

**B cells promote inflammatory T cell responses during the initiation of central
nervous system autoimmune disease**

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Abstract

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Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) mediated by autoreactive T cells specific for myelin proteins. A recent development in MS treatment is the discovery that B cell depletion with Rituximab reduced the numbers of lesions and new relapses in MS patients. The role of B cells has been studied in the animal model of MS, experimental autoimmune encephalomyelitis (EAE), but results from these studies have been variable and contradictory, and the contribution of B cells to CNS autoimmune disease is still unclear. We examined the role of B cells in C3H mice and found that both C3HeB/Fej and C3H.SW μ MT mice had a reduced incidence of EAE, suggesting an important role for B cells in the initiation of disease. B cells were the predominant MHC class II⁺ cells in the healthy CNS and were able to secrete cytokines, indicating that they could be influencing T cell responses before inflammation is initiated. Myelin-specific T cells were able to migrate to the CNS in the absence of B cells, but were unable to initiate immune responses. The ability of the early

infiltrating T cells to secrete cytokines and initiate the recruitment of additional T cells to the CNS from the periphery before onset of EAE was defective in B cell deficient mice. Recruitment of T cells from the periphery constituted the majority of the increase in T cell number in the CNS of wildtype mice prior to onset. *In vitro*, B cells preferentially reactivated effector Th1 cells and not Th17 cells in the absence of IL-1 β . Induction of EAE with Th1- or Th17-skewed cells led to reduced numbers of IFN- γ - and IL-17-producing cells in the brains of B cell deficient mice after onset of EAE. However, there was a greater effect on the numbers of IFN- γ -producing cells, and in B cell deficient recipients of Th1-skewed cells, the localization of inflammation changed to permit inflammation in the brain. These studies indicate that B cells are important for T cell responses in the initiation of CNS autoimmunity, and can influence the localization of inflammation in EAE.

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List of Abbreviations

APC	Antigen-presenting cell
BBB	Blood-brain barrier
BCR	B cell receptor
CD	Cluster of differentiation
CNS	Central nervous system
CSF	Cerebrospinal fluid
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
eLF	Ectopic lymphoid follicle
HEV	High endothelial venule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LT	Lymphotoxin
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NMO	Neuromyelitis optica
PCR	Polymerase chain reaction
PLP	Proteolipid protein
PP-MS	Primary progressive multiple sclerosis
RR-MS	Relapsing-remitting multiple sclerosis
SP-MS	Secondary progressive multiple sclerosis
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper cell
TNF	Tumor necrosis factor
TLO	Tertiary lymphoid organ
Treg	Regulatory T cell

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Chapter 1: Introduction

Anatomy and Immunology of the Central Nervous System

The central nervous system (CNS) is comprised of the brain and spinal cord. The major divisions of the brain are the forebrain, containing the cerebrum, the midbrain, containing the brainstem, and the hindbrain, containing the cerebellum. The brain and spinal cord are protected by multiple barriers, including bone (skull and vertebral column), and the meninges, the fluid-filled membranes that cushion the tissue. The three layers of the meninges are comprised of the dura mater, the outermost membrane, the arachnoid mater in the middle, and the pia mater that adheres to the surface of the brain and spinal cord. The subarachnoid space is the space between the arachnoid and pia mater, and is filled with cerebrospinal fluid (CSF) (1).

In the CNS, half of the cells are neurons, the information-processing cells, and the other half of the cells are glia, the cells that support neuron function (2). The white matter of the CNS is made up of the axons of the neurons that are covered in a myelin sheath, and the grey matter is made up of the soma, or cell bodies of neurons. Glial cells include oligodendrocytes, astrocytes, and microglia. Oligodendrocytes are cells that form the insulating myelin sheath around axons in the CNS, a critical function that allows rapid conduction of signals between neurons. Astrocytes provide structural support for neurons and regulate the flow of ions and other molecules in the extracellular space. Microglia are resident phagocytic cells of the CNS that scavenge for cellular debris and pathogens.

An important mechanism protecting the CNS is the blood brain barrier (BBB), a complex system of endothelial cells and membrane layers surrounding the CNS microvessels

that separates the circulating blood from the CSF. The BBB is highly selective, allowing diffusion of small hydrophobic molecules such as O₂ and CO₂ while restricting entry of large or hydrophilic molecules, pathogens, and most circulating cells. This selective permeability is due to the presence of specialized tight junctions made up of transmembrane and cytoplasmic proteins linked to the actin skeleton that connect the endothelial cell monolayer of CNS vessels. A basement membrane made up of laminins and pericytes surrounds the endothelial vessels. An additional barrier called the glia limitans is formed by end foot processes extending from astrocytes. The glia limitans restricts entry of cells or molecules into the CNS parenchyma from the perivascular space surrounding blood vessels as well as from the subarachnoid space through the pia mater. Collectively, these layers and membranes constitute the BBB (3).

Another important feature of the CNS, the ventricular system, is a connected system of internal brain cavities through which CSF continuously flows after it is produced in the choroid plexus. Each ventricle contains a choroid plexus, which consists of a layer of epithelial cells folded into villi surrounding capillaries (1). These cells actively transport ions and metabolic waste in and out of the CSF as it is made, and the tight junctions between cells of the epithelial layer form the blood-CSF barrier. Similar to the BBB, the blood-CSF barrier functions to prevent the majority of substances from entering the CNS. CSF flows from the ventricles into the subarachnoid space, where it is mainly resorbed into venous blood. There is also some evidence that CSF can drain into the cervical lymph nodes, which could allow antigens from the CNS to be presented to T cells in the periphery and stimulate immune responses (1).

Immune responses in the CNS are more carefully regulated than those in the periphery (4). The normal CNS tissue is highly suppressive to inflammation, with very low levels of co-stimulatory factors, adhesion molecules, and pro-inflammatory mediators (5). Constitutive

expression of CD95 ligand in the healthy CNS promotes apoptosis of activated T cells (6). However, under pathological conditions, the BBB becomes compromised and is more accessible to inflammatory cell entry, and various resident cell types become activated and promote inflammation (7). Normal immune surveillance of the CNS is believed to involve circulating memory or activated T and B cells that enter the healthy CNS in low numbers. If T cells encounter their cognate antigen-presenting cells within the CNS, they are reactivated, and an inflammatory response may be initiated. Local CNS antigen-presenting cells are thought to include perivascular dendritic cells and macrophages, and activated microglia, which can rapidly upregulate MHC class II and co-stimulatory molecule expression (6). During immune responses, astrocytes can also become activated and secrete pro-inflammatory cytokines and chemokines that promote permeabilization of the BBB and recruitment of additional immune cells from the periphery (8).

Multiple Sclerosis

Overview of multiple sclerosis

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system, estimated to affect up to two million people worldwide. Hallmarks of MS include focal inflammatory infiltrates, demyelinating plaques, and axonal damage. While the etiology of MS is not known, it is widely considered to be an autoimmune disease. Recent genome-wide association studies (GWAS) provided strong support for this notion by identifying >50 susceptibility loci associated with MS of which the vast majority represent genes with immune cell function (9). A long-standing hypothesis is that environmental factors, such as infectious agents, trigger MS in genetically susceptible individuals by promoting the activation of myelin-specific T cells that normally circulate in the periphery in a tolerant state. Once activated, these

T cells can enter the CNS and initiate an autoimmune response. A major role for CD4⁺ T cells in this process is supported by the strong association of genetic susceptibility to MS is with MHC class II alleles that present antigen to CD4⁺ T cells. However, a role for MHC class I alleles that present antigen to CD8⁺ T cells was also confirmed by the GWAS studies, suggesting a complex pathogenesis (9). Other factors influencing susceptibility to MS include ethnicity, vitamin D levels, smoking, and Epstein-Barr virus infection (10, 11).

Heterogeneity of the clinical course in MS

Patients with MS can be subcategorized based on distinct clinical patterns. About 85% of patients with MS develop relapsing-remitting MS (RR-MS), during which patients experience episodes of neurological disability but return to baseline following relapse. Approximately 50% of these patients develop a clinically distinct form of disease referred to as secondary progressive MS (SP-MS) within 10 years of onset of RR-MS (12). In SP-MS, relapses still occur but clinical disability worsens over time and brain atrophy steadily increases (13). The reasons for this shift to increasing clinical disability and lack of return to baseline are not known. Interestingly, even though patients may convert from RR-MS to SP-MS at different times following the initial diagnosis, once a patient converts to SP-MS, the overall increase in disability and in brain atrophy proceed at the same rate (14). This suggests that there is a mechanistic shift in the pathogenesis that occurs when a patient converts to SP-MS, and mechanisms underlying progressive disease have recently been proposed (14). While inflammatory lesions detected by MRI during RR-MS decrease in SP-MS, some inflammatory immune cells may still be engaged in tissue destruction but are not detected by imaging because the blood brain barrier is no longer permeable to gadolinium.

There is also a form of MS called primary progressive (PP-MS), which comprises approximately 10-20% of all MS cases. Patients with PP-MS lack the acute exacerbations seen in patients with RR-MS; instead, they develop steadily worsening clinical disability from the time of their initial diagnosis (15). Patients with PP-MS respond poorly to most commonly used immune-modulating MS therapeutics (16), suggesting that a different pathogenic pathway may be at play in these patients compared to those with RR-MS.

Inflammatory patterns differ in patients with MS

The locations of lesions within the CNS are the major determinant of clinical signs, and these are also variable among patients. The majority of lesions are found in the brain, particularly in the periventricular white matter, cerebellum, brainstem, and optic nerves. Many patients exhibit lesions in the spinal cord as well as the brain, while 2-10% of patients exhibit inflammation in the spinal cord and optic nerves without extensive involvement of the brain (referred to as opticospinal MS) (17, 18). Opticospinal MS has particularly high prevalence in patients with MS of Asian descent, while patients with MS from North America tend to exhibit the more common pattern of brain lesions (19). These distinct localization patterns suggest that mechanisms promoting inflammation in the brain may be distinct from lesions promoting inflammation in the spinal cord.

Patients with MS exhibit diverse pathological features

The CNS pathology in MS is characterized by inflammatory lesions, demyelination, remyelination, neurodegeneration, and glial scar formation (14). Most MS lesions are dominated by T cells and macrophages. Beyond these fairly common characteristics, substantial heterogeneity is observed in the CNS pathology. Criteria have been developed that allow the lesions seen in patients with MS to be divided into four categories (Patterns I-IV).

The criteria are based on the distribution of myelin loss, plaque geography and extension, pattern of oligodendrocyte injury, and immunopathological evidence of immunoglobulin and activated complement deposits (20). Patterns I and II lesions share a perivenous distribution of plaques dominated by T cells and macrophages with preservation of oligodendrocytes. The main difference between pattern I and pattern II lesions is the presence of immunoglobulin and complement deposits in pattern II lesions. In contrast, patterns III and IV are associated with oligodendrocyte cell death. In pattern III, oligodendrocytes undergo apoptosis while in pattern IV, non-apoptotic cell death is observed.

One of the most important findings to come from these studies is that multiple active plaques analyzed from an individual belong to the same pattern, indicating a lack of intra-individual heterogeneity. This suggests that the pathological patterns may reflect distinct pathogenic pathways occurring in different individuals rather than the evolution of lesions over time within an individual (20). This notion emphasizes the importance of developing biomarkers to stratify patients with MS into distinct groups so that treatment protocols are appropriately matched to the pathogenic mechanisms relevant to their disease.

Developing therapies to treat or prevent MS requires an in-depth understanding of the pathogenesis of the disease. Mechanistic studies in MS are complicated because central nervous system tissue is difficult to access and immune responses within this tissue cannot be easily monitored. Therefore, animal models are essential in defining the mechanisms underlying MS.

Experimental Autoimmune Encephalomyelitis

Overview of EAE

The pathogenic mechanisms that lead to the development of MS have been widely studied using the animal model experimental autoimmune encephalomyelitis (EAE). The origin of the model dates back to 1925 with the discovery that rabbits immunized with human spinal cord homogenate exhibited spinal cord inflammation (21). EAE has been studied in a variety of animal species, including mouse, rat, rabbit, guinea pigs, and non-human primates, but rodents are currently the most commonly used animals in EAE studies. EAE is induced by stimulating CD4⁺ T cell-mediated immunity to myelin proteins, either by immunization with myelin antigens in complete Freund's adjuvant or by adoptive transfer of myelin-specific T cells (22, 23). Self-reactive CD4⁺ T cell responses toward myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) have been most extensively studied. EAE has been very useful for investigating the immunological mechanisms that contribute to CNS autoimmune disease. The synthesis of many studies indicates that the following events comprise the pathogenic pathway that leads to EAE, and presumably MS (Figure 1.1)(24). CD4⁺ T cells are activated in the periphery and express cell-surface activation markers that facilitate their extravasation across the blood brain barrier. Upon entering the CNS, the CD4⁺ T cells are reactivated by myelin epitopes presented by dendritic cells (DCs), which may (25) or may not (26, 27) be sufficient for T cell reactivation. Activation of macrophages and microglia as well as myelin damage occurs during this initial inflammatory response. The number of MHC class II⁺ antigen presenting cells (APCs) capable of presenting antigen to CD4⁺ T cells then increases dramatically within the CNS, largely due to recruitment of circulating monocytes, which differentiate into inflammatory DCs and macrophages upon

entering the tissue (28). The blood brain barrier becomes increasingly permeable, allowing naïve T cells to enter the CNS, some of which are specific for myelin epitopes distinct from that of the initial infiltrating T cell population. Recognition of myelin antigen by these naïve T cells leads to a phenomenon known as determinant-spreading in which chronic inflammation is propagated by continuous generation of APCs and diversification of the myelin-specific T cell response (29, 30). Pathogenic CD8⁺ T cells and B cells also contribute to ongoing inflammation, while regulatory subsets of CD4⁺ T cells, CD8⁺ T cells and B cells promote recovery from disease activity. The balance between pathogenic and regulatory cells likely determines the outcome of inflammation. A major factor that has been suggested to influence this balance is the gut microflora, which can promote both pathogenic and regulatory cell development (31).

Heterogeneity in EAE models

No single model replicates the full spectrum of inflammatory mechanisms and neurodegeneration seen in MS, just as individual patients manifest only a subset of the diverse features of the disease. Therefore, EAE models that exhibit distinct pathology and clinical signs can be valuable tools to investigate different aspects of the disease. Reflecting the heterogeneity in the clinical course of MS patients, the clinical symptoms in EAE models can follow a monophasic or chronic course, or, in the case of SJL/J mice, exhibit a relapsing-remitting disease (32, 33). NOD and Biozzi ABH mice may recapitulate some of the mechanisms involved in secondary progressive MS as these mice develop EAE with a relapsing remitting course followed by a chronic progressive course (34, 35). The generation of mice expressing transgenic T cell receptors (TCRs) specific for myelin antigens has produced a new toolset to study CNS autoimmunity as EAE occurs spontaneously in many of these models (36). In some

models, the incidence of spontaneous EAE occurs with varying frequency that is influenced by the microbial exposure in the environment in which the mice are housed (37). Recently, the presence of commensal bacteria has been suggested to influence the development of spontaneous EAE in MOG-specific TCR transgenic mice (38). The frequency of spontaneous EAE is significantly increased in many TCR transgenic models when regulatory T cells are eliminated from the TCR transgenic mice by crossing them to the RAG^{-/-} background (39, 40). While the majority of spontaneous EAE seen in TCR transgenic mice develop monophasic or chronic classic EAE, a TCR transgenic model on the SJL/J background develops spontaneous relapsing remitting EAE; these mice also exhibit variable clinical EAE symptoms during each relapse (41).

There are many similarities between the pathology of lesions seen in EAE and MS; however, an important difference between most EAE models and MS is the clinical manifestation of disease. Instead of the heterogeneous clinical presentation seen in MS, the clinical signs in most EAE models are typically manifested as ascending flaccid paralysis. This clinical presentation is referred to as “classic” EAE, and reflects the fact that inflammatory cells predominantly infiltrate the spinal cord with a relative lack of inflammation in the brain in these models. There are a few EAE models, however, in which mice develop parenchymal inflammation in the brain (with or without accompanying spinal cord inflammation) (42-44). Mice with brain inflammation exhibit clinical signs that include leaning, rolling, and ataxia, referred to as “atypical EAE”. Studies of both atypical and classic EAE models have identified some mechanisms that lead to inflammation in the brain versus the spinal cord, and may help illuminate mechanisms underlying the varying lesion localization patterns among patients with MS.

CD4⁺ T cell subsets and cytokines in EAE and MS

While both CD4⁺ and CD8⁺ T cells are known to contribute to the pathogenesis of EAE, the roles of CD4⁺ T cells have been studied more extensively. The pathogenic T cells mediating inflammation in EAE were originally thought to be Th1 cells, whose signature cytokine is IFN- γ . T cell clones that produce IFN- γ , and Th1 cells generated *in vitro* are able to induce EAE upon adoptive transfer (45, 46). Additionally, IFN- γ is secreted by CNS-infiltrating T cells in EAE (47, 48). Furthermore, a deficiency in T-bet, a transcription factor that is essential for expression of IFN- γ , was reported to confer resistance to EAE (49). Based on observations from EAE, it was suggested that MS is also mediated by Th1 cells, and this notion was supported by detection of IL-12 (a growth factor for Th1 cells) and IFN- γ in MS lesions (50). There are many activities of IFN- γ that could be pathogenic in the CNS. IFN- γ induces MHC class II expression and activates CNS resident microglia and macrophages. IFN- γ also induces production of certain chemokines such as CXCL9 and CXCL10 that are important for inflammatory cell recruitment (51). However, subsequent observations emerged that undermined the paradigm that IFN- γ is the major pathogenic cytokine in EAE, particularly the observations that IL-12^{-/-} (52, 53) and IFN- γ ^{-/-} mice (54) remain very susceptible to development of EAE.

The discovery that IL-23^{-/-} mice are very resistant to EAE led to the proposal that T cells that produce IL-17 (Th17 cells) may represent the pathogenic T cell subset in EAE, as IL-23 is a Th17-promoting cytokine (55). Subsequent studies lent considerable support to this idea (56). Th17 cells can be generated *in vitro* from naïve T cells using IL-6, TGF- β , and IL-23 to induce the transcription factor ROR γ t. IL-23 is critical for stabilization of IL-17 production and pathogenicity of Th17 cells in EAE (57). IL-17 production in the CNS induces production of

the neutrophil-recruiting chemokines CXCL1 and CXCL2, potentially by astrocytes (58). IL-17-producing cells have been detected in both EAE and MS lesions (59), and various studies have shown a reduced incidence, severity, and delayed onset of EAE in the absence of IL-17A or its receptors (60-63). As a result of such studies, trials in patients with MS are underway to investigate the therapeutic potential of IL-17 neutralization. Initial data from a clinical trial administering anti-IL-17A neutralizing antibody to patients with RR-MS reported reduced lesion activity and a trend towards reduced relapse rates (64). However, not all studies are consistent with an essential role for IL-17 in EAE. No major decrease in EAE susceptibility was observed when the activities of both IL-17A and IL-17F were neutralized (65).

A confounding factor in studies exploring the role of Th1 and Th17 cells in EAE is the plasticity observed in their cytokine phenotype. Th17 cells can co-express IL-17 and IFN- γ as well as their respective transcription factors ROR γ t and T-bet (66, 67). These dual-expressing T cells have been suggested to exhibit enhanced pathogenicity in EAE (67). There is also some evidence that Th1 cells can co-express IL-17 (68), and IL-17⁺IFN- γ ⁺ T cells have been identified in MS brains (69). A fate-mapping EAE study using IL-17A reporter mice showed that up to two-thirds of CNS-infiltrating T cells had at some point expressed IL-17A and converted to IFN- γ producers (70). These so-called “ex-Th17” cells down-regulated IL-17 and ROR γ t and up-regulated the IL-12 receptor in an IL-23-dependent manner.

Although neither IL-17 nor IFN- γ are required for the induction of EAE, another T cell cytokine, GM-CSF, appears to play a critical role in EAE development. GM-CSF^{-/-} mice are resistant to EAE (71), and GM-CSF production by T cells is required for their pathogenicity (72). Recently, two papers strengthened the evidence for the critical role of GM-CSF in EAE by demonstrating that GM-CSF production by both Th1 and Th17 cells was required for EAE

induction (73, 74). GM-CSF^{-/-} T cells were able to infiltrate the CNS initially, but these cells did not accumulate to levels seen in wildtype mice. T cell production of GM-CSF seems to be influenced by multiple cytokines. IL-23 and IL-1 β upregulated T cell production of GM-CSF, while IL-12, IL-27, and IFN- γ inhibited GM-CSF production. Although ROR γ t was implicated as an important transcription factor for GM-CSF expression, the presence of ROR γ t was not essential for GM-CSF production under certain conditions (particularly *in vitro*) (73, 74). GM-CSF could promote inflammation in the CNS in many ways. GM-CSF mobilizes Ly6C^{hi} monocytes from the bone marrow into the bloodstream, facilitating their infiltration into the CNS during EAE where they differentiate into inflammatory DCs and macrophage (28). GM-CSF is also required during EAE for generation of migratory CD103⁺ DCs in lymph nodes that strongly promote the differentiation of CD4⁺ T cells into effector T cell subsets (75). GM-CSF may also upregulate MHC class II and pro-inflammatory cytokine expression in microglia, macrophages, and dendritic cells (73, 74, 76). Elevated levels of GM-CSF are found in the CSF of patients with active MS (77), and clinical evaluation of the safety of a GM-CSF-neutralizing antibody in patients with MS is ongoing (see: <http://clinicaltrials.gov/show/NCT01517282>).

IL-17 and IFN- γ signaling influence localization of inflammation to the brain

Both IFN- γ and IL-17 appear to play important roles in differentially regulating inflammatory responses in the brain and spinal cord. Mice that are genetically deficient in IFN- γ or IFN- γ R exhibit a high incidence of atypical EAE (42, 78-80). This change in clinical manifestation reflects an increase in the localization of inflammatory cells in the brain, suggesting that IFN- γ may in fact inhibit inflammation in the brain during CNS autoimmunity. Interestingly, some studies have shown that IFN- γ signaling enhances spinal cord

inflammation, indicating that this cytokine may exert opposite effects in the brain compared to the spinal cord (78).

Our laboratory recently developed a model of EAE that exhibits both brain and spinal cord inflammation that does not involve ablation of IFN- γ signaling. Immunization of C3HeB/Fej mice with recombinant rodent MOG induces a high frequency of atypical EAE characterized by proprioception defects, rolling, and ataxia, as well as limp tail in some mice (44). Consistent with these clinical signs, extensive parenchymal brain inflammation is observed in addition to spinal cord inflammation. In contrast, only classic EAE is seen when the MHC congenic strain C3H.SW is immunized with MOG, suggesting that the T cells primed in C3HeB/Fej mice are responsible for inducing the distinct inflammatory pattern. The CD4⁺ T cells in C3HeB/Fej mice recognize two different epitopes of MOG, MOG₇₉₋₉₀ (I-E^k restricted) and MOG₉₇₋₁₁₄ (I-A^k restricted). Adoptive transfer of each population separately demonstrated that MOG₇₉₋₉₀-specific T cells induced inflammation localized predominantly in the spinal cord, while MOG₉₇₋₁₁₄-specific T cells preferentially induced inflammation in the brain (44). Interestingly, the polyclonal population of T cells primed by immunization with MOG that were specific for MOG₉₇₋₁₁₄ exhibited a significantly higher Th17:Th1 ratio than T cells that responded to MOG₇₉₋₉₀. The difference in Th17:Th1 ratio was observed both in the T cells that infiltrated the CNS during EAE and in the periphery before disease induction, suggesting that this might be an intrinsic property of these epitope-specific T cells.

To test the idea that the Th17:Th1 ratio of CNS infiltrating T cells influenced where inflammation localizes in the CNS, the Th17:Th1 ratio was altered prior to adoptive transfer by incubating the T cells in either IL-23 or IL-12. Incubating T cells of either specificity with IL-23 triggered inflammation in the brain and spinal cord, while incubation with IL-12 directed

inflammation toward the spinal cord rather than the brain (44). Interestingly, the Th17:Th1 ratio, rather than the absolute number of Th17 or Th1 antigen-specific T cells present in either CNS microenvironment, appeared to be the major determinant of whether inflammation occurred in the brain or was restricted to the spinal cord. Thus, brain inflammation would not occur even if there were high numbers of Th17 cells in the brain as long as Th1 cells predominated in the infiltrating population.

When a low Th17:Th1 ratio in the transferred population prevented induction of inflammation in the brain, immunochemical analyses showed that transferred T cells were confined primarily to the meninges in the brain of mice with classic EAE, while parenchymal infiltration was observed in the spinal cord (44). When the Th17:Th1 ratio was >1 , the T cells invaded the parenchyma in both the brain and spinal cord. Consistent with the hypothesis that the brain might be more susceptible to an inhibitory effect of IFN- γ signaling than the spinal cord, real time PCR data showed that the expression of IFN- γ Rb is fivefold higher in the brain than in the spinal cord. Together these data suggest that the Th17 cells, and possibly IL-17 itself, may overcome an inhibitory signal mediated by IFN- γ that dampens inflammatory responses in the brain.

Additionally, in the study described above, atypical disease induced by the transfer of Th17-skewed cells was converted to classical disease upon administration of a soluble IL-17 receptor-fusion construct that blocks IL-17 signaling (44). In support of the notion that IL-17 exerts a pro-inflammatory influence in the brain, Domingues et al. reported that mice develop atypical EAE symptoms after the transfer of Th17 cells but develop classic symptoms after transfer of Th1 cells (81). Further evidence of the specific role of IL-17 came from a study that showed that IFN- $\gamma^{-/-}$ T cells, which induce atypical disease when transferred into wildtype

hosts, instead induced classic disease when transferred into IL-17RA^{-/-} mice (79). Collectively, these studies support the idea that IL-17 signaling promotes inflammation in the brain but is not required for inflammation in the spinal cord.

T cell trafficking to the CNS

T cells can enter the CNS via several different routes of migration. One route, thought to be particularly important for the initial T cells infiltrating the CNS is from the blood via the choroid plexus into the CSF, via the blood-CSF barrier. This migration route requires T cells to cross the tight junctions of the choroid plexus epithelial layer in order to enter the subarachnoid space (82). T cells can also enter the subarachnoid space through meningeal vessels, which have a less complex structure than the postcapillary venules of the parenchyma (83). Once in the subarachnoid space, T cells may encounter antigen-presenting cells (84). If T cells are sufficiently reactivated in the subarachnoid space, they may be able to enter the parenchyma via the glia limitans. The other major route of migration is from the blood directly into the perivascular space of the CNS parenchyma, which requires crossing the blood brain barrier (1). Encounter with APCs in this perivascular space then allows entry into the parenchyma. The BBB has more stringent requirements for immune cell extravasation, and is a less efficient entry point for T cells. Thus, it has been suggested that a “first wave” of T cells initially enters the subarachnoid space via the blood-CSF barrier in the brain (82). Upon reactivation of the first wave of CNS-antigen-specific T cells within the subarachnoid space, various processes occur that lead to the upregulation of chemokines and adhesion molecules on endothelial cells and the permeabilization of the blood brain barrier, facilitating rapid recruitment and entry of the “second wave” of T cells and other immune cells from the periphery (3). These cells may

enter through the BBB directly into the parenchymal perivascular space of the brain or spinal cord.

The adoptive transfer model has been an important tool for studying T cell migration to the CNS. Upon adoptive transfer into host animals, CNS antigen-specific T cells have been shown to first home to the peripheral lymphoid organs and upregulate certain chemokine receptors (85). This results in a latency period before T cells are observed in the CNS, and T cells begin to enter the CNS in detectable numbers approximately 3 days post-transfer. All activated T cells are capable of entering the CNS, regardless of antigen specificity (86). However, if no antigen-specific signal is provided by MHC class II-expressing cells to the T cells in the CNS, the T cells do not persist (87). After the first wave of CNS-antigen-specific T cells is reactivated in the subarachnoid space and the blood brain barrier is permeabilized, non-CNS-antigen specific T cells and naïve T cells are able to enter the CNS readily (87).

T cell extravasation into the CNS is mediated by various chemokines and adhesion molecules. T cells in the blood are induced to slow their velocity and “roll” along vessel walls via T cell PSGL-1 interactions with P- and E-selectins on the endothelial cells. High affinity interactions mediated by T cell integrins VLA-4 and LFA-1 and their ligands VCAM-1 and ICAM-1 on the endothelium induce adhesion, which is facilitated by interactions with chemokines displayed on the luminal side of the vasculature near sites of inflammation (3). Chemokine/chemokine receptor interactions activate T cell integrins and promote extravasation via tight junctions across the endothelium into the inflamed tissue (82). TNF receptor 1 signaling is required for entry of MOG-specific T cells into the CNS parenchyma by inducing VCAM-1 expression on astrocytes, which form the glia limitans barrier between the perivascular space and the parenchyma (88).

Both human and mouse Th1 and Th17 cells are known to express different chemokine receptors. The receptors CXCR3 and CCR5 are preferentially expressed on Th1 cells, while CCR6, the receptor for CCL20, is preferentially (though not exclusively) expressed on Th17 cells (82). Adoptive transfer of wildtype and CCR6^{-/-} MOG-specific Th17 cells into wildtype recipients showed that the initial migration of T cells into the CNS was CCR6-dependent. However, CCR6^{-/-} T cells were able to infiltrate the CNS after inflammation was already established (89). This study also demonstrated strong expression of CCL20 by epithelial cells of the choroid plexus in both healthy and EAE mice, and CD45⁺ cells were observed to accumulate at the choroid plexus barrier in the absence of CCR6 expression. These results suggested that CCR6⁺ Th17 cells may be required for initial T cell infiltration, which occurs via the choroid plexus in the brain.

Additionally, two studies showed that the α 4 integrin (a subunit of VLA-4) was more highly expressed on Th1 compared to Th17 cells (90, 91). The studies suggested that α 4 integrin was required for infiltration of the spinal cord by both Th1 and Th17 cells, but not for infiltration of the brain by Th17 cells. Both Th1 and Th17 cells were found to express CD11a (α L integrin, a subunit of LFA-1) (90). LFA-1 may be used by Th17 cells to access the CSF and periventricular compartments in the brain via the choroid plexus in a VLA-4-independent manner.

After CNS-antigen-specific T cell reactivation occurs, many additional molecules are induced that promote recruitment and entry of other immune cells into the parenchyma. Pro-inflammatory cytokines including IL-1 β , IL-6, TNF- α , IL-12, IL-17, IFN- γ , and GM-CSF are expressed that drive ongoing inflammation. CCL2, CCL4, and CXCL10 recruit monocytes and macrophages (92). CXCL1, CXCL2, and MMP12 recruit neutrophils. Matrix

metalloproteinases (MMPs) such as MMP3, MMP8, and MMP9, are enzymes that can degrade the extracellular matrix and may facilitate cellular entry into the parenchyma (82). The high numbers of inflammatory cells infiltrating the CNS mediate destruction of the myelin sheath and oligodendrocyte death.

B cells

B cells are adaptive immune cells that play many different roles in immune responses, including antibody secretion and antigen presentation. They have been shown to be important in several autoimmune diseases including multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus. B cells develop in the bone marrow, rearranging their variable immunoglobulin (Ig) genes and emerging into the blood as immature IgM⁺ B cells. The maturation of naïve B cells into IgM⁺ IgD⁺ cells occurs in the periphery, where B cells enter follicles of secondary lymphoid organs. Upon encounter with antigen and T cell help in the follicles, B cells undergo germinal center reactions, which lead to clonal expansion, somatic hypermutation of V_H genes, class switch recombination at the IgH locus, and affinity maturation to select for increased affinity of a B cell receptor (BCR). Activated B cells then differentiate into memory B cells or antibody-secreting plasmablasts and plasma cells. Plasmablasts are the precursors of short-lived and long-lived plasma cells. Short-lived plasma cells develop from B cells that have not undergone affinity maturation, whereas long-lived plasma cells develop from germinal center B cells that have undergone affinity maturation, leading to secretion of high affinity antibodies (93). In addition to antibody secretion, B cells are critical for the initiation of T cell immune responses (94), particularly in response to protein antigens (95). B cells internalize antigen through the BCR, then process and present the antigen via MHC molecules to antigen-specific T cells. B cells can also present antigens that are

nonspecifically endocytosed through fluid phase pinocytosis, although this method is much less efficient (96). B cells secrete many cytokines, and they are a major producer of lymphotoxin, which is critical for normal development and organization of lymphoid tissues (97).

B cells in Multiple Sclerosis

B cells have long been known to play a role in multiple sclerosis as antibody-secreting cells. Early studies demonstrated increased intrathecal production of immunoglobulins (Ig) in the CSF of most MS patients (98), and the identification of clonotypic B cells in MS lesions suggests an ongoing humoral immune response within the CNS (99). Although the antigenic targets of the intrathecal antibody response have not been unequivocally defined, evidence from MS patients and various animal studies indicates that myelin proteins including MOG and MBP are potential targets (100). Anti-MOG antibodies are found in MS lesions and have been shown to induce demyelination and exacerbate EAE (101-103). The presence of Ig in the CSF of MS patients correlates with more severe disease activity (104, 105). In patients with MS, particularly those patients whose lesion pattern was defined by antibody deposition and B cell enrichment, plasma exchange was found to be beneficial in reducing neurological disability (106).

More recently, an antibody-independent role for B cells has been uncovered in MS. Clinical trials with Rituximab, or anti-CD20, demonstrated that selective B cell depletion in patients with RR-MS resulted in significant reduction in new focal inflammatory lesions and clinical relapses (107, 108). CD20 depletion does not eliminate plasma cells, and no reduction in antibodies was seen 6 months after B cell depletion in MS patients. This suggests that B cells play a pathogenic cellular role in MS in addition to autoantibody production. B cells are commonly found at increased levels in the CSF of MS patients (109), and a higher proportion

of B cells relative to monocytes in the CSF correlates with more rapid disease progression (110). In Rituximab-treated RR-MS patients, numbers of T cells in the CSF were reduced after treatment (111). Additionally, in samples taken from patients with RR-MS after Rituximab treatment, CD4⁺ and CD8⁺ T cells showed reduced levels of proliferation and cytokine production upon stimulation, relative to samples from the same patients before treatment (112).

B cells in EAE

The role of B cells in EAE has been more controversial, and appears to be highly dependent on the mouse strain and myelin antigen used in each study. On the B10.PL background, immunization with MBP peptide or adoptive transfer of MBP-specific T cells resulted in a similar incidence and severity of EAE in both wildtype and B cell deficient (μ MT) mice, but the B cell deficient mice failed to spontaneously recover (113, 114). In μ MTB10.Q^{-/-} mice on a C57BL/10 background, the incidence and severity of EAE induced by immunization with rat MOG protein was decreased compared to wildtype mice (115). On the C57BL/6 background, immunization with rat MOG protein or MOG₃₅₋₅₅ peptide resulted in a similar incidence of EAE in both wildtype and μ MT mice; however, μ MT mice were resistant to EAE induced by immunization with human MOG protein (116-118). While rat and mouse MOG proteins are identical, human MOG differs from rat MOG at several sites, and the functional difference in the encephalitogenic epitope MOG₃₅₋₅₅ is believed to be at position 42, where human MOG has a proline instead of a serine (118). The human MOG₃₅₋₅₅ epitope was shown to be less encephalitogenic, leading to the hypothesis that B cells might be more important for EAE induction when the encephalitogenic epitope is weak (118). The transfer of either activated MOG-specific B cells or serum from MOG-primed wildtype mice restored the ability of μ MT mice to develop EAE induced by immunization with human MOG protein (119).

Many recent studies have also shown variable incidence and severity of EAE in anti-CD20 B cell depleted mice. In SJL/J mice, B cell depletion suppressed spontaneous relapsing-remitting EAE that is characterized by activation of MOG-specific T and B cells (41). In contrast, in B10.PL MBP-TCR transgenic mice, B cell depletion accelerated development of spontaneous EAE (120). In C57Bl/6 mice, B cell depletion before or after onset of mouse MOG protein-induced EAE resulted in reduced severity (121). In these same mice, B cell depletion before or after onset led to exacerbated mouse MOG₃₅₋₅₅ peptide-induced EAE (121). In contrast, another study in which EAE was induced in C57Bl/6 mice by immunization with MOG₃₅₋₅₅ peptide found dramatically different results when B cells were depleted either before or after disease induction. Depletion of B cells before immunization significantly worsened disease severity, while depletion after onset of EAE significantly ameliorated disease severity (122). Although a previous study had identified an IL-10 producing regulatory B cell subset in EAE (123), this B cell depletion study helped to clarify the opposing roles that B cells can play at different stages in disease pathogenesis. The regulatory B cells appear to play a greater role during T cell priming, while the pathogenic B cells play a greater role in ongoing EAE (122). The protection provided by the regulatory B cell subset has been attributed both to the immunosuppressive production of IL-10, as well as the maintenance of Tregs via expression of glucocorticoid-induced TNFR ligand (120, 124). The antibody-independent pathogenic functions of B cells are not well defined, but may include antigen presentation, cytokine production, or other support of T cell functions.

Support for a pathogenic role of B cells in EAE has emerged from the combination of two genetically-engineered mouse models. Mice expressing a transgenic TCR specific for MOG in C57BL/6 mice develop a very low incidence of spontaneous EAE (although 47%

develop spontaneous optic neuritis) (40), and C57BL/6 mice in which a heavy chain from a MOG-specific antibody was knocked into the Ig locus do not develop clinical disease. When these mice were bred together, however, the incidence of spontaneous EAE significantly increased. B cells were shown to enhance MOG-specific T cell activation in this model (125, 126). Interestingly, inflammation primarily targeted the optic nerve and spinal cord in these mice, suggesting that this may serve as a model of optospinal MS. In a separate model of spontaneous relapsing-remitting EAE (RR-EAE) in SJL/J mice expressing a transgenic MOG-specific TCR, relapses alternated between targeting cerebellum, brainstem, spinal cord, and optic nerve tissue (41). In these RR-EAE mice, B cells that secreted anti-MOG antibodies were expanded from the endogenous repertoire, with evidence of ongoing germinal center reactions in the cervical lymph nodes (38, 41). B cell depletion suppressed RR-EAE in these mice, providing support for the pathogenic role of B cells.

B cells as antigen presenting cells

B cells have been shown to influence T cell responses in MS and EAE in various studies and have been suggested to act as antigen presenting cells. In a human study, naïve and memory B cells were isolated from the peripheral blood of RR-MS patients and co-cultured with autologous T cells from the same patients. Both naïve and memory B cells from RR-MS patients were able to induce CD4⁺ T cell proliferation and IFN- γ production in response to MOG at higher levels than naïve or memory B cells from healthy donors (127). In one CD20-mediated B cell depletion study in EAE, CD4⁺ T cells were isolated from the lymph nodes of depleted or control mice after onset of EAE and incubated with MOG peptide and splenic B cells from control mice with EAE. CD4⁺ T cells from B cell-depleted mice were found to proliferate less than those from control mice (122). Similar results were also found in a separate

study in which anti-CD20 depletion prevented EAE induction; after immunization with MOG protein, T cells from depleted mice showed decreased proliferation and IL-17 production (128). A third study in which anti-CD20 B cell depletion was utilized in EAE found that immunization with mouse MOG protein, but not MOG₃₅₋₅₅ peptide, activated B cells and allowed them to activate T cells in response to MOG *in vitro* (121). Collectively, these studies demonstrate that B cells have the potential to act as antigen presenting cells, but it has not been conclusively determined that this function is required in MS or EAE.

B cells as cytokine producing cells

B cells can also influence T cell activity by secreting various pro- and anti-inflammatory cytokines. As mentioned above, regulatory B cells secrete IL-10 to limit inflammation. However, B cells from MS patients were found to secrete significantly less IL-10 than healthy donors (129). B cells have been shown to produce lymphotoxin, IL-6, TNF- α , IL-12, IFN- γ , IL-2, IL-4, and GM-CSF (130, 131). B cells can make different subsets of cytokines depending on the local environment. B cells were found to make either Th1- or Th2-promoting cytokines when cultured with those cell types (130). Additionally, the activation status of B cells and the method of stimulation influence their cytokine production. B cells activated through the BCR combined with CD40 ligation express higher levels of TNF- α , lymphotoxin, and IL-6 compared with B cells stimulated by CD40 ligation alone (132). An additional “third signal” stimulus via TLR engagement induced abnormally high levels of TNF α and lymphotoxin in B cells from MS patients compared to healthy donors (112). Activated, but not naïve B cells can also make some IL-1 β and IL-23 (133, 134).

B cell cytokine production has been shown to promote T cell function. IL-12 production by B cells stimulates IFN- γ production by Th1 cells (134, 135). In CD4⁺ and CD8⁺ T cell

cultures from MS patients, proliferation and cytokine production was enhanced when activated B cell supernatants were added. This effect was reversed with neutralization of lymphotoxin and TNF α , confirming that these B cell cytokines promoted T cell activation (112). Another study in mice with EAE found that *in vitro*-stimulated B cells from the spleen produced high levels of IL-6 (136). This study created mice with a B cell-specific IL-6 deficiency using bone marrow chimeras, and found that these mice had a similar incidence and onset of EAE but significantly reduced severity. In mice lacking B cell IL-6 production, there was a reduced production of IL-17 in spleen T cells, suggesting that IL-6 production by B cells promotes Th17 responses. Additionally, the study found that *in vitro*-stimulated B cells from MS patients secreted elevated levels of IL-6; however, after Rituximab treatment, the B cells that repopulated these patients secreted normal IL-6 levels (136). While the B cell IL-6 production influenced EAE severity later in disease, other cytokines produced by B cells may have effects at different stages of disease.

Tertiary Lymphoid Organs

Tertiary lymphoid organs (TLOs), or ectopic lymphoid follicles (eLFs), are highly organized structures resembling secondary lymphoid organs that form in tissues during chronic inflammation. TLOs contain B cell follicles and T cell areas, lymphatic vessel and high endothelial venules (HEVs), and lymphoid chemokines, and may serve as sites for T and B cell activation, driving chronic inflammation (137). The formation of TLOs begins with clusters of activated B and T cells in the inflamed tissue. These cells express lymphotoxin, which could drive lymphoid chemokine synthesis and structural organization into follicles with T cell zones. Ectopic follicle structures are seen in autoimmune diseases such as rheumatoid arthritis, chronic inflammatory diseases such as ulcerative colitis, and certain chronic infectious diseases

(137). Approximately 30-40% of patients with secondary progressive MS have been shown to have ectopic follicles in the cerebral leptomeninges, and the presence of these follicles is linked to more severe disease (138). Ectopic follicles have also been found in several EAE models. In one model, B cell aggregates were detected in both the meninges and parenchyma of the cerebellum and spinal cord as early as 15 days after disease onset (139). These B cell aggregates frequently developed into TLOs in the chronic phase of disease, based on strict guidelines for characterization of TLOs including B and T cell compartmentalization, HEVs, and one additional characteristic feature, such as follicular dendritic cells, reticulin fibers, or plasma cells. This study also confirmed that T cells are proliferating within the TLOs, suggesting that these structures could be important for driving inflammation (139). In another EAE model, adoptive transfer of MOG-TCR transgenic Th17 cells induced the formation of ectopic follicle-like structures composed of B cell clusters surrounded by T cells and encapsulated by collagen fibers (140). Mice receiving Th1 cells did not develop these eLFs, and their formation was partially dependent on IL-17 and podoplanin expression by Th17 cells.

Remaining Questions

The general processes that occur during T cell-mediated inflammation in MS and EAE are described above. There are still many events that are not well understood, particularly the earliest events in the initiation of disease. These preclinical events are difficult to study due to the low cell numbers involved at early time points. However, improving our understanding of these early events might contribute to potential prevention of MS relapses in the future. Specifically, understanding the earliest reactivation events that take place when the first T cells infiltrate the CNS is crucial because these events initiate the inflammatory cascade. Additionally, we know that the Th17:Th1 ratio of these infiltrating cells determines whether

inflammation localizes to the brain, but it is not known whether this ratio can be influenced during reactivation by cells within the CNS. Finally, B cells have been shown to be important players in MS, but many EAE models do not reflect a strongly pathogenic role for B cells, preventing the investigation of this pathogenic role. Most studies have focused on the role of B cells after onset of EAE, and an earlier role during preclinical disease is not known; it is also not known whether B cells are present within the CNS before disease onset. These questions will be addressed by the work that follows.

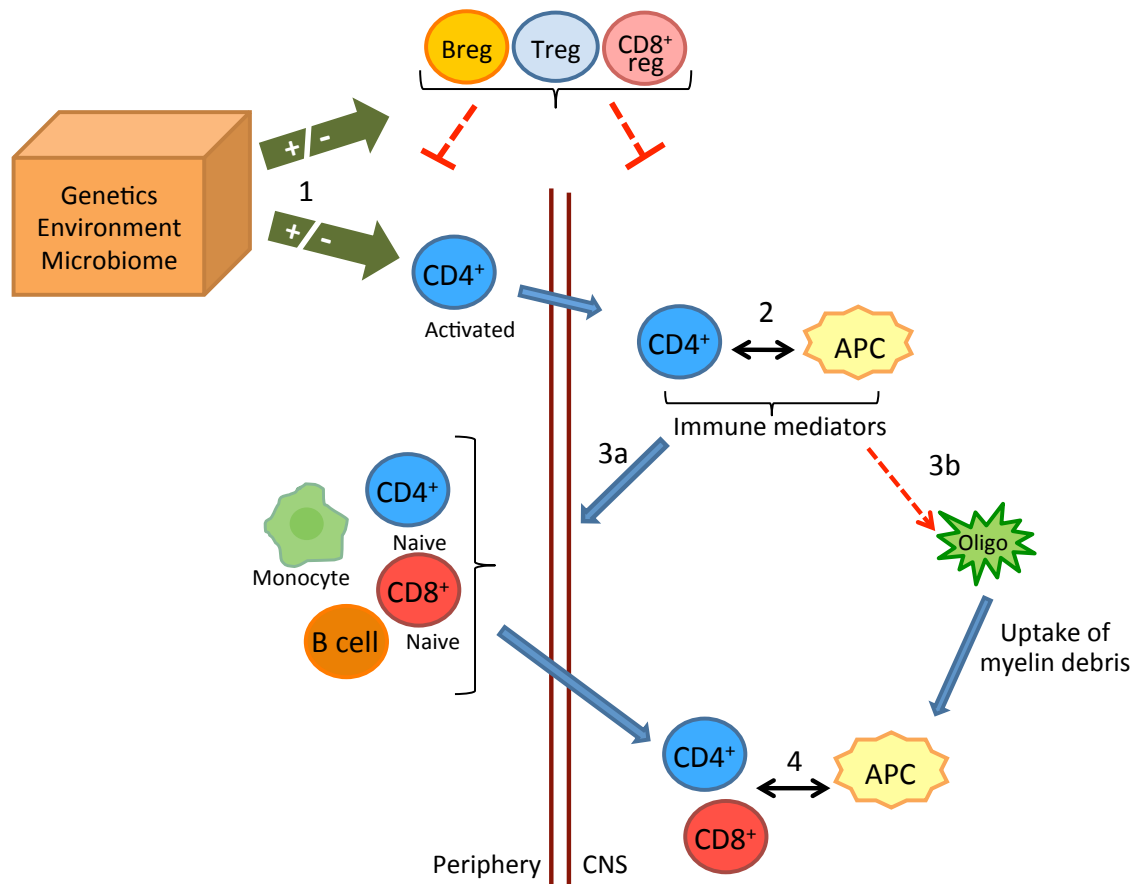


Figure 1.1. Model of CD4⁺ T cell-initiated CNS autoimmunity

The sequential steps proposed for the pathogenesis of CD4⁺ T cell-initiated disease are indicated by numbers. CD8⁺ T cell initiated autoimmunity may occur, but is not included in this schematic. (1) Genetic and environmental factors both promote myelin-specific CD4⁺ T cell activation and influence the type and efficacy of the corresponding immunoregulatory response mediated by regulatory (reg) CD4⁺ and CD8⁺ T cells and B cells. (2) Activated CD4⁺ T cells enter the CNS and are re-activated by resident APCs, triggering production of inflammatory mediators. (3) These mediators promote (a) localized inflammation of the blood brain barrier (BBB) that facilitates recruitment of naïve CD4⁺ and CD8⁺ T cells, B cells, and monocytes to the CNS, and (b) may directly damage myelin and/or oligodendrocytes. (4) Determinant spreading occurs as APCs presenting epitopes derived from myelin debris activate newly recruited T cells with different myelin specificities. Dashed lines indicate pathways not yet verified with experimental evidence.

Chapter 2: Characterizing the phenotypes of B cells within the central nervous system

Introduction

B cells are known to be important effector cells in both MS and EAE, and may have several functions including antibody secretion, antigen presentation, and cytokine production. B cells, plasmablasts, and plasma cells have all been identified in the CNS of MS patients (141). These cell types can be differentiated on the basis of their CD19 and CD138 (Syndecan-1) expression. B cells are CD19⁺ CD138⁻, plasmablasts are CD19⁺ CD138⁺, and plasma cells are CD19⁻ CD138⁺ (142). B cells also express certain molecules that are downregulated upon differentiation into plasmablasts such as MHC class II and CD79b, or Ig β , a co-receptor for the BCR.

The CSF of MS patients was found to contain approximately 5% CD19⁺ cells on average; half were CD138⁻ B cells and half were CD138⁺ plasmablasts (143). The majority of these B cells were found to be memory B cells based on CD27 expression, a marker that is only found on human B cells. Mouse B cells do not have any single reliable marker of the memory phenotype. The chemokines CXCL10, CXCL12, CXCL13, which are all upregulated in MS lesions, are thought to regulate B cell traffic to the inflamed CNS (144, 145). Levels of CXCL13 in the CSF of MS patients were found to correlate with numbers of B cells, plasmablasts, and plasma cells (145). CXCL13 is also associated with the development of ectopic lymphoid follicles (142).

In the normal CNS, B cells are thought to be present in low numbers, but not enough to have a significant effect on any immune processes that may be occurring (146). B cells have

been shown to migrate across the healthy BBB and may do so more readily than T cells (147). An important B cell survival factor, BAFF, is constitutively expressed by astrocytes in the healthy CNS (148). CXCL12 is also constitutively displayed on blood vessels in the normal brain (145). Whether B cells in the CNS are functional before inflammation has been initiated has not been studied. To investigate whether B cells play a role in the preclinical stage of disease, we first characterized the phenotypes of B lineage cells in the CNS of naïve mice.

Results

Characterization of the MHC class II⁺ populations in the healthy CNS

One important function that B cells could perform during the effector stage of EAE is to serve as antigen presenting cells (APCs) to the CD4⁺ T cells that infiltrate the CNS. APCs are required within the CNS because previously activated T cells must see myelin antigen on resident antigen presenting cells in order to be reactivated; an important consequence of this reactivation is the secretion of immune mediators. To determine the extent to which B cells comprise the MHC class II⁺ APC population in the CNS before inflammation occurs, we analyzed the monocyte population from well-perfused, naive C3HeB/Fej mice by flow cytometry. Surprisingly, we found that the majority of cells in the MHC class II⁺ population in both the brain and spinal cord were CD19⁺ B cells (Figure 2.1). In fact, in the naïve spinal cord, over 90% of the Class II⁺ cells were B cells. The remaining Class II⁺ cells in the brain and spinal cord mostly consisted of F4/80⁺CD11c⁺ cells (Figure 2.1C). The predominance of B cells suggests that they could be contributing to antigen presentation in the non-inflamed CNS. Therefore, CNS B cells may influence T cells entering the CNS during the initial phase of EAE induction.

To confirm that the CD19⁺ cells we identified were unquestionably B cells, we examined the MHC class II⁺ populations in the brain and spinal cord of naïve C3HeB/Fej μ MT mice, which lack B cells. The brain and spinal cord of μ MT mice contained very small populations of MHC class II⁺ cells (Figure 2.2A), and none of these cells were CD19⁺ B cells (Figure 2.2B). We also tested the efficiency of our perfusions, to determine whether contamination from the blood could explain the surprising number of B cells we found in the CNS of naïve wildtype mice. We injected naïve wildtype mice with CFSE-labeled splenocytes and isolated monocytes from the brains of injected mice that were either perfused or not perfused. In the CD19⁺ B cell gate, we found that half of the cells in the unperfused brains were CFSE⁺ cells from the blood, while the CD19⁺ cells from perfused brains were all CFSE⁻ (Figure 2.2C). This confirmed that our perfusions efficiently removed all blood contaminating cells from the CNS, indicating that the B cells we identified in the CNS were tissue-resident cells.

Comparison of MHC class II⁺ populations in the CNS of healthy or EAE mice

We next compared the MHC class II⁺ populations in the CNS of healthy mice and mice with EAE. As expected, there was a large increase in the MHC class II⁺ population during EAE (Figure 2.3A). This increase was due to the infiltration of a large number of F4/80⁺CD11c⁺ monocytes and dendritic cells from the periphery, a critical inflammatory process during EAE onset (Figure 2.3B and 2.3C). As a result of this infiltration, the relative proportion of CD19⁺ B cells in the MHC class II⁺ population decreased in EAE, suggesting that B cells are no longer the predominant antigen-presenting cells. However, the total number of B cells increased in both the brain and spinal cord during EAE (Figure 2.4), indicating that they could still play an important role during disease progression. We observed similar cell numbers in the CNS of

mice with EAE that was induced either by immunization with MOG or by adoptive transfer of MOG-specific T cells (data not shown). We also examined the activation status of B cells in the CNS of naïve and EAE mice by staining for CD80 and CD86, co-stimulatory molecules that are typically upregulated upon activation. CNS B cells from both naïve and EAE mice expressed very low levels of CD80 and CD86, although there was a slight increase in expression during EAE (Figure 2.5). In contrast, B cells found in the CSF of MS patients tend to express high levels of CD80 and CD86 (142).

Identification of a plasmablast-like population in the CNS of healthy mice

Because our data indicate that CD19⁺ cells comprise the majority of MHC class II⁺ cells in the naïve CNS, we examined their phenotype in greater detail. By analyzing CD19 expression among CD45⁺ cells, we detected two distinct populations of CD19⁺ cells in the brain and spinal cord that were differentiated by levels of CD45 and CD19 expression (Figure 2.6A). The CD45^{hi}CD19^{hi} cells displayed a typical B cell phenotype of MHC class II⁺ (I-A^k), CD79b⁺ (Igβ), and CD138⁻ (Syndecan-1). The CD45^{int}CD19^{int} cells displayed a plasmablast-like phenotype of I-A^{k lo/-}, CD79b⁻, and CD138⁺. The CD45^{hi}CD19^{hi} B cells were found to have high expression of surface IgM, while the majority CD45^{int}CD19^{int} plasmablasts had low surface IgM expression (Figure 2.6B). To confirm that this population, which has not been described previously, was not restricted to the C3H mouse strain, we also examined the brain monocyte populations of naïve B10.PL mice. We found that the majority of the MHC class II⁺ cells in the naïve B10.PL brain, as in the C3H brain, were CD19⁺ B cells (Figure 2.7A). We also found that the B10.PL brain contained similar proportions of the two CD19⁺ populations, and that the plasmablast-like population was present (Figure 2.7B).

To further understand this plasmablast population, we compared the CD138⁺ population in the healthy CNS with the corresponding population in the periphery, as well as the CD138⁺ population in mice with EAE. The CD138⁺ cells in the healthy CNS had a CD45^{int} phenotype, while those in the spleen and blood had a CD45^{hi} phenotype (Figure 2.8A). Unlike the plasmablasts in the healthy CNS, the CD138⁺ cells in the spleen and blood expressed MHC class II and CD79b (data not shown). Additionally, the plasmablasts in the spleen expressed higher levels of CD86 than those in the healthy CNS (Figure 2.9A). These differences between plasmablasts found in the CNS compared with plasmablasts in the periphery suggest that they are distinct populations, and that the population in the CNS may be generated locally.

In the CNS of mice with EAE, the CD138⁺ population was also found to be distinct from the healthy CNS population. The CNS CD138⁺ population in EAE had a CD45^{hi} phenotype (Figure 2.8A), and was CD19⁻ and CD86⁺ (Figure 2.9B), a phenotype characteristic of plasma cells. We observed a loss of CD45^{int}CD19^{int} cells in the CNS of mice with EAE (Figure 2.8B). It is possible that these CNS plasmablasts differentiate into plasma cells during EAE, which would explain the change in phenotype of the CD138⁺ population.

To determine whether the plasmablasts that we detected in the healthy CNS were functional, we tested their ability to secrete antibodies in an ELISPOT assay. We plated CNS and spleen cells from healthy mice or mice with EAE in ELISPOT wells coated with anti-IgG. We were able to detect some IgG producing cells in the healthy CNS, and higher numbers in the CNS of mice with EAE (Figure 2.10). However, the frequency of cells that secreted antibody was very low, suggesting that the plasmablasts may not be highly active in the naïve CNS.

Analysis of cytokine production by B lineage cells within the CNS

In addition to antigen presentation, B cells may also contribute to the inflammatory milieu by producing cytokines. To investigate this possibility, we sorted B cells, plasmablasts, CD45^{hi}CD11b/c⁺ cells, and microglia from the CNS of naïve and EAE mice, and measured cytokine mRNA levels directly *ex vivo* (without stimulation). B cells were pooled from the CNS of multiple mice, yielding detectable levels of mRNA for several cytokines. IL-10, GM-CSF, IL-6, and IL-1 β transcripts were not detected in B cells sorted from the CNS of either naïve or EAE mice (data not shown). Interestingly, IL-12 p35 was expressed by CNS B cells from both naïve and EAE mice at significantly higher levels than by CD11b/c⁺ cells (Figure 2.11A). Naïve CNS plasmablasts also expressed IL-12 p35. TNF α mRNA expression was also detected in CNS B cells from both naïve and EAE mice, although more TNF α was produced by microglia than B cells during EAE (Figure 2.11B). In naïve mice, CNS B cells constitutively expressed significantly higher levels of IL-12 p35 and TNF α than B cells in the blood, indicating that the functional phenotype of CNS B cells differs from B cells in the periphery. These data suggest that cytokine production by both CNS B cells and plasmablasts could affect T cells as they infiltrate the CNS.

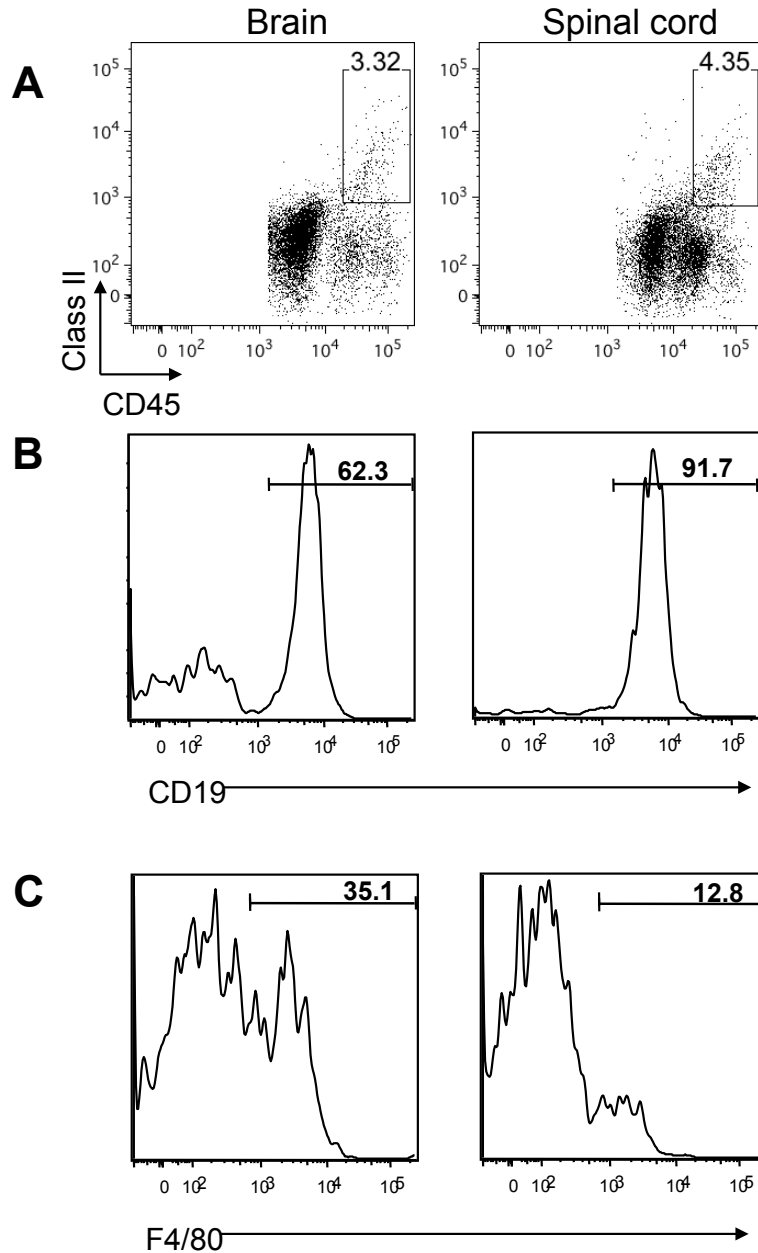


Figure 2.1. B cells are the predominant MHC class II⁺ cells in the healthy CNS

Brain and spinal cord mononuclear cells were isolated from naïve C3HeB/Fej mice. Well-perfused CNS tissue was pooled from 3-6 mice per sample for detection of small populations. (A) Representative staining of CNS mononuclear cells gated on CD45⁺ cells showing the MHC class II⁺ (I-A^{k+}) gate from the brain or spinal cord of naïve mice. Lower panels show representative histograms of (B) CD19 and (C) F4/80 through the MHC class II⁺ gate. Similar results were obtained in at least 4 independent experiments.

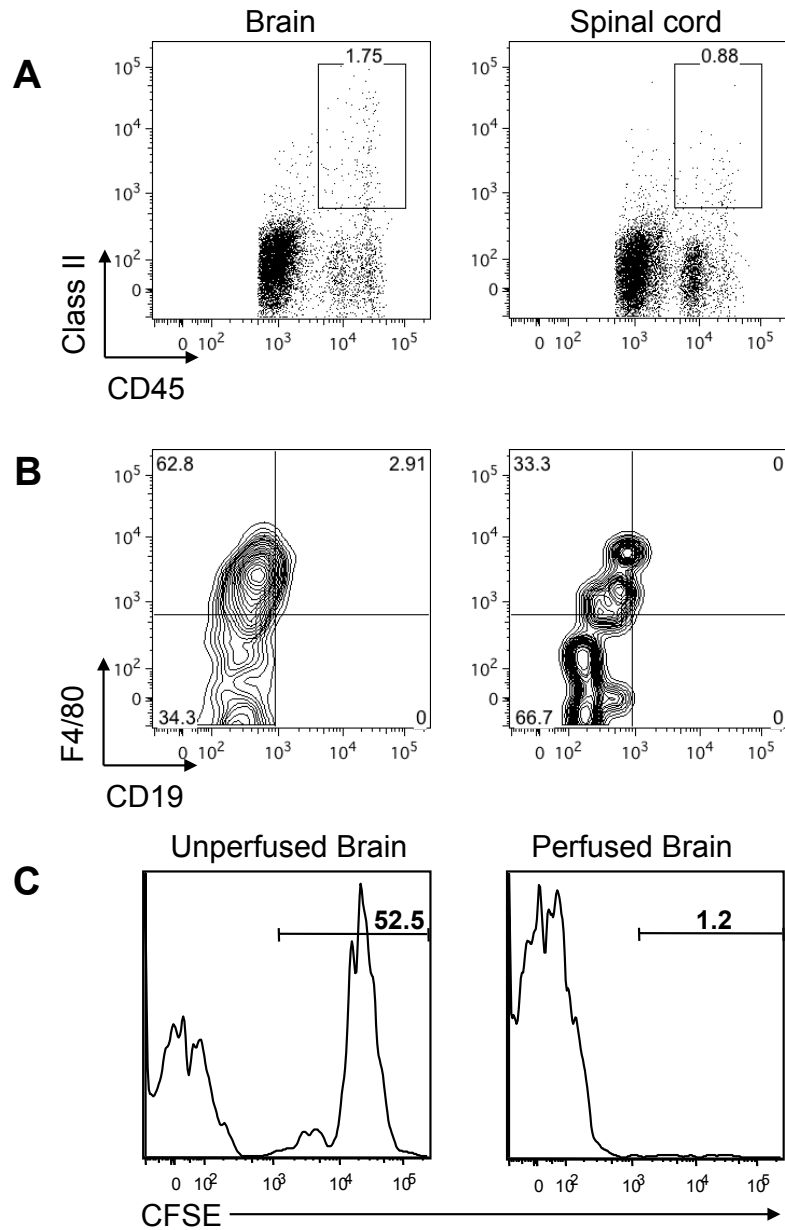


Figure 2.2. Controls show that the CNS B cell population is legitimate

(A) Brain and spinal cord mononuclear cells were isolated from naïve C3HeB/Fej μ MT mice. Tissue was pooled from 3-6 mice per sample for detection of small populations. Representative staining of CNS mononuclear cells gated on CD45⁺ cells showing the MHC class II⁺ gate from the brain or spinal cord. (B) CD19 and F4/80 staining in the brain and spinal cord of μ MT mice is shown through the MHC class II⁺ gate. (C) CNS monocytes were isolated from unperfused or perfused wildtype mice after injecting CFSE-labeled splenocytes. Data is gated on CD45⁺Class II⁺CD19⁺ B cells. In the perfused brain, less than 2% of the B cells are CFSE labeled, which is at the level of background seen in mice without any CFSE labeled cells injected, confirming that our perfusion method successfully removes all circulating blood lymphocytes from the CNS. Representative of at least two independent experiments.

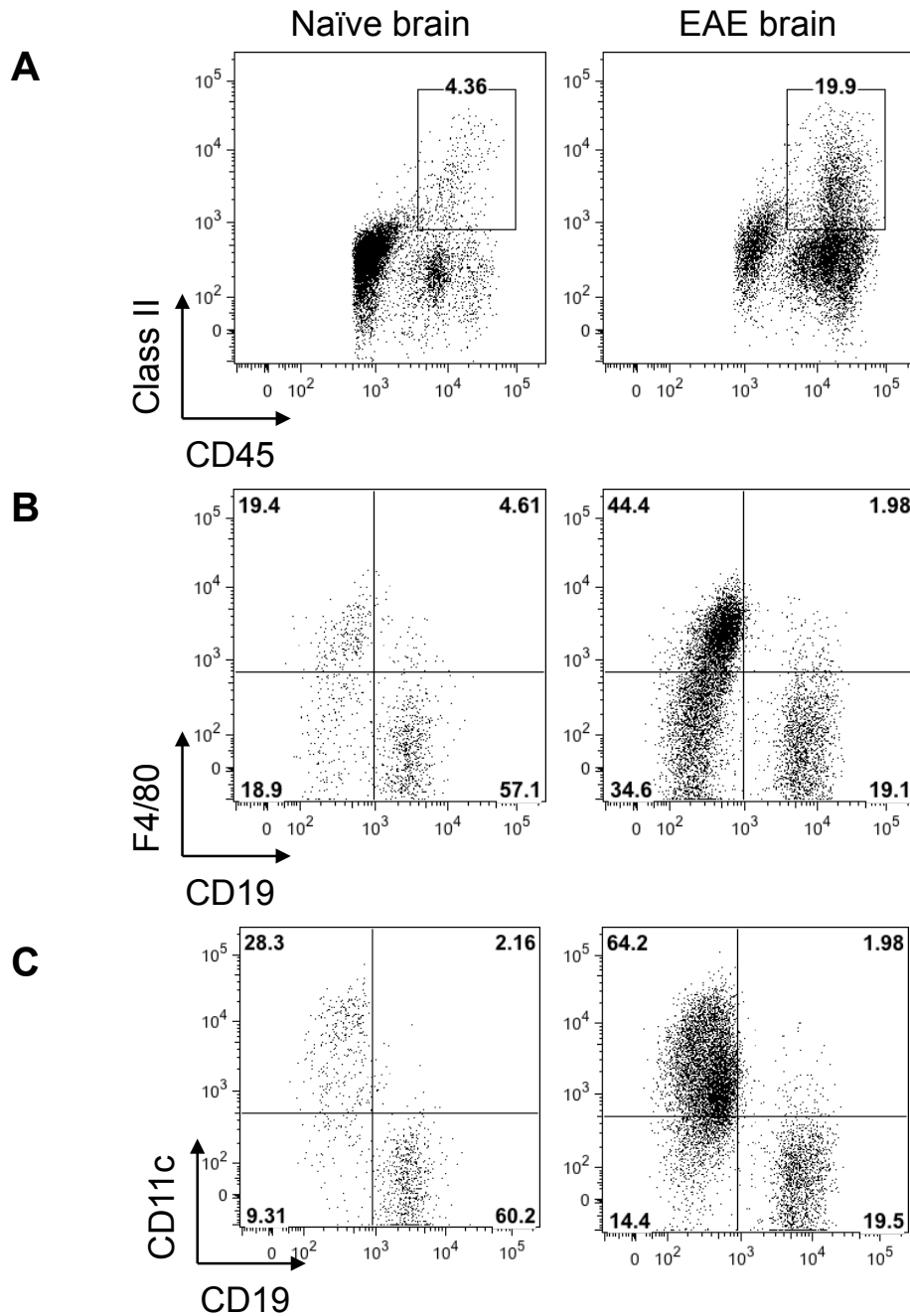


Figure 2.3. The MHC class II⁺ population changes during EAE

Brain and spinal cord mononuclear cells were isolated from either naïve C3HeB/Fej mice or mice with EAE. Well-perfused CNS tissue was pooled from 3-6 mice per sample for detection of small populations. **(A)** Representative staining of CNS mononuclear cells gated on CD45⁺ cells showing the MHC class II⁺ (IAk⁺) gate (top panel) from the brain of naïve or EAE mice. Lower panels show **(B)** CD19 and F4/80, or **(C)** CD19 and CD11c staining through the MHC class II⁺ gate. Similar results were obtained in at least three independent experiments.

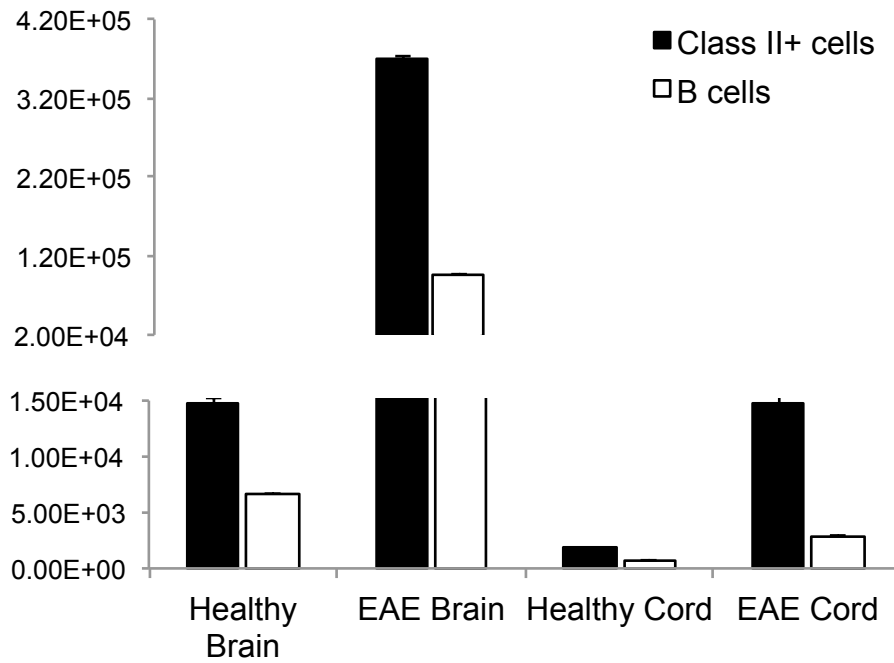


Figure 2.4. Total MHC class II⁺ and B cell numbers in the healthy and EAE CNS

Quantification of the numbers of MHC class II⁺ cells and B cells in the brain and spinal cord of naïve mice or mice with EAE shown in Figures 2.1 and 2.3. Representative of three experiments.

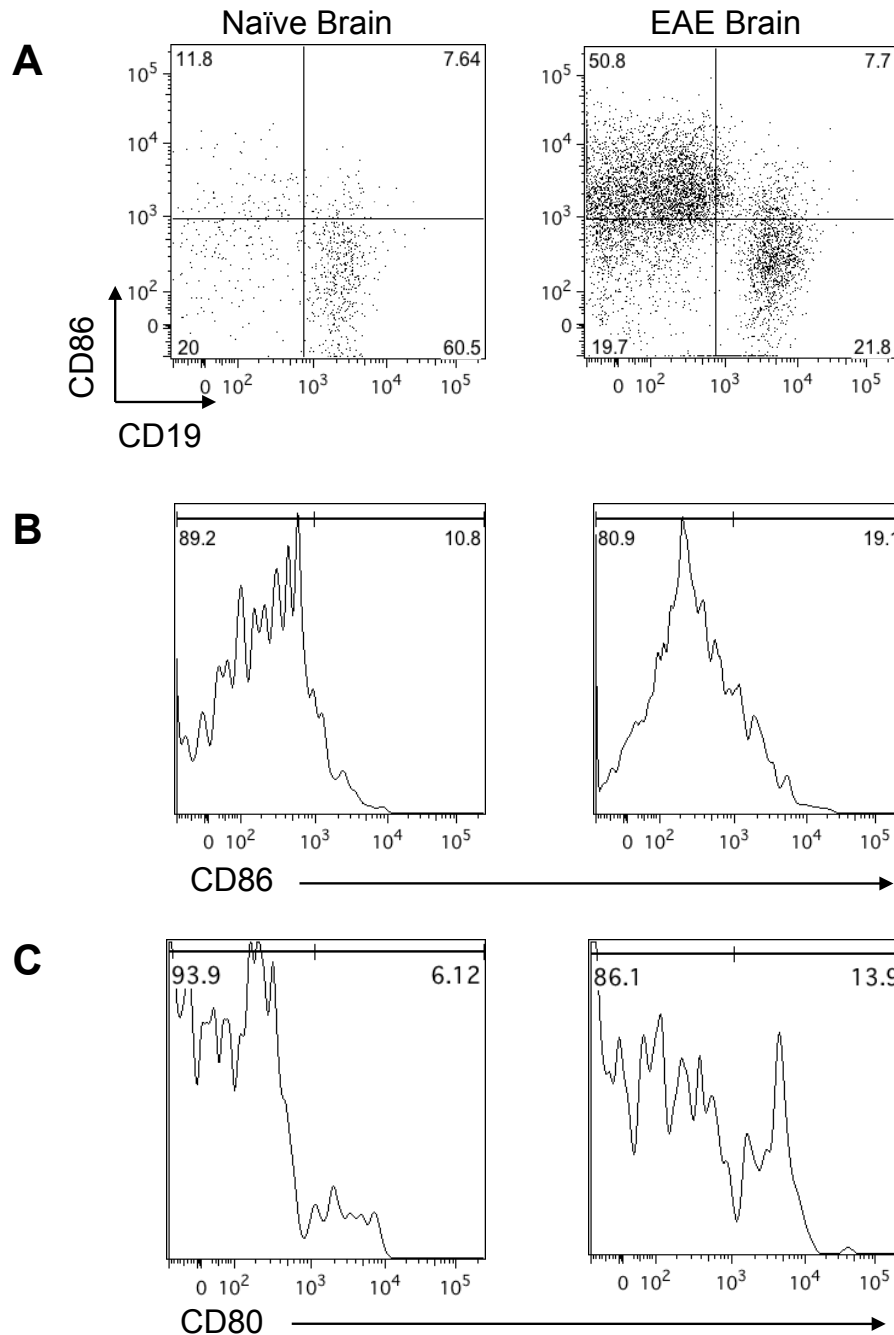


Figure 2.5. B cells in the CNS are not activated

Brain mononuclear cells were isolated from well-perfused naïve or EAE mice. **(A)** Representative staining of CNS mononuclear cells gated on CD45⁺Class II⁺ cells showing CD19 and CD86 staining from naïve or EAE brains. Lower panels show **(B)** CD86 or **(C)** CD80 staining through the CD19⁺ B cell gate from naïve or EAE brains. Representative of at least two experiments.

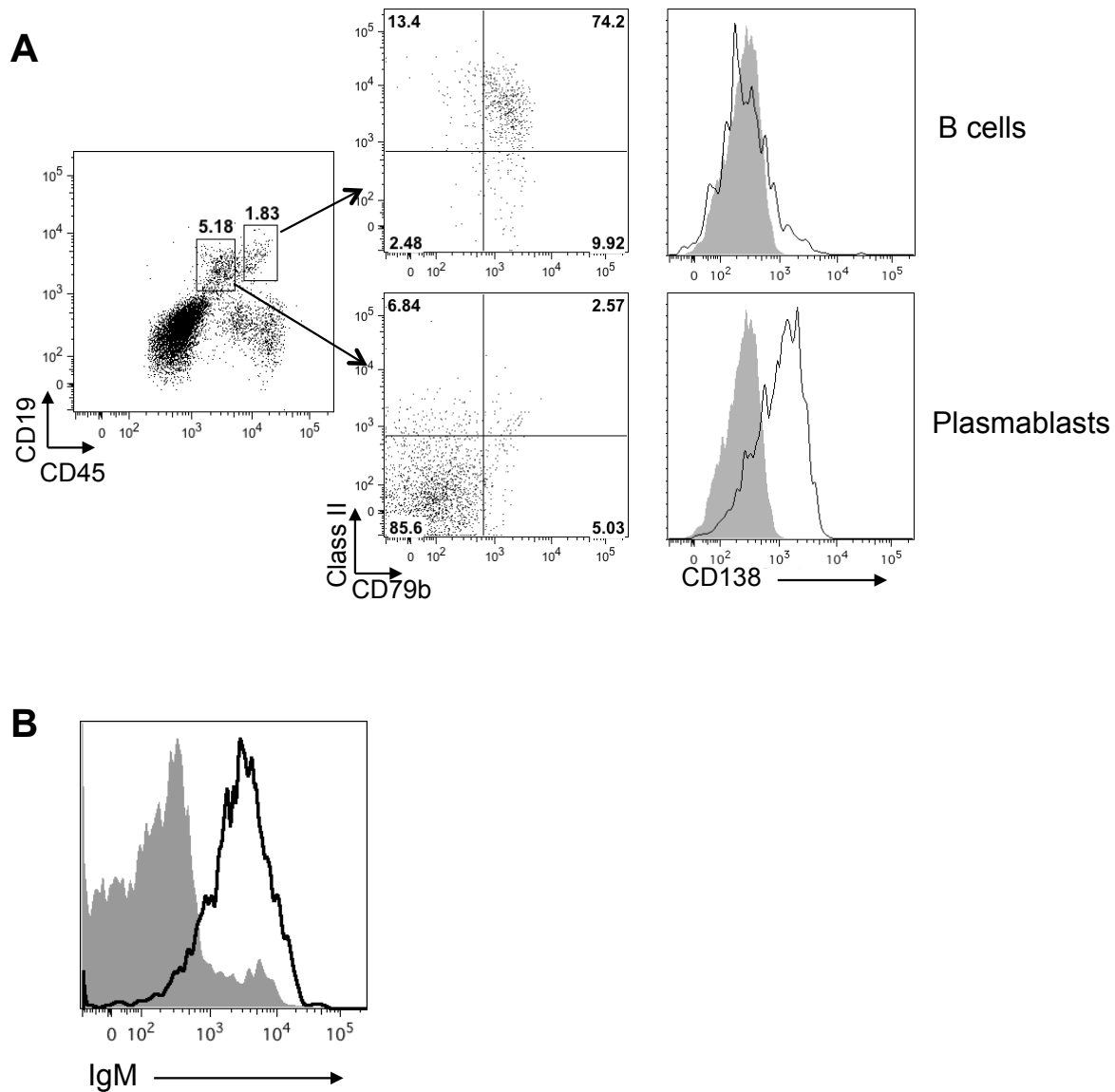


Figure 2.6. Two $CD19^+$ populations identified in the healthy CNS

CNS mononuclear cells were isolated from well-perfused, naïve C3HeB/Fej mice, and tissues were pooled from 3-6 mice. **(A)** Representative staining of CNS cells gated on $CD45^+$ showing two distinct $CD45^+CD19^+$ populations in the brain of naïve mice. Cells in the $CD45^{hi}CD19^{hi}$ gate are Class II⁺, $CD79b^+$ and $CD138^-$ (top middle and right panels) while cells in the $CD45^{int}CD19^{int}$ gate are Class II⁻, $CD79b^-$, and $CD138^+$ (bottom middle and right panels). Similar results were obtained in at least 5 independent experiments. **(B)** Representative histogram showing IgM staining through the $CD19^{hi}$ B cell gate (black line) or the $CD19^{int}$ plasmablast gate (grey).

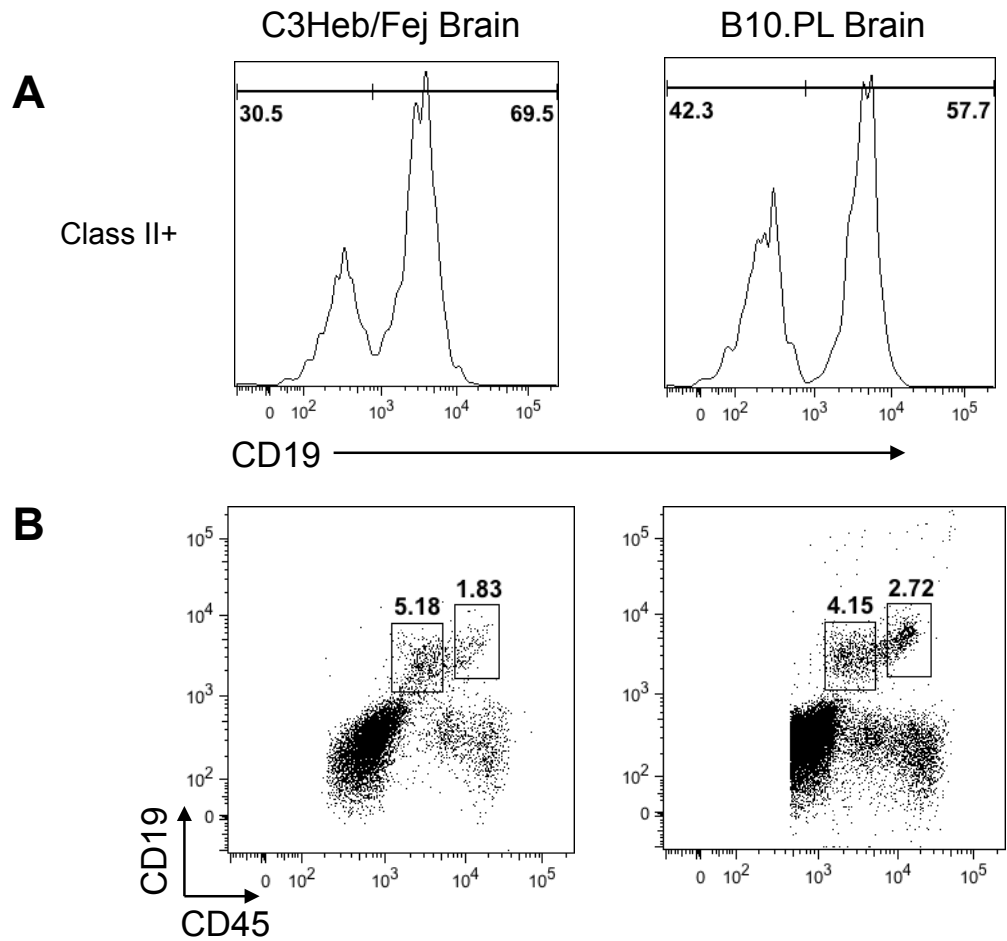


Figure 2.7. CD19⁺ phenotypes are similar in the CNS of C3H and B10.PL mice

Mononuclear cells were isolated from the brains of well-perfused, naïve C3HeB/Fej or B10.PL mice. **(A)** Representative staining through the MHC class II⁺ gate showing the percentage CD19⁺ in the naïve brain. **(B)** CD45⁺CD19⁺ populations are shown through the CD45⁺ gate. Representative of three experiments.

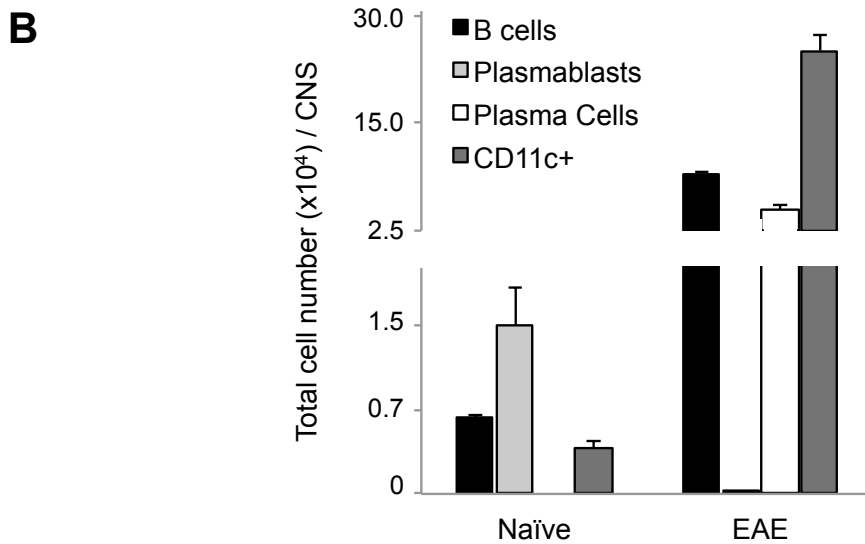
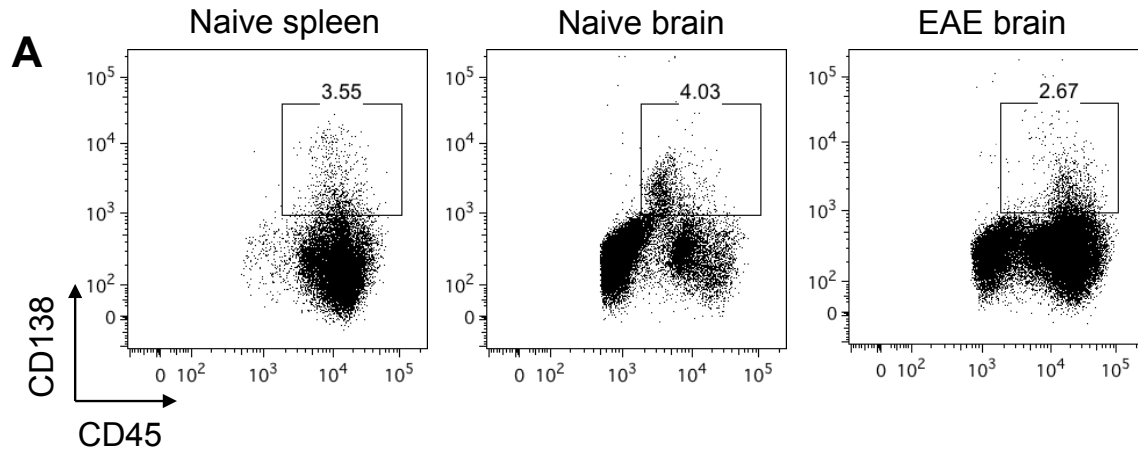


Figure 2.8. CD138 populations and quantification of B lineage cells in the CNS

Brain and spinal cord mononuclear cells were isolated from well-perfused naïve C3HeB/Fej mice or mice with EAE. Splenocytes were isolated from naïve mice. **(A)** Representative staining of splenocytes and CNS mononuclear cells gated on CD45⁺ cells showing the CD138⁺ populations. **(B)** Total numbers (means, SEM) of CD45^{hi}CD19^{hi} B cells, CD45^{int}CD19^{int} plasmablasts, CD45^{int}CD19⁻ plasma cells, and CD45^{hi}CD11c⁺ cells in CNS of naïve and EAE mice. Similar numbers were detected in EAE mice induced both actively and passively, from at least 3 independent experiments.

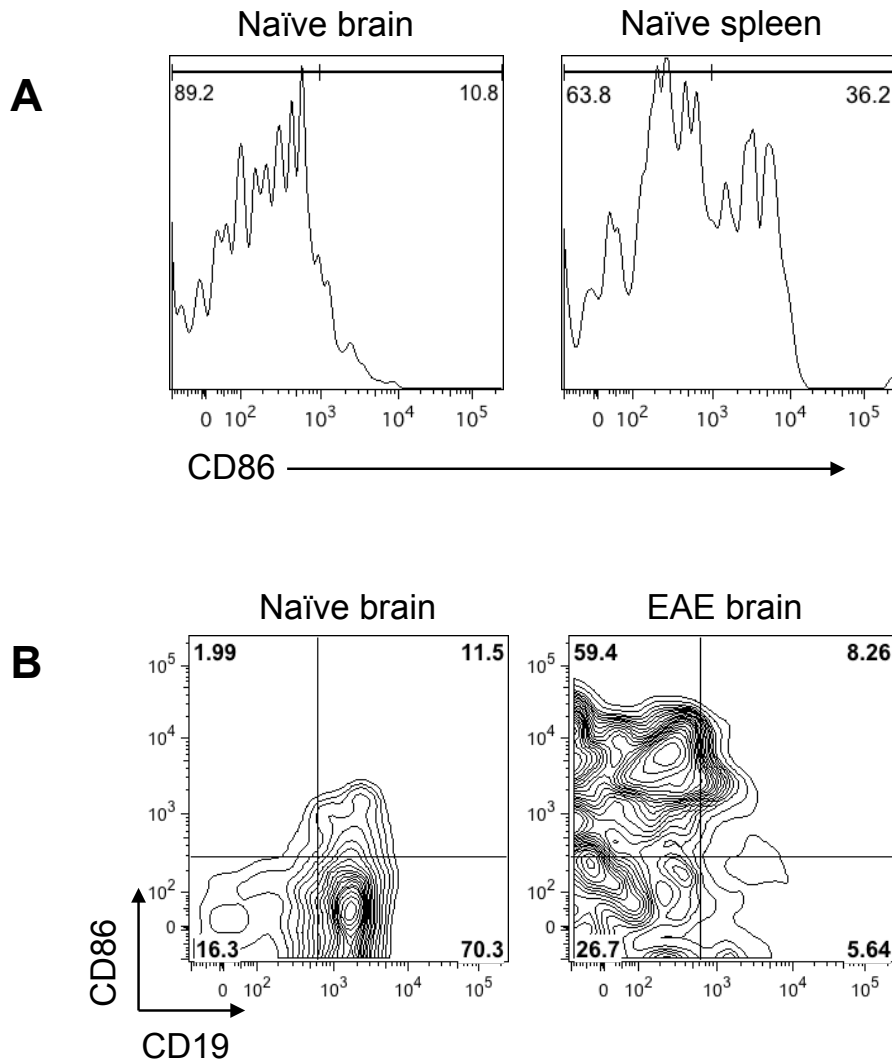


Figure 2.9. Activation status of CD138⁺ populations in the CNS and spleen

Brain and spinal cord mononuclear cells were isolated from well-perfused naïve C3HeB/Fej mice or mice with EAE. Splenocytes were isolated from naïve mice. **(A)** Representative staining of splenocytes and CNS mononuclear cells gated on CD45⁺CD138⁺ cells showing the CD86 expression. **(B)** Representative staining of CNS mononuclear cells from brains of naïve or EAE mice gated on CD45⁺ cells showing CD19 and CD86 expression. Representative of at least two independent experiments.

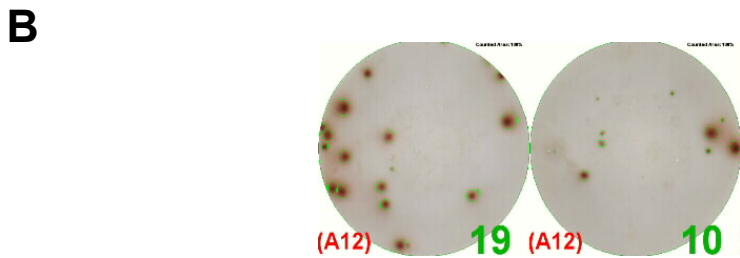
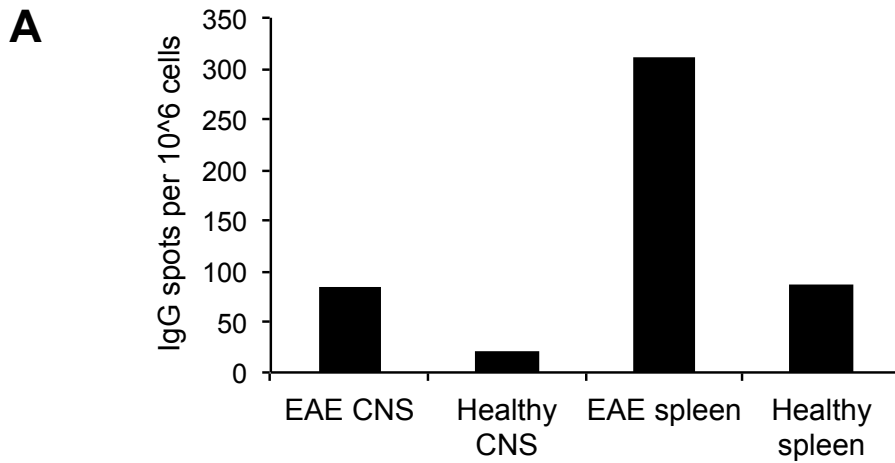


Figure 2.10. IgG antibody secretion by CNS cells from healthy or EAE mice

CNS mononuclear cells and splenocytes were isolated from well-perfused naïve mice or mice with EAE. Cells were plated in ELISPOT wells for detection of total IgG secretion with anti-IgG antibodies. **(A)** Total numbers of IgG spots per million cells is shown for each sample. **(B)** Representative ELISPOT well images are shown for cells from the healthy CNS. Representative of two independent experiments.

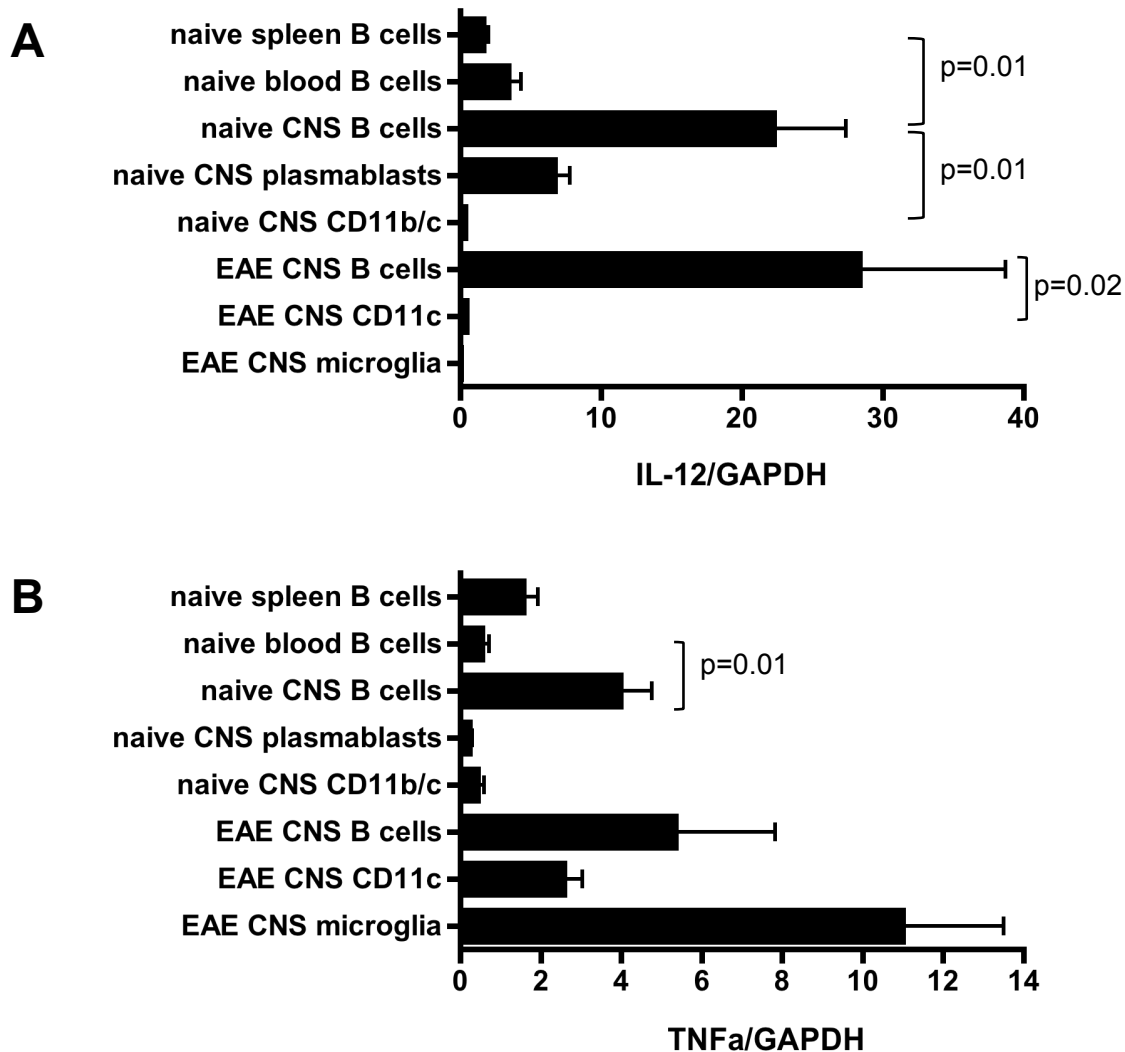


Figure 2.11. Cytokine production by B cells from the CNS of healthy and EAE mice

CNS mononuclear cells were isolated from well-perfused naïve mice or mice with passive EAE. B cells, plasmablasts, CD11b/c⁺ cells, and microglia were FACS-sorted. For each sample, 100,000 cells were isolated from CNS tissue pooled from 3-5 mice. Spleen and blood B cells were also purified from naïve mice. Cells were frozen immediately after sorting for mRNA isolation. Relative abundance of IL-12p35 or TNFα is shown. Relative transcript values are normalized to GAPDH transcript levels as quantified by real time PCR analysis. (n=3-4 samples per group, Student's *t* test.)

Chapter 3: B cells promote preclinical recruitment of T cells to the CNS during initiation of EAE and influence the Th17:Th1 ratio

Introduction

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system (CNS) that presents with a heterogeneous disease course and pathology. A widely used animal model of MS, experimental autoimmune encephalomyelitis (EAE) exhibits many similarities to MS and is induced by activation of T cells specific for myelin proteins, either by immunization with myelin antigens or by adoptive transfer of myelin-specific CD4⁺ T cells. The activated CD4⁺ T cells traffic to the CNS where they must be reactivated by local antigen presenting cells in order to initiate an inflammatory cascade resulting in demyelination and axonal loss. Both myelin-specific Th1 and Th17 cells can induce EAE and the different effector functions of these T cell subsets results in recruitment of distinct types of inflammatory cells that mediate different forms of tissue damage (58). We recently described a unique EAE model in C3HeB/Fej mice in which inflammation targets the brain as well as the spinal cord (44). However, inflammation in the brain occurred only when the antigen-specific Th17 cells outnumbered the Th1 cells in the infiltrating population, while spinal cord inflammation was not regulated by the Th17:Th1 ratio.

In addition to the different types of effector T cells, the potential contribution of B cells to the pathogenesis of MS has received increasing attention, in part because of the demonstration of clinical efficacy of B cell depletion with rituximab in decreasing the number of lesions and relapses in MS patients (107). A role for B cells was first suggested in the

production of antibodies that bind myelin components, mediating inflammation and demyelination. More recently, both pathogenic and regulatory roles for B cells have been demonstrated in EAE, which is reflected in the highly variable incidence and disease course of EAE seen in different strains of mice on the μ MT background (113, 115, 117, 149). In a double-transgenic model with both MOG-specific T cells and B cells, there was an increase in activated, cytokine-producing T cells compared to the TCR-transgenic alone, suggesting that B cells may function as antigen presenting cells in the activation of pathogenic T cells (125, 126). Another study demonstrated the disparate roles of B cells in EAE by depleting B cells with CD20 mAb at various times during disease induction; depletion of an IL-10-secreting regulatory B cell subset before induction exacerbated disease while depletion of pathogenic B cells after EAE onset decreased the severity of disease (122). The pathogenicity of B cells during disease progression could result from direct antigen presentation to T cells, or indirect support of T cell activity through co-stimulation or secretion of immunomodulatory cytokines that can influence the local inflammatory milieu. In MS, rituximab-treated patients had reduced numbers of T cells in the cerebrospinal fluid (111) as well as reduced numbers of cytokine-producing and proliferating T cells among PBMCs restimulated *ex vivo* (112), suggesting a pathogenic role for B cells in modulating T cell responses. However, the nature of this pathogenic role is not well understood, particularly in the earliest phase of T cell entry into the CNS.

Results

B cell-deficient mice have a significantly reduced incidence of EAE

To investigate the role of B cells in the neuropathogenesis of EAE models with distinct clinical manifestations, we compared rMOG-induced EAE in μ MT C3HeB/Fej and μ MT

C3H.SW mice with wildtype controls. Our previous studies of rMOG-induced EAE in these MHC congenic mice found that C3HeB/Fej (H-2^k) mice develop a severe, rapidly progressing form of atypical EAE characterized by proprioception defects, spasticity, and hyperreflexivity, with parenchymal brain and spinal cord inflammation. In contrast, C3H.SW (H-2^b) mice develop classic, chronic EAE characterized by tail and hind limb paralysis with parenchymal inflammation only in the spinal cord (44). In the active induction model, upon immunization with rat rMOG protein, the B cell deficient mice on both backgrounds had a significantly reduced incidence of EAE (Table 3.1), indicating a role for B cells in the pathogenesis of both atypical and classic EAE. The μ MT C3HeB/Fej and C3H.SW mice that did develop EAE had a similar day of onset, clinical course, severity, and clinical signs compared to the wildtype controls. Immunohistochemical analyses of CNS tissue did not reveal significant differences in the inflammatory index between wildtype and μ MT mice (Figure 3.1). This suggests that B cells may be more important for the initiation of EAE, and play a lesser role in ongoing inflammation in C3H mice.

To contribute to initiation of EAE, B cells could play a role in the initial steps of the pathogenesis of EAE, either by influencing T cell priming in the periphery and/or T cell reactivation within the CNS. In order to assess the role of B cells during Th1 and Th17 priming, both C3HeB/Fej wildtype and μ MT mice were immunized with recombinant MOG in CFA (with pertussis toxin). Splenocytes were restimulated after seven days *ex vivo* with rMOG, MOG₇₉₋₉₀, or MOG₉₇₋₁₁₄, and numbers of antigen-specific IL-17 and IFN- γ producing cells were determined by ELISPOT. Compared to wildtype mice, significantly fewer T cells from μ MT mice produced IL-17 in response to both MOG₇₉₋₉₀ and MOG₉₇₋₁₁₄, and significantly fewer T cells produced IFN- γ in response to MOG₉₇₋₁₁₄ (Figure 3.2). These data indicate that

the presence of B cells facilitates T cell priming into effector cells *in vivo*. B cells could affect priming by acting as antigen presenting cells, or by maintaining normal lymphoid architecture. Alternatively, the low incidence of EAE in μ MT mice could reflect a role for B cells during the effector stage of disease. To investigate the role of B cells during the effector stage (and bypass the priming phase), we induced passive EAE in C3HeB/Fej WT and μ MT mice by adoptive transfer of T cells isolated from rMOG-immunized WT mice and re-stimulated *in vitro* with MOG₉₇₋₁₁₄. In these experiments, the ratio of Th17:Th1 cells in the transferred population was approximately 1:1. As seen in actively induced EAE, the incidence of EAE was significantly reduced in μ MT compared to wildtype recipients (Table 3.1), indicating that B cells play an important role in the effector stage of disease independent of their role during T cell priming.

Kinetics of T cell entry into the CNS

In the adoptive transfer model, a delay between the transfer of CNS antigen-specific T cells and detection of these cells in the CNS has been previously observed (85). In order to determine the kinetics of entry of donor T cells into the CNS in our adoptive transfer EAE model, we transferred genetically marked (Thy1.1⁺) cells from MOG-immunized donors into wildtype recipients and isolated CNS cells at various timepoints post-transfer. We were able to detect donor CD4⁺ T cells in significant numbers beginning on day 4 post-transfer. There was a slight increase in donor T cell number by day 5, followed by a rapid increase by day 7, the average day of EAE onset (Figures 3.3A and 3.4A). We also compared the brain and spinal cord cells separately on day 5 post-transfer to determine whether there was differential preclinical migration. We found that while the CD4⁺ population in the brain was approximately 40-50% Thy1.1⁺ donor cells, the CD4⁺ population in the spinal cord was only 10% donor cells

(Figure 3.3B). These data suggested that the donor cells initially migrate to the brain, likely via the choroid plexus, and are later recruited to the spinal cord.

B cells promote the preclinical increase in CNS T cell number during EAE induction

Because B cells comprise the majority of the MHC class II⁺ cells in the naive CNS, we hypothesized that they may influence the earliest events in EAE induction that occur directly after T cells infiltrate the CNS. To test this, we transferred activated T cells isolated from rMOG-immunized mice into wildtype and μ MT recipients and compared the numbers of donor T cells in the CNS on day 4 (pre-clinical) and day 7 (average day of EAE onset in wildtype mice). On day 4 post-transfer, comparable numbers of donor T cells were found in the CNS of both wildtype and μ MT recipients, indicating that B cells are not required for the initial migration of T cells into the CNS (Figure 3.4A and 3.4B). There was no detectable increase in host T cell number in the CNS of wildtype and μ MT mice on day 4 compared to naïve mice (Figure 3.4C). In the periphery, approximately 1 million donor T cells were found in the spleen on day 4 post-transfer in both wildtype and μ MT recipients (Figure 3.4D). By day 7 post-transfer, the donor T cell number in the CNS had increased one hundred-fold relative to day 4 in wildtype recipients at onset of EAE (Figure 3.4A), and the donor cells made up the vast majority of T cells in the wildtype CNS at this timepoint (Figure 3.4B). In contrast, donor T cell numbers decreased on day 7 in the CNS of μ MT recipients (which have not developed EAE) relative to day 4 (Figure 3.4A). There was also no significant increase in the number of host T cells in the CNS of μ MT recipients on day 7 (Figure 3.4C). These data indicate that initial donor T cell infiltration to the CNS is not impaired in μ MT mice. However, in the absence of B cells, infiltrating T cells fail to initiate events that lead to an increase in T cell number and ultimately to EAE.

Adoptively-transferred T cells are thought to visit peripheral lymphoid organs to upregulate migration-related markers and become competent to enter the CNS (85). We considered the possibility that in the absence of B cells, this process may not occur normally, and that the donor T cells we observed in the CNS of μ MT mice on day 4 post-transfer were adhering to the inner surface of the CNS blood vessels but had not crossed the blood brain barrier. To test whether the donor T cells had crossed the blood brain barrier, we used a CD4 antibody protection assay that has been previously described (150). This assay takes advantage of the antibody-exclusion function of the blood brain barrier, so that any cells on the CNS side of the blood brain barrier will not be available for binding of serum antibody. To perform the assay, on day 4 post-transfer of Thy1.1⁺ CD4⁺ donor cells into wildtype and μ MT recipients, we injected an unlabeled anti-CD4 antibody (clone GK1.5) i.v., perfused the mice after an hour and isolated the CNS mononuclear cells, then analyzed the expression of CD4 on the donor cells using two non-competing labeled anti-CD4 antibodies (GK1.5 and RM4-4). The RM4-4 antibody was used to identify all CD4⁺ cells within the CNS, and Thy1.1 identified the donor population. Donor cells that were attached to the inner vessel wall and available for unlabeled GK1.5 binding were blocked from staining by the labeled GK1.5 antibody (Figure 3.5A). Donor cells that had crossed the blood brain barrier were protected from unlabeled GK1.5, and stained positive for labeled GK1.5. In the CNS of both wildtype and μ MT recipients, the majority of the donor cells were found to be GK1.5⁺, indicated that they had crossed the blood brain barrier and were protected from the injected antibody (Figure 3.5B). In the blood, as expected, none of the cells were protected, and a smaller percentage of cells in the spleen were protected from antibody binding. These data confirm that the donor T cells that we observed in

the CNS of both wildtype and B cell deficient recipients on day 4 post-transfer were competent to enter the CNS and had crossed the blood brain barrier.

Identifying mechanisms responsible for the preclinical increase in CNS T cell number

The increase in T cell number in the CNS during the preclinical stage of EAE is usually attributed to a combination of proliferation of the initial infiltrating T cells and recruitment of a second wave of T cells from the periphery. Both events may be influenced by the reactivation of the initial wave of T cells by CNS antigen presenting cells. To determine if proliferation of T cells that initially infiltrate the CNS is impaired in μ MT mice, we examined the levels of BrdU incorporation in the donor Thy1.1⁺ CD4⁺ T cell population in the CNS of both wildtype and μ MT recipients between day 4 and 5 post transfer. BrdU incorporation in donor T cells within the CNS was comparable between WT and μ MT recipients during this time, indicating that B cells do not influence proliferation of the initial infiltrating T cells (Figure 3.6A). The percent of BrdU⁺ T cells in both wildtype and μ MT recipients was slightly but not significantly increased in the CNS compared to blood and spleen. Thus, most of the donor T cell proliferation observed in the periphery and CNS likely reflects the *in vitro* stimulation prior to T cell transfer rather than encounter with antigen *in vivo*. The similar extent of BrdU incorporation in donor T cells in the CNS of WT and μ MT recipients between days 4 and 5 post transfer suggests that proliferation does not account for the increase in donor T cells in the CNS of WT mice observed at day 7 post-transfer. Other studies have reported T cell proliferation within the CNS that is comparable to the levels seen in our model (151, 152). We also investigated whether B cells influenced donor CD4⁺ T cell survival by analyzing AnnexinV⁺ donor T cells in the CNS of WT and μ MT recipients on day 5 post-transfer. The

percent of AnnexinV⁺ donor T cells was similar in WT and μ MT recipients, indicating that T cell survival in the CNS is not significantly enhanced by the presence of B cells (Figure 3.6B).

Because proliferation and apoptosis of donor T cells in the CNS were comparable in WT and μ MT mice, we hypothesized that the increase in donor T cells in the CNS of WT mice on day 7 post-transfer resulted from recruitment of additional donor T cells from the periphery. To address this question, we utilized the sphingosine-1-phosphate receptor modulator FTY720, which rapidly depletes circulating lymphocytes from the blood by sequestering them in secondary lymphoid tissues, and has been shown to be effective in EAE models (153). We injected wildtype mice with FTY720 or vehicle once daily beginning on day 4 after transfer of donor Thy1.1⁺ CD4⁺ T cells, and analyzed the numbers of Thy1.1⁺ CD4⁺ T cells in the CNS on day 4 (untreated) and day 7 (vehicle vs FTY720). We observed a large increase in donor T cells in the CNS of vehicle-treated (control) WT mice between day 4 and day 7 (Figure 3.7). In contrast, there were significantly fewer donor T cells on day 7 in mice treated with FTY720 compared to vehicle-treated mice. These data indicate that recruitment of T cells from the pool of donor cells found in the periphery is the primary mechanism underlying the increase in donor T cell number in the CNS that precedes EAE onset. Therefore, recruitment, and not enhanced proliferation or survival accounts for the increase in CNS donor T cells and may be impaired in the absence of B cells.

Infiltrating T cells do not trigger production of inflammatory mediators in μ MT mice

We hypothesized that the failure to recruit peripheral T cells during preclinical EAE in μ MT recipients is due to impaired reactivation of the initial T cells that infiltrated the CNS. The reactivation of the initial wave of T cells entering the CNS is believed to induce the production of multiple cytokines, including IL-17, IFN- γ , GM-CSF, and TNF- α . These cytokines act on

various cell types within the CNS including astrocytes and endothelial cells of the blood brain barrier, which normally limit T cell entry into the CNS. The T cell cytokines increase expression of adhesion molecules, integrin ligands, and chemokines, all of which are important for recruiting additional cells from the periphery. To test the hypothesis that impaired T cell reactivation in μ MT mice prevents upregulation of genes mediating recruitment, we first confirmed that genes reported to be induced in other EAE models (82) were also induced in C3HeB/Fej mice during EAE. Gene expression was compared by RT-PCR between WT mice with EAE induced by adoptive transfer of MOG-specific T cells and WT mice that received polyclonal CD4⁺ T cells activated *in vitro* with anti-CD3 and anti-CD28 (healthy controls). After identifying genes induced in the brains of mice with clinical EAE, we determined which of these genes were induced in preclinical WT C3HeB/Fej mice (five days post-transfer of MOG-specific CD4⁺ T cells) compared to healthy controls (Table 3.2). Using these genes as indicators of T cell reactivation, we compared their expression in the brains of WT and μ MT mice five days after transfer of CD4⁺ MOG-specific T cells. To ensure that tissue was harvested from mice at the same preclinical stage, half of each brain was used for RT-PCR analyses, and cells were isolated from the other half of each brain to determine donor T cell numbers by flow cytometry. Only brains from WT and μ MT recipients with comparable numbers of donor CD4⁺ T cells (2000-5000 cells) were used for gene expression analyses. Strikingly, the expression of all selected genes was significantly reduced in the brains of μ MT recipients compared to WT recipients (Figure 3.8 and Table 3.3). Impaired gene expression levels were observed for pro-inflammatory T cell- and non-T cell-derived cytokines, cell surface molecules involved in adhesion and transmigration of the blood brain barrier, and chemokines involved in recruitment of additional cells from the periphery. Expression of a few

genes was induced in μ MT mice relative to healthy controls, but their level of expression was always significantly less than that seen in WT recipients (Table 3.3). The greater variability in gene expression in WT mice likely reflects differences in the extent of T cell reactivation at this preclinical time point. These observations suggest that although equal numbers of donor T cells are initially present in the μ MT CNS, their reactivation is significantly impaired in the CNS in the absence of B cells, resulting in an inability to recruit additional T cells from the periphery.

B cells preferentially reactivate Th1 cells in vitro

One mechanism by which B cells could influence T cell reactivation is to function as APCs for T cells infiltrating the non-inflamed CNS. To test this possibility, we first investigated whether B cells can process and present rMOG to previously activated CD4⁺ MOG-specific T cells skewed toward a Th1 or Th17 phenotype. Resting B cells (CD19⁺ CD43⁻) sorted from spleens of naïve C3HeB/Fej mice were co-cultured overnight with sorted MOG-specific CD4⁺ Th1 or Th17 effector cells and rMOG. The non-B cell fraction of the sorted splenocytes (containing dendritic cells and macrophages) as well as unsorted splenocytes were used as control APCs in separate co-cultures with the MOG-specific Th1 and Th17 cells. Reactivation of MOG-specific IL-17- and IFN- γ -producing cells by the different types of APCs was quantified by ELISPOT. The fraction of cells containing dendritic cells and macrophages efficiently reactivated T cells that had been skewed to either a Th1 or Th17 phenotype. In contrast, resting B cells reactivated the Th1-skewed but not the Th17-skewed cells (Figure 3.9A). To determine if activated B cells could reactivate Th17 cells, naïve splenic B cells were stimulated with LPS and anti-CD40 prior to culturing with the T cells. A small increase in Th17 cell reactivation was observed when activated versus resting B cells were used as APCs; however, the extent of Th17 reactivation was still significantly reduced compared to the

reactivation observed when non-B cells were used as APCs (Figure 3.9A). We confirmed that the activated B cells expressed higher levels of MHC class II and CD86, and that the expression of these molecules was similar in the co-cultures of Th1 and Th17 cells (Figure 3.9B).

We then tested whether the addition of various cytokines would increase Th17 reactivation by B cells. The addition of IL-23 and IL-6 to the co-cultures of B cells with T cells had no effect on Th17 cell activation (Figure 3.10A). However, addition of exogenous IL-1 β restored IL-17 production by the B cell/Th17 co-cultures in an antigen-specific manner without affecting the B cell/Th1 co-cultures (Figure 3.10A and 3.10B). We found that the B cells in our co-cultures were making very low amounts of IL-1 β compared to the non-B cell APCs (Figure 3.10C). Additionally, we tested whether blocking IL-1 β signaling using the anti-IL-1R antibody would prevent Th17 reactivation by the non-B cell APCs. We observed a 50% reduction in Th17 reactivation when IL-1 β signaling was blocked, but no difference in Th1 reactivation (Figure 3.10D). These data indicate that *in vitro*, B cells preferentially reactivate Th1 cells in response to MOG, and are not able to reactivate Th17 cells in the absence of IL-1 β .

B cells influence the Th17:Th1 ratio in the CNS

Based on the ability of B cells to reactivate Th1 but not Th17 cells *in vitro*, we hypothesized that the absence of B cells in μ MT mice may have a greater effect on EAE induced by transfer of Th1 cells compared to Th17 cells. To determine how the presence of B cells affected EAE induced by Th1 and Th17 cells individually, we transferred MOG-specific T cells that were skewed to either a Th17:Th1 ratio of \sim 1:8 (Th1-skewed) or \sim 3:1 (Th17-skewed). The incidence of disease was significantly reduced in C3HeB/Fej μ MT mice after transfer of either Th1- or Th17-skewed cells (Table 3.4). This reduced incidence is similar to that observed

when the transferred T cells exhibited a Th17:Th1 ratio of 1:1 (Table 3.1), and likely reflects the loss of the majority of the MHC class II⁺ APCs in the CNS. As we have previously observed, Th1-skewed cells induced classic EAE and Th17-skewed cells induced atypical EAE in wildtype recipients. In contrast, in the μ MT recipients that did develop EAE, both Th1- and Th17-skewed cells induced predominantly atypical EAE (Figure 3.11). This shift from classic to atypical EAE when Th1-skewed cells were transferred into μ MT instead of wildtype mice correlated with a significant increase in the Th17:Th1 ratio of cells isolated from the brain of μ MT recipients with EAE (Figure 3.12A). Consistent with our *in vitro* observation that B cells preferentially reactivate Th1 cells compared to Th17 cells, there were significantly fewer IFN- γ -producing T cells in the brains of μ MT recipients that received Th1-skewed cells (Figure 3.12B). The number of IL-17-producing T cells was also decreased in μ MT mice, but the difference was not significant. These data suggest that B cells preferentially promote IFN- γ production in the CNS of mice that received Th1 cells.

A similar trend of an increased Th17:Th1 ratio was observed in the brains of μ MT recipients that received Th17-skewed cells compared to wildtype, although this difference was not statistically significant (Figure 3.13A). The numbers of both IFN- γ and IL-17-producing T cells were significantly decreased in the brains of μ MT recipients that received Th17 cells (Figure 3.13B). However, the fold decrease in IFN- γ producing cells was significantly greater than the fold decrease in IL-17-producing cells in the brains of μ MT relative to WT recipients (7.6 ± 1.6 vs 3.7 ± 0.7 ; $P=0.04$, Student's *t* test). Overall, these data suggest that B cells promote both IFN- γ and IL-17 production in the CNS of Th17 recipients, but may promote IFN- γ production more strongly.

B cell deficient CNS cells are competent APCs in vitro

The above data shows reduced numbers of IFN- γ - and IL-17-producing cells in the CNS of B cell deficient mice with EAE. This suggests that B cells are important for reactivating Th1 and Th17 cells in the CNS. To support this idea, we wanted to confirm that the other APCs in the CNS were functional in B cell deficient mice. We isolated CNS mononuclear cells from naïve wildtype or μ MT mice and co-cultured them with purified MOG-specific Th1 or Th17 cells *in vitro*. We determined the numbers of antigen-specific IFN- γ - and IL-17-producing cells in the co-cultures by ELISPOT. We found similar levels of Th17 reactivation by CNS APCs from both wildtype and μ MT mice (Figure 3.14A), indicating that the APCs in the μ MT CNS are functional. We found a slight decrease in reactivation of Th1 cells by the μ MT CNS APCs. This could reflect the loss of the B cell contribution to Th1 reactivation. However, it is difficult to draw strong conclusions from these *in vitro* data, because the cells are concentrated in small wells that do not reflect the tissue architecture or environment in the CNS. We also confirmed that the dendritic cells in the μ MT CNS express similar levels of MHC class II, CD40, CD80, and CD86 compared to wildtype (Figure 3.15). This indicates that the decrease in Th1 and Th17 activation in the μ MT CNS is due to the lack of B cells, not any defect in the other APC populations.

Limitations of bone marrow chimeras in C3H EAE models

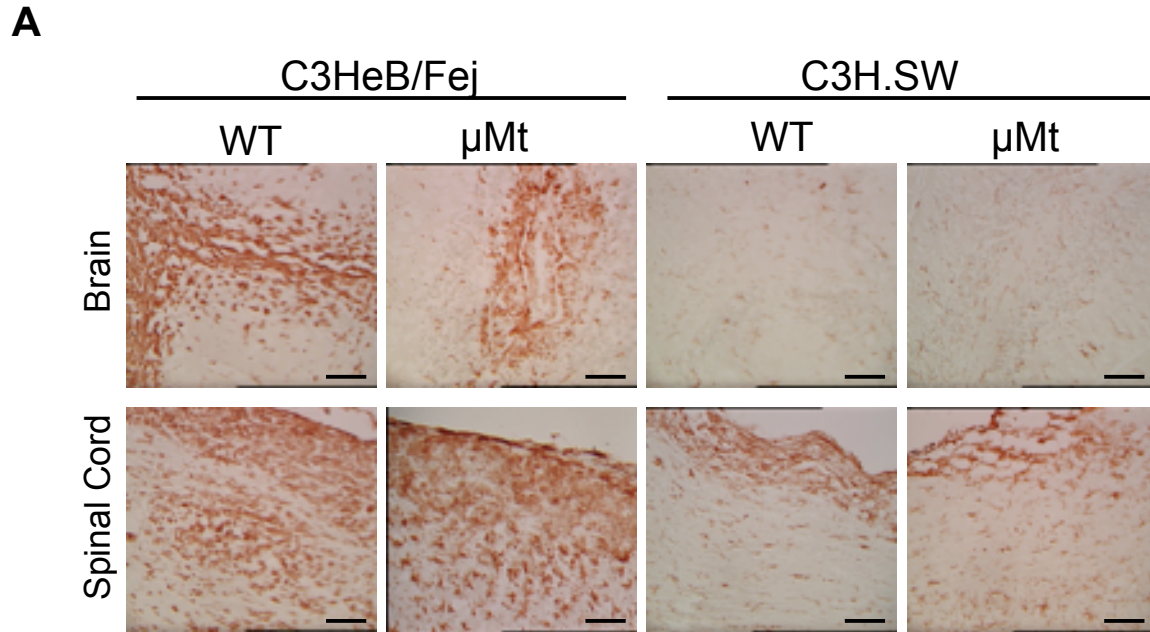
To determine whether the antigen presentation function of B cells is specifically required for efficient induction of EAE, we generated a mixed MHC bone marrow chimera that removes the antigen presentation function of the B cells while preserving other functions. A 1:1 ratio of C3HeB/Fej (H-2^k) μ MT and C3H.SW (H-2^b) wildtype donor bone marrow was transferred into F1 recipients, so that the SW B cells would provide all functions of B cells

except for antigen presentation to Fej T cells (Figure 3.16). Positive control chimeras were generated by transferring C3HeB/Fej wildtype and C3H.SW wildtype donor bone marrow into F1 recipients. Negative control chimeras were generated by transferring only C3HeB/Fej μ MT donor bone marrow into F1 recipients. MOG-specific C3HeB/Fej CD4⁺ T cells were adoptively transferred into each set of chimeras after 8 weeks of reconstitution. Unexpectedly, all chimeras had 100% incidence of EAE (Table 3.5), and the progression was rapid and very severe. To further investigate this surprising result, we analyzed the CNS mononuclear populations of naïve control chimeras at 8 weeks post-reconstitution. Compared to naïve wildtype C3HeB/Fej mice, the CNS of the naïve chimeras appeared to have low level inflammation, with increased numbers of MHC class II⁺ cells, CD45⁺CD11b⁺ cells (dendritic cells and macrophages), and fewer resting microglia (Figure 3.17). These data suggest that the chimeras were already in a state primed for inflammation, perhaps due to the highly sensitive nature of C3H mice in response to irradiation (unpublished observations). The increased numbers of antigen presenting cells present in the chimeras may have been able to overcome the lack of B cells, permitting development of EAE in the μ MT chimeras. The inability to create chimeras on the C3H background that are dependent on the presence of B cells for EAE development precludes further investigation in the chimeric mice.

Induction	Strain	Genotype	Incidence	Onset	Max score	Recovery
Active ^A	C3H/Fej	WT	88% (7/8)	18 ± 5	5.0 ± 0.3	0/7
Active	C3H/Fej	μMT	33% (5/15) ^C	21 ± 4	4.9 ± 0.5	0/5
Active	C3H.SW	WT	100% (6/6)	14 ± 1	3.1 ± 0.1	3/6
Active	C3H.SW	μMT	50% (5/10) ^D	15 ± 4	3.6 ± 0.2	3/5
Passive ^B	C3H/Fej	WT	96% (29/30)	6.7 ± 0.9	4.6 ± 1.3	0/29
Passive	C3H/Fej	μMT	21% (6/28) ^E	6.6 ± 0.5	4.3 ± 1.9	0/6

Table 3.1. EAE in C3H μMT mice

C3HeB/Fej or C3H.SW wildtype and μMT mice were either ^Aimmunized with rat rMOG (active) or were ^Brecipients of *in vitro*-activated lymphocytes from MOG-immunized donors (passive) in order to induce EAE. Assessment of clinical EAE includes the number of mice that developed disease, the day of disease onset and maximal clinical score (mean ± SEM) among mice with EAE, and the number of mice that recovered from EAE. Significant differences between wildtype vs μMT mice for each group are indicated; ^C*P*=0.01, ^D*P*=0.03, ^E*P*<0.0001, Chi-squared test.



B

Relative Inflammation in the CNS of μ Mt Mice

Strain	Brain	Spinal Cord
C3HeB/Fej WT	12.3 \pm 2.5	10.5 \pm 2.1
C3HeB/Fej μ Mt	11.3 \pm 1.5	8.3 \pm 4
C3H.SW WT	5 \pm 0	9.5 \pm 0.7
C3H.SW μ Mt	3.5 \pm 0.5	6.5 \pm 2.1

Figure 3.1. Inflammation in the CNS of wildtype or μ MT mice with EAE

(A) Immunohistochemistry of F4/80⁺ macrophages/microglia in the brain and spinal cord of C3HeB/Fej and C3H.SW wildtype and μ MT mice at onset of rMOG-induced active EAE. Representative of 5 mice per group. Scale bars, 50 μ m for all panels. (B) Inflammatory index \pm s.d. in the brain and spinal cord of wildtype and μ MT mice with EAE was quantified using F4/80⁺ stained sections from 3 mice per group.

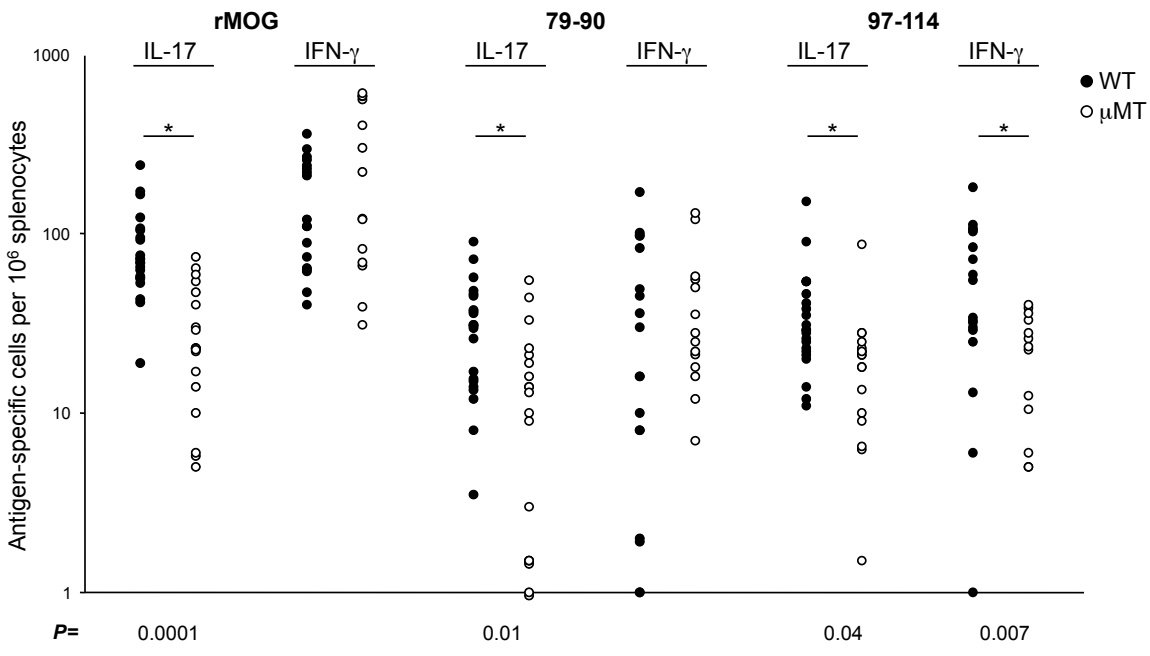


Figure 3.2. MOG-specific T cell priming is altered in μMT mice

Splenocytes were isolated from C3HeB/Fej wildtype and μMT mice 7 days after immunization with rat rMOG and plated into anti-IFN-γ and anti-IL-17-coated ELISPOT wells directly *ex vivo* with or without rMOG protein, or the peptides MOG₇₉₋₉₀ or MOG₉₇₋₁₁₄. ELISPOT plates were developed after overnight incubation to determine the number of antigen-specific IL-17 or IFN-γ secreting cells per million splenocytes. Significance determined by student's *t* test.

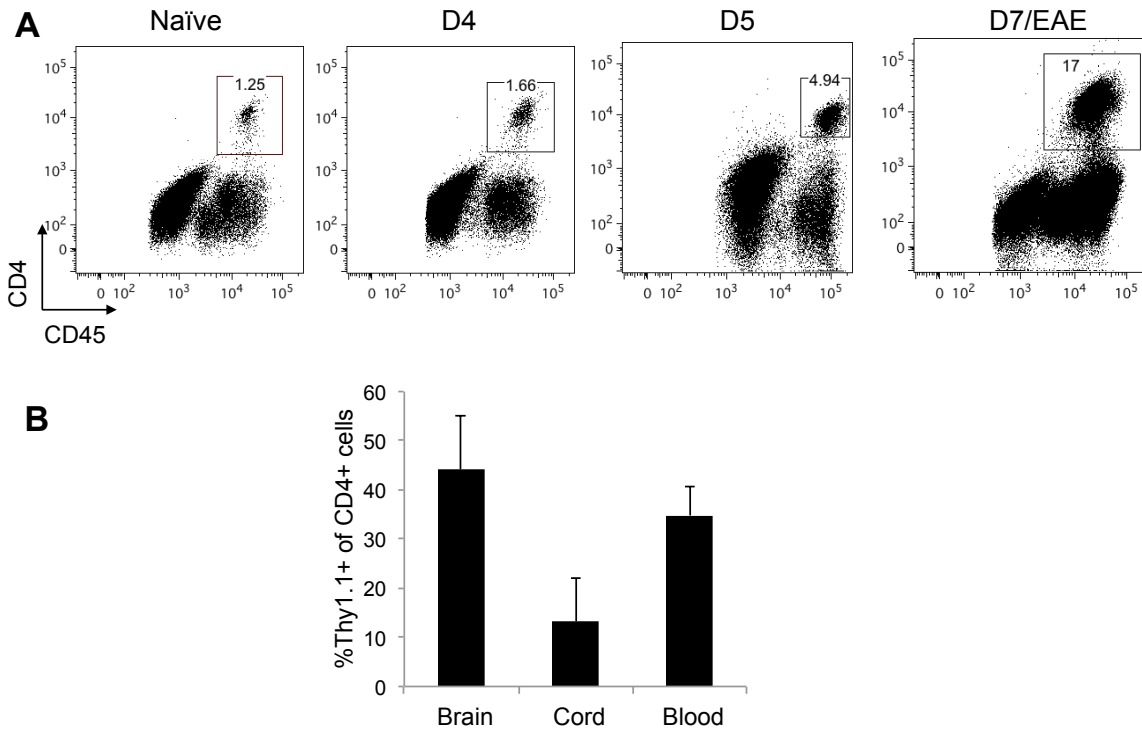


Figure 3.3. Kinetics of T cell entry into the brain and spinal cord in preclinical EAE

Brain and spinal cord mononuclear cells were isolated from well-perfused naïve mice or at specific time points post-transfer from Thy1.2 mice that had received MOG-specific Thy1.1⁺ T cells. **(A)** Representative staining gated on CD45⁺ cells showing CD4⁺ gates in the CNS of mice at various points after transfer of donor cells. **(B)** Percentage of donor cells within the CD4⁺ population in the brain, spinal cord, or blood on day 5 after transfer. Representative of at least 4 experiments.

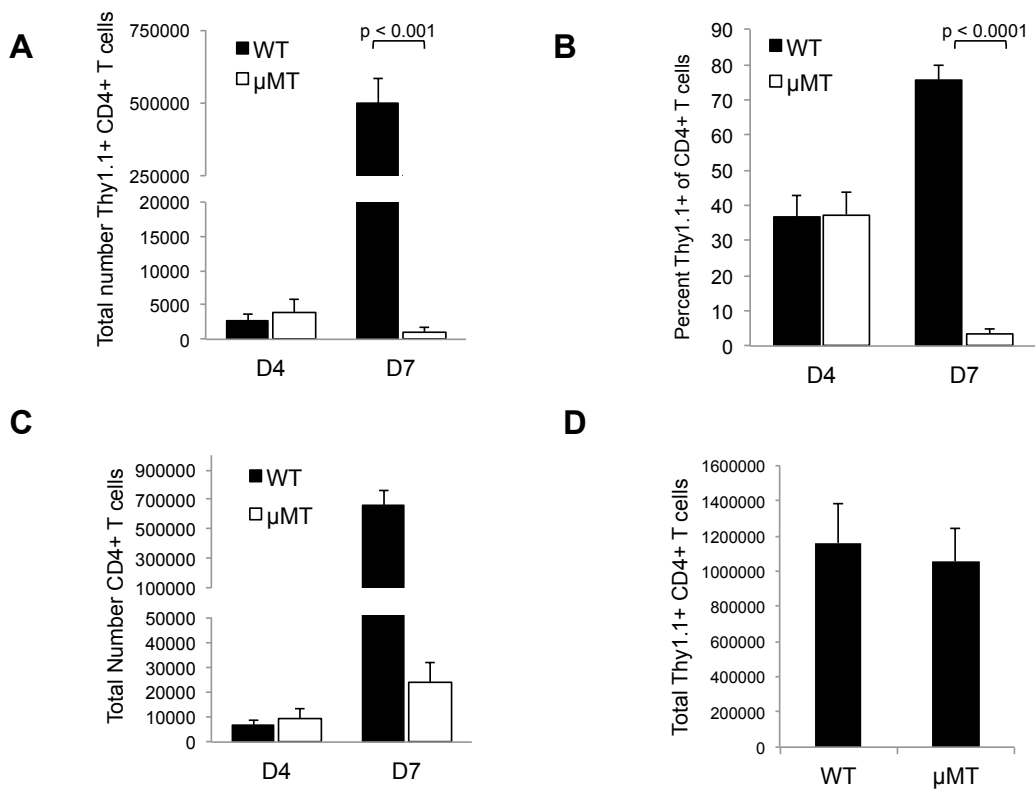


Figure 3.4. Preclinical MOG-specific T cell numbers fail to increase in the CNS of B cell deficient mice

Genetically marked (Thy1.1⁺) T cells from MOG-immunized donors were activated *in vitro* for three days and transferred into wildtype or μMT Thy1.2 recipients. CNS mononuclear cells were isolated on day 4 (preclinical) and day 7 (EAE onset in wildtype recipients) post transfer and analyzed for (A) total number of donor CD4⁺ Thy1.1⁺ T cells, (B) percent of Thy1.1⁺ donor cells among gated CD4⁺ T cells, and (C) total number CD4⁺ cells (host and donor combined) in the CNS. (D) Numbers of Thy1.1⁺ CD4⁺ donor cells were analyzed in the spleens of recipient mice on day 4 post transfer. (means, SEM, n=5 mice per group, Student's *t* test).

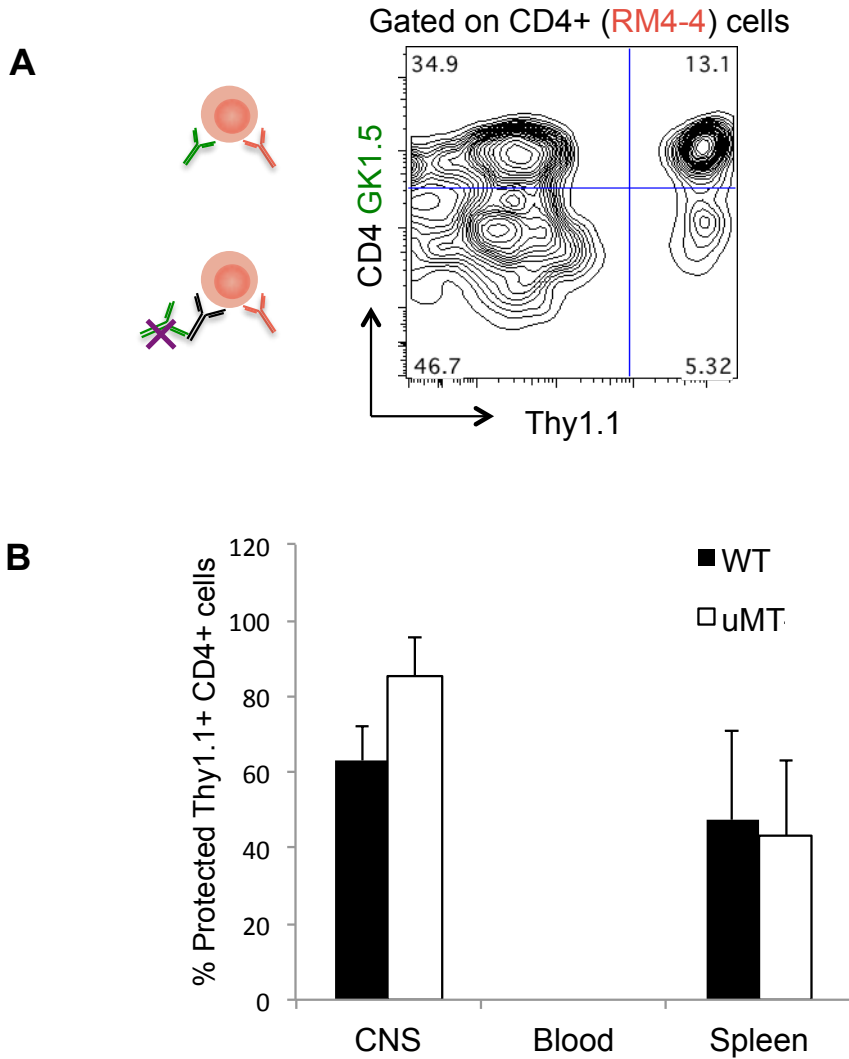


Figure 3.5. Donor T cells cross the blood brain barrier into the CNS on day 4

Thy1.1⁺ T cells from MOG-immunized donors were activated *in vitro* for three days and transferred into wildtype or μ MT Thy1.2 recipients. On day 4 post transfer, unlabeled anti-CD4 antibody (clone GK1.5) was injected i.v. into recipient mice. CNS mononuclear cells were isolated after 10 minutes and analyzed with labeled anti-CD4 antibody clones GK1.5 and RM4-4. **(A)** Labeling and gating strategy is shown. Donor cells are identified using RM4-4 and Thy1.1. Cells that were not bound by unlabeled serum GK1.5 antibody (black) are positive for both anti-CD4 clones GK1.5 (green) and RM4-4 (red) and are considered “protected” by the BBB, within the CNS. **(B)** Percent of the Thy1.1⁺ donor cells that are protected from serum antibody are shown in the CNS, blood, and spleen of recipient mice. Representative of two independent experiments.

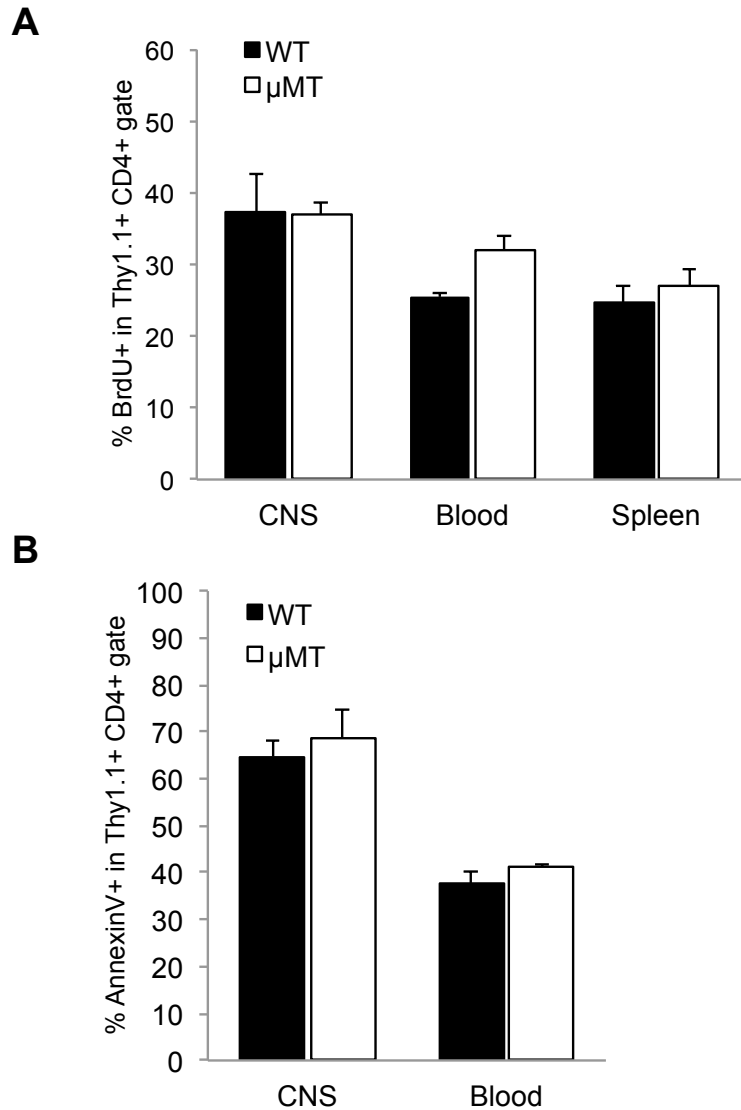


Figure 3.6. T cells have similar levels of proliferation and apoptosis in the CNS of wildtype and B cell deficient recipients

Thy1.1⁺ T cells from MOG-immunized donors were activated *in vitro* for three days and transferred into wildtype or μMT Thy1.2 recipients. BrdU was injected on day 4 post transfer and CNS cells were isolated on day 5 post transfer and analyzed for frequency of (A) BrdU⁺ or (B) AnnexinV⁺ cells among gated Thy1.1⁺ CD4⁺ donor cells (means, SEM, n=4). Representative of three independent experiments.

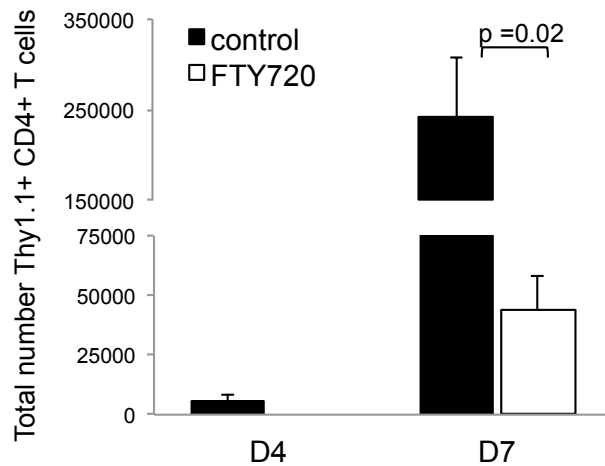


Figure 3.7. FTY720 blocks the increase in CNS donor T cell number by preventing recruitment of cells from the periphery

Thy1.1⁺ T cells from MOG-immunized donors were activated *in vitro* for three days and transferred into wildtype recipients. FTY720 was injected daily starting on day 4 post transfer. CNS cells were isolated on day 4 (before injection), as well as on day 7 post transfer from both treated and control recipients. The total number of CNS CD4⁺ Thy1.1⁺ donor T cells was determined. (means, SEM, n=4 per group, representative of 3 independent experiments, Student's *t* test).

Gene	D5	EAE	Forward primer (5'-3')	Reverse primer (5'-3')
IL-17A	Y	Y	CTCAAAGCTCAGCGTGTCCAAACA	TATCAGGGTCTTCATTGCGGTGGA
IFN- γ	Y	Y	AACGCTACACACTGCATCTTGG	GCCGTGGCAGTAACAGCC
GM-CSF	Y	Y	CACCCGGCCTTGGAAGCATGTAGA	GCATGTCATCCAGGAGGTCA
TNF α	Y	Y	GCACAGAAAGCATGACCCG	GCCCCCATCTTTTGGG
IL-6	Y	Y	GCTACCAAAGTGGATATAATCAGG	CCAGGTAGCTATGGTACTCCAGAA
IL-1 β	Y	Y	TGTAATGAAAGACGGCACACC	TCTTCTTTGGGTATTGCTTGG
IL-10	N	Y	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT
IL-12 p35	N	N	AAATGAAGCTCTGCATCCTGC	TCACCCTGTTGATGGTCACG
IL-23 p19	N	Y	GAACAAGATGCTGGATTGCAGAG	TGTGCGTTCAGGCTAGCA
VCAM	N	Y	TGGTGAAATGGAATCTGAACC	CCCAGATGGTGGTTTCCTT
ICAM	Y	Y	CCCACGCTACCTCTGCTC	GATGGATACCTGAGCATCACC
E-selectin	Y	Y	TCCTCTGGAGAGTGGAGTGC	GGTGGTCAAAGCTTCACAT
P-selectin	Y	Y	GTGCAGAGCGGTCAAATGC	CTGAGAGCTTTCTTAGCAGAGC
iNOS	N	Y	TTGATGTGCTGCCTCTGGTCTTGC	AGCTCCTGGAACCACTCGTACTT
CCL1	N	Y	CCCCTGAAGTTTATCCAGTGTTA	GCAGCTTTCTCTACCTTTGTTCA
CCL2	Y	Y	CCACTCACCTGCTGCTACT	TCTGGACCCATTCTTCTTG
CCL3	N	Y	TGTACCATGACACTCTGCAAC	CAACGATGAATTGGCGTGGA
CCL4	Y	Y	TCTTGCTCGTGGCTGCCT	GGGAGGGTCAGAGCCCA
CCL5	N	Y	TTTGCCTACCTCTCCCTCG	CGACTGCAAGATTGGAGCACT
CCL6	N	Y	CAAAGAAGGGCATGGAAGTCTG	ATCCCTTAGGACCGTGATCAAC
CCL7	Y	Y	CAAAGAAGGGCATGGAAGTCTG	ATCCCTTAGGACCGTGATCAAC
CCL9	N	Y	CTCACAACCACGGACCTAC	TGTGTAGTCATTGAGGTCAGC
CCL19	N	Y	TGTGGCCTGCCTCAGATTAT	AGTCTCCGCATCATTAGCAC
CCL20	Y	Y	TTTTGGGATGGAATTGGACAC	TGCAGGTGAAGCCTTCAACC
CXCL1	Y	Y	CTGCACCCAAACCGAAGTC	AGCTTCAGGGTCAAGGCAAG
CXCL2	Y	Y	CGCTGTCAATGCCTGAAG	GGCGTCACACTCAAGCTCT
CXCL9	Y	Y	AATGCACGATGCTCCTGCA	AGGTCTTTGAGGGATTTGTAGTG
CXCL10	Y	Y	GCCGTCATTTTCTGCCTCA	CGTCCTTGCGAGAGGGATC
MMP3	N	Y	TTGTTCTTTGATGCAGTCAGC	GATTTGCGCCAAAAGTGC
MMP8	N	Y	AACGGGAAGACATACTTCTTCATA	TGGGTTTCATGGATCTTCTTTG
MMP9	N	Y	ACGACATAGACGGCATCCA	GCTGTGGTTCAGTTGTGGTG

Table 3.2. List of genes analyzed by RT-PCR for expression in the brain

The mRNA expression levels of these genes were tested in wildtype recipients of MOG-specific T cells on day 5 post-transfer or after onset of EAE. Genes indicated with (Y) are induced at the time point listed. Primer sequences are listed for each gene.

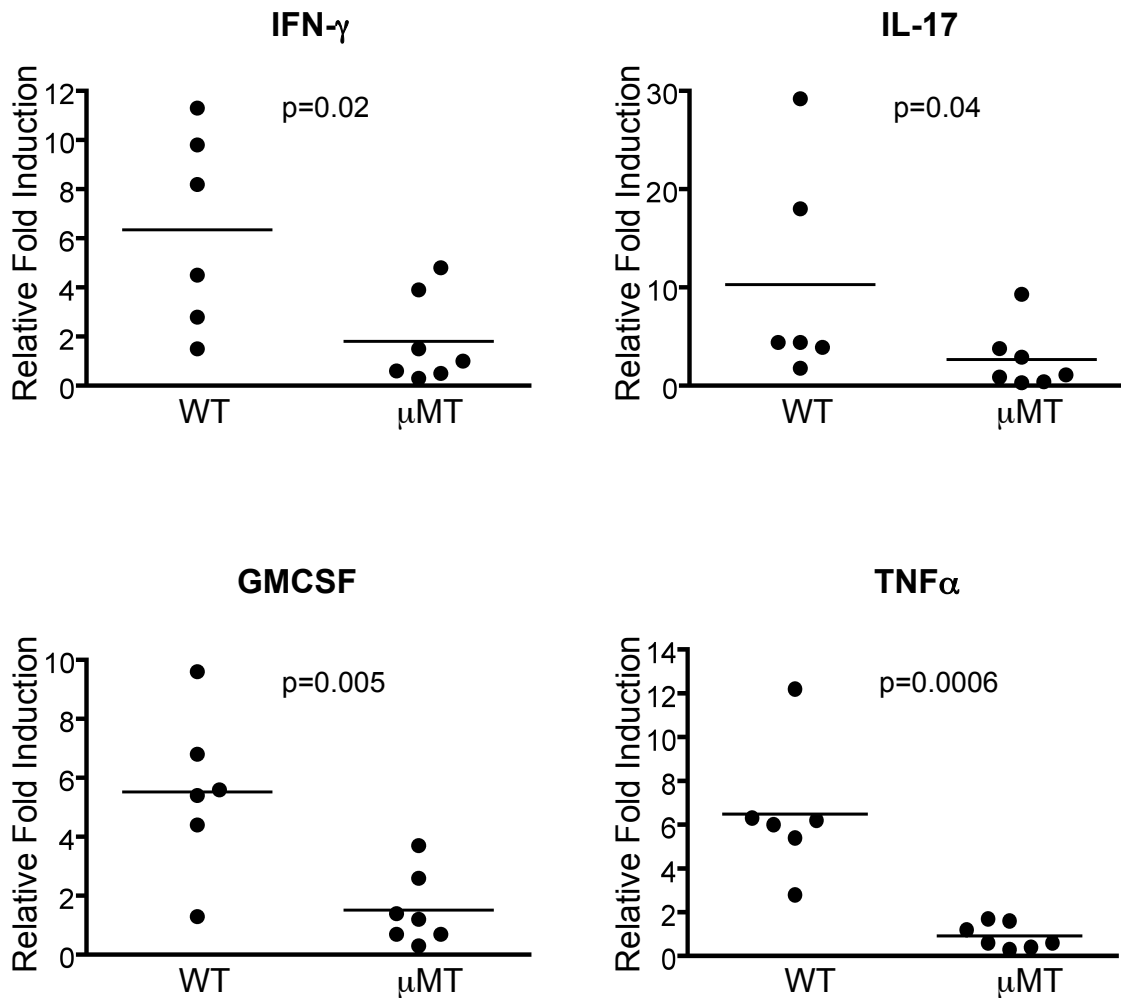


Figure 3.8. Cytokine production is not induced in the CNS of μ MT T cell recipients compared to wildtype recipients

Genetically marked (Thy1.1⁺) T cells from MOG-immunized donors were activated *in vitro* for three days and transferred into wildtype or μ MT Thy1.2 recipients. Five days post-transfer, the brain of each recipient was taken and cut in half at the midline. One half was used to analyze the number of transferred (Thy1.1⁺) T cells in the tissue and the other half was frozen and RNA harvested for real time PCR analysis. Data shown is the mRNA fold induction of cytokines IFN- γ , IL-17, GMCSF, and TNF α in the brain of wildtype or μ MT recipients relative to healthy brain tissue. Samples were obtained in at least 3 independent experiments. Significant differences between wildtype and μ MT recipients are indicated with the corresponding *P* values, Mann-Whitney non-parametric *t* test.

Gene	Wildtype	μ MT	P value
CXCL1	17.5 \pm 3.7	1.9 \pm 0.4	0.001
CXCL2	3.8 \pm 0.9	0.9 \pm 0.2	0.004
CXCL9	17.2 \pm 2.9	5.5 \pm 1.8	0.01
CXCL10	126.4 \pm 27.2	12.3 \pm 3.3	0.001
CCL2	47.4 \pm 12.8	5.9 \pm 2.1	0.008
CCL4	2.8 \pm 0.4	0.8 \pm 0.1	0.001
CCL7	19.2 \pm 4.8	1.9 \pm 0.4	0.002
CCL20	3.8 \pm 0.9	1.1 \pm 0.3	0.01
IL-1 β	2.1 \pm 0.4	0.3 \pm 0.06	0.002
IL-6	13.1 \pm 3.3	1.7 \pm 0.4	0.004
E-selectin	22.4 \pm 7.8	5.3 \pm 2.4	0.01
P-selectin	49.4 \pm 14	11.6 \pm 4.1	0.01
ICAM	3.9 \pm 0.5	0.9 \pm 0.1	0.001

Table 3.3. Genes involved in T cell recruitment are not upregulated in the CNS of μ MT T cell recipients compared to wildtype recipients

Genetically marked (Thy1.1⁺) T cells from MOG-immunized donors were activated *in vitro* for three days and transferred into wildtype or μ MT Thy1.2 recipients. Five days post-transfer, the brain of each recipient was taken and cut in half at the midline. One half was used to analyze the number of transferred (Thy1.1⁺) T cells in the tissue and the other half was frozen and RNA harvested for real time PCR analysis from mice with comparable numbers of Thy1.1⁺ T cells in the CNS. Data shown is the mRNA fold induction (mean \pm SEM) of various chemokines, cytokines, and adhesion molecules in the brain of wildtype or μ MT recipients relative to healthy brain tissue. Results are the sum of at least 3 independent experiments, $n \geq 6$ mice per group. Significant differences between wildtype and μ MT recipients are indicated with the corresponding *P* values, Mann-Whitney non-parametric *t* test.

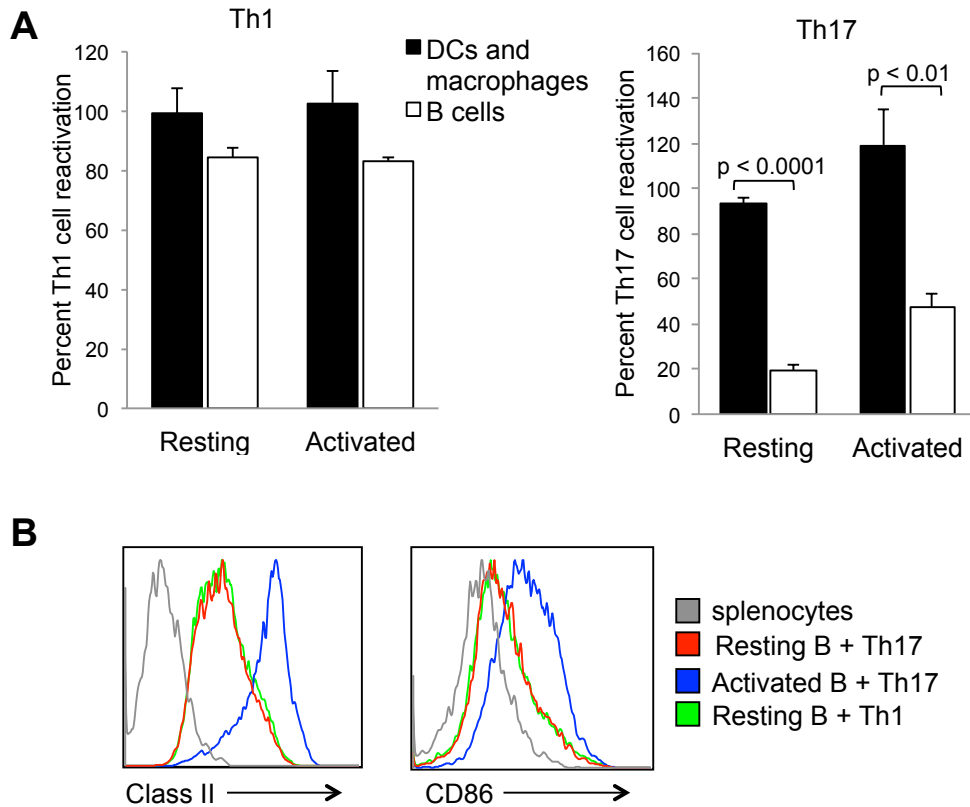


Figure 3.9. Reactivation of effector Th1 and Th17 cells *in vitro*

Effector Th1 and Th17 cells were generated and rested down. CD4⁺ T cells and naïve spleen B cells or dendritic cells and macrophages were then FACS-sorted. Purified CD4⁺ Th1 or Th17 cells were co-cultured overnight with various antigen presenting cell types, with or without rMOG. Antigen-specific IL-17 or IFN- γ production was detected by ELISPOT. **(A)** Th1 or Th17 cells were co-cultured overnight with spleen B cells or dendritic cells and macrophages. Where indicated, the APCs were stimulated with LPS and anti-CD40 after sorting (activated). Data shown is calculated from the number of antigen-specific spots produced by either Th1 or Th17 cells in response to the indicated APC and normalized to the number of antigen-specific IFN- γ or IL-17 spots obtained by co-culture of either Th1 or Th17 cells with unsorted, naïve splenocytes (100%). **(B)** The activation status of the B cells from the co-cultures was confirmed; histograms showing Class II and CD86 expression are shown through the B cell gate. Results shown are representative of at least 3 independent experiments. Significant differences are indicated, Student's *t* test.

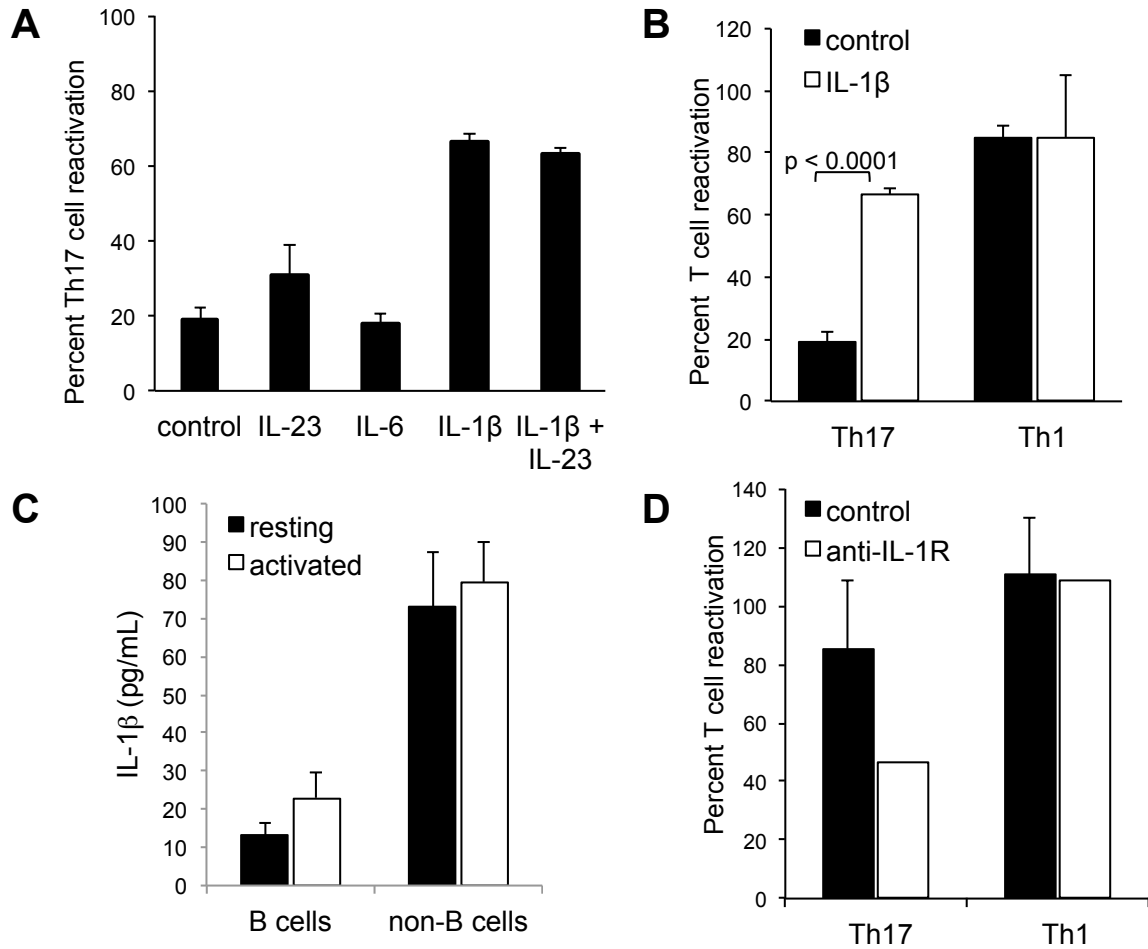


Figure 3.10. IL-1 β promotes the reactivation of Th17 cells by B cells *in vitro*

Effector Th1 and Th17 cells were generated and rested down. CD4⁺ T cells and naïve spleen B cells or dendritic cells and macrophages were then FACS-sorted. Purified CD4⁺ Th1 or Th17 cells were co-cultured overnight with various antigen presenting cell types, with or without rMOG. Antigen-specific IL-17 or IFN- γ production was detected by ELISPOT. **(A)** Th17 or **(B)** Th17 and Th1 cells were co-cultured with resting spleen B cells with or without IL-23, IL-6, and/or IL-1 β . Control indicates the absence of added cytokine. **(C)** IL-1 β production by B cells or DCs and macrophages in the conditions from Figure 3.9 was determined by ELISA. **(D)** Th17 and Th1 cells were co-cultured with non-B cell APCs with or without anti-IL-1R blocking antibody. Results shown are representative of at least 3 independent experiments. Significant differences are indicated, Student's *t* test.

Recipient	Incidence	Day of Onset	Max Score
Th1: WT	0.94 (16/17)	6.7	5.5
Th1: μ MT	0.37 (3/8)	7.0	5.2
Th17: WT	0.97 (32/33)	6.2	5.5
Th17: μ MT	0.37 (9/24)	5.7	4.8

Table 3.4. EAE in wildtype and μ MT recipients of Th1- or Th17-skewed cells

Th1 and Th17 cells were generated by culturing spleen and lymph node cells from rMOG-immunized mice with MOG₉₇₋₁₁₄ and IL-12 or IL-23 + α -IFN- γ for three days *in vitro*, followed by adoptive transfer into C3HeB/Fej wildtype or μ MT recipients. Assessment of clinical EAE includes the number of mice that developed disease, the day of disease onset and maximal clinical score among mice with EAE. Significant differences were found between wildtype and μ MT recipients of both Th1- and Th17-skewed cells, $P < 0.001$, Fisher's exact test.

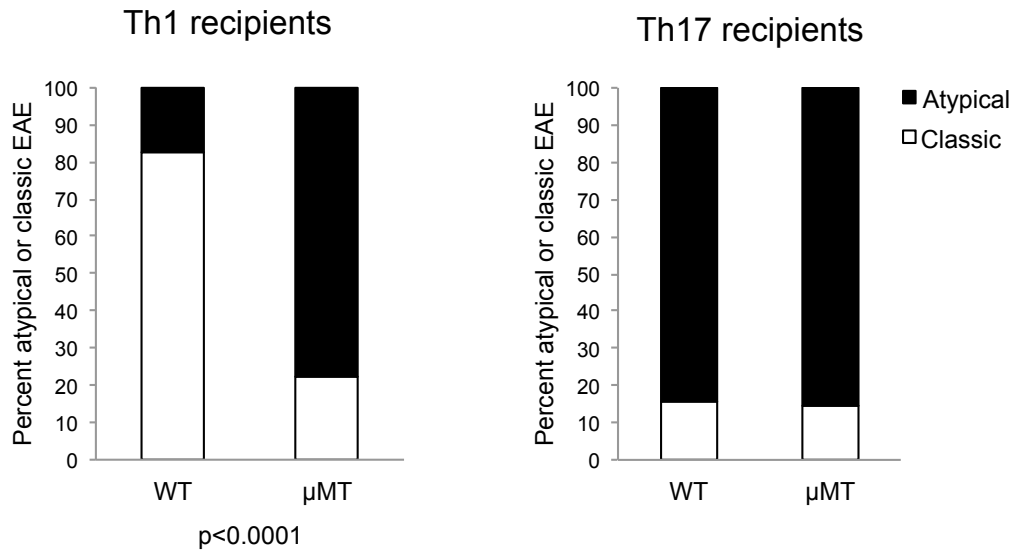


Figure 3.11. Th1 cells induce classic EAE in wildtype recipients and atypical EAE in B cell deficient recipients

Th1 and Th17 cells were generated by culturing spleen and lymph node cells from rMOG-immunized mice with MOG₉₇₋₁₁₄ and IL-12 or IL-23 + α -IFN- γ for three days *in vitro*, followed by adoptive transfer into C3HeB/Fej wildtype or μ MT recipients. The percentages of classic or atypical symptoms in recipient mice that developed EAE are shown (n=5-24 per group). B cell deficient recipients of Th1 cells had significantly more atypical EAE than wildtype recipients, $P < 0.0001$, Fisher's exact test.

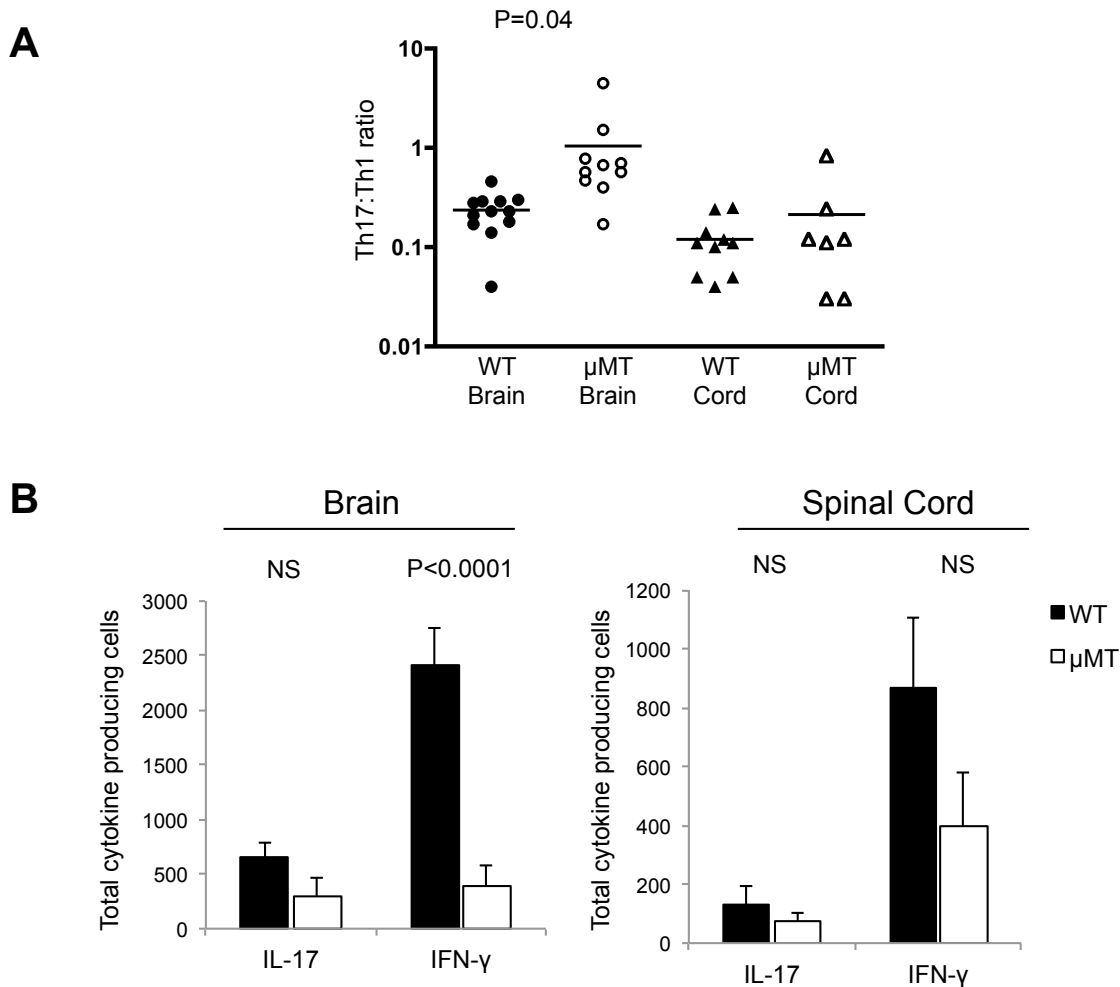


Figure 3.12. B cell deficient recipients with Th1-mediated EAE have higher Th17:Th1 ratios in the brain compared to wildtype recipients

Th1 cells were generated by culturing spleen and lymph node cells from rMOG-immunized mice with MOG₉₇₋₁₁₄ and IL-12 for three days *in vitro*, followed by adoptive transfer into C3HeB/Fej wildtype or μ MT recipients. At onset of EAE, CNS mononuclear cells were isolated from the brain and spinal cord and plated in ELISPOT wells coated with α -IL-17 or α -IFN- γ , with or without MOG₉₇₋₁₁₄, in order to calculate the number of antigen specific IL-17 or IFN- γ producing cells. **(A)** The ratios of IL-17/IFN- γ producing cells (Th17:Th1 ratio) as well as **(B)** the total numbers of IL-17 or IFN- γ producing cells within the CNS were calculated in mice with Th1-induced EAE (means, SEM). Results are the sum of at least 3 independent experiments. Significant differences between wildtype and μ MT recipients are indicated, Student's *t* test.

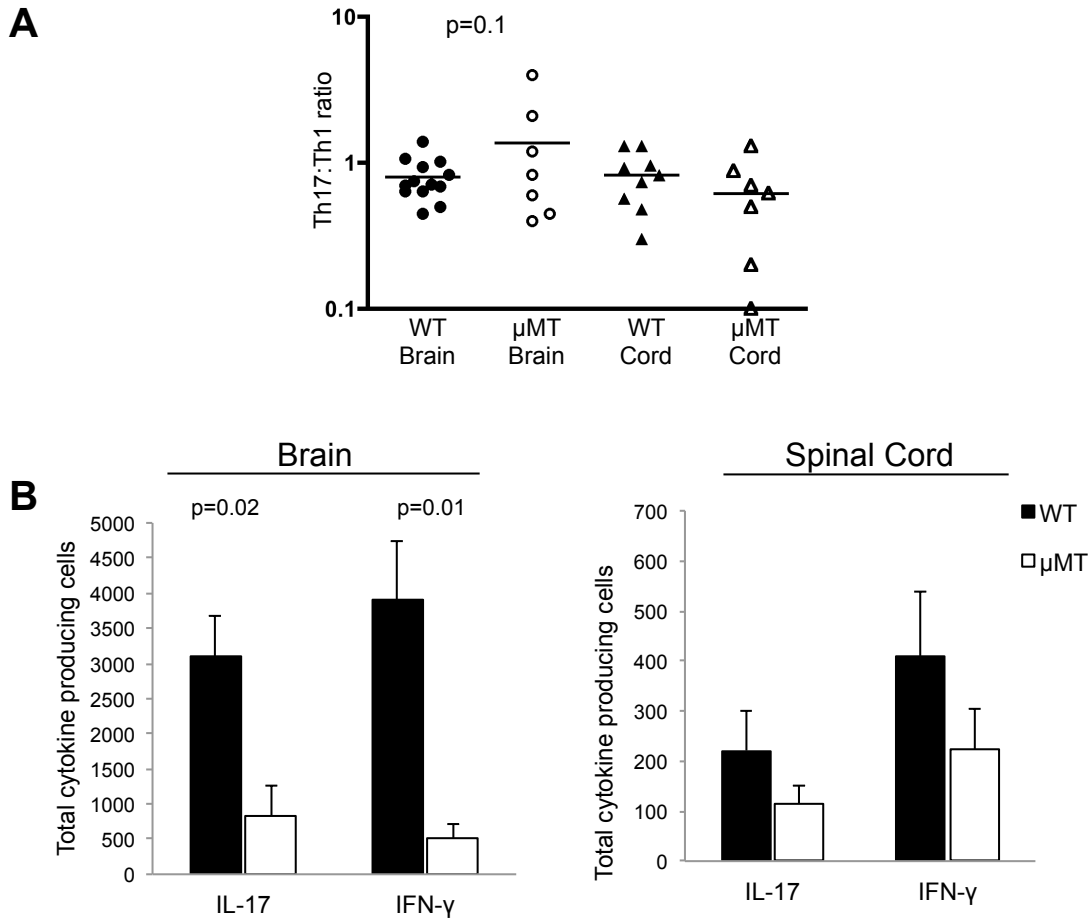


Figure 3.13. B cell deficient recipients with Th17-mediated EAE have fewer IL-17 and IFN- γ producing cells in the brain compared to wildtype recipients

Th17 cells were generated by culturing spleen and lymph node cells from rMOG-immunized mice with MOG₉₇₋₁₁₄ and IL-23 + α -IFN- γ for three days *in vitro*, followed by adoptive transfer into C3HeB/Fej wildtype or μ MT recipients. At onset of EAE, CNS mononuclear cells were isolated from the brain and spinal cord and plated in ELISPOT wells coated with α -IL-17 or α -IFN- γ , with or without MOG₉₇₋₁₁₄, in order to calculate the number of antigen specific IL-17 or IFN- γ producing cells. **(A)** The ratios of IL-17/ IFN- γ producing cells (Th17:Th1 ratio) as well as **(B)** the total numbers of IL-17 or IFN- γ producing cells within the CNS were calculated in mice with Th17-induced EAE (means, SEM). Results are the sum of at least 3 independent experiments. Significant differences between wildtype and μ MT recipients are indicated, Student's *t* test.

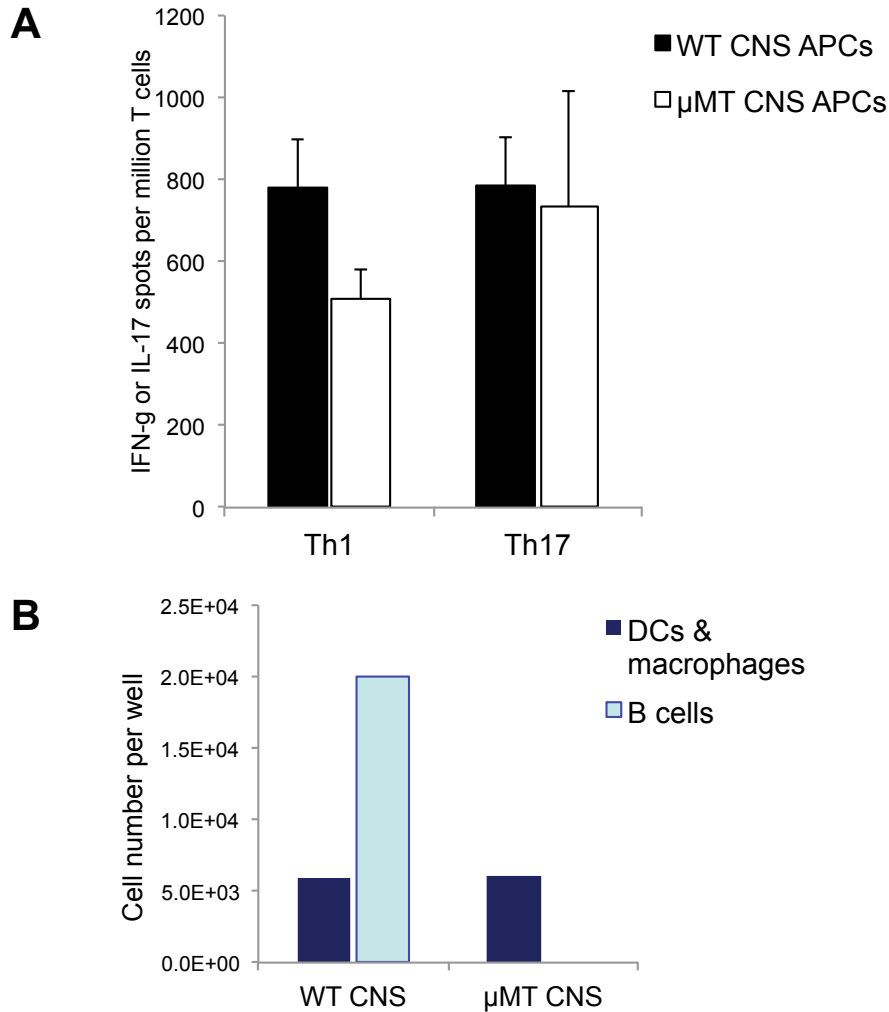


Figure 3.14. Non-B cell APCs are functional in the B cell deficient CNS

Effector Th1 and Th17 cells were generated and rested down. CNS mononuclear cells from naïve wildtype or μ MT mice were isolated and analyzed for expression of CD19 and CD11b. Numbers of APCs for each culture were normalized to the number of CD11b⁺ cells in the sample. APCs were co-cultured with Th1 or Th17 cells overnight with or without rMOG. Antigen-specific IL-17 or IFN- γ production was detected by ELISPOT. **(A)** Numbers of antigen-specific IL-17 or IFN- γ producing cells per million T cells in co-cultures with either wildtype or μ MT CNS APCs. **(B)** Quantification of the number of B cells or CD11b⁺ DCs and macrophages in each APC population for the purpose of normalizing APC numbers. Representative of two independent experiments.

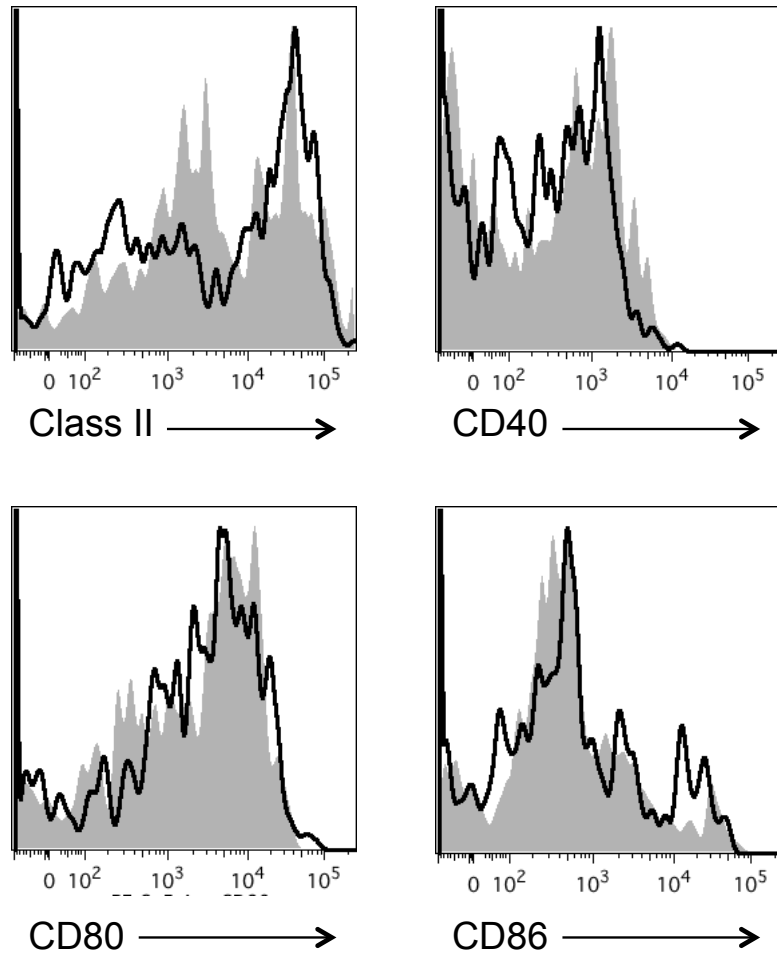


Figure 3.15. DCs have normal expression of activation markers in the μ MT CNS

CNS mononuclear cells were isolated from well-perfused naive wildtype or μ MT mice and analyzed by flow cytometry. Cells were gated on CD45⁺ CD11c⁺ dendritic cells and expression of MHC class II, CD40, CD80, and CD86 is shown. Wildtype, black line, μ MT, grey. Representative of two experiments.

<u>Donor bone marrow</u>	<u>Host</u>	<u>APCs present for Fej T cells</u>
1. C3H/Fej WT + C3H.SW		DCs, macrophages, B cells
2. C3H/Fej μ MT + C3H.SW	C3H Fej x SW F1	DCs, macrophages
3. C3H/Fej μ MT (negative control)		DCs, macrophages

Figure 3.16. Schematic for generation of mixed-MHC bone marrow chimeras

Mixed MHC bone marrow chimeras were generated using combinations of C3HeB/Fej (H-2^k) wildtype or μ MT bone marrow and C3H.SW (H-2^b) wildtype bone marrow with F1 hosts. 8 weeks post-reconstitution, EAE was induced in the different chimeras by adoptive transfer of CD4⁺ MOG-specific T cells from C3HeB/Fej mice.

Chimera	EAE Incidence	Average Day of Onset	Average Max Clinical Score
Fej WT + SW WT	100% (8/8)	7.3	5.3
Fej μ MT + SW WT	100% (7/7)	6.3	5.5
Fej μ MT	100% (5/5)	8.4	5.1

Table 3.5. Adoptive transfer EAE in mixed-MHC bone marrow chimeras

EAE was induced in various mixed-MHC bone marrow chimeras as described in Figure 3.16. Assessment of clinical EAE includes the number of mice that developed disease, the day of disease onset and maximal clinical score among mice with EAE. Similar results were obtained in three independent experiments.

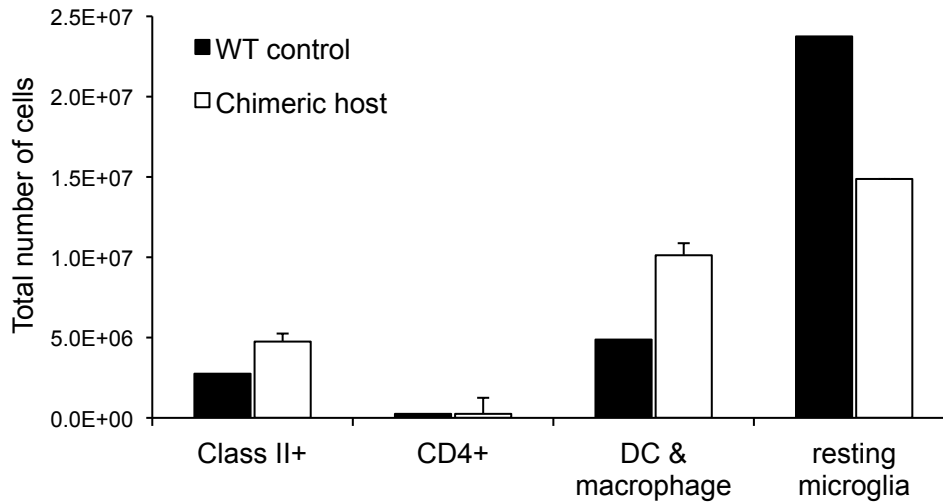


Figure 3.17. Naïve C3H bone marrow chimeras show evidence of CNS inflammation

Mixed MHC bone marrow chimeras were generated as described in Figure 3.16. CNS mononuclear cells were isolated from naïve wildtype (positive control) chimeras 8 weeks post-reconstitution, before EAE was induced. CNS cells were also isolated from naïve C3HeB/Fej wildtype mice as a control. Flow cytometric analysis of CNS cells through the CD45⁺ gate was used to determine the total numbers of MHC class II⁺, CD4⁺, CD11bhi (DC & macrophage), and CD11bint (resting microglia) cells from each mouse. Representative of two independent experiments.

Chapter 4: Concluding remarks and outstanding questions

This work has investigated the role of B cells in CNS autoimmune disease.

Understanding the contributions of B cells to CNS autoimmunity is of great interest due to studies showing the efficacy of B cell depletion with Rituximab in MS patients (107, 108). Rituximab does not deplete plasma cells (107), indicating that B cells play an important non-antibody-mediated role in disease development, perhaps by promoting T cell effector function (112). Rituximab has been shown to deplete B cells from both the periphery and the CSF(111), and it is not clear whether B cells exert a pathogenic effect in one or both compartments. Our studies provide evidence that B cells within the CNS play an important role in the pathogenesis of EAE by contributing to reactivation of the infiltrating T cells. In C3HeB/Fej and C3H.SW mice, we discovered that B cells are important for the initiation of EAE. We observed that B cells are the predominant MHC class II⁺ cell in the healthy CNS and play a crucial role in T cell reactivation and subsequent recruitment within the CNS during preclinical EAE. We also identified a global effect of B cells on both Th1 and Th17 cells, but found that B cells preferentially promote Th1 reactivation and can influence the localization of inflammation.

Previous studies using B cell-deficient (μ MT) mice in EAE reported conflicting results, suggesting that the impact of different B cell functions may vary in different systems. B cells appeared to enhance pathogenicity in EAE models employing certain strain and antigen combinations (115, 117, 118), but exerted a regulatory role or had no impact in other models (113, 116, 118). In our studies, C3H μ MT mice have a significantly reduced incidence of EAE induced with rodent MOG protein, both by active immunization and by adoptive transfer. B cells are important for initiation of EAE in both the C3H.SW (H-2^b) and C3HeB/Fej (H-2^k) strains, even with different immunodominant epitopes presented in these strains (MOG₃₅₋₅₅ or

MOG₇₉₋₉₀ and MOG₉₇₋₁₁₄). The highly reduced incidence of EAE in our model suggested that B cells are important in the initiation of EAE, leading to our focus on the preclinical stages of disease. It is possible that regulatory B cells are less effective in these strains, which allowed us to uncover a role for B cells in reactivation of infiltrating T cells during the initial stages of EAE. By performing the majority of our studies in the adoptive transfer model, we focused on the role of B cells within the CNS.

Surprisingly, B cells are the predominant MHC class II⁺ cells in the healthy CNS. The presence of B cells in the healthy CNS suggests that they could influence the initial effector T cells infiltrating the CNS, possibly through antigen presentation or cytokine production. After onset of EAE, although the total number of B cells increases, the relative proportion of B cells in the MHC class II⁺ gate decreases with the influx of inflammatory monocytes and dendritic cells. Despite this relative decrease, B cells could still play a role in promoting the ongoing course of disease. The majority of B cells in the CNS of both naïve and EAE mice were found to be resting B cells, and we showed that resting splenic B cells are able to present MOG and activate effector T cells *in vitro*.

We also unexpectedly identified a population of plasmablasts in the healthy CNS. We were able to clearly distinguish the two populations of B cells and plasmablasts based on CD138, CD79b, and Class II expression. While the lack of MHC class II expression suggests that the plasmablasts are not functioning as antigen presenting cells, we were able to detect a limited amount of IgG antibody secretion from cells isolated from the healthy CNS. The function of these plasmablasts in the naïve CNS is still unclear and warrants further investigation. We found that the CNS plasmablasts had a different phenotype from plasmablasts found in the spleen and blood, suggesting that the population in the CNS may

have been generated locally. Additionally, the population identified in the healthy CNS was not found in the CNS of mice with EAE, and we instead observed a population of plasma cells. It would be interesting to determine whether the plasmablasts differentiate into plasma cells during EAE, or whether the plasmablasts undergo cell death and the plasma cells are newly differentiated from B cells.

We determined that the B cells in the CNS produced cytokines, and the relative levels of cytokine production suggested that the CNS B cells differ from peripheral B cells. Specifically, B cells in the CNS expressed IL-12 p35 and TNF α , and were the major producers of IL-12 p35 in naïve mice. B cell production of these cytokines may function to promote T cell effector function during the initiation of immune responses in the CNS. IL-12 produced by B cells *in vitro* has been shown to stimulate IFN- γ production from Th1 cells (134, 135). B cells expressed similar levels of IL-12 p35 in both the naïve and EAE CNS. In agreement with this, we did not see an upregulation of IL-12 p35 in the whole brain tissue on day 5 post transfer relative to naïve mice (Table 3.2), and thus were not able to analyze whether IL-12 p35 upregulation was defective in the absence of B cells. IL-12 p35 has not been shown to be significantly upregulated in the CNS until several days after onset of EAE (55). However, because B cells do express IL-12 when the initial T cells enter the CNS, the cytokine could still be influencing their effector function.

TNF α and lymphotoxin (LT) have been shown to be produced in high amounts by stimulated B cells from MS patients, and B cell supernatants promoted CD8⁺ and CD4⁺ T cell proliferation and IFN- γ production in a TNF/LT-dependent manner (112, 129). Although other cytokines have been shown to be produced by highly activated B cells (129, 133, 136), we did not detect IL-6, GM-CSF, IL-1 β , or IL-10 mRNA from unstimulated CNS B cells directly ex

vivo. To confirm that the cytokine production by naïve B cells influences T cell effector function in the CNS, B cell specific genetic depletion of various cytokines would be an important tool. While we did not detect lymphotoxin in the small numbers of cells that we isolated from the CNS, it has been shown to be constitutively expressed by B cells (154). In whole brain tissue from μ MT mice, we did not see a decreased level of lymphotoxin expression compared to wildtype mice; however, we cannot rule out the possibility that lymphotoxin expression by B cells within specific microenvironments may affect T cell function. Lymphotoxin production by B cells is known to promote normal lymphoid architecture development, may contribute to the development of ectopic follicles, and may also support the function of certain cell types including subcapsular sinus macrophages (155). The role of lymphotoxin within the CNS is not yet understood, and further studies are important to define its role in EAE and MS.

The antigen presentation and cytokine production functions of CNS B cells could affect the reactivation of infiltrating T cells, but are not likely to influence their initial migration to the CNS. As expected, upon investigation of the ability of T cells to enter the CNS before onset of EAE, we found that T cells are equally able to migrate to the CNS in μ MT mice. Importantly, B cells do appear to be critical for the initiation of immune responses in the CNS, and the subsequent rapid increase in T cell number that precedes onset of EAE in wildtype mice. This increase, occurring between day 4 and day 7 post-transfer, consisted primarily of donor T cells. We investigated these preclinical processes by analyzing brain mRNA expression just after donor T cells had entered the CNS, to determine the effects of the initial T cell reactivation. The μ MT brains were defective in upregulating expression of T cell cytokines and many other genes, including chemokines and adhesion molecules. Both IL-17- and IFN- γ -induced

chemokines and other cytokines were affected in the absence of B cells. The upregulation of these cytokines and chemokines was critical for the recruitment of additional T cells from the pool of donor cells remaining in the periphery. Accordingly, we found that recruitment, and not proliferation, constituted the majority of the increase in T cell number in the CNS on day 7 in wildtype mice. Effector T cells are capable of producing cytokine upon reactivation without significant proliferation, particularly in the uninflamed CNS, which is a hostile environment for proliferation (156). These data suggest that B cells promote the reactivation and cytokine production of the initial T cells entering the CNS, thus leading to the upregulation of various factors that facilitate rapid recruitment of large numbers of donor T cells from the periphery prior to onset of EAE.

The results described above suggest, but do not conclusively show antigen presentation function by B cells. We attempted to determine whether the antigen presentation function of B cells was required in EAE using bone marrow chimeras, but found that the C3H mice are highly sensitive to irradiation, leading to inflammatory conditions in the reconstituted chimeras even before disease induction that precluded the need for B cells. An earlier study suggested that dendritic cells are sufficient as APCs to initiate T cell responses in EAE (25); however, the requirement for DCs in EAE is still controversial (26, 27, 157). Generating mice on the C3H background lacking MHC class II expression on B cells would allow further studies into the requirement for B cell antigen presentation.

We also examined the effects of B cells on Th1 and Th17 cells *in vitro* and *in vivo*. Our *in vitro* observations showed that naïve B cells can process and present rMOG to effector Th1 cells, but not Th17 cells. We further found that the inability to activate Th17 cells was due to a lack of IL-1 β production by B cells. IL-1 β is known to promote IL-17 production (158), and the

B cells in our studies did not make IL-1 β . A short-term stimulation with LPS and anti-CD40 did not significantly increase the amount of IL-1 β produced by the B cells and correspondingly did not restore their ability to reactivate Th17 cells. In contrast to our data showing preferential B cell reactivation of effector Th1 cells, the quantification of MOG-primed Th1 and Th17 cells in the spleens of wildtype and B cell deficient mice suggested that B cells may be important for the priming of Th17 cells in response to rMOG. Several other studies have also suggested a possible role for B cells in Th17 priming (128, 136). However, we found reduced priming of MOG₉₇₋₁₁₄-specific Th1 and Th17 cells in B cell deficient mice. Additionally, T cell priming is known to be affected by the altered lymphoid architecture in μ MT mice (159), and we therefore cannot draw strong conclusions from our priming data.

In the healthy CNS, B cells are not activated, and although IL-1 β is present in the CNS during ongoing EAE, its expression is not induced until after the initial T cells enter the CNS and become reactivated. This led us to investigate whether Th1- and Th17-induced EAE would be differentially affected by the lack of resting B cells in the CNS. We found that while both Th1- and Th17-induced EAE were affected by the presence of B cells, with significantly reduced incidence and fewer cytokine-producing cells in the CNS, there was a greater effect on Th1-induced EAE and IFN- γ producing cells overall. TNF α production by B cells may promote both Th1 and Th17 effector function, while IL-12 production by B cells may specifically promote Th1 effector function.

B cells do promote both preclinical IL-17 production (in our 1:1 transfers) as well as T cell IL-17 production in EAE (in the Th17 transfers), suggesting that B cells have global effects on T cell activation whether or not they are able to directly reactivate Th17 cells. Additionally, while the ratios that determine localization of inflammation are generated early, B cells may be

able to promote Th17 activation during ongoing disease. Naïve T cells encountering CNS B cells during a more chronic disease course could potentially become Th1 or Th17 cells, as activated B cells were able to generate Th1 and Th17 cells in response to MOG *in vitro* (121). However, there was still a relatively greater loss of IFN- γ producing cells in μ MT mice receiving Th17 cells, although the Th17:Th1 ratio did not change significantly. The wildtype mice receiving Th17 cells had brain inflammation and thus the slightly increased ratio in the brains of μ MT recipients could not affect the clinical manifestation. In contrast, in Th1-mediated EAE, the preferential loss of IFN- γ producing cells in the absence of B cells caused a change in the localization of inflammation, permitting inflammation in the brain. This indicates that B cells can influence lesion localization during initiation of EAE.

Our studies support a pathogenic role for B cells in EAE and MS, and suggest that B cells could be influencing the initiation of T cell responses by antigen presentation or cytokine production (Figure 4.1). Further studies are needed to determine the specific requirements for each of these functions in EAE. Additionally, B cells could play other roles later in disease, such as influencing the formation of ectopic follicles that promote ongoing inflammation. The role for B cells in the initiation of immune responses could be clinically relevant during newly occurring MS relapses. In light of these studies, it would be interesting to determine whether Rituximab treatment could have different effects if Th1 or Th17 cells are dominant. These effects would likely depend on the timing of treatment, as the preferential effect on Th1 cells may be seen early in new relapses. In conclusion, this work has demonstrated a clear role for B cells early in the development of CNS autoimmunity and may lead to a better understanding of how Rituximab treatment can be optimized to treat patients with MS.

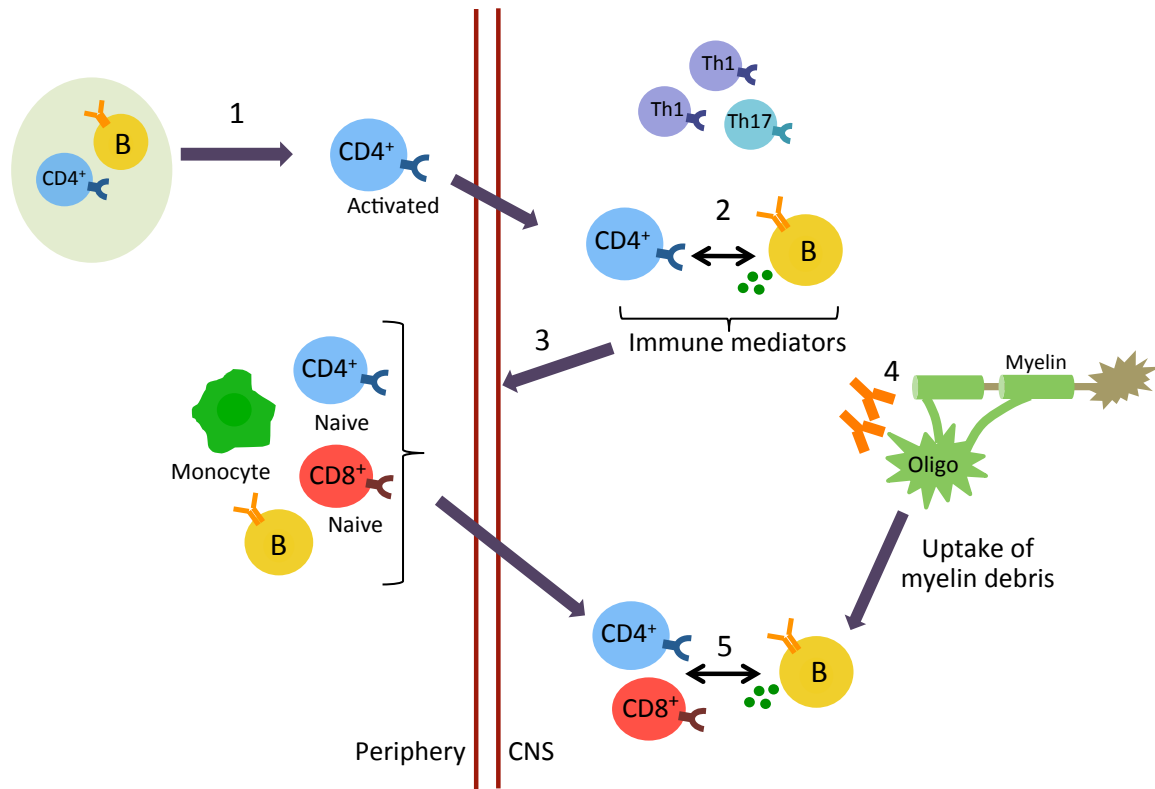


Figure 4.1. Model of the pathogenic roles of B cells in CNS autoimmunity

B cells may influence many of the steps involved in the initiation of inflammation in EAE and MS. (1) B cells can promote T cell priming in the spleen and lymph nodes. (2) Once activated $CD4^+$ T cells enter the uninflamed CNS, they encounter MHC class II⁺ cells, and B cells are the predominant class II⁺ cells. B cells may directly reactivate T cells or cytokine production (IL-12, $TNF\alpha$) by the B cells might promote T cell function. B cells preferentially reactivate Th1 cells in the absence of IL-1 β and may influence the Th17:Th1 ratio, which affects localization of inflammation. (3) Cytokines produced by the T cells in response to reactivation promote activation of endothelial cells at the blood brain barrier, upregulation of chemokines, and subsequent recruitment of additional immune cells from the periphery. (4) Antibodies secreted by plasma cells can mediate demyelination. (5) B cells may influence the activation of naïve T cells entering the CNS later in disease progression, and this activation could take place in ectopic lymphoid follicle structures, whose formation depends in part on lymphotoxin produced by B cells.

Chapter 5: Materials and Methods

Mice

C3HeB/Fej, C3.SW-H-2^b/SnJ (C3H.SW), B6.129S2-Igh-6^{tm1Cgn}/J and B10.PL mice were purchased from The Jackson Laboratory and maintained in a specific pathogen free facility at the University of Washington. To generate μ MT mice, the B6.129S2-Igh-6^{tm1Cgn}/J strain was backcrossed to C3HeB/Fej and C3H.SW for 12 generations. The Institutional Animal Care and Use Committee at the University of Washington approved all procedures.

Recombinant MOG protein and peptides

Recombinant rat MOG protein (1-125) was produced in *Escherichia coli* and purified as previously described (160). MOG peptides 79-90 (GKVALRIQNVRF) and 97-114 (TCFFRDHSYQEEAAVELK) were synthesized by GenScript.

Active EAE Induction

Active EAE was induced by immunizing 8-12 week old mice subcutaneously with 100 μ g of rMOG in complete Freund's adjuvant (CFA) containing 1 mg/ml of heat-killed mycobacteria (Sigma), accompanied by two injections of 200 ng pertussis toxin (List Biological Laboratories), as previously described (23). Animals were observed daily for clinical signs. We scored the severity of EAE as follows: grade 1, paralyzed tail, hindlimb claspings, hyperactivity; grade 2, head tilt, hindlimb weakness; grade 3, one paralyzed leg, mild body leaning; grade 4, two paralyzed legs, moderate body leaning; grade 5, forelimb weakness, severe body leaning; grade 6, hunched, breathing difficulty, body rolling; grade 7, moribund. Atypical EAE was

determined by the presence of one or more of the following symptom(s): hyperactivity, head tilt, body leaning and rolling.

Passive EAE induction

Cells were isolated from spleen and lymph nodes of wildtype mice 7 days after rMOG immunization and cultured at 1×10^7 cells per ml for three days with MOG₉₇₋₁₁₄ (10 μ M). For transfers of 1:1 Th17:Th1 cells, we included 10 ng/ml rIL-23 (R&D). To skew cells toward a Th1 phenotype, we included 10 ng/mL IL-12 (eBioscience). To skew cells toward a Th17 phenotype, we included 10 ng/mL IL-23 (R&D) and 10 μ g/mL anti-IFN- γ (XMG1.2, eBioscience). Viable cells were isolated from a Lympholyte gradient (Cedarlane) and intraperitoneally injected (2×10^7 cells per mouse) into sublethally irradiated (250 rads) mice. For certain experiments, we further purified the CD4⁺ T cells using a CD4⁺ T cell isolation kit and an AutoMACS separator (Miltenyi). 5×10^6 CD4⁺ T cells were intraperitoneally injected into non-irradiated mice. The severity of EAE was scored as described above.

Mouse perfusion

After sedation with 250 μ l of a mixture of ketamine/xylazine (6.5 mg/ml ketamine and 0.44 mg/ml xylazine) and a lethal dose of B Euthanasia (300 μ l of 5 mg/ml), mice were gravity perfused with at least 40 mL cold heparinized PBS using a 23G needle through the left ventricle.

Perfusion control

Splenocytes isolated from naïve wildtype mice were labeled with CFSE (1 μ M, Molecular Probes) and 2×10^7 cells were transferred i.v. into naïve recipients. Half of the mice were perfused as described above, while the other half were not perfused, and CNS mononuclear cells were isolated from all mice. Cells were analyzed for expression of CD45, CD19, and CFSE to determine whether cells observed in the perfused CNS were contaminating cells from the blood.

Immunohistochemistry

We stained 7- μ m frozen sagittal sections from perfused CNS tissue for F4/80 (BM8, Invitrogen) and used Vectastain (Vector) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma) for detection. For image analysis, we stained four sections per mouse, encompassing each region of the brain and spinal cord, and photographed lesions using PixelLink digital-camera software. The inflammatory index was calculated by grading the level of F4/80⁺ cellular infiltration in each region (1, leptomeningeal infiltration; 2, submeningeal infiltration; 3, moderate parenchymal infiltration; 4, severe parenchymal infiltration) and then adding together the grades from each region for a total index for the brain or spinal cord of each mouse.

Isolation of CNS mononuclear cells

Mononuclear cells were isolated from the CNS after cardiac perfusion with PBS as previously described (161). Briefly, brain and spinal cord were dissociated through sterile stainless steel mesh and centrifuged at 4°C for 10 min at 3000 rpm. Cell pellets were then resuspended in

30% Percoll, overlaid onto 70% Percoll, and centrifuged without brake at 20°C for 20 min at 2600 rpm. Cells were collected from the 30%-70% Percoll interface.

Flow cytometry

Cells were incubated with Fc block (clone 2.4G2; eBioscience) in 5% normal mouse serum for 15 min at room temperature, washed and stained with mAbs for 30 min at 4°C. mAbs for CD45 (30-F11), CD19 (1D3), F4/80 (BM8), CD11b (M1/70), CD11c (N418), CD40 (1C10), and CD4 (GK1.5 and RM4-4) were from eBioscience. mAbs for MHC class II (I-A^k; 11-5.2), CD4 (RM4-5), Thy1.1 (OX7), CD79b (HM79b), IgM (DS-1), CD138 (281-2), CD80 (16-10A1), CD86 (GL1), and CD43 (S7) were from BD Biosciences. BrdU and AnnexinV staining kits were purchased from BD Biosciences.

CD4 antibody blocking assay

Thy1.1⁺ T cells from MOG-immunized donors were activated *in vitro* for three days and transferred into wildtype or μ MT Thy1.2 recipients. On day 4 post transfer, unlabeled anti-CD4 antibody (clone GK1.5) was injected i.v. into recipient mice (50 μ g per mouse). CNS mononuclear cells were isolated after 10 minutes and analyzed with labeled anti-CD4 antibody clones GK1.5 and RM4-4.

FTY720 injection

Thy1.1⁺ T cells from MOG-immunized donors were activated *in vitro* for three days and transferred into wildtype Thy1.2 recipients. Starting on day 4 post-transfer, 3 mg/kg FTY720 or

vehicle (5% DMSO) was injected intraperitoneally daily. CNS mononuclear cells were isolated from mice on day 7 for analysis.

Enzyme-linked immunosorbent spot assays

Cells were plated in duplicate wells of 96-well Enzyme-linked immunosorbent spot (ELISPOT) plates (Millipore) and ELISPOT assays were carried out according to BD Biosciences protocols and analyzed on an ImmunoSpot Analyzer (CTL). IFN- γ -specific mAb pairs, IL-17-specific (TC11-18H10) and biotinylated IL-17-specific (TC11-8H4.1) mAbs were from BD Biosciences. Background spots obtained by plating T cells in the absence of exogenous antigen were subtracted from the total number of spots with antigen. For detection of IL-17 and IFN- γ producing cells in the CNS of mice with EAE, total mononuclear cells isolated separately from the brains and spinal cords of perfused mice (typically $1-10 \times 10^5$ cells per well) were plated with or without MOG₉₇₋₁₁₄. For co-cultures of Th1 and Th17 effector cells with either CNS or spleen APCs, T cells were plated at 5×10^4 cells per well, spleen APCs were plated at 5×10^5 cells per well, and CNS APCs were plated to normalize the number of non-B cell APCs to 5000 cells per well.

Th17:Th1 ratio

The Th17:Th1 ratio is determined by restimulating brain or spinal cord mononuclear cells in an ELISPOT plate overnight with or without MOG₉₇₋₁₁₄ (10 μ M) and calculating numbers of antigen-specific IL-17 and IFN- γ spots.

Anti-IgG ELISPOT

CNS mononuclear cells were isolated from naïve mice or mice with EAE and plated in duplicate wells of 96-well Enzyme-linked immunosorbent spot (ELISPOT) plates (Millipore) and ELISPOT assays were carried out according to BD Biosciences protocols and analyzed on an ImmunoSpot Analyzer (CTL). Anti-IgG capture and detection antibodies were used for detection of IgG secretion.

T cell priming

Wildtype or μ MT mice were immunized with rMOG in CFA and injected with pertussis toxin. Splenocytes were isolated 7 days after immunization and plated at 1×10^6 cells per well in anti-IL-17 or anti-IFN- γ coated wells of an ELISPOT plate with or without rMOG (20 μ g/ml), MOG79-90 (10 μ M), or MOG97-114 (10 μ M). After 16 hours culture, IL-17 and IFN- γ producing cells were detected as described above.

Th1 and Th17 cell generation for co-cultures

Cells were isolated from spleen and lymph nodes of wildtype mice 7 days after rMOG immunization and cultured at 1×10^7 cells per ml for three days with MOG₉₇₋₁₁₄ (10 μ M). For Th1-skewed cells, we included 10 ng/mL IL-12 (eBioscience). For Th17-skewed cells, we included 10 ng/mL IL-23 (R&D) and 10 μ g/mL anti-IFN- γ (XMG1.2, eBioscience). Cells were split after 3 days and maintained in culture without MOG₉₇₋₁₁₄ for an additional 4 days with the addition of 10 U/ml IL-2. Viable cells were isolated from a Lympholyte gradient, stained for CD4, and sorted on a FACS Aria cell sorter (BD).

Th1/Th17 reactivation

Effector Th1 and Th17 cells were generated and rested down as described above. CD4⁺ T cells and naïve spleen B cells (CD19⁺ CD43⁻) or non-B cell APCs (CD11c⁺ and CD11b⁺) were then FACS-sorted. Purified CD4⁺ Th1 or Th17 cells were co-cultured overnight with B cells, non-B cell APCs (DCs and macrophages), or unsorted splenocytes, with or without 25 µg/ml rMOG. Where indicated, APCs were stimulated with 10 µg/mL anti-CD40 (R&D) and 20 µg/ml LPS for 4 hours prior to culture. We included the cytokines IL-23 (10 ng/ml, R&D), IL-6 (20 ng/ml, eBioscience), and IL-1β (10 ng/ml, eBioscience), and neutralizing anti-IL-1R antibody (20 µg/ml, BD, 35F5) as indicated. Antigen-specific IL-17 or IFN-γ production was detected by ELISPOT as described. The percent reactivation was calculated from the number of antigen-specific spots produced by either Th1 or Th17 cells in response to the indicated APC and normalized to the number of antigen-specific IFN-γ or IL-17 spots obtained by co-culture of either Th1 or Th17 cells with unsorted, naïve splenocytes (100%).

IL-1β ELISA

Supernatants from B cell and T cell co-cultures were plated in 96 well plates coated with anti-IL-1β (B122, eBioscience) and incubated overnight at 4 degrees. IL-1β was detected with biotinylated anti-IL-1β (eBioscience) and Streptavidin-HRP. Recombinant IL-1β (eBioscience) was used as a standard.

Generating polyclonally-activated T cells for PCR controls

CD4⁺ T cells were isolated from naïve splenocytes using CD4⁺ T cell isolation kits and an AutoMACS separator (Miltenyi). 2×10^7 CD4⁺ cells were cultured at 1×10^6 cells/ml with anti-

CD3/anti-CD28 Dynabeads for 48 hours according to Invitrogen protocols. After beads were removed, viable cells were adoptively transferred (5×10^6 per mouse) into sublethally irradiated mice. Mice were perfused 5 days post transfer and CNS tissue was harvested for RNA isolation.

CNS B cell sorting

CNS mononuclear cells were isolated from perfused naïve wildtype mice or from mice at onset of EAE induced by adoptive transfer of CD4⁺ MOG-specific T cells (1:1 Th17:Th1) into non-irradiated recipients. Tissue was pooled from 3-5 mice, and approximately 1×10^5 B cells (CD45^{hi} CD19^{hi} CD11b⁻), plasmablasts (CD45^{int} CD19^{int} CD11b⁻), CD45⁺ CD11b⁺/CD11c⁺ cells, and microglia (CD45^{int} CD11b⁺) were purified from CNS tissue using a FACS Aria cell sorter (BD). B cells were also purified from the spleen and blood of naïve mice. Cell pellets were immediately snap frozen for RNA isolation.

Real-time RT-PCR

From sorted CNS cells, total RNA was isolated using the RNeasy Micro kit (Qiagen). From brain tissue isolated from mice on day 5 after adoptive transfer, total RNA was isolated using the RNeasy Lipid Tissue Midi kit (Qiagen). cDNA was generated using the Superscript III first strand synthesis system (Invitrogen). Real time quantitative PCR was performed in triplicate using SYBR Green PCR master mix and an AB7300 or Viia7 (Applied Biosystems). All data were normalized to *GAPDH*. Data were analyzed using the comparative Ct method to obtain relative quantitation values. Fold induction for day 5 brain tissue was calculated relative to

healthy brain tissue from mice that had received anti-CD3/anti-CD28 activated T cells. Mouse primer sequences are listed in Table 3.2.

Mixed MHC bone marrow chimeras

Cells (1×10^7) were isolated from femurs of C3HeB/Fej (H-2^k) wildtype or μ MT and C3H.SW (H-2^b) wildtype mice. T and B cells were depleted using Dynabeads (Invitrogen). 5-10 $\times 10^6$ cells at a 2:1 SW:Fej ratio were transferred i.v. on day 0 into lethally irradiated (1,000 rads on day -1) C3HeB/Fej x C3H.SW F1 hosts. Recipients were provided neomycin sulfate (2 mg/ml; Sigma) in their drinking water from day -2 to day 21. 8 weeks post-reconstitution, mice were used for adoptive transfer EAE induction of CD4⁺ MOG-specific T cells from C3HeB/Fej donors.

Statistics

Statistical analysis was performed using Prism software (GraphPad). Significance between groups was determined using either Fisher's exact test, Chi squared test, Student's *t* test, or Mann Whitney non-parametric *t* test, as indicated. A *P* value of less than 0.05 was considered significant.

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