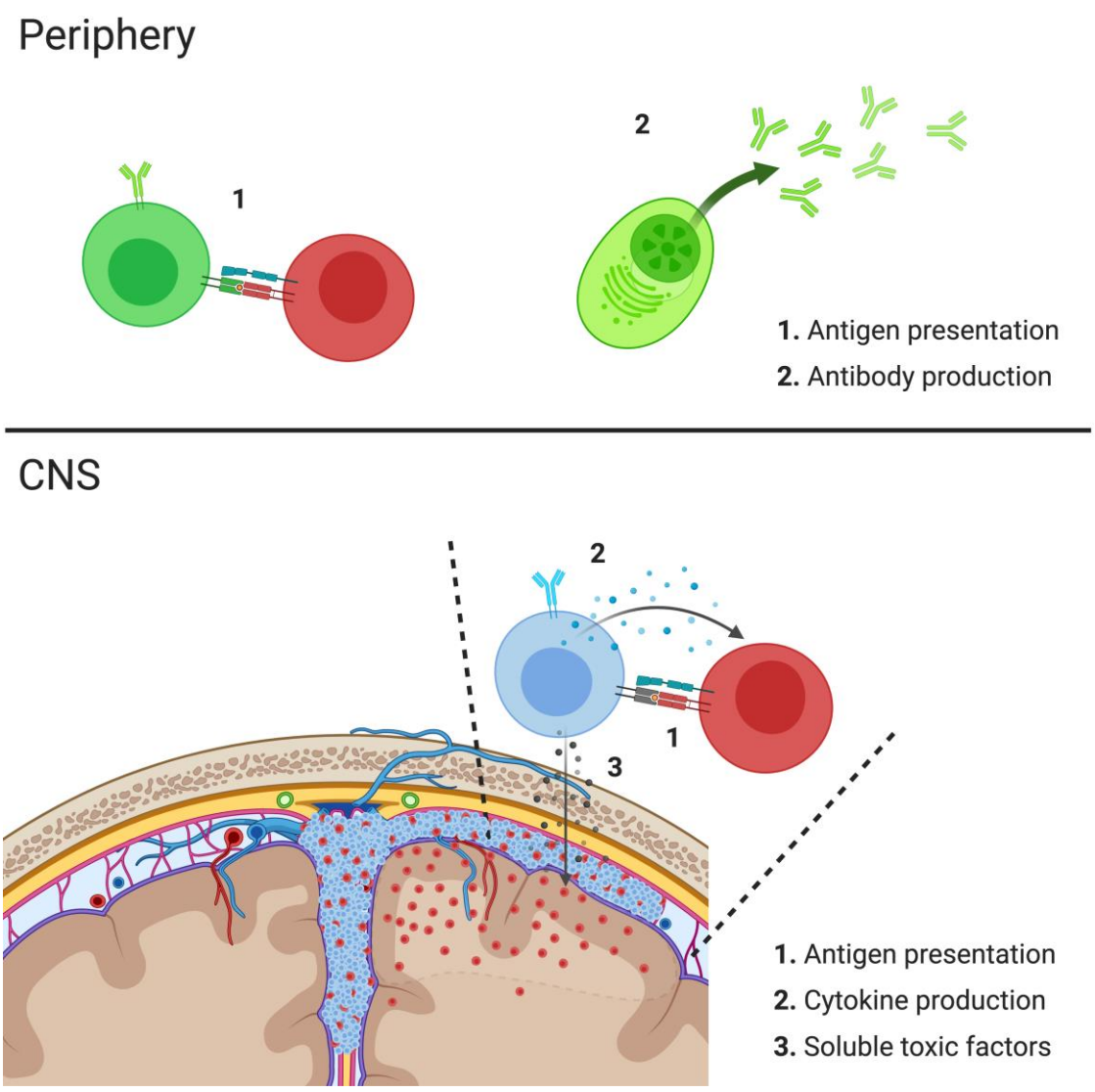


Figure 5.1 | Proposed mechanism of B cell involvement in CNS autoimmunity.

Two distinct populations of B cells contribute to CNS autoimmunity. In the periphery, myelin-specific B cells (green) can active myelin-specific T cells (red) via cognate interactions. In turn, myelin-specific B cells acquire T cell help, producing a GC response that can generate high-affinity, class-switched, short-lived memory B cells, and autoantibody-secreting plasma cells. In contrast, in the CNS non-specific B cells (blue) within the meninges promote disease pathogenesis by three probable mechanisms: 1) reactivating myelin-specific T cells, 2) producing proinflammatory cytokines (IL6, GM-CSF, TNFα, and lymphotoxin), and 3) secreting soluble toxic factors. This figure was created with BioRender.com.

5.2 Concluding remarks and implications to MS

The data presented in this thesis demonstrates that our current hypothesis of how B cells contribute to disease within the inflamed CNS in anti-myelin autoimmunity is inherently flawed. Previous assumptions were based on what we knew about $CD4^+$ T cells, however, it is clear that B and T cells have fundamentally different relationships within the inflamed CNS. Furthermore, while it is still unclear how B cells contribute to the local pathology within the inflamed CNS, the work in this thesis suggests they are involved in disease pathogenesis. This is based on their location adjacent to demyelinating lesions, correlation to disease severity (217), and observation that CNS pathology is reduced following their depletion within the inflamed CNS. Although I was unable to preferentially deplete meningeal B cells, the currently completed and future sc-RNA-seq experiments from our lab will hopefully help identify features unique to meningeal B cells that may reveal how to more specifically target these cells. Currently, the only approved therapy for progressive MS is anti-CD20 B cell depleting therapies. While they have been effective at reducing lesions within the brain, clinical relapses, and disability progression (3, 5, 6, 168), they still result in an untargeted depletion of all $CD20^+$ expressing B cells. By selectively targeting the pathogenic populations of B cells in MS, this will allow for the maintenance of regulatory B cell populations or B cells that can then go on to mount an immune response to infections. Currently I hypothesize that *standard follicular B cells recruited to the CNS become activated within the inflammatory milieu and contribute to disease through production of proinflammatory cytokines, neurotoxic factors, and antigen presentation to T cells*. Future work from the Kerfoot laboratory will build on these findings to characterize meningeal B cell clusters and determine how they contribute to disease.

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Appendix

Appendix A | Statement of permission for the use of mice for experimental research.

The use of mice for experimental procedures were conducted in compliance with the animal use protocol 2015-090 and 2019-123 held by Dr. Steven Kerfoot, principal investigator at Schulich School of Medicine and Dentistry in the department of Microbiology and Immunology at the University of Western Ontario in London, Ontario, Canada.

eSirius3G

Western

PI :	Kerfoot, Steven
Protocol #	2015-090
Status :	Approved (w/o Stipulation)
Approved :	02/16/2016
Expires :	02/01/2020
Title :	B cell and T cell activation and differentiation in the initiation of immune responses and autoimmunity

eSirius3G

Western

PI :	Kerfoot, Steven
Protocol #	2019-123
Status :	Approved (w/o Stipulation)
Approved :	02/01/2020
Expires :	02/01/2024
Title :	B cell and T cell activation and differentiation in the initiation of immune responses and autoimmunity

Appendix B | Permission for reproducing a published manuscript in Chapter 3.

Yodit Tesfagiorgis, BMSc
PhD Candidate, Microbiology and Immunology
University of Western Ontario
London, ON, Canada

January 25, 2021

Dear Ms. Tesfagiorgis,

The American Association of Immunologists, Inc., grants permission to reproduce figures from "Activated B Cells Participating in the Anti-Myelin Response Are Excluded from the Inflamed Central Nervous System in a Model of Autoimmunity that Allows for B Cell Recognition of Autoantigen" contingent on the following conditions:

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Curriculum Vitae

Yodit Tesfagiorgis, BMS

Education

2015-21 **PhD Candidate**, *University of Western Ontario,* Microbiology and Immunology.
Thesis: Meningeal B cells: determining their characteristics and susceptibility to therapeutic depletion in an animal model of Multiple Sclerosis
Supervisor: Dr. Steven M. Kerfoot

2011-15 **BMSc Honors,** *University of Western Ontario*, Microbiology and Immunology.
Minor: Medical Cell Biology
Thesis: Characterizing B cells in the inflamed spinal cord of an animal model of Multiple Sclerosis
Supervisor: Dr. Steven M. Kerfoot

Major Awards

2018-21 **endMS Doctoral Studentship Award,** *Multiple Sclerosis Society of Canada*
$66,000 CAD

2016-18 **Ontario Graduate Scholarship**, *University of Western Ontario*
$30,000 CAD

2012-14 **Heaslip Scholarship,** *University of Western Ontario*
$45,000 CAD

Honors and Awards

2020 **CIHR National Health Research Poster Presentation,** Honourable Mention
University of Manitoba

2020 **Top 20 in the 3-Minute Thesis Competition,** *University of Western Ontario*

2018,19 **endMS Scholar Program for Researchers IN Training (SPRINT)**
Multiple Sclerosis Society of Canada

2019 **endMS Conference**, Poster Presentation Award, *Calgary Alberta*

2019 **Infection and Immunity Research Forum**, Poster Presentation Award
London ON

2019 **Canadian Society of Immunology,** Poster Presentation Award**,** *Banff Alberta*

2019 **Canadian Society of Immunology**, Travel Award, *Banff Alberta*

2016,17, 19 **Dr. Frederick W. Luney Graduate Travel Award**, *University of Western Ontario*

2017 **Infection and Immunity Research Forum**, Poster Presentation Award
London ON

2016 **Infection and Immunity Research Forum,** 1st place Oral Presentation Award

2016 **endMS Summer School,** *University of British Columbia*

2013,14,15 **Dean's Honor List**, *University of Western Ontario*

2011-12 **The Western Scholarship of Excellence,** *University of Western Ontario*

Employment

Teaching Assistant:

2019-20 Immunology Course, 3300B. Class size = 150
- 1 lecture – "Immunoglobulin Receptors: BCR gene rearrangement"

2019-20 Immunology Lab Course, 3620G. Class size = 76
- Monitor students throughout experiments, grade lab reports

2018-19 Immunology Lab Course, 3620G. Class size = 80
- Monitor students throughout experiments, grade lab reports
- 1 lecture – "TNF Bioassay"

2017-18 Microbiology and Immunology for nurses, 3820A. Class size = 351
- Create case studies for Bacteria and Virus midterms.
- 2 lecture hours – Topic: "Innate Immunity" and "B cell Immunity"

2016-17 Microbiology and Immunology for nurses, 3820A. Class size = 320
- Create case studies for the Bacteria and Virus midterms.

Work Study Student:

2012-2013 Department of Microbiology and Immunology, *University of Western Ontario*
- Supervisor: Dr. John K. McCormick

Community and Volunteer Activities

2019-2020 **Graduate Appointments Committee Representative**
University of Western Ontario

2019-2020 **Graduate Committee Student Representative**, *University of Western Ontario*

2019-2020 **Schulich Graduate Student Department Representative**,
University of Western Ontario

2015-2020 **Canadian Society of Immunology University Representative**
University of Western Ontario

2015-2019 **Observer in the department of Neurology**, MS clinic
University Hospital, London

2018-19 **Sponsorship Liaison**, *Infection and Immunity Research Forum (IIRF)*

2017-19 **Graduate Student Representative**, *University of Western Ontario*

2018 **Graduate Representative**, *Canadian Society of Immunology Council Meeting*

2017-18 **President of the Western Triathlon Club**, *University of Western Ontario*

2016-18 **Committee Member**, *Infection and Immunity Research Forum (IIRF)*

2015-16 **Microbiology and Immunology Social Committee**
University of Western Ontario

2013-15 **VP Events – Western Biochemistry Club**, *University of Western Ontario*

2013-15 **Co-President/Co-Founder of Microbiology and Immunology Student Association (MISA)**, *University of Western Ontario*

Mentorship

Undergraduate Students:

2019-20 **Robbie Jin**. "Characterizing B cells in autoimmunity"

2017-18 **Yomna El-Sakka**. "Characterizing CNS autoimmunity induced by haMOG and bMOG"

2016-17 **Alicia Dakins**. "Characterizing CNS pathology in novel EAE models"

2015-16 **Sarah Zhu**, "Investigation of MOG-specific B cells in the CNS of EAE mice"

Publications

Peer Reviewed Publications:

1. Laramee A.S., Raczkowski H.L, Shao P, Batista C.R., Shukla D, Xu L.S., Haeryfar S.M.M., Tesfagiorgis Y, Kerfoot S.M., and DeKoter R.P. 2020. Opposing Roles for the Related ETS-family Transcription Factors Spi-B and Spi-C in Regulating B Cell Differentiation and Function. *Front. Immunol*. 11: 841

2. Jain RW, Parham KA, Tesfagiorgis Y, Craig HC, Romanchik E, and Kerfoot SM. 2018. Autoreactive, low-affinity T cells preferentially drive differentiation of short-lived memory B cells at the expense of germinal center maintenance. *Cell Rep.* 25; 3342-3355.e5.

3. Whittaker Hawkins R, Patenaude A, Dumas A, Jain R, Tesfagiorgis Y, Kerfoot S, Matsui T, Gunzer M, Poubelle P, Larochelle C, Pelletier M, and Vallières L. 2017. ICAM1+ neutrophils promote chronic inflammation via ASPRV1 in B cell-dependent autoimmune encephalomyelitis. *JCI Insight.* 2: e96882.

4. Tesfagiorgis Y, Zhu SL, Jain R, and Kerfoot SM. 2017. Activated B cells participating in the anti-myelin response are excluded from the inflamed central nervous system in a model of autoimmunity that allows for B cell recognition of autoantigen. *J. Immunol*. 199: 449-457.

5. Dang AK, Tesfagiorgis Y, Jain RW, Craig HC, and Kerfoot SM. 2015. Meningeal infiltration of the spinal cord by non-classically activated B cells in associated with chronic disease course in a spontaneous B cell-dependent model of CNS autoimmune disease. *Front. Immunol*. 6: 470.

Manuscripts in Preparation:

1) Campden R*, Edwards T*, Tesfagiorgis Y*, and Berrigan, L. A systematic review of how cognitive impairment impacts quality of life in Multiple Sclerosis. *In Preparation*.
   * Signifies all authors contributed equally to this manuscript
2) Tesfagiorgis Y and Kerfoot SM. B cell clusters within the meninges of central nervous system autoimmunity can be targeted for depletion over time resulting in reduced pathology. *In Preparation*.
3) Shin AE, Tesfagiorgis Y, Good HJ, Zhang L, Kerfoot SM, Sherman PM, Howlett CJ, Wang TC, and Asfaha S. F4/80^{+}Ly6C^{high} macrophages are key to cancer initiation in colitis. *In Preparation*.

Presentations at professional meetings

1. Tesfagiorgis Y and Kerfoot SM. Characterizing a unique B cell subset in an animal model of Multiple Sclerosis. *CSHRF 2020*. Winnipeg, MB, Canada. 2020-06-09.
    - Virtual conference, only 5% of PhD candidates are asked to attend
2. Tesfagiorgis Y and Kerfoot SM. Meningeal B cells in an animal model of Multiple Sclerosis: characterizing their phenotype and susceptibility to therapeutic depletion. *NRD 2020*. London, ON, Canada. 2020-02-21.
3. Tesfagiorgis Y and Kerfoot SM. Meningeal B cells: their phenotype and susceptibility to therapeutic depletion. *endMS Conference*. *Calgary, AB, Canada*. 2019-12-8
4. Tesfagiorgis Y and Kerfoot SM. B cell aggregates, their susceptibility to therapeutic depletion. *The 14th Annual Infection and Immunity Research Forum. London, ON, Canada.* 2019-11-08.
5. Campden R*, Edwards T*, Tesfagiorgis Y*, and Berrigan, L. The relationship between quality of life and cognition in MS: A systematic review. *Consortium of Multiple Sclerosis Centers, Seattle, WA, USA*. 2019-05-29.
    - *** Signifies all authors contributed equally to this abstract**.
6. Tesfagiorgis Y, Jain RW, Parham, KA, and Kerfoot SM. Does manipulation T cell affinity to myelin antigen result in differences in CNS autoimmunity. *Canadian Society of Immunology 2019, Banff, AB, Canada*. 2019-04-13.
    - **This abstract is selected for a platform oral presentation**.
7. Tesfagiorgis Y, and Kerfoot SM. B cell aggregates, their phenotype within the inflamed CNS, and their susceptibility to therapeutic depletion. *The 13th Annual Infection and Immunity Research Forum, Stratford, Canada.* 2018-10-11.
    - **This abstract was selected for a platform oral presentation.**
8. Tesfagiorgis Y, Zhu S, Jain RW, Kerfoot SM. Characterizing B and T cell infiltrates in the inflamed central nervous system of an animal model of Multiple Sclerosis. *Canadian Society of Immunology 2018, London, ON, Canada.* 2018-06-01.
9. Tesfagiorgis Y, Zhu S, Jain RW, Kerfoot SM. Activated B Cells Participating in the Anti-Myelin Response Are Excluded from the Inflamed Central Nervous System in a Model of Autoimmunity that Allows for B Cell Recognition of Autoantigen. *London Health Research Day, London Convention Centre, London, ON, Canada.* 2018-05-10.
10. Tesfagiorgis Y, Zhu S, Jain RW, and Kerfoot SM. Activated B cells participating in the anti-myelin response are excluded from the inflamed central nervous system in a model of autoimmunity that allows for B cell recognition of autoantigen. *CNS Research Day, London Ontario*, 2018-04-17.
11. Tesfagiorgis Y, Zhu S, Jain R, and Kerfoot SM. Deciphering the differences between B and T cell recruitment to the inflamed CNS. *2017 Infection and Immunity Research Forum, London, ON.* 2017-10-27.
12. Tesfagiorgis Y, Zhu SL, Jain RW, and Kerfoot SM. (2017). Activated myelin-specific B cells, contrary to T cells, are excluded from the inflamed CNS in an animal model of central nervous system autoimmunity. *London Health Research Day, London Convention Centre, London, ON, Canada.* 2017-03-28.

13. Tesfagiorgis Y, Zhu SL, Jain RW, and Kerfoot SM. (2017). B and T cells follow fundamentally different recruitment mechanisms to the inflamed CNS in an animal model of CNS autoimmunity. *MS Rounds*. *London Health Science Center-University Hospital*. 2017-03-13.
    - **This was an oral presentation during Clinical Rounds.**
14. Tesfagiorgis Y, Zhu S, and Kerfoot SM. B and T cells are fundamentally different in their recruitment to the inflamed CNS. *endMS Conference 2016. Toronto, ON.* Dec. 6-9, 2016.
15. Tesfagiorgis Y, Sarah L. Zhu and Steven M. Kerfoot. (2016). B and T cells are fundamentally different in their recruitment to the inflamed CNS. *Annual Infection and Immunity Research Forum, London, ON.* 09/16.
    - **This abstract was selected for a platform oral presentation.**
16. Tesfagiorgis Y, Dang AK, Craig HC, and Kerfoot SM. Characterization of the activation history and antigen specificity of B cells found within meningeal B cell clusters in central nervous system autoimmunity. *Annual meeting of the Canadian Society for Immunology, Ottawa, ON*. April 1-4, 2016.
17. Tesfagiorgis Y, Dang AK, Craig HC, and Kerfoot SM. (2016). Characterization of the activation history and antigen specificity of B cells found within meningeal B cell clusters in central nervous system autoimmunity. *London Health Research Day 2016, London Convention Centre, London, ON, Canada.* 03-2016.
18. Tesfagiorgis Y, Dang AK, Craig HC, and Kerfoot SM. Characterization of the activation history and antigen specificity of B cells found within meningeal B cell clusters in central nervous system autoimmunity. *Infection and Immunity Research Forum, 2015 Infection and Immunity Research Forum, London, ON.* September 2015.