LYMPHOCYTES IN MS AND EAE: MORE THAN JUST A CD4+ WORLD

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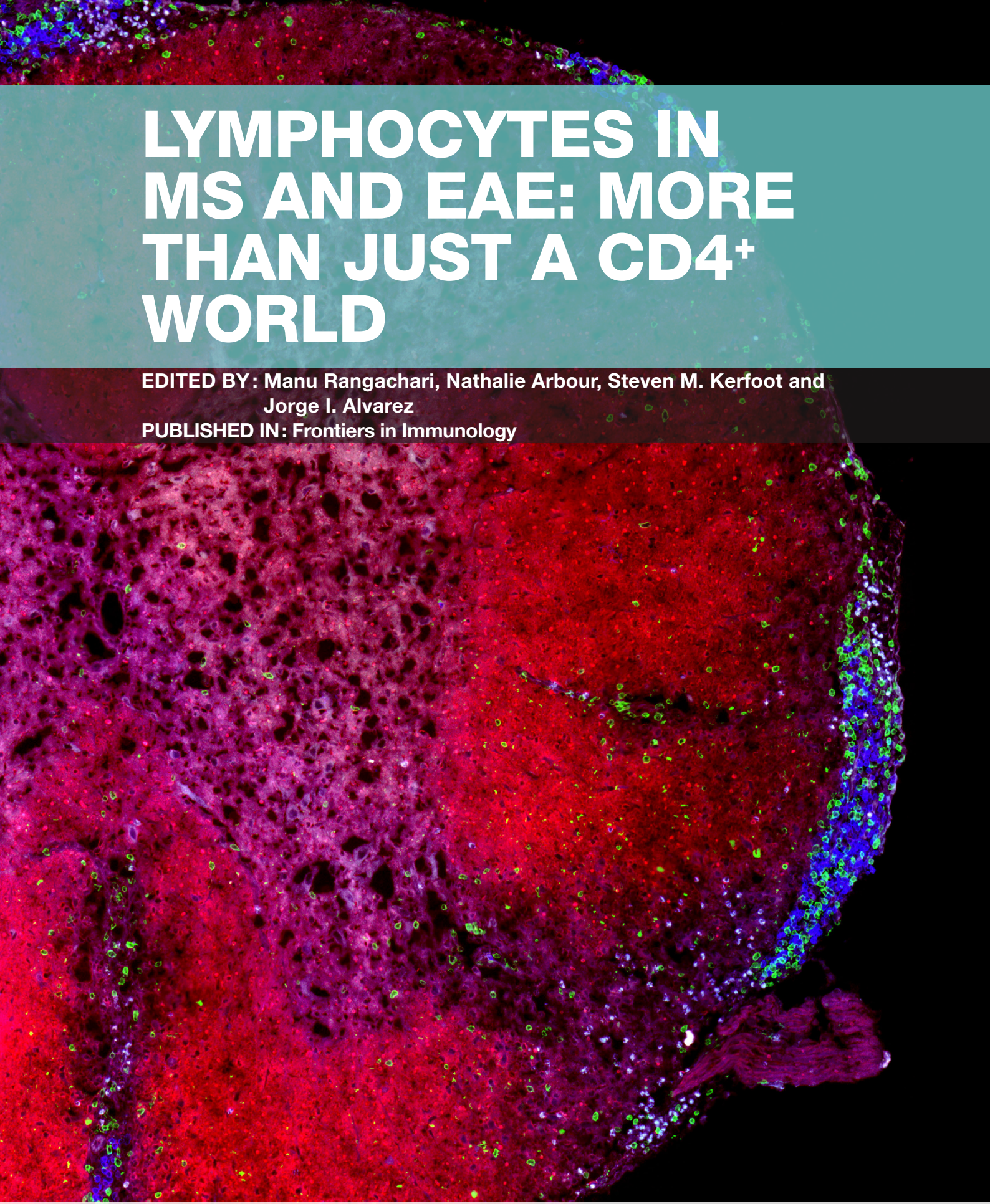

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LYMPHOCYTES IN MS AND EAE: MORE THAN JUST A CD4$^+$ WORLD

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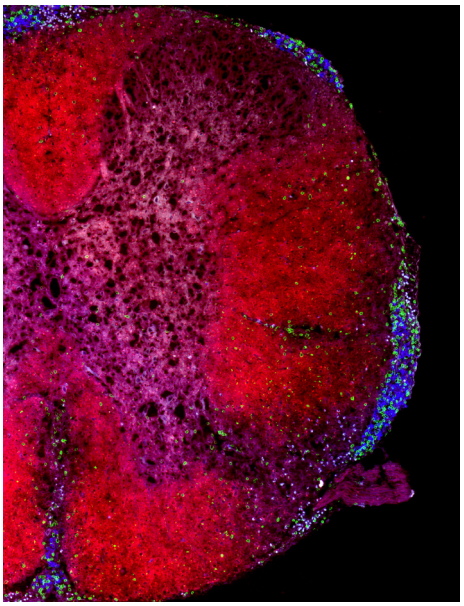
Multiple sclerosis is degenerative disease of the central nervous system (CNS) in which myelin destruction and axon loss leads to the accumulation of physical, cognitive, and mental deficits. MS affects more than a million people worldwide and managing this chronic disease presents a significant health challenge. Multiple lines of evidence indicate that MS is an autoimmune disorder in which immune cells launch an inflammatory attack targeting myelin antigens. Indeed, myelin-reactive T cells and antibodies have been identified in MS patients and in animal models (namely experimental autoimmune encephalomyelitis, or EAE) that recapitulate many features of human disease. Animal model studies have demonstrated that T cells are both necessary and sufficient to initiate and sustain CNS autoimmunity. However, most MS animal models rely on the role played by CD4$^+$ T cells and partially replicate the multiple aspects of MS pathogenesis. Thus, research in the past has focused heavily on the contribution of CD4$^+$ T cells to the disease process; searching PubMed for "MS AND CD4" yields twice the results as corresponding searches for "CD8" or "B cell" and four times that for "NK cells".

While CD4$^+$ T cells may represent the minimum requirement to mediate CNS autoimmunity, it is clear that the immune response underlying human MS is far more complex and involves numerous other immune cells and subsets. This is well illustrated by the observation that MS patients treated with an anti-CD4 depleting antibody did not gain any clinical benefits whereas removal of several lymphocyte subsets using an anti-CD52 depleting antibody has been shown to impede disease progression. In particular, the pathogenic role(s) of non-CD4$^+$ T cell lymphocytes is relatively poorly understood and under-researched, despite evidence that these subsets contribute to disease pathology or regulation. For example, the observed oligoclonal expansion of CD8$^+$ T cells within the CNS compartment supports a local activation. CD8$^+$ T cells with polarized cytolytic granules are seen in close proximity to oligodendrocytes and demyelinated axons in MS tissues. The presence of B cells in inflammatory lesions and antibodies in the CSF have long been recognized as features of MS and Rituximab, a B cell depleting therapy, has been shown to be highly effective to treat MS. Intriguingly, the putative MS therapeutic reagent Daclizumab may function in part through the expansion of a subset of immunoregulatory NK cells. NKT and γδ T cells may also play a role in CNS autoimmunity, given that they respond to lipid antigens and that myelin is lipid-rich. While different animal



Spinal cord inflammation and pathology in a spontaneous model of experimental autoimmune encephalomyelitis in mice. Mice expressing mutant T and B cell receptors against myelin oligodendrocyte glycoprotein (MOG) developed signs of autoimmune disease at approximately 5 wks of age (See Dang et.al. in this issue). Immune cell infiltration of spinal cords was assessed by immunofluorescent histology. B cells (B220, Blue) were largely restricted to the meninges, often forming clusters adjacent to regions of reduced myelin (Red) staining. T cells (CD3, Green) were found in association with B cells and throughout the grey and white matter, concentrating in demyelinating lesions.

Image by Steven Kerfoot

models recapitulate some of these aspects of human disease, identifying appropriate models and measures to investigate the role of these less well-understood lymphocytes in MS remains a challenge for the field.

This Frontiers research topic aims to create a platform for both animal- and human-focused researchers to share their original data, hypotheses, future perspectives and commentaries regarding the role of these less-well understood lymphocyte subsets ($CD8^+$ T cells, B cells, NK cells, NK T cells, γδ T cells) in the pathogenesis of CNS autoimmunity.

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EDITORIAL
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Editorial: Lymphocytes in MS and EAE: More Than Just a CD4$^+$ World

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Keywords: multiple sclerosis, experimental autoimmune encephalomyelitis, CD4$^+$ T cell, CD8$^+$ T cell, B cell, gamma-delta T cell, MAIT cell, NK cell

Editorial on the Research Topic

Lymphocytes in MS and EAE: More Than Just a CD4$^+$ World

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Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) that affects nearly two million people worldwide. Disease onset can occur at a young age, leaving sufferers with a significantly reduced quality of life. The fact that there is no cure for MS, plus the fact that its animal model experimental autoimmune encephalomyelitis (EAE) is considered a "classic" model of CD4$^+$ T cell-triggered autoimmunity, has led MS pathogenesis to be an area of intense investigation in the past few decades. Numerous lines of evidence indicate that MS is driven by CD4$^+$ T lymphocyte-mediated mechanisms (1–3). Polymorphisms in the HLA class II region are by far the strongest genetic link to MS (4). Moreover, the majority of currently available MS disease-modifying therapies are believed to act by modulating inflammatory CD4$^+$ T cell responses, although in many cases the effects on other immune cell types are under-studied and may be at least as important. Furthermore, while current immunomodulatory drugs are able to reduce the frequency and severity of MS relapses, they are relatively ineffective in progressive forms of the disease (5). Attempted blockade of CD4$^+$ T cells using anti-CD4-depleting antibody therapy did not produce clinical benefits to patients with MS (6). In contrast, more global immunosuppressive or immunomodulatory approaches reduce the number of relapses and disease progression of MS patients (7). Furthermore, numerous publications have presented data generated from autopsy material obtained from human patients supporting the notion that other cell types are involved (8–11). There is thus an urgent need to expand our view of immune-related pathogenesis in human disease. Despite evidence that lymphocytes such as B cells and CD8$^+$ T cells play a role, their contributions are much less well studied experimentally compared to those of CD4$^+$ T cells. In this Special Topic, we promote this expanded view of disease pathogenesis by presenting articles that examine the role played by lymphocytes other than CD4$^+$ T cells in MS and its experimental models.

The B cell-depleting reagents rituximab and ocrelizumab have shown success against relapsing/remitting (12) and even progressive MS (13). Thus, it is no surprise that this collection features several submissions considering different aspects of potential B cell contributions to CNS autoimmunity. Both Claes et al. and Michel et al. survey what is known about B cells in MS and discuss potential pathogenic mechanisms including antibody production, cytokine secretion, antigen presentation to T cells, and the promotion of disease from within the CNS. Claes et al. additionally present a detailed review of how B cell subpopulations and effector functions are altered both by broadly

specific disease-modifying therapies such as interferon-beta and glatiramer acetate as well as by rituximab and ocrelizumab. B cell infiltration into the CNS in MS was a particular focus of the Michel et al. review, and this was further expanded upon by two additional reviews in this special topic. Blauth et al. describe the signals that may permit B cell entry to the CNS, such as CXCL13, VLA-4, and ICAM. Additionally, they discuss recent evidence suggesting that B cells can also exit the CNS so as to undergo additional affinity maturation in peripheral lymphoid tissues, as well as data indicating that the meninges can support differentiation of CNS-specific B cells independently of the periphery. Once in the CNS, B cells have been described to form aggregates in the meninges akin to tertiary lymphoid organ-like structures, and these are the subject of a review from Pikor et al. In particular, they discuss recent evidence suggesting that antigen-experienced T and B cells accumulate in these structures to promote CNS inflammation.

A common theme of these reviews is the uncertainty regarding the neuropathogenic role of B cells in MS. Interestingly, evidence from the two primary research articles in this issue present a challenge to the hypothesis that autoreactive B cell responses are propagated within meningeal aggregates of lymphocytes. First, in an attempt to identify antigenic targets of B cell-mediated destruction in MS, Willis et al. cloned the IgV heavy and light chains of CNS-infiltrating B cell clones from six MS patients to generate putative CNS-reactive recombinant antibodies. Surprisingly, using various approaches (binding to candidate antigen, CNS cell lines, or antigen array) no CNS- or MS-specific antigen targets could be identified. Second, Dang et al. present data showing that the presence of B cells in spinal cord-associated meningeal clusters correlates with chronic symptoms in a B cell-dependent model of spontaneous EAE. Intriguingly, however, these "cluster B cells" have a naïve phenotype with little evidence of Ig class switching and the clusters themselves do not bear the features of structured lymphoid follicles, suggesting that the simple presence of B cells in the meninges is sufficient to promote disease progression.

The remaining B cell-centric articles in this issue focus on other potential pathogenic mechanisms. B cells almost certainly shape the autoimmune response through the secretion of inflammatory cytokines such as TNFα, lymphotoxin, and GM-CSF, as described by Li et al. In addition, B cells can present antigen to T cells and modulate their properties. Márquez and Horwitz discuss the possibility that Epstein-Barr virus (EBV)-infected B cells preferentially elicit Th1 responses in the CNS. It is tempting to speculate that exposure to EBV, which is an environmental factor strongly associated with MS (14), influences disease outcomes by altering B cell activity. Furthermore, the effectiveness of B cell depletion therapy in MS may be due in part to reduced effector T cell function. While the "immune helper" functions of B cells in MS have been intensely investigated, the classic role of B cells as antibody-secreting cells cannot be neglected. Indeed, the presence of oligoclonal IgG bands in CSF has long been a clinical biomarker of MS and is still used as a differential diagnostic tool (15). Khorooshi et al. detail what is known about antibody-mediated pathogenic mechanisms in CNS autoimmunity. They pay particular attention to the role of anti-aquaporin 4 antibodies in neuromyelitis optica—a disease that has only recently been recognized as being independent of MS. They present a scheme in which these antibodies cross a disrupted blood–brain barrier and target astrocytes for complement-mediated destruction in a T cell-independent manner.

$CD8^+$ T cells are present in MS tissue at all stages of disease. They can greatly outnumber $CD4^+$ T cells in lesions, perivascular cuffs, and normal-appearing white matter. Furthermore, unlike $CD4^+$ T cells that mostly remain restricted to the perivascular space, $CD8^+$ T cells infiltrate deep into the CNS parenchymal lesions (16). Salou et al. delineate some of the current lines of investigation into the role of $CD8^+$ T cells in MS, such as ongoing efforts to characterize the antigenic repertoire of these cells, as well as recent advances in the study of $CD8^+$ T cells using EAE and the importance of IL-17-producing $CD8^+$ T cells in pathogenesis. Yang et al. describe the pathogenic function of $CD8^+$ T cells in peripheral neuropathies such as Guillain–Barré syndrome, with a particular emphasis on a mouse model that features $CD8^+$ T cell-driven inflammation in the peripheral sciatic, trigeminal, and facial nerves. By contrast, Sinha et al. argue that the role of regulatory $CD8^+$ T cells (T_{reg}) in CNS autoimmunity deserves further attention. Importantly, the frequency of $CD8^+$ T_{reg} is diminished during MS relapse, and the authors discuss findings suggesting that the drug glatiramer acetate may act, in part, by augmenting the $CD8^+$ T_{reg} response. Finally, Ignatius Arokia Doss et al. describe a transgenic mouse strain (1C6) in which both $CD4^+$ and $CD8^+$ T cells bear T cell receptor specificity to myelin antigen. The 1C6 transgene is on the NOD background, on which mice develop a relapsing-to-progressive pattern of EAE that models the form most commonly seen in human MS. The authors propose an approach in which 1C6 $CD8^+$ T cells are stimulated *ex vivo* with distinct differentiation stimuli, prior to adoptive transfer. This would allow one to dissect the relative contributions of IFNγ-producing, IL-17-positive, and potentially even regulatory $CD8^+$ T cells to CNS autoimmunity.

While B cells and $CD8^+$ T cells represent two-thirds of the lymphocytic "Holy Trinity," there is an array of other lymphocyte subsets with innate immune-like properties that have been posited to play a role in MS. Edwards et al. and Malik et al. provide an overview of what is known about γδ T cells in MS and EAE, respectively. These cells, which are found principally in skin and mucosal tissues, readily produce IL-17-associated cytokines and thus may be important mediators of inflammation in the CNS. The role of natural killer cells in disease may be Janus-like, with inflammatory $CD16^+CD56^{dim}$ cells being increased during MS relapse while the $CD16^{dim/-}CD56^{bright}$ subset predominates during remission (Edwards et al.). Treiner and Liblau report what is known about recently identified mucosal-associated invariant T (MAIT) cells. These are lymphocytes with innate properties that, as their name suggests, are located in mucosal tissues such as in the gut. While studies in mouse EAE have suggested that MAIT cells are anti-inflammatory, the picture is less clear in humans as MS immunotherapy appears to affect the frequency of peripheral MAIT cells. However, as MAIT cells may proliferate in response to commensal microbial antigens (17), it is tempting to speculate that they may provide part of the answer to the question of

how the microbiome can influence autoimmune inflammatory diseases such as MS.

The past decades have seen remarkable progress in unraveling the complex and diversified immune mechanisms that contribute to MS pathobiology. Nevertheless, the precise etiology of MS remains elusive. Furthermore, despite an increasing number of immunomodulatory or immunosuppressive therapies altering relapsing-remitting MS, there is a pressing need for effective treatments for progressive disease. The more expanded view of disease pathology presented in this Special Topic may prove to be the key for the next generation of MS therapies.

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ORIGINAL RESEARCH
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Meningeal infiltration of the spinal cord by non-classically activated B cells is associated with chronic disease course in a spontaneous B cell-dependent model of CNS autoimmune disease

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We characterized B cell infiltration of the spinal cord in a B cell-dependent spontaneous model of central nervous system (CNS) autoimmunity that develops in a proportion of mice with mutant T and B cell receptors specific for myelin oligodendrocyte glycoprotein. We found that, while males are more likely to develop disease, females are more likely to have a chronic rather than monophasic disease course. B cell infiltration of the spinal cord was investigated by histology and FACs. $CD4^+$ T cell infiltration was pervasive throughout the white and in some cases gray matter. B cells were almost exclusively restricted to the meninges, often in clusters reminiscent of those described in human multiple sclerosis. These clusters were typically found adjacent to white matter lesions and their presence was associated with a chronic disease course. Extensive investigation of these clusters by histology did not identify features of lymphoid follicles, including organization of T and B cells into separate zones, $CD35^+$ follicular dendritic cells, or germinal centers. The majority of cluster B cells were IgD^+ with little evidence of class switch. Consistent with this, B cells isolated from the spinal cord were of the naïve/memory $CD38^{hi}$ $CD95^{lo}$ phenotype. Nevertheless, they were $CD62L^{lo}$ and $CD80^{hi}$ compared to lymph node B cells suggesting that they were at least partly activated and primed to present antigen. Therefore, if meningeal B cells contribute to CNS pathology in autoimmunity, follicular differentiation is not necessary for the pathogenic mechanism.

Keywords: B cells, EAE, demyelination, inflammation, meninges

Introduction

The best evidence supports the hypothesis that multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) driven by immune cells targeting myelin antigens. The autoimmune response results in chronic inflammation of the CNS, demyelination, destruction of axons, and neurodegeneration over an extended period of time (1, 2). The presence of infiltrating immune cells in MS CNS tissues, including potentially myelin-specific T and B cells, supports a primary immune

etiology for disease (3–6), as do studies that have identified genes associated with the immune system, immune regulation, and antigen presentation as the primary genetic risk factors for MS (7). Deliberate induction of myelin-targeting autoimmunity in animal models results in CNS inflammation and pathology that recapitulates some features of human MS, although the degree to which these models resemble human disease varies depending on how autoimmunity is induced and on the species or strain of animal used (8–10).

Outside of the laboratory, the vast majority of immune responses incorporate antigen targeting by multiple subpopulations of both T and B cells. However, in recent decades, research interest has focused heavily on $CD4^+$ T cells and this is particularly true of MS research. Considerable advances in our understanding of how B cells collaborate with $CD4^+$ T cells during the initiation and development of a response highlight the importance of understanding immune responses as a whole. When both T and B cells are involved in target recognition, germinal center (GC) formation is the typical result. GCs are the source for long lived, high affinity B cell and antibody responses fundamental to normal, complex protective, and pathogenic immunity (11). While best known for their role in antibody production, it is becoming increasingly apparent that B cells are also important regulators and modulators of the immune response through the production of cytokines (12, 13) and presentation of antigen to T cells (14). As antigen-presenting cells (APCs), B cells very efficiently take up antigen that binds to their specific B cell receptor (BCR) and process it for presentation to T cells. The resulting "cognate" interactions between T and B cells specific for the same or physically linked antigen are foundational to the development of most complex immune responses (15, 16), almost certainly including those underlying organ-specific autoimmune diseases like MS (4).

Considerable evidence supports an important role for B cells in addition to T cells in driving MS pathology. The presence of antibodies in the cerebrospinal fluid (CSF) and B cells in the CNS infiltrate has long been recognized as features of disease (4, 5). More recently, targeted depletion of CD20-expressing B cells using humanized anti-CD20 antibodies was shown to very effectively reduce inflammatory signs and relapses in MS (17). This ignited interest in B cells as therapeutic targets. However, a more recent trial of a soluble recombinant version of the cytokine receptor TACI (TACI-Fc), which depletes B cells through the inhibition of the cytokines BAFF and APRIL, was halted early due to indications that treatment increased relapse rate (18). Anti-CD20 and TACI-Fc deplete B cells through very different mechanisms and target different subsets of cells (19). CD20 is not expressed by antibody-producing plasma cells and therapeutic benefit of anti-CD20 was observed well prior to any reduction in antibody levels. By contrast, TACI-Fc does target plasma cells in addition to mature B cells. Further, as both T cells and neurons express receptors for BAFF (20, 21), the effects of this drug likely extend well beyond B cell depletion. Therefore, while antibodies may contribute to pathology (1), the primary B cell contribution to MS is through some other mechanism(s), perhaps via APC function or cytokine modulation of the autoimmune response. Furthermore, the effectiveness of B cell depletion via anti-CD20 (17) suggests that their pathogenic role is ongoing and drives chronic disease. The lack of benefit and perhaps pathogenic effects of TACI-Fc confuses the issue, and highlights the need to identify pathogenic and protective B cell subpopulations and their roles in CNS autoimmunity.

It is also not clear where, anatomically, B cells exert their pathogenic function. There has been considerable recent interest in clusters of B and T cells observed in the meninges in post-mortem studies of MS brains, often in direct association with demyelinating lesions (3, 6, 22). Some studies have focused on the potential similarity of these structures to secondary lymphoid organs, suggesting that they may perform similar functions in propagating immune responses from within the inflamed CNS (23, 24). Understanding the pathogenic contributions of B cells and the role played by meningeal clusters to ongoing disease will require models that appropriately recapitulate a complex anti-myelin immune response.

Animal models with induced anti-myelin autoimmunity are referred to by the umbrella term "experimental autoimmune encephalomyelitis" (EAE). Currently, the most commonly used versions of this model are induced through immunization with short peptides mimicking dominant $CD4^+$ T cell epitopes derived from myelin protein antigens. By their design, these models severely limit the involvement of other lymphocytes, including B cells that would normally participate in antigen targeting. Immunization with larger protein antigens can overcome this limitation (25). Alternatively, B cells may contribute to several non-immunization-based models. CNS autoimmunity can develop "spontaneously" (sEAE) in a proportion of mice with enhanced anti-myelin activity due to expression of mutant antigen-specific receptors (26–28). In most cases, enhanced anti-myelin immunity is restricted to T cells. However, two groups (29, 30) independently reported that disease occurs with much greater incidence in mice expressing both a transgenic T cell receptor (TCR) specific for myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide (26) and a BCR heavy chain knock-in mice that, when paired with an appropriate light chain, also confers specificity for MOG protein on ~20% of B cells (31). This model, with predetermined B as well as T cell recognition of the myelin autoantigen, may therefore be valuable for investigations of the B cell collaboration with autoimmune T cells to promote CNS autoimmune disease.

Here, we characterize B cells participating in spontaneous CNS autoimmune disease in 2D2 IgH^{MOG} mice. We observe that sEAE can follow either a monophasic or chronic disease course and that this correlates with ongoing inflammation in the spinal cord and with formation of meningeal clusters of T and B cells in particular. However, we found little evidence that the B cells in meningeal clusters are activated in a conventional sense. Finally, only in a very rare case did we find any evidence of development of follicular features in meningeal clusters, indicating that if clusters do contribute to ongoing pathology in CNS autoimmunity, the relatively unorganized form must represent the minimum requirement for disease.

Materials and Methods

Mice

Wild type C57Bl/6 and 2D2 TCR transgenic (26) mice were purchased from Jackson Laboratories. IgH^{MOG} MOG-specific BCR knock-in mice (32) were received as a gift from Dr. Hartmut Wekerle. Genotyping was accomplished using the following primers: 2D2: f-GCG GCC GCA ATT CCC AGA GAC ATC CCT CC, r-CCC GGG CAA GGC TCA GCC ATG CTC CTG; IgH^{MOG}: f-GGA TTG CAC GCA GGT TCT CCG, r-CCG GCC ACA GTC GAT GAA TCC. All mice were housed under specific pathogen-free conditions at the West Valley Barrier Facility at Western University Canada. Animal protocols (#2011-047) were approved by the Western University Animal Use Subcommittee.

Antibodies for Flow Cytometry and Histology

The following antibodies were purchased from BD Biosciences: anti-CD4-V450 (RM4-5), anti-CD45R-V450 (RA3-6B2), anti-CD45R-APC-Cy7 (RA3-6B2), anti-CD45R-A647 (RA3-6B2), anti-CD138-BV421 (281-2), anti-CD19-BV711 (1D3), anti-CD95-PE-Cy7 (Jo2), anti-Bcl6-A647 (K112-91), anti-IgG1-APC (A85-1), anti-CD62L-A700 (MEL-14), anti-CD35-biotin (8C12), anti-CD49D (R1-2), anti-CD62P (RB40.34), anti-IgM-APC (II/41), and anti-CD80-PE (16-10A1). The following antibodies were purchased from BioLegend: anti-CD4-A647 (RM4-5), anti-CD3e-FITC (145-2C11), and anti-rabbit DyLight 649 (Poly4064). The following antibodies were purchased from eBioscience: anti-CD4-PE-Cy5 (RM4-5), anti-PNAd-A488 (MECA-79), anti-CD38-PE (90), anti-IgM-PE-Cy5 (II/41), anti-IgD-APC (11-26c), anti-IgD eF450 (11-26c), anti-CD279-Biotin (RMP1-30), anti-CD273-Biotin (TY25), anti-F4/80-Biotin (BM8), Streptavidin-APC-eF780, and Streptavidin-APC. Anti-Ki-67 (SP6) unconjugated was purchased from Thermo Scientific. FluoroMyelin Red for myelin staining was purchased from Invitrogen.

Spontaneous 2D2 IgH^{MOG} EAE Model

$2D2^{+/-}$ $IgH^{MOG+/-}$ double mutant mice were generated as the F1 generation of $2D2^{+/-}$ mice crossed with $IgH^{MOG+/+}$ mice. Where indicated, some mice received a single i.v. injection of 250 ng pertussis toxin (PTX – List Biological Laboratories, Inc.) between 31 and 33 days of age. Clinical disease was monitored daily and was scored as follows: 0, no clinical signs; 1, tail paralysis; 2, tail paralysis and hind limb weakness; 3, hind limb paralysis; and 4, complete hind limb paralysis and front limb weakness. Half points were given for intermediate scores.

Flow Cytometry

Flow cytometry analysis of T cells and B cells harvested from mouse lymph nodes and spinal cords was performed, as previously described (25). Briefly, spinal cord and lymph nodes, including inguinal, axillary, and cervical lymph nodes, were harvested from mice after perfusion with ice cold PBS. Individual spinal cords were additionally dissociated through a wire mesh after which myelin was removed using a Percoll (GE Healthcare Life Sciences) gradient. Leukocytes were collected at the 37/90% Percoll interface.

Both lymph node and isolated spinal cord cell suspensions were blocked with an anti-Fc-γ receptor (CD16/32 2.4G2) in PBS containing 1% FBS before further incubation with the listed combination of staining antibodies. Dead cells were excluded by staining with the Fixable Viability Dye eFluor506 (eBioscience). Flow cytometry was performed on a LSRII cytometer (BD Immunocytometry Systems) and analyzed with FlowJo software (Treestar).

Immunofluorescent Histology

Spinal cords and lymph nodes were extracted from mice and prepared, as previously described (25). Briefly, whole lymph nodes and spinal cords were fixed in periodate–lysine–paraformaldehyde (PLP) and subsequently passed through sucrose gradients to protect from freezing artifacts. Lymph nodes were frozen whole in OCT (TissueTek) media. Spinal cords were cut into five to nine evenly spaced pieces and arranged in order prior to freezing in OCT. Serial cryostat sections (7 μm) were blocked in PBS containing 1% Bovine Serum Albumin, 0.1% Tween-20, and 10% rat serum before proceeding with staining. Sections were mounted with ProLong Gold Antifade Reagent (Invitrogen) and stored at −20°C. Tiled images of whole spinal cord sections (20×) were imaged using DM5500B fluorescence microscope (Leica).

Image and Statistical Analyses

The size of meningeal clusters in images of diseased spinal cords was analyzed using ImageJ software. PRISM software was used for all statistical analysis. Unless otherwise stated, single comparisons were performed using a Student's *t*-test and multiple comparisons were performed by ANOVA followed by a Tukey *post hoc* test.

Results

Disease Incidence in 2D2 IgH^{MOG} Double Mutant Mice

We followed mice bearing mutant TCR and BCR specific for MOG autoantigen for the development of CNS autoimmune disease. Mice demonstrating overt signs of physical disability were defined as "sick." Consistent with the previous descriptions (29, 30, 33), a proportion of unmanipulated $2D2^{+/-}$ $IgH^{MOG+/+}$ mice (here after described as 2D2 IgH^{MOG}) developed sEAE (**Figure 1A**). No disease was observed in either 2D2 (TCR) or IgH^{MOG} (BCR) single mutant mice (Not Shown); it is clearly demonstrating that antigen recognition by both T and B cells contributes to disease development in double mutant mice. Interestingly, males were significantly more likely to develop disease than females, although there was no difference in the time of onset (**Table 1**). Although previous studies did not note gender differences, the incidence data presented by Krishnamoorthy et al. (30) suggest a similar trend in male bias.

Overall incidence was highly variable over the study period. Initially, 39% of unmanipulated mice developed signs of disease (**Figure 1A**, Timepoint 1), but over ~2 years of study incidence fell to 0% (Timepoint 2) but later rose to nearly 100% incidence

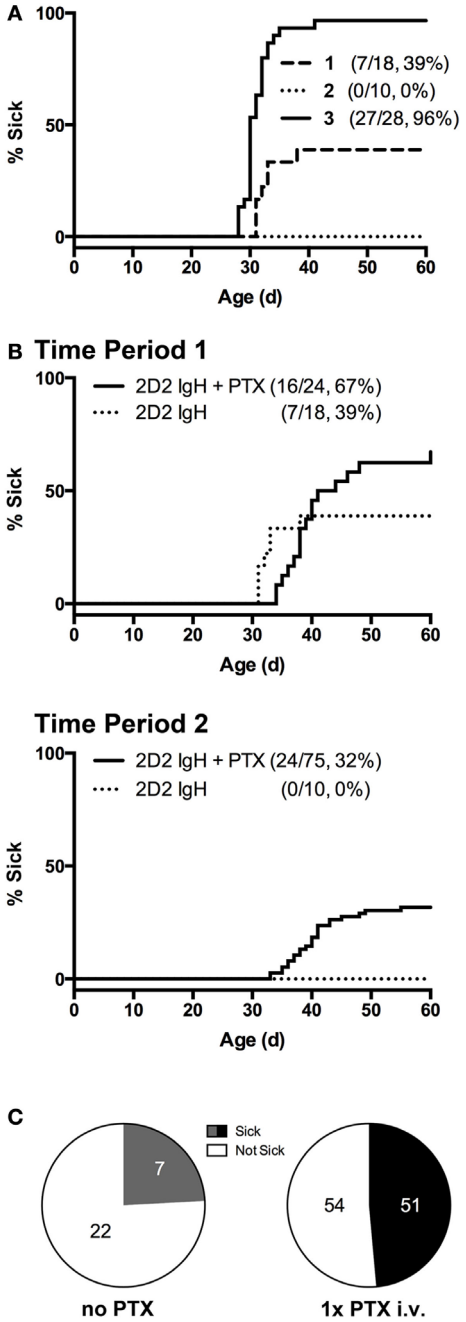

FIGURE 1 | Incidence of spontaneous CNS autoimmune disease (sEAE) in 2D2 IgH^{MOG} mice. **(A)** Disease onset curves for three representative sequential 4- to 6-month time-periods (Timepoint 1, 2, and 3) selected from the ~2-year period of study. The percent of mice in each group to demonstrate signs of disability as determined by the disease scoring system (see Materials and Methods) is shown (% Sick) **(B,C)** PTX administration increases disease incidence. **(B)** Single injections of 250 ng PTX i.v. were administered to ~32 days old 2D2 IgH^{MOG} mice, which were subsequently followed for onset of disease compared to unmanipulated mice. **(C)** Fraction of diseased mice in PTX-untreated and -treated mice, restricted to times when the overall incidence was below 80%. Significantly more PTX-treated mice developed disease as determined by Chi-square analysis ($p = 0.0003$, df = 13.13,1).

(Timepoint 3). Season has previously been identified as a factor contributing to susceptibility to EAE in a different induced model (34), but did not explain the variance observed in the case of our 2D2 IgH^{MOG} colony. We also excluded obvious changes in environmental factors, such as food or alterations in animal care. Differences in animal housing, largely attributed to differences in microbial exposure, are well known to impact EAE models and spontaneous models, in particular, both between institutions and within the same colony (27, 35). We did not investigate commensal bacteria in our own studies, but unexplained changes in microbiota over time may be the underlying reason for the dramatic shifts in incidence we observed within our colony. Nevertheless, this suggests that, like human MS, spontaneous CNS autoimmune disease in 2D2 IgH^{MOG} mice is variable and influenced by environmental factors.

Pertussis toxin (PTX) is commonly used in the induction of several models of immunization-induced EAE, particularly in C57Bl/6 mice. While the disease-promoting mechanism(s) are not entirely clear (36–38), PTX represents an antigen non-specific pathway to promote disease. Indeed, PTX was shown to increase incidence in a similar model of otherwise spontaneous CNS autoimmunity that develops in mice expressing a transgenic TCR to myelin basic protein (27). In our hands, we similarly found that a single i.v. injection of 250 ng PTX was sufficient to significantly increase disease incidence in 2D2 IgH^{MOG} mice during periods of lower disease incidence (<80%) (**Figures 1B,C**). Disease in PTX-treated mice was otherwise indistinguishable from that in mice that did not receive PTX (not shown). The mechanism by which it promotes disease induction is not yet clear, but we (37) and others (36, 39) have shown that PTX has innate immunomodulatory effects. Therefore, PTX may act as a surrogate for environmental factors that promote development of CNS autoimmune disease.

2D2 IgH^{MOG} Mice Develop Either Monophasic or Chronic Disease

2D2 IgH^{MOG} mice were evaluated daily for disease severity. Of those that showed signs of disease (defined as "sick"), the majority of mice had severe disability of the tail, hindlimbs, and partial involvement of the forelimbs, reminiscent of other EAE models and consistent with previous descriptions of this model (29, 30). Unlike for disease incidence (see above), there was no difference between males and females in maximum disease severity (**Table 1**). We observed that disease typically followed one of two courses; after the initial acute phase some mice largely recovered with little evidence of ongoing disability while others showed little sign of recovery. Therefore, we grouped mice that survived past 21 days post disease onset (i.e., that had not been used experimentally or been euthanized early due to severe disease) into one of two groups based on their final disease status: (1) chronic – mice with no more than 1 point recovery after the acute phase as determined by the standard 5 point score system, and 2) Monophasic – mice that recovered at least 1 point on the severity scale and had a final score <2 (**Figure 2**). Further evaluation of these populations showed that while the timing of disease onset was not different between them, the chronic group attained a significantly higher maximum disease score (**Table 2**).

TABLE 1 | Disease profiles in 2D2 IgH^{MOG} mice by gender.

	n=	Sick	Incidence	Day onset	SEM	Max score	SEM
Male	154	95	61.7%	36.7	(±0.52)	2.55	(±0.107)
Female	137	68*	49.6%	36.6	(±0.68)$^{n.s.}$	2.43	(±0.143)$^{n.s.}$

**p = 0.0387, df = 4.275,1 by Chi squared analysis. Note that mice that did not develop disease were not included in analysis of onset and severity.*
n.s., not significant.

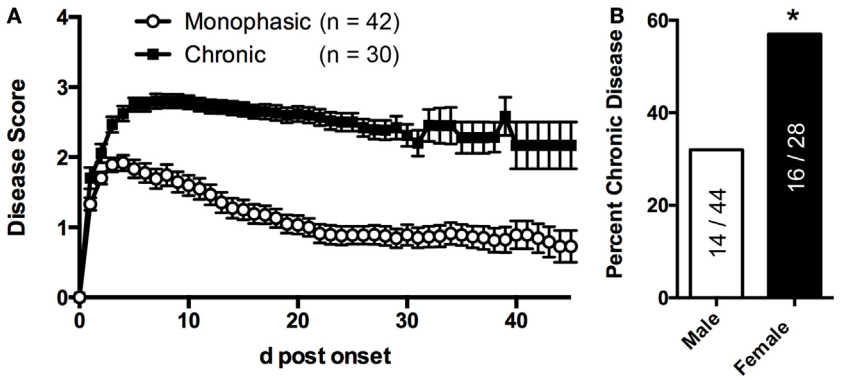

FIGURE 2 | sEAE in 2D2 IgH^{MOG} mice can follow a monophasic or chronic disease course. **(A)** 2D2 IgH^{MOG} mice were allowed to develop CNS autoimmune disease and severity was evaluated daily. Mice that developed signs of CNS autoimmune disease for at least 21 days (excluding mice that had been used experimentally or were euthanized prior to 21 days) were divided into "monophasic" or "chronic" categories based on the following criteria: chronic – no more than 1 point recovery after the acute phase as determined by the standard 5 point score system. Monophasic – recovery of at least 1 point on the severity scale and a final score <2. **(B)** Compared to male mice, a greater proportion of female mice develop chronic disease as determined by Chi-square analysis ($p = 0.0004$, df 12.65,1).

TABLE 2 | Monophasic and chronic disease profiles in 2D2 IgH^{MOG} mice.

	n=	Day onset	SEM	Max score	SEM
Monophasic	42	38.2	(±0.62)	2.26	(±0.117)
Chronic	30	35.8	(±1.47)$^{n.s.}$	2.98	(±0.106)***

****p < 0.001.*
n.s., not significant.

This indicates that while the populations were separated into the chronic or monophasic groups based principally on their status at the end of the study, the differences between groups manifested themselves earlier in the acute phase of disease (**Figure 2A**). Interestingly, while males were more likely to develop disease (see above), females were more likely to have a chronic disease course (**Figure 2B**).

Characterization of the B Cell Response in 2D2 IgH^{MOG} Mice

Previous studies employing the 2D2 IgH^{MOG} model focused primarily on T cell activation (29, 30, 33) and information about B cell activation in this or other spontaneous models is very limited. Immune responses that incorporate both T and B cell recognition of antigen typically result in a GC response. Consistent with this, significantly more CD95^{hi} CD38^{lo} GC B cells were present in lymph nodes harvested from sick 2D2 IgH^{MOG} mice (~3 weeks post onset) compared to wild type or age-matched 2D2 IgH^{MOG} that did not develop sEAE (**Figures 3A,B**). It should be noted that, with disease progression and severity, we observed lymph node atrophy and in some cases little remaining GC response could be detected (not shown).

Evaluation of Spinal Cord Pathology in 2D2 IgH^{MOG} Mice

2D2 IgH^{MOG} mice were sacrificed for histological evaluation of CNS pathology. No evidence of pathology or inflammation was evident in the CNS of wild type mice (**Figure 4A**) or 2D2 IgH^{MOG} mice that did not develop disease (not shown). Consistent with previous descriptions of this model (29, 30), there was little evidence of inflammation in the brains of 2D2 IgH^{MOG} mice that developed disease (not shown). By contrast, extensive and profound pathology was observed in the spinal cord. Evaluation of tissue harvested from mice in the acute phase of disease (<11 days post onset) revealed that extensive infiltration by CD4$^+$ T cells (**Figure 4**, compare **Figure 4A** – wild type to **Figure 4B** – acute 2D2 IgH^{MOG}) was associated with regions of reduced myelin staining (**Figures 4B,C**, inset box *ii*, middle) and F4/80$^+$ macrophage/activated microglia (**Figure 4C**, bottom). CD4$^+$ T cells were also observed in the gray matter in some mice and in these cases myelin staining of the gray matter was often altered compared to healthy mice (**Figure 4C** top, inset box *i*).

B cell infiltration of the spinal cord was almost exclusively restricted to the meninges although rare cells could be found in the white matter lesions. Meningeal B cells often formed clusters in close association with CD4$^+$ T cells (**Figures 4B,C**, inset box *ii*) reminiscent of lymphoid clusters described in human MS tissue (3, 6, 22). Similar clusters were also reported in other

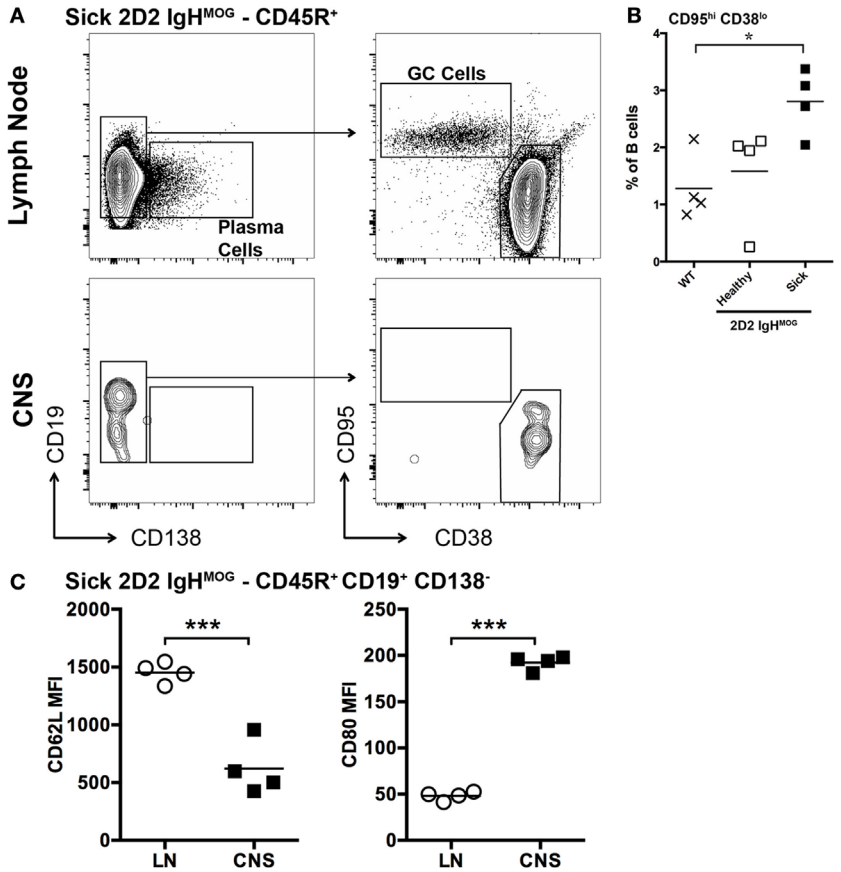

FIGURE 3 | Characterization of B cells in the lymph nodes and spinal cords of 2D2 IgH^{MOG} mice with sEAE. **(A)** Lymph nodes and spinal cords were harvested from healthy wild type mice as well as 2D2 IgH^{MOG} mice that had either developed disease (sick – 20–30 days post onset) or not (healthy – age matched). Cells were prepared and analyzed by FACS. CD45R$^+$ cells were first selected. An example of the gating strategy to identify Plasma cells [**(A)** – left] and B cells with a GC phenotype [**(A)** – right] from the CD45R$^+$ pool is shown for lymph node and spinal cord cells isolated from a single sick mouse. **(B)** Quantification of CD95^{hi} CD38^{lo} germinal center B cells in the lymph nodes of wild type and healthy or sick 2D2 IgH^{MOG} mice. Each symbol represents an individual mouse. $^*p < 0.05$. **(C)** Comparison of CD62L (left) and CD80 (right) expression by CD45R$^+$ CD19$^+$ CD138$^-$ B cells isolated from lymph nodes or the CNS of the same mouse. Each symbol represents an individual sick mouse. $^{***}p < 0.001$ as determined by paired Student's *t*-test. One representative of two experiments shown.

investigations of this model (29, 30, 33), indicating that they are a consistent feature of disease in 2D2 IgH^{MOG} mice. These clusters were very often in direct association with regions of white matter demyelination and CD4$^+$ T cell infiltration (**Figure 4B**). Pathology was not restricted to any particular region of the spinal cord as in some cases the entire spinal cord was involved, while in others inflammation was restricted to either distal (lumbar) or proximal (cervical) regions (not shown).

B Cell Infiltration of the Spinal Cord is Associated with Chronic Disease

We next evaluated spinal cord pathology later in disease (>20 days post disease onset) in mice with either a chronic or monophasic disease course (as defined above). White matter pathology in monophasic mice was very limited, in that there was little CD4$^+$ T cell infiltration or demyelination (**Figure 5A**). By contrast, ongoing white and gray matter inflammation by CD4$^+$ T cells and white matter demyelination was clearly evident in mice with chronic disease (**Figure 5B**) demonstrating that continued disability in these mice reflects active and ongoing inflammation, rather than permanent injury incurred during the initial attack. Large meningeal clusters containing T and B cells were also common in these mice. Nevertheless, despite reduced white matter involvement, small meningeal clusters were sometimes also present in monophasic mice (**Figure 5A**). However, subsequent analysis confirmed that meningeal clusters were both more numerous (**Figure 5C**) and larger (**Figure 5D**) in chronic vs. monophasic mice. Further, independent of disease course classification the size of meningeal clusters correlated with disease severity (**Figure 5E**). This, combined with the common spatial association between clusters and underlying regions of demyelination, suggests that these structures may contribute to ongoing chronic CNS autoimmune disease in 2D2 IgH^{MOG} mice.

Characterization of B Cells in Meningeal Clusters

To begin to dissect the role that B cells play in spinal cord pathology in sEAE, we evaluated the activation phenotype of

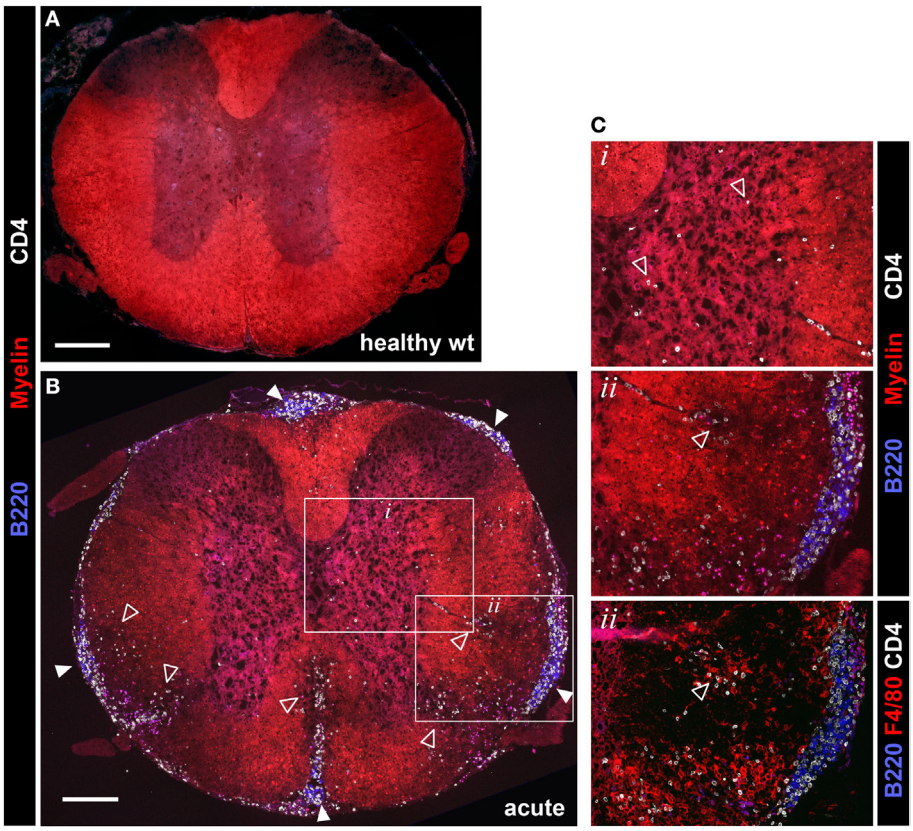

FIGURE 4 | Evaluation of spinal cord pathology in 2D2 IgH^{MOG} mice. Mice were sacrificed in the acute phase of disease (<11 days post onset) and spinal cord pathology was evaluated by immunofluorescent histology. Sections were stained for myelin and invading CD4$^+$ T cells and B220$^+$ B cells. [**(A)** – wild type health control, **(B)** – 2D2 IgH^{MOG} acute disease]. Infiltrating T cells (open triangles) were evident in the gray matter of diseased mice [see **(B)** inset box *i*, shown at higher magnification in **(C)**, top panel]. Clusters containing B220$^+$ B cells and CD4$^+$ T cells (closed triangles) were clearly apparent in the meninges of diseased mice, while no B cells were found in healthy spinal cords [compare **(A)** with **(B)**]. Meningeal clusters were often adjacent to areas of demyelination and CD4$^+$ T cell infiltration of the white matter (open triangles). Ongoing parenchymal invasion by T cells and macrophages/activated microglia were clearly evident, associated with regions of demyelination [open triangles, see enlarged image and serial section stained with F4/80, inset box ii, **(C)**]. Representative images shown (n = 5 wt, n = 5 acute phase 2D2 IgH^{MOG}, minimum three sections taken from different regions of each spinal cord). Scale bars represent 200 μm.

infiltrating B cells. FACS analysis of lymphocytes isolated from spinal cords revealed that B cells are almost exclusively CD38^{hi} CD95^{lo}, consistent with naïve or memory lymph node B cells (**Figure 3A**). However, compared to lymph node B cells with a similar CD38^{hi} CD95^{lo} phenotype, spinal cord B cells had significantly lower expression of CD62L and higher expression of CD80 (**Figure 3C**), indicating at least some level of non-classical activation, perhaps to present antigen. Cluster B cells were further characterized by histological examination of spinal cord tissue. We focused on spinal cords from chronic mice (see above) with evidence of ongoing disease activity. Consistent with a potential role for B cells in presenting antigen to T cells in clusters, T and B cells were found in close physical association with each other (**Figures 6A,B**). Subsequent staining confirmed that T cells in clusters were almost exclusively CD4$^+$ T cells. However, we were surprised to find that CD8$^+$ T cells were minor yet common component of the T cell infiltrate of white and gray matter (**Figure 6A**). This was not the case in the acute phase of disease (not shown). Although CD8$^+$ T cells are known to infiltrate the CNS in human MS and contribute to some animal models of CNS autoimmunity (2, 35), we did not expect their presence in the 2D2 IgH^{MOG} model as the 2D2 TCR is derived from an MHC class II-restricted CD4$^+$ T cell (26). However, CD8$^+$ T cells were shown to infiltrate the CNS and participate in pathology in a similar model that makes use of a different MOG$_{35-55}$-specific TCR on the NOD background. Although the TCR in this model was similarly derived from a CD4$^+$ T cell, CD8$^+$ T cells were found to express the transgenic TCR and recognize the MOG$_{35-55}$ peptide presented on MHC class I (40).

We further investigated cluster B cells for evidence of activation. While Ki67$^+$ cells were detectable within clusters as well as in the white matter, very few of them were co-stained with B cell markers (**Figure 6B**). Instead, the large majority of proliferating Ki67$^+$ cells were T cells (not shown). CD138$^+$ Plasma cells were not apparent in clusters (**Figure 6C**). Finally, cluster B cells were investigated for evidence of class switch. Virtually, all B cells expressed IgD (**Figure 6D**) and IgM, but not IgG1 (not shown).

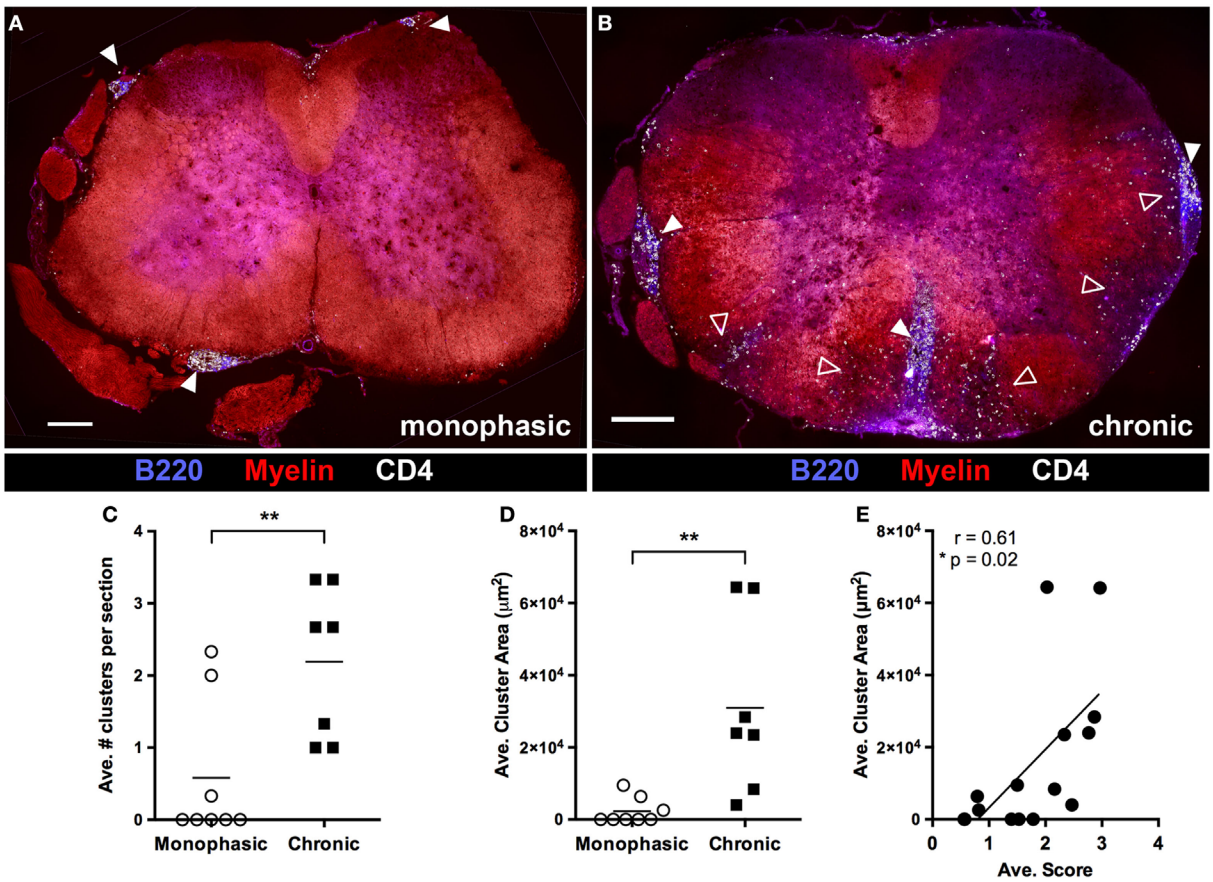

FIGURE 5 | B cell infiltration of the spinal cord is associated with chronic disease and increased disease severity. At study endpoints (~4 weeks post disease onset), spinal cords were harvested for evaluation of pathology by immunofluorescence histology. Spinal cords from mice deemed to have either a "Monophasic" **(A)** or "Chonic" **(B)** disease course (as defined in **Figure 2**) were evaluated for demyelination, CD4+ T cell infiltration and meningeal cluster formation. Solid arrowheads indicate meningeal clusters. Open arrowheads indicate regions of white matter demyelination and infiltration by CD4+ cells. Scale bars represent 200 μm. The number of meningeal clusters per section **(C)** and cluster area **(D)** was evaluated using Image J software. Three sections from different regions of the spinal cord were evaluated from each mouse. $^{**}p < 0.01$ as determined by Student's *t*-test. Each symbol represents the average value per section from an individual mouse. **(E)** Cluster size was correlated with average disease score (as a measure of overall disease severity) for each individual mouse included in the study. Each symbol represents an individual mouse. A two tailed Pearson *r* test was performed to test for correlation.

It has been suggested that meningeal clusters in the CNS of MS patients may function as the so-called tertiary lymphoid tissues that recapitulate the structure and function of secondary lymphoid organs, such as lymph nodes and spleen (22). We therefore evaluated meningeal clusters in chronic 2D2 IgH^{MOG} mice for features of lymphoid follicles. With the exception of a single cluster in one mouse from the chronic group (**Figure 6E**), there was no evidence that T and B cells were organized into separate zones as occurs in lymphoid tissue (see **Figures 4B, C, 5** and **6A–D** for examples). Neither evidence of CD35+ follicular dendritic cells (FDCs) (**Figure 6F**) nor Bcl-6+ staining (GC B cells or T follicular helper Tfh cells) (not shown) was evident in any cluster that we examined. Finally, while some evidence of PNAd staining was apparent in the single cluster with evidence of T and B cell organization (**Figure 6F**), perhaps indicating development of specialized high endothelial venules, PNAd staining was not evident in any other cluster that we examined (not shown). Therefore, while it is possible that given sufficient time a proportion of meningeal clusters may attain some features of organized lymphoid tissue, the majority of clusters remain largely unorganized. If, as their association with demyelinating regions suggests, these clusters do contribute to the pathology of CNS autoimmunity, the less organized form must represent the minimum requirement for the pathogenic mechanism.

Discussion

Here, we characterize a spontaneous model of CNS autoimmunity that depends on both T and B cell recognition of the myelin autoantigen. We are aware of only three previously published studies using this 2D2 IgH^{MOG} model. The original descriptions came from independent studies from Bettelli et al. (29) and Krishnamoorthy et al. (30) that focused principally on characterizing T cell activation as well as lesion distribution, which they found to be limited to the optic nerve and spinal cord. A third study made creative use of a version of this model

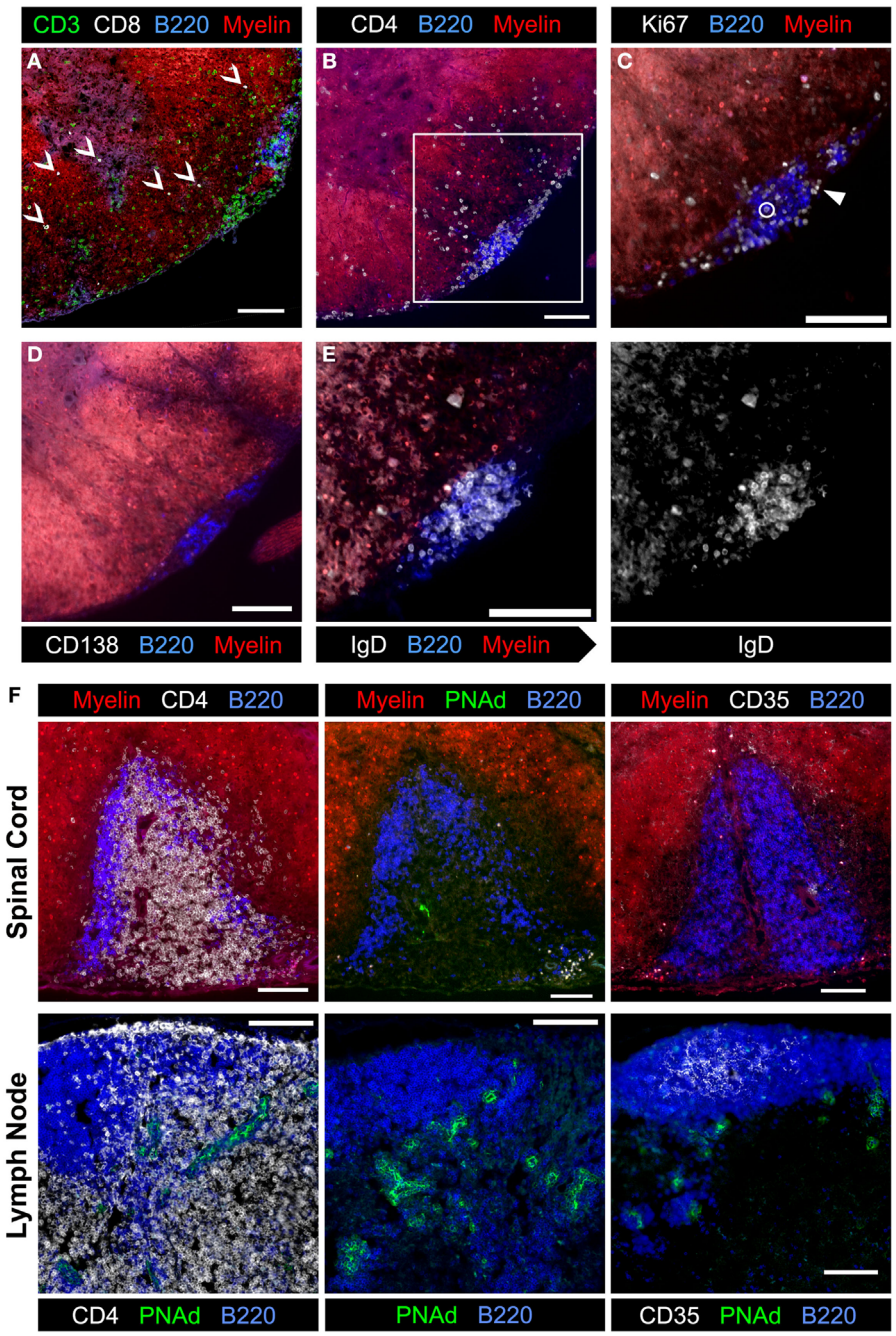

FIGURE 6 | Evaluation of meningeal clusters in spinal cords from 2D2 IgH^{MOG} mice with chronic sEAE. Serial sections of spinal cord tissue from mice determined to have chronic disease (see Figure 2) were stained by immunofluorescence to characterize infiltrating immune cells. Images of one representative cluster from a single mouse ($n = 7$) are shown **(A–E)**. Scale bars represent 100 μm. **(A)** CD3$^+$ CD8$^+$ T cells were a common but minor component of the white matter infiltrate (open arrowheads), but only very rarely in meningeal clusters. **(B)** CD4$^+$ cell infiltration into a region of demyelination adjacent to a meningeal cluster composed of B220$^+$ B cells and CD4$^+$ T cells. Inset box indicates the magnified region shown in subsequent serial sections. **(C)** Ki67$^+$ cells in cell cycle were evident in meningeal clusters and in the affected white matter. The large majority of Ki67$^+$ cells did not co-stain with B220 (example – closed arrowhead), with only very rare exceptions (open circle). **(D)** Little to no evidence of CD138$^+$ plasma cells was observed in association with meningeal clusters. **(E)** Nearly, all B220$^+$ B cells in meningeal clusters co-stained with IgD and therefore not class-switched (gray-scale of IgD channel alone shown on right). **(F)** Evaluation of meningeal clusters for evidence of features of lymphoid follicles. (Top) images of the single cluster from a 2D2 IgH^{MOG} mouse with chronic disease to show evidence of T and B cell organization into different separate regions (left, top – compare to B cell follicle and T cell zone separation in a healthy naïve lymph node, bottom) and differentiation of specialized high endothelial venules (middle top – compared to extensive PNAd staining in the lymph node, bottom). Little to no evidence of CD35$^+$ follicular dendritic cells (right, top – compared to extensive follicular staining in the lymph node, bottom) was apparent in this meningeal cluster. For each stain listed above, between four and seven individual mice with chronic disease were evaluated, choosing sections with the most developed clusters.

to demonstrate that antibody production by MOG-specific B cells was not important to disease initiation, which instead was linked to antigen presentation by B cells to T cells (33). As discussed in more detail below, our findings presented here are largely consistent with these previous reports. We extend these studies by focusing on the B cell response and on characterizing infiltrating B cells and meningeal clusters in the diseased spinal cord.

The primary goal of our study was to characterize the B cell response in this B-cell-dependent model of CNS autoimmune disease. As expected, measurable GC responses were detected in lymph nodes from sick mice, although this was not true in all cases due to lymph node atrophy. Nevertheless, a GC response driven by interactions between MOG-specific T and B cells is presumably the mechanism for B cell-dependent disease initiation, as B cell presentation of antigen has been shown to be essential in a similar B cell-dependent mouse model (33). Of greater interest to therapeutic intervention in autoimmunity is the potential role of B cells in propagating ongoing disease, which may occur from within the inflamed CNS. With the exception of the involvement of $CD8^+$ T cells, which were relatively common infiltrates of the spinal cord parenchyma in chronic but not acute disease, inflammation and pathology was qualitatively similar over the course of active disease. We observed extensive B cell infiltration of the meninges wherever white matter pathology was apparent, often forming clusters with $CD4^+$ T cells. By contrast, white matter pathology was largely absent in mice that had recovered from monophasic disease. It is not clear if this reflects myelin repair or if extensive demyelination did not occur in these mice and disability was a reflection of inflammation, rather than actual tissue destruction.

Contrary to our initial expectations, B cells in meningeal clusters showed little indication of activation, with no evidence of class switch and little proliferation compared to infiltrating $CD4^+$ T cells. By FACS, spinal cord B cells were $CD38^{hi}$ $CD95^{lo}$, consistent with naïve or memory cells, although elevated CD80 in particular suggests a degree of activation. Further investigation will be required to determine if B cells upregulate CD80 once in the meninges or if $CD80^{hi}$ B cells are selectively recruited. It should be noted that B cell follicles in secondary lymphoid tissues are largely populated by naïve B cells, and therefore an unactivated phenotype would be expected if meningeal clusters do indeed represent lymphoid structures. However, with the possible exception of the single cluster in a mouse with chronic disease described above, we did not observed any other evidence of typical follicular features in the meningeal clusters that formed in sick 2D2 IgH^{MOG} mice. We previously observed similar clusters in a model of EAE induced by immunization with a protein antigen based on mouse MOG (25). Again, there was no evidence of follicular differentiation in these clusters. Together, these models suggest that cluster formation is common in models that incorporate B cell recognition of the autoantigen. This is not an absolute requirement, however, as small clusters could still form in mice with mutant BCR incapable of recognizing MOG or in mice immunized with the standard short $MOG_{35–55}$ peptide (25). White matter demyelination and inflammation were much reduced compared to what we observed in either MOG protein-induced disease or in sick 2D2 IgH^{MOG} mice, which also feature greater meningeal B cell infiltration.

Models of CNS autoimmunity that incorporate target recognition by more than just $CD4^+$ T cells, such as 2D2 IgH^{MOG} mice or EAE models induced with protein antigen, represent significant improvements over peptide-induced models that are used most commonly today as they allow for a more normal and complex response. Responses to "real" antigens recruit multiple immune targeting and effector processes. Nevertheless, it is not clear that these models more accurately represent human MS. Focusing specifically on investigations of infiltrating B cells in MS, lineage analysis suggest that B cells isolated from CNS represent a population derived from the GC response and subsequently selected from the peripheral pool (4, 41, 42). FACS studies suggest that CSF B cells are enriched for $CD27^+$ cells (4, 43, 44), which in humans is considered a marker of memory. Therefore, these studies suggest that at least some B cells in human disease were previously activated. As there is no equivalent memory marker in mice, it is not currently possible to directly compare these observations to our own of the 2D2 IgH^{MOG} model.

Histological studies of human post-mortem tissue have been inconsistent in finding meningeal clusters (3, 6, 45–48). When clusters have been observed (3, 6, 45), more examples of follicular differentiation were reported than we observed in our mouse models [here and in Ref. (25)]. Nevertheless, our observations suggest that, given time, it is possible that meningeal clusters can attain at least some features of true follicles. Human studies were performed on tissues from patients who had disease for many years and in most cases decades (22), and despite their focus on the most follicle-like structures, it is clear that most meningeal clusters in MS remained largely unorganized (22, 23), consistent with our observations in both mouse models. Furthermore, human studies were almost exclusively of progressive disease, where neurodegeneration occurs even though there is less evidence of active inflammation (2). It is not clear that these observations are relevant to the earlier active inflammatory stage of disease that is likely a better correlate to EAE. The rarity of tissue from this earlier stage of disease and of spinal cord tissue will make direct comparison very difficult. Nevertheless, as the best evidence supports the hypothesis that MS is an autoimmune disease targeting myelin antigens, holistic models of CNS autoimmune disease that more completely involve the immune system have the best chance of revealing important fundamental pathogenic B cell mechanisms that drive ongoing disease.

The apparent contradictory effects of B cell depletion by anti-CD20 (17) vs. TACI-Fc (18) in human MS highlights the urgent need to better understand the complex biology of these cells in autoimmune disease and the immune response in general. Both anti-CD20 and TACI-Fc target IL-10-producing Breg cells (19, 49). However, they have very different activity on plasma cells and therefore antibody production (19), as well as T cell biology (20). There are likely additional B cell subsets with differential susceptibility to depletion by each reagent. Indeed, IgM^+

memory B cells are more dependent on BAFF and therefore to depletion by TACI-Fc than class-switched memory cells (50). Anatomical location may also affect susceptibility to depletion by either reagent. Work to decipher this will have to rely heavily on models, such as the 2D2 IgH^{MOG} mice, as in human patients usually only the circulating and more rarely CSF pools can be accessed. Our models suggest that antigen-specific B cells contribute to disease but that, unexpectedly, the B cell infiltrate in the CNS are not activated in a way that we would expect based on studies of antigen-specific activation in lymphatic tissue. Further work will be required to identify subsets of B cells, how they contribute to pathology or protection from disease, and their susceptibility to different methods of therapeutic intervention.

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Investigating the Antigen Specificity of Multiple Sclerosis Central Nervous System-Derived Immunoglobulins

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The central nervous system (CNS) of patients with multiple sclerosis (MS) is the site where disease pathology is evident. Damaged CNS tissue is commonly associated with immune cell infiltration. This infiltrate often includes B cells that are found in multiple locations throughout the CNS, including the cerebrospinal fluid (CSF), parenchyma, and the meninges, frequently forming tertiary lymphoid structures in the latter. Several groups, including our own, have shown that B cells from distinct locations within the MS CNS are clonally related and display the characteristics of an antigen-driven response. However, the antigen(s) driving this response have yet to be conclusively defined. To explore the antigen specificity of the MS B cell response, we produced recombinant human immunoglobulin (rIgG) from a series of expanded B cell clones that we isolated from the CNS tissue of six MS brains. The specificity of these MS-derived rIgG and control rIgG derived from non-MS tissues was then examined using multiple methodologies that included testing individual candidate antigens, screening with high-throughput antigen arrays and evaluating binding to CNS-derived cell lines. We report that while several MS-derived rIgG recognized particular antigens, including neurofilament light and a protocadherin isoform, none were unique to MS, as non-MS-derived rIgG used as controls invariably displayed similar binding specificities. We conclude that while MS CNS resident B cells display the characteristics of an antigen-driven B cell response, the antigen(s) driving this response remain at large.

Keywords: multiple sclerosis, B cell, autoantibody, autoantigen

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INTRODUCTION

Multiple sclerosis (MS) is the most common neurological disease affecting young adults. MS is an inflammatory disease of the central nervous system (CNS) characterized by immune cell infiltration and demyelination of the brain and spinal cord that leads to physical disability (1). Although the cause of the demyelination is not entirely clear, many studies have implicated T cells as the dominant immune cell type contributing to disease pathology. However, growing evidence also suggests that B cells play an active role in the disease (2). A recent ENCODE study (3) implicated B cells second only to T cells among the cell types most affected by MS susceptibility genes. B cells are found at sites of tissue injury in the CNS. They are also found in the CSF, white matter lesions, gray matter, and in the meninges, where

they form lymphoid-like tissue aggregates (4) that associate with proximal tissue damage (5). Furthermore, they are responsible for the production of the oligoclonal immunoglobulin bands (OCB) in the spinal fluid that are a hallmark of the disease. Their roles as both effective antigen-presenting cells (6) and immune response regulators (7) have recently been appreciated. Finally, B cell depletion, which has emerged as a beneficial therapeutic approach for MS, confirms that B cells contribute to MS pathology (8).

A number of autoimmune demyelinating diseases of the CNS are associated with a robust B cell response, and in several cases, antigens implicated in this response have been identified. Neuromyelitis optica (NMO) serves as a prototypical example of demyelinating CNS autoimmunity associated with B cells. Most NMO patients produce antibodies [both serum immunoglobulin (9) and CSF-derived IgG (10)] that bind the water channel aquaporin-4 (AQP4). These antibodies have been shown to be derived in part from a clonally expanded B cell pool located within the CSF (11, 12). Other examples of B cell-related autoimmune demyelinating CNS conditions are pediatric MS and acute disseminated encephalomyelitis (ADEM), where antibodies to myelin oligodendrocyte glycoprotein (MOG) have been identified (13, 14).

During subacute and chronic active infections of the CNS such as Lyme neuroborreliosis or subacute sclerosing panencephalitis (SSPE), OCB are found in the CSF and resolve when the infection is cleared. In SSPE, brain-derived, recombinant immunoglobulin can be specifically absorbed by the causative virus, namely, the measles virus (15). The humoral immune response in MS shares many similarities with that seen in SSPE, NMO, and other inflammatory diseases of known cause. The MS CSF often includes elevated immunoglobulin levels and OCB, both of which are derived from B cells residing in the CSF and CNS tissue (16, 17). The CNS B cells in SSPE, NMO, and MS display the characteristics of an antigen-driven response, with high levels of clonal expansion and somatic hypermutation in IgG variable regions, all of which are consistent with post-germinal center activation (12, 18–20). However, in contrast to SSPE and many infectious encephalopathies, the antigen target of the CNS-associated immunoglobulin is not known in MS. Given these similarities and the clear evidence for an antigen-driven response displayed by MS CNS resident B cells, the identification of the autoantibody targets in MS is of substantial interest.

The search for specific autoantibodies in MS has been an area of focus for decades, but the antigens targeted by MS autoantibodies have remained elusive. Many studies have focused on serum antibodies given their accessibility and that serum autoantibodies have been identified in several diseases. Myelin basic protein (MBP) autoantibodies are detected in a very small subset of MS patients (21). MOG autoantibodies appear to be reliably found in a small subset of patients with MS (14) that are primarily pediatric. More exhaustive lists of candidate MS antigens can be found in a number of valuable reviews (22, 23). Numerous candidate autoantibody targets have been reported [reviewed in Ref. (2, 24, 25)], but none have met all the criteria that would allow for widespread acceptance as a genuine disease-associated MS autoantibody. These criteria would, at the very least, include such characteristics as disease specificity, reproducible detection among different laboratories, and different patient cohorts and disease relevance in terms of diagnosis, prognosis, or contribution to immunopathology. Newly identified candidate antigens of interest include contactin-2 (26), ATP-sensitive inward rectifying potassium channel KIR4.1 (27), and sperm-associated antigen 16 (28), all of which are undergoing validation. Although a number of serum-derived antibody targets, such as MOG, can be found in small subsets of MS patients, most of those identified in serum have failed to be sensitive and specific markers for the disease. Some candidate autoantigens appear to be enriched in (29) or restricted to the CSF relative to serum, such as recombination signal binding protein for immunoglobulin kappa J region (RBPJ) (30). These autoantigens also represent a small subset of patients that have not yet defined a unique clinical phenotype.

To date, no antigen has emerged as a validated and widely accepted "MS antigen." We reasoned that the recombinant IgG (rIgG) derived from the clonally expanded and antigen experienced B cells that populate sites of tissue damage in the MS CNS are likely to represent the most enriched sources of disease-relevant antibody. Accordingly, we sought to explore the specificity of such MS CNS-derived immunoglobulin. To this end, we produced rIgG from a series of clonally expanded CNS-derived B cells from different MS CNS specimens and controls. The rIgGs were then screened against previously implicated candidate antigens as well as with high throughput approaches that multiplex large sets of antigens such as whole protein arrays and CNS-derived cell lines. In all of the screening approaches, an effort was made to maximize preservation of conformational and post-translational epitopes. This study, to our knowledge, represents the first time that such a technically demanding approach utilizing recombinant antibodies from CNS lesion-derived B-cells has been employed toward antigen discovery in MS.

MATERIALS AND METHODS

Ethics Statement

Patient-derived specimens did not include personally identifiable private information or intervention or interaction with an individual and were accordingly collected under an exempt protocol approved by the Human Research Protection Program at Yale School of Medicine.

Subject Specimens

Tissues were dissected at autopsy from six subjects with clinically defined MS. Five of the six subjects had a progressive clinical course and one had a relapsing remitting clinical course. Collected tissues included lesions and meningeal follicles. Our group previously reported the characteristics of the B cells that infiltrated these specimens (18, 31). Control tissues, which harbored robust B cell infiltrates, included germ cell tumors and muscle tissue from patients with inclusion body myositis (IBM), both of which have been previously described by our group (32, 33).

Laser Capture Microdissection and B Cell Variable Region Cloning

Central nervous system tissue was sectioned at 12 μm on a microtome/cryostat, mounted onto a glass slide then fixed

in 75% ethanol for 30 s. For the identification and capture of individual B lineage cells, the tissue was stained with mouse anti-CD20 or anti-CD38 antibodies (Accurate Chemical & Scientific) after fixation, then counterstained with poly-horseradish peroxidase anti-mouse IgG (Ivax Diagnostics). The tissue was then dehydrated in consecutive washes of 75, 95, and 100% ethanol then xylene. Cells were captured with a PixCell IIe laser capture microdissection instrument and CapSure Macro caps (Arcturus) and immediately stored at −80°C. RNA was isolated with the Absolutely RNA Nanoprep Kit (Stratagene) according to the manufacturer's protocol. B cell variable regions were cloned and analyzed according to procedures that we have previously reported (18, 33).

Recombinant IgG Synthesis and Purification

Multiplex PCR was used to amplify the immunoglobulin variable heavy chain (VH) and variable light chain regions (VL). These products were subsequently directionally sub-cloned behind the CMV promoter into a pcDNA3.3- or pCEP4-based vector constructed in-house to harbor the human immunoglobulin IgG_1 heavy chain and kappa constant domains, respectively. The heavy chain vector was modified to contain a C-terminal affinity tag (HA-hemagglutinin). Expression and purification of the recombinant whole human IgG was performed with protocols that we have previously described (34). Recombinant IgGs (rIgG) were prepared from the matched variable heavy (VH) and light regions (VL) derived from either laser captured single cells or by matching the most highly expressed VH and VL clones from each library.

Solid Phase Immunosorbent Assays

Solid phase ELISA was performed to evaluate rIgG recognition to a number of candidate antigens. These assays were performed using an approach that we have previously described (21). Similarly, the DELFIA assay for the detection of antibody binding to MBP was performed using an approach that we have previously described (35).

ProtoArray

ProtoArray Human Protein Microarrays version 5.0 (Life Technologies), containing approximately 9,400 unique full-length human proteins, were used. The assay was performed according to the manufacturer's instructions as we have previously described (30). Briefly, protein microarray slides were probed with rIgG pools (normalized for total IgG content) by overnight incubation at 4°C. Bound rIgG was detected with an Alexa Fluor 647-conjugated goat anti-human IgG (Life Technologies). The arrays were then scanned using a GenePix 4200A (Molecular Devices) fluorescent microarray scanner and analyzed with GenePix software. The standard score (*Z*-score) for binding to each antigen was determined using the Immune Response Profiling function within Prospector software (Life Technologies). The selection criteria applied for binding to be considered positive was a *Z*-score >3.

Cell-Based Antibody Binding Assays

The cell-based assay for MOG binding was performed with Jurkat cells that were transfected to express a fusion protein that included the extracellular domain of human MOG linked to GFP. Antibody binding was then measured using an approach as we have previously described (13).

Cell lines were prepared for surface binding screening using methods we have previously described (34). Briefly, cells were incubated with each recombinant antibody at a concentration of 5 μg/ml, and then incubated with a polyclonal goat anti-human IgG AlexaFluor 488-labeled antibody (Life Technologies) to detect binding. Cells were resuspended in BD Cytofix (BD Biosciences) and stored at 4°C in the dark until being analyzed by flow cytometry with a FACSCalibur flow cytometer (BD Biosciences). Median fluorescence intensity (MFI) was used to assess binding of MS-derived and control rIgG to the CNS and control cell lines. Similarly, intracellular staining was performed in the same manner as that described for surface binding except for the addition of the permeabilization step, which was facilitated using Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions.

RESULTS

Generation of Recombinant IgGs from MS Brain

To explore the specificity of the antibodies produced by CNS-derived B cells, we prepared rIgG from immunoglobulin variable region sequences derived from MS and control tissues. The MS cohort included rIgG constructed from MS autopsy tissue specimens from six subjects, MS-A thru MS-F (**Table 1**). Five of the six subjects were female and one male, with ages ranging from 34 to 65 years at the time of death. Five of the MS subjects had a progressive course, while one had relapse-remitting MS. Disease duration ranged from 2 to 20 years. Six recombinant antibodies (Table S1 in Supplementary Material) were derived from clones present in MS-A; four recombinant antibodies each

TABLE 1 | Subject demographics and source of MS and control tissue.

Case	Age (years)	Gender	Clinical course	Disease duration (years)	Source
MS-A	43	F	Progressive MS	20	Autopsy
MS-B	34	F	Progressive MS	2	Autopsy
MS-C	39	F	Progressive MS	13	Autopsy
MS-D	38	F	Relapsing remitting MS	n.a.	Autopsy
MS-E	65	M	Progressive MS	n.a.	Autopsy
MS-F	49	F	Progressive MS	14	Autopsy
GCT	<18	M	Intracranial germinoma	n.a.	Resection
IBM-A	>40	M	Inclusion body myositis	n.a.	Biopsy
IBM-B	>40	M	Inclusion body myositis	n.a.	Biopsy

n.a., data not available.

were derived from clones present in MS-C and MS-D and a single recombinant antibody was derived from each of the MS-B, MS-E, and MS-F tissues. All of the MS-derived rIgGs were constructed from clonally expanded cells that displayed evidence of affinity maturation including class switching and the accumulation of somatic mutations (Table S2 in Supplementary Material). The control rIgGs were derived (**Table 1**) from either an intracranial germinoma (GCT-A) or muscle tissue from two different patients with inclusion body myositis (IBM) (IBM-A and IBM-B). We previously demonstrated that the B lineage cells infiltrating both the tumor and muscle tissue (32, 33) shared antigen-driven characteristics that were similar to those representing the MS cohort (Table S2 in Supplementary Material). Specifically they had class switched to IgG, had accumulated somatic mutations, and were remarkably clonally expanded into families that included numerous clonal variants. Nine recombinant antibodies (Table S1 in Supplementary Material) were derived from clones present in the germinoma (GCT-A1 thru GCT-A9) and three each from the two IBM specimens (IBM-A1-3 and IBM-B1-3). Finally, a well-described (36) monoclonal antibody that recognizes MOG, which we humanized, was included in the control cohort (h8-18C5) (Table S1 in Supplementary Material).

Screening MS rIgG for Binding to Candidate Antigens

To investigate the specificity of CNS-derived antibodies, we began by screening against candidate antigens that have previously been implicated in MS. A DELFIA and an ELISA assay were performed to test MBP (37) and contactin (26), respectively. Differences in binding to MBP and contactin between the MS and control rIgG were unremarkable (*not shown*). We also used an ELISA assay to assess binding of MS and control rIgG to the intracellular protein neurofilament light (NF-L) (38). Ten MS-derived antibodies that were tested showed modest binding to NF-L (**Figure 1**) while three antibodies (MS-B1, MS-C2, and MS-C4) displayed strong binding with absorbance values that exceeded the mean +2SD of the control data set (*benchmark for strong positive binding*). The difference between the MS and the control group was significant ($p = 0.0018$, *Mann–Whitney test*). However, binding was not restricted to MS-derived antibodies as a germinoma-derived antibody (GCT-A6) was also positive, indicating a lack of specificity for MS in the rIgG cohorts.

We also examined binding to MOG; autoantibodies to MOG have recently been described in a small subset of MS patients (13), in pediatric MS (14) and in NMO (39). MOG binding was evaluated using a cell-based assay that preserves conformational epitopes and, accordingly, has become a widely accepted approach for detection of such antibodies (13). Robust binding by the humanized monoclonal anti-MOG monoclonal antibody (h8-18C5) was recorded (**Figure 2**). The clear recognition of MOG by our humanized h8-18C5 demonstrates that our recombinant expression system produces fully functional whole human IgG and did not introduce any artifacts that might confound native specificity. Applying this approach to the MS- and germinoma-derived rIgG demonstrated that none of these antibodies recognized MOG expressed on the surface of the cells (**Figure 2**; Figure S1 in Supplementary Material).

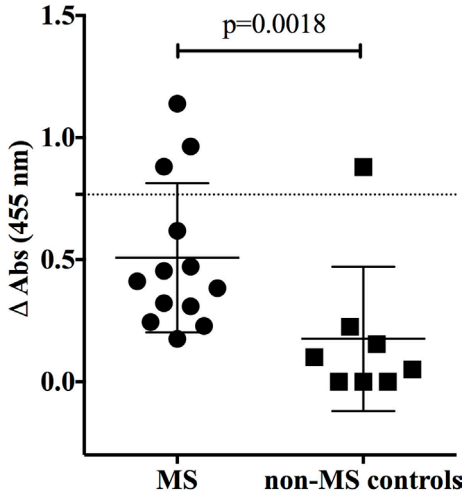

FIGURE 1 | MS and control-derived rIgG binding to neurofilament light (NF-L) by solid phase ELISA. MS-derived rIgG ($n = 13$) and control rIgG ($n = 8$) derived from a germinoma were tested by solid phase ELISA for binding to NF-L. The specific samples included in the assay are shown in the Supplementary Material. Each dot or square represents the binding of a single rIgG. The dashed line indicates the mean +2 SD of the control germinoma-derived cohort (0.76). Values above this line were determined to be positive (95% CI). To correct for non-specific binding, the reported ELISA signal (ΔOD) was calculated by subtracting the signal generated by binding to glyceraldehyde 3-phosphate dehydrogenase (GADPH) from that of the NF-L. The mean and SD are shown for each data set. Statistical differences are indicated when significant. Data associated with each rIgG for the MS and control groups are shown in the Supplementary Material.

Screening MS rIgG Reactivity with High-Throughput Protein Arrays

Having shown no specificity for the MS-derived rIgG to several candidate antigens, we sought to expand the search by using an unbiased library of antigens that could be screened in a high-throughput manner. To this end, we examined the rIgG specificity from the MS and control cohorts with a commercially available protein array composed of approximately 9,400 unique full-length human proteins that were expressed in a system such that the products included some physiologic post-translational modifications and processing. The rIgGs from both the MS and controls groups were pooled so that three rIgG were included on each array during the initial scouting to maximize efficient use of the arrays. A total of three MS and three control arrays were run. Target antigens that were identified by at least one MS antibody pool that did not react with any of the control groups are shown in Figure S2 in Supplementary Material. In most instances, antigen targets were found on a single MS array; however, several were found on two of the three MS arrays. Of these, protocadherin gamma subfamily C, three (PCDHGC3), transcript variant three was of particular interest as a candidate autoantigen as protocadherin isoforms, include extracellular domains, are predominantly expressed in the nervous system and have been implicated in human neurological disorders (40, 41). Given their attractive role as candidate MS antigens we investigated this specificity further. To do this we tested binding to PCDHGC3 protein by ELISA

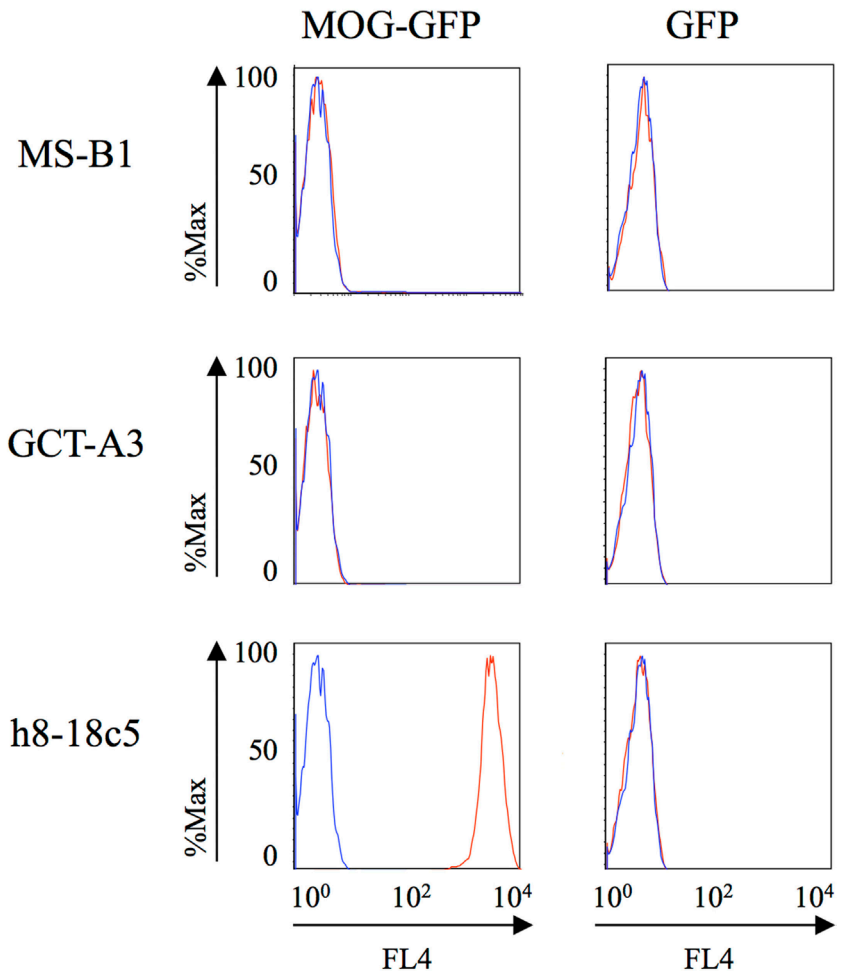

FIGURE 2 | MS and control-derived rIgG binding to MOG detected with a cell-based assay. Representative binding of MS (MS-B1) or germinoma control-derived (GCT-A3) rIgG to Jurkat cells transfected with MOG-GFP (left column) or GFP alone (right column). Histograms show the MFI of transfected cells gated on those that were positive for both GFP and a florescent anti-human secondary antibody (red). The blue histograms show secondary antibody alone. A humanized monoclonal antibody, h8-18c5, specific for human MOG served as a positive control for the Jurkat–MOG–GFP binding. FACS data for additional rIgGs from the MS and control groups are shown in the Supplementary Material.

with individual rIgGs rather than pooled mixtures (**Figure 3**). The MS-derived rIgG MS-C2 that was present in the pool that bound PCDHGC3 on the ProtoArray (MS array 2) bound to the protein. However, binding was not observed for the individual rIgGs present on the second array that also identified this target (MS array 3). Furthermore, the difference between the MS and the control group was not significant ($p = 0.3432$, *Mann–Whitney test*) and binding was not restricted to MS-derived antibodies as a germinoma-derived antibody (GCT-A10) also was positive in the ELISA, indicating a lack of specificity for MS in the rIgG cohorts.

Screening MS rIgG for Binding to Human CNS-Derived Cell Lines

Limitations of the ProtoArray for autoantigen discovery include the underrepresentation of membrane proteins on the array that would have an extracellular domain accessible to antibody and the possibility of altered structural conformation. Cell-based assays can circumvent such restraints. Increased binding to extracellular components of oligodendrocyte precursor and neuronal-derived cell lines by MS serum immunoglobulin compared to healthy controls has been reported (42). Thus, to complement the array data and address its limitations we screened the MS and control rIgG by flow cytometry for binding to extracellular antigens present on the surface of the CNS-derived cell lines including a human oligogendroglioma cell line (HOG) and a human neuroblastoma cell line (SKNSH). Applying this approach, we found that none of the MS or control rIgG tested bound to the surface of either of the CNS cell lines (Figure S1 in Supplementary Material).

We were also interested to test whether the rIgGs would recognize antigens that reside in the cell cytoplasm as CNS resident antibodies may be exposed to such antigens during tissue damage. Flow cytometry was used to screen for intracellular binding of the rIgGs to the HOG cell line. All of the rIgGs from the MS and control cohorts bound to the permeabilized cells (Figure

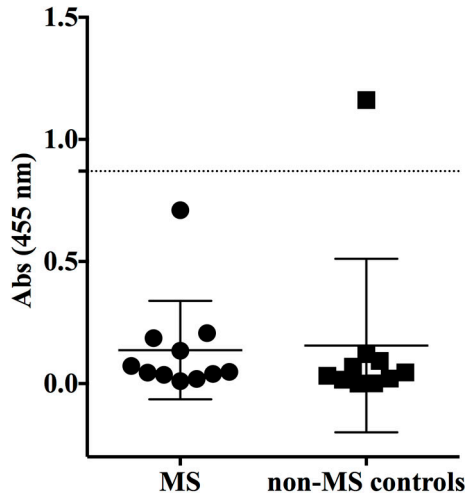

FIGURE 3 | MS and control-derived rIgG binding to protocadherin gamma (PCDHGC3) by solid phase ELISA. MS-derived rIgG (n = 11) and control rIgG (n = 10) derived from a germinoma and muscle tissue were tested by solid phase ELISA for binding to protocadherin. The specific samples included in the assay are shown in the Supplementary Material. Each dot or square represents the binding of a single rIgG. The dashed line indicates the mean +2 SD of the control-derived cohort (0.87). Values above this line were determined to be positive (95% CI). The mean and SD are shown for each data set. Statistical differences are indicated when significant. Data associated with each rIgG for the MS and control groups are shown in the Supplementary Material.

S3 in Supplementary Material). A similar pattern of binding was obtained with the 293T cell line (*not shown*). Overall, there were no significant or remarkable distinctions between the flow cytometry MFI histograms of the two cell lines for both the MS and control rIgGs, indicating that intracellular components are frequent non-specific targets of antibodies.

DISCUSSION

The purpose of our work was to investigate the antigen specificity of the humoral immune response in MS. We focused on those B cells that reside at the site of tissue damage in MS brain as this compartment likely represents an enrichment of disease-associated antibodies compared to serum and to the CSF. Two studies have leveraged a similar approach to examine the specificity of antibody-secreting cells present in the MS CSF (43, 44). While these studies suggested that MS-derived B cells recognize myelin, also derived from MS brain, the specific myelin component could not be identified. Moreover, no reactivity was observed to the major myelin protein antigens, MBP, and MOG. Using CNS tissue-derived B cell products, we found that MS-derived antibodies did recognize a number of candidate antigens, but when challenged with a matched set of appropriate controls the MS specificity did not persist. We carefully selected control rIgG that shared the same properties as the MS-derived rIgG. In studies outside of our MS program, we have characterized the B cells that infiltrate particular solid CNS tumors and the muscle tissue of patients with myositis. In both instances, similar to what we observed in MS, the tissue-enriched B cell repertoire was class switched, clonally expanded and somatically hypermutated. Clones were selected from these repertoires in the same manner as those selected from our MS cohort. In light of these considerations, we suggest that the set of controls we employed are superior to controls produced by naïve B cells or random memory B cells from the circulation. In spite of using candidate antigens coupled with systems biology approaches, we did not identify a validated target for the B cells that reside in the MS CNS tissue. Overall our study highlights the difficulty inherent in antigen discovery approaches, but provides a methodological road map for improvement in the field with emerging technology. We postulate that the target of MS CNS B cells may be: an undiscovered common antigen that may include post-translational modification; antigen(s) possibly exposed through tissue damage; a collection of target antigens that vary among smaller MS population subsets and/or vary based on compartments (serum/CSF) or an infectious agent in the brain itself that would not be identified using our current screening approaches.

Antibody-independent mechanisms may help to explain the pathological contribution of B cells to MS. However, the antigen-driven characteristics of MS CNS B cells still point toward a role for antibody-dependent mechanisms. It is intriguing that B cells have been shown to form structures that resemble ectopic germinal centers in the meninges of MS patients (5, 18, 45) where they display all of the characteristics of an antigen-driven response. Similar organized structures have also been found in a number of autoimmune disease tissues (46) and often in different solid tumors (33). As an example, tertiary lymphoid structures have been identified in the thymus tissue of myasthenia gravis (MG) patients (47), where they have been shown to contribute to MG autoantibody production. With respect to MS, the question remains as to the target of the B cell response. Are the B cells directed toward a CNS target that is involved in disease initiation or is the B cell response generated as an indiscriminate secondary response to the dead tissue, rather than the cause of the pathology in the first place? In the case of organized tumor immune cell infiltrates, it is expected that there is not an underlying immune dysregulation that would be expected in autoimmune conditions. Given the similarity between these organized infiltrates, this leaves the possibility open that the process in MS may not entirely be a product of abnormal immune regulation. Are the antibodies in the CNS a normal immune response to the ongoing tissue damage that occurs? This possibility could provide an explanation for the B cell response in MS CNS tissue that it is part of apoptotic cell recognition, secondary to the disease pathology and part of a normal response.

Antigen discovery efforts are not without limitations and ours in not an exception. First, we employed LCM to confirm VH and VL domains were endogenously paired. However, this approach provides a low yield of paired VH and VL domains from single cells derived from autopsy tissue, so we chose to also pair domains based on their representation in the respective libraries. We acknowledge that pairing of VH and VL domains based on their dominant distribution in the repertoire is not a guarantee that they were naturally paired in a single

cell; however, it represents the best possible means toward obtaining rIgG from autopsy tissue with current technology. High-throughput technology to pair heavy and light chains from single cells (48) was not available when our study began and this technology is currently limited to use with cells in suspension, which is not possible with cryopreserved autopsy tissue. Pairing based on the most highly represented VH and VL domains was successfully demonstrated in a vaccination setting (49). Furthermore, "knock in" transgenic mice, which express the VH from the 8-18C5 anti-MOG antibody, produce antibody using the endogenous light chain repertoire of the host, and a large fraction of the antibodies recognize MOG regardless of the light pairing, demonstrating the dominant contribution of the VH in target binding (50).

The second limitation concerns the antigen sources. Although our screening strategy was thorough, it was not exhaustive. We focused on proteins since they constitute antigenic targets more frequently than other molecules. In testing individual antigens, we employed ELISA, which allows for rapid testing of multiple samples, and cell-based assays where physiological epitopes are better emulated. Our use of cell lines offers the advantage of presenting multiple cell surface candidate antigens that are present in the CNS. However, low or sparse endogenous expressional levels of proteins on the cell surface or altered expression can affect antibody binding (51) to such cells. Using tissue offers deliberate sourcing of compartments but also can present technical challenges including antigen recovery in fixed tissue used in immunohistochemistry and low antigen abundance when performing western blots or immunoprecipitations.

The protein array, we employed, offers the advantage of highly enriching low abundance proteins that might not be detectable when presented in other formats such as tissue. Furthermore, inclusion of whole proteins offers the potential for increased sensitivity and specificity compared to peptide arrays and phage display libraries. However, the arrays that we used include some shortcomings, such as an abundance of intracellular proteins and an absence of comprehensive post-translational modifications. Moreover, certain surface proteins that have been implicated in demyelination such as contactin-1 and 2, contactin-associated protein-1 and neurofascin-155 were absent from the panel. Our array results were negative for several surface proteins (catenins, integrins, tetraspanins, claudins) that could be considered biologically plausible targets (52) and were also negative for myelin-associated proteins, as well as for the previously identified intracellular targets of CSF antibodies, myelin-associated enzyme CNPase and RBPJ (30, 53, 54). Positive array hits primarily included several intracellular proteins. Abundant, intracellular protein autoantigens may be useful biomarkers only after extensive validation and scrutiny. Our cell-based assays comparing extracellular and intracellular binding clearly illustrate lack of specificity of the latter. Second, intracellular proteins can be present in many cell types, and therefore lack the tissue specificity that is often associated with validated autoantigens. Thirdly, the question of access to circulating antibodies to intracellular proteins cannot be easily answered. Overall, both our data and other whole protein array studies suggest that IgG specificity may vary among subsets of MS patients (30, 55, 56).

CONCLUSION

A comprehensive method to systematically characterize and screen disease-associated immunoglobulins is needed. Such an ideal system is not yet available, but as one is developed it should include biologically relevant whole proteins presented in their native biological state, that is, with endogenous post-translational modifications and process-dependent modifications that can occur during apoptosis or necrosis. Inclusion of surface proteins should be emphasized. Non-protein antigens would also be required, such as lipids, carbohydrates, and other small molecules. Human-derived antigens represent a priority, but environmental antigen sources such as pathogens and viruses cannot be excluded. Technology is emerging that is approaching these goals through expressing the human genome (57) or virome (58) for the purpose of antibody screening (59). Continued development of these technologies will likely include tertiary structure and post-translational modifications that are important in the formation of many epitopes. Particular focus on MS antigens should start with well-characterized CNS tissue and CSF from early and progressive disease. Now that links between the CNS, CSF, cervical lymphnodes, and peripheral B cells are better understood (19, 60–62), the isolation and examination of particular B lineage subsets in the circulation will be of value. Next generation B cell antibody sequencing now allows comprehensive sequencing of B cell populations to create a repertoire that can be used to guide selection of clones for antigen screening. This can now be coupled with single cell approaches to pair the native VH and VL. The use of animal models to test and validate the contribution of MS-derived immunoglobulin to pathology should be leveraged. Finally, large scale, multi-center studies involving a number of investigators are best suited to tackle this expensive and high-risk endeavor.

AUTHOR CONTRIBUTIONS

The original hypothesis was conceived by DH and KO who also co-directed the project. The study was initiated and designed by DH and KO. The experiments were designed and data were collected by KO, SW, and DH. Data were analyzed and interpreted by SC, PS, AC, KO and SW. SW, SC, PS, and KO wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2015.00600

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REVIEW
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B Cells Are Multifunctional Players in Multiple Sclerosis Pathogenesis: Insights from Therapeutic Interventions

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Multiple sclerosis (MS) is a severe disease of the central nervous system (CNS) characterized by autoimmune inflammation and neurodegeneration. Historically, damage to the CNS was thought to be mediated predominantly by activated pro-inflammatory T cells. B cell involvement in the pathogenesis of MS was solely attributed to autoantibody production. The first clues for the involvement of antibody-independent B cell functions in MS pathology came from positive results in clinical trials of the B cell-depleting treatment rituximab in patients with relapsing-remitting (RR) MS. The survival of antibody-secreting plasma cells and decrease in T cell numbers indicated the importance of other B cell functions in MS such as antigen presentation, costimulation, and cytokine production. Rituximab provided us with an example of how clinical trials can lead to new research opportunities concerning B cell biology. Moreover, analysis of the antibody-independent B cell functions in MS has gained interest since these trials. Limited information is present on the effects of current immunomodulatory therapies on B cell functions, although effects of both first-line (interferon, glatiramer acetate, dimethyl fumarate, and teriflunomide), second-line (fingolimod, natalizumab), and even third-line (monoclonal antibody therapies) treatments on B cell subtype distribution, expression of functional surface markers, and secretion of different cytokines by B cells have been studied to some extent. In this review, we summarize the effects of different MS-related treatments on B cell functions that have been described up to now in order to find new research opportunities and contribute to the understanding of the pathogenesis of MS.

Keywords: multiple sclerosis, B cell subtypes, therapy, antibodies, cytokines, costimulation, antigen presentation

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INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), characterized by demyelination in white and gray matter regions, axonal degeneration, and gliosis (1). MS is the most common chronic neurological disease in young adults affecting more women than men (three to one) with an incidence of 7/100,000 and a prevalence of 120/100,000 in Northern Europe (1). The diagnosis of MS is mostly preceded by a clinically isolated syndrome (CIS), which

is the first clinical manifestation of a demyelinating disease that has not met the criteria of MS yet (2). Different clinical subtypes of MS are described. About 80% of the patients present with relapsing-remitting (RR) MS, which is characterized by disease exacerbations with periods of functional improvement (3). Over time, about 60% of the RRMS patients develop secondary progressive (SP) MS (4). About 10–20% of MS patients show progressive accumulation of disability from onset, referred to as primary progressive (PP) MS (2). According to the revised definitions of MS, above mentioned MS subtypes can present themselves in an inactive and active form (2). The underlying process of disease progression is not completely understood (5). Most MS therapies are primarily designed as treatment for RRMS patients, where there is marked inflammation.

Current data support the conceptual idea of MS as a complex heterogeneous disease caused by interactions between the environment, genetic susceptibility, and a dysbalanced immune system (6–8). Traditionally, T cells were considered as critical immune components required for the induction of MS pathogenesis. Recently, compelling evidence is present highlighting B cells as central components of the disease as well (9, 10). Autoreactive T cells are activated in the periphery most likely via molecular mimicry or bystander activation and home through a disrupted blood–brain barrier (BBB) to the CNS, where they are reactivated by antigen-presenting cells. This triggers the production of different mediators, such as chemokines and cytokines, by T cells, microglia, and other cells of the CNS. This will in turn initiate the recruitment of other inflammatory cells, including B cells and macrophages. B cells have the ability to cross the BBB and undergo stimulation, antigen-driven affinity maturation, and clonal expansion (11). The inflammatory reaction of T, B, and other immune cells leads to demyelinated lesions throughout the CNS (3).

As B cell involvement in MS has become more evident in recent years, more data have been collected concerning the effects of B cells in MS pathogenesis. Proof of B cell involvement in MS is described thoroughly further on in the review. Both B cell subtype distribution and B cell effector functions are important contributors to the disease. These processes are first described in more detail in order to fully understand how these processes are affected in MS patients and modulated by different MS treatments.

B Cell Subtype Distribution in MS

B cell development starts in the bone marrow where a hematopoietic stem cell evolves into an immature $CD19^+$ B cell (**Figure 1**) (12). Transitional B cells ($CD19^+CD38^{++}CD24^{++}$ or $CD19^+CD27^-IgD^+CD38^+$) enter the circulation and mature into naive B cells ($CD19^+IgD^+CD27^-$). Upon antigen recognition, naive B cells proliferate into short-lived plasma blasts ($CD19^+CD138^{++}$ or $CD19^+CD27^+CD38^{++}$) or plasma cells ($CD38^+CD138^+$) that produce low-affinity antibodies for a few days or further mature into memory B cells ($CD19^+CD27^+$) in a germinal center (GC) reaction. A proportion of memory B cells remains non-class-switched memory cells ($CD19^+IgD^+CD27^+$), while others lose their immunoglobulin (Ig)D expression following isotype switching ($CD19^+IgD^-CD27^+$). This classically results in the surface expression of IgG, IgA, or IgE, although a small proportion of memory B cells preserve IgM surface expression, namely IgM only memory B cells ($CD19^+IgD^-CD27^+IgM^{++}$) (13–17). A proportion of the memory B cells further matures into plasma blasts and long-lived plasma cells.

T cell subtypes important for providing help in the GC reactions are follicular helper T cells (TFH), follicular regulatory T cells (TFR), but also Th17 cells that can all induce or regulate GC formation and isotype switching (18–21). Regulatory B cells (Bregs) have been identified more recently by their function in immune regulation via the production of IL-10 (22, 23). Bregs could be enriched from transitional B cells, $CD27^+$ memory B cells and plasma cells. Surface markers to characterize Bregs are still not clearly defined, although in humans CD24, CD38, CD5, and CD1d are mostly used (24–26).

Compositional changes of B cell subtypes in the peripheral blood (PB) are evidenced, shifting the balance toward more pro-inflammatory responses and less regulation. It is thought that memory B cells, plasma blasts and plasma cells preferentially cross the disrupted BBB and migrate into the CNS of MS patients, where they dominate the B cell pool and exert different effector functions (11, 27–35). During MS relapses, the percentage of PB memory B cells is increased (36). As TFH and TFR cells contribute to a normal GC response wherein potential autoantibodies are eliminated, the altered TFH and TFR function observed in MS patients can result in an inadequate GC response and the production of autoantibodies in the PB (18, 19).

In contrast to an increased percentage of memory B cells in PB, the proportion of Bregs was decreased in MS patients, while unchanged compared to healthy donors in other studies (37–40). Breg function was shown to be preserved as no differences were observed between MS patients and healthy donors in the ability of Bregs to inhibit proliferation of $CD4^+CD25^-$ T responder cells (40).

B Cell Effector Functions

B cells exert multiple effector functions, which are relevant to the pathogenesis and therapy of MS (9). First, B cells differentiate into antibody-secreting plasma blasts and plasma cells and produce antigen-specific antibodies (**Figure 2**). IgG from MS patients caused demyelination and axonal damage in a complement-dependent manner when using both *in vivo* and *in vitro* models (41, 42). Plasmapheresis and immunoadsorption in order to remove antibodies and complement factors already showed promising results as treatment for MS patients with steroid-resistant relapses (43, 44). In MS, different antibody targets have been described, including myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), neurofilament, sperm-associated antigen 16 (SPAG16), coronin-1a, heat shock proteins, and other components of the CNS, emphasizing the diversity and complexity of the antibody response (45–54). An extensive review on different antibody targets is found in Ref. (45).

Second, B cells form GC-like structures, ectopic lymphoid follicles, outside of secondary lymphoid organs at sites of inflammation (**Figure 2**). These follicles harbor a local source of class-switched Igs that contribute to the immune response and are detected as oligoclonal bands (OCB) in the cerebrospinal fluid (CSF) of MS patients (55–57). These OCB in the

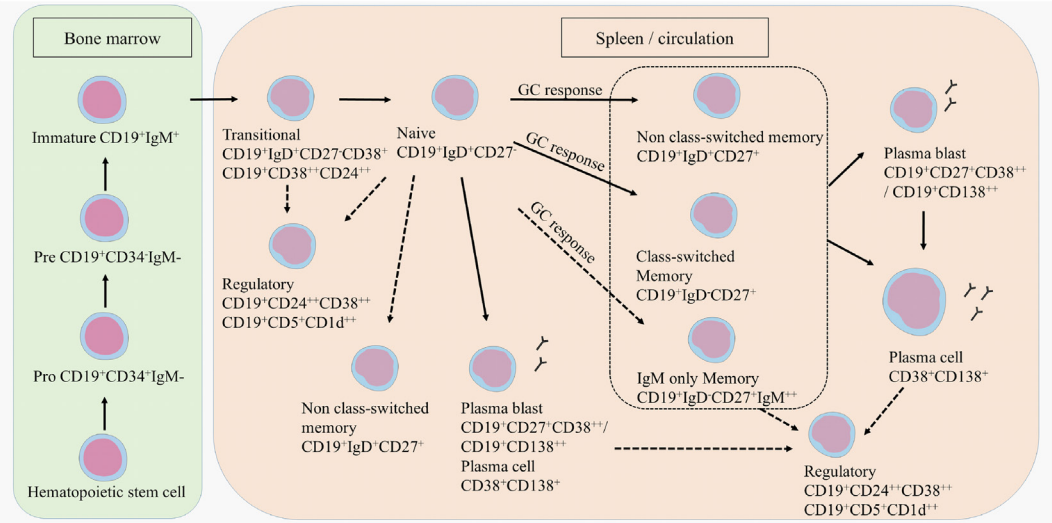

FIGURE 1 | B cell development. B cells develop in the bone marrow and enter the circulation as transitional B cells. B cells remain naive until they encounter an antigen after which they differentiate into plasma blasts, short-lived plasma cells, or further mature into class-switched or non-class-switched memory B cells in a GC response. However, non-class-switched memory B cells can also be formed independent of a GC. A proportion of the memory B cells further develops into plasma blasts and/or plasma cells. Regulatory B cells are characterized within the transitional, naive, memory, and plasma blast or plasma cell population. Potential developmental routes are indicated with the dotted lines.

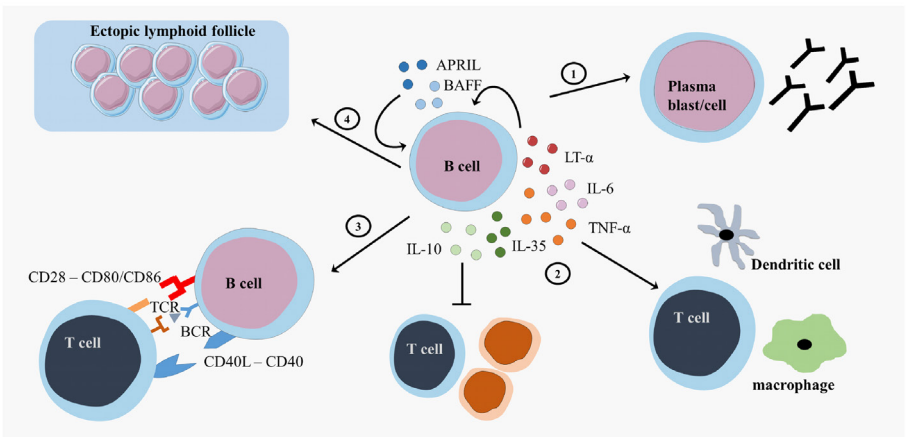

FIGURE 2 | B cell effector functions. B cells exert different effector functions. B cells evolve into plasma blasts or plasma cells and produce antibodies (1). B cells produce different pro-inflammatory cytokines (lymphotoxin (LT)-α, tumor necrosis factor (TNF)-α, interleukin (IL)-6 or regulatory cytokines (IL-10, IL-35)) that influence other immune cells (2). B cells present antigens to T cells and provide costimulatory signals in order to induce appropriate T cell responses (3). B cells form ectopic lymphoid follicles that support the inflammatory responses (4). CD, cluster of differentiation; CD40L, CD40 ligand; APRIL, a proliferation-inducing ligand; BAFF, B cell activating factor; TCR, T cell receptor; BCR, B cell receptor.

CSF of MS patients were one of the first findings for B cell involvement in MS (58, 59). Intrathecal B cells are the local source for these OCB in the CSF, contributing to inflammation, and the destruction of the myelin sheet in the CNS (60). B cells migrate to the CNS using surface markers such as C–X–C motif receptor (CXCR)3, CXCR5, and CC chemokine receptor (CCR)5. The CNS has a fostering environment in which the production of CXCL10 and CXCL13 attracts B cells (61). In the meninges of MS patients, these migrated B cells form ectopic GC structures (57).

Third, B cells serve as highly effective and selective antigen-presenting cells leading to optimal antigen-specific T cell expansion, memory formation, and cytokine production (**Figure 2**) (62–64). After antigen binding by the B cell receptor (BCR), the antigen is internalized, processed, and expressed on the surface of the B cells as a complex with major histocompatibility complex

(MHC)-I or II molecules. Additional to antigen-presentation molecules, costimulatory molecules, such as CD80, CD86, and CD40, are expressed on B cells and contribute to optimal T cell activation (65). Myelin reactive peripheral B cells can induce $CD4^+$ T cell responses in a proportion of MS patients (66). Additionally, B cell expression of the costimulatory molecules CD80 and CD86 is higher in MS patients than healthy controls (67, 68).

Finally, B cells support or regulate effector immune functions via the secretion of different cytokines (**Figure 2**). B cell activation factor (BAFF) and A Proliferation-Inducing Ligand (APRIL) are important survival factors for B cells and plasma cells, thereby maintaining the B cell pool (69). BAFF expression is upregulated in active and inactive MS lesions (70, 71). Maintaining BAFF expression within certain limits in order to balance pro-inflammatory and regulatory B cell subtypes can be an important feature for MS therapies. B cells support pro-inflammatory functions through secretion of tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and lymphotoxin alpha (LT-α) and exert regulatory functions via the production of IL-10 and IL-35 (22, 23, 72–75). In healthy individuals, transitional B cells perform regulatory functions by producing IL-10, thereby suppressing antigen-mediated T cell activity (26). Within the $CD27^+$ memory B cell and plasma cell population, IL-10 and IL-35 producing Bregs can be enriched, showing that more mature B cells can also have regulatory functions next to antibody production and T cell activation (23, 25, 76–78). B cells from MS patients showed an increased production of IL-6, an increased LT-α/IL-10 ratio and increased LT-α and TNF-α production after stimulation *in vitro* (70). In addition, B cells from untreated MS patients secreted more pro-inflammatory IL-6 and less regulatory IL-10 than those from healthy controls (37, 79, 80).

Additional Proof of B Cell Involvement in MS

Additional proof of B cell involvement in MS came from analysis of BCR sequences and genetic and animal studies. Analysis of Ig heavy chain variable sequences (VH) of intrathecal B cells from MS patients showed a restricted usage of Ig VH gene segments, pointing to a chronic antigen-driven B cell response in MS patients (81–83). Genetic studies in MS identified susceptibility genes that show a strong association with B cell function, such as HLA-DRB1*1501, HLA-DRB5*0101, and HLA-DQB1*0602 (84). Also observations from clinical trials of the B cell-depleting anti-CD20 monoclonal antibody rituximab indicated the importance of antibody-independent B cell functions in the pathogenesis of MS. These clinical studies showed an unchanged level of total Ig and a decrease in CSF T cell numbers, providing additional proof that B cells highly interact with T cells in MS (85–87).

Other information about the involvement of B cells in the pathogenesis of MS is available from experimental autoimmune encephalomyelitis (EAE), the animal model of MS. The role of B cells in EAE has long-time been neglected as B cells are not essential contributors to EAE models based on peptide immunization. More recent studies using recombinant MOG protein immunization have highlighted the role of B cells in EAE induction and pathology (88). The dual role of B cells in EAE was indicated by the use of anti-CD20 treatment, as disease exacerbation was evident when depleting Bregs before EAE induction while disease severity decreased when depleting memory B cells after EAE induction (22, 89, 90). B cells were essential for the generation of optimal pathogenic $CD4^+$ T cell responses and differentiation of MOG specific T-helper (Th)1 and Th17 cells (91). In B cell deficient mice, EAE induction by adoptive transfer of activated T cells was reduced and reactivation of infiltrated T cells was impaired (92). Further, B cell-specific MHC class II-deficient mice were resistant to EAE induction and exhibited diminished Th1 and Th17 responses (93). Hence, B cells can promote EAE induction by acting as antigen-presenting cells. Moreover, B cell antigen presentation was proven to be crucial for maximal disease in EAE, further emphasizing the importance of B cells in MS pathogenesis (94).

Recently, a direct link between peripheral and intrathecal B cells was demonstrated. Clonally expanded autoreactive B cells with signs of affinity maturation were, next to the CSF, found in the PB of MS patients (82, 95). Further, expanded B cell clones were found both in the PB/draining cervical lymph nodes and the CSF, indicating a complex crosstalk between the periphery and the CNS in MS pathogenesis (27, 96). Exchange of B cells between the CSF and the PB may suggest that B cells carry antigen from the CNS to peripheral secondary lymphoid organs (11). Primed T cells then migrate to the CNS where residing B cells may further promote T cell activation. These data underline the importance of using therapeutics based on the inhibition of B cell transmigration into the CNS or that induce peripheral B cell depletion (11, 27, 96, 97). Additionally, autoreactive B cells can be removed from the B cell pool via both a central and a peripheral checkpoint. It seems that especially the peripheral tolerance checkpoint is defective, as shown by the equal proportion of polyreactive and anti-nuclear transitional B cells in MS patients and healthy donors (normal central B cell tolerance) and the increased proportion of mature naive B cells from MS patients reactive toward peripheral and CNS self antigens (defective peripheral B cell tolerance) (98). This defect is probably due to impaired Treg function that leads to the accumulation of autoreactive B cells (99). All these observations strengthen the idea that PB B cells contribute to the pathogenic B cell pool present in the CNS of MS patients and are involved in MS pathogenesis both by antibody-dependent and -independent B cell functions. Thus, investigating PB B cells and the effects of treatment on peripheral B cell functions may contribute to our understanding of the pathogenesis of MS (80, 85–87).

This review summarizes how current MS treatments influence B cell functions. At the moment, numerous FDA approved MS treatments or drugs in clinical trials can be subdivided in first-, second- and third-line therapies (**Tables 1–3**). Generally established first-line therapies include interferon-beta (IFN-β) and glatiramer acetate (GA), while fingolimod and natalizumab are considered to be second-line treatments. The recently approved oral drugs teriflunomide and dimethyl fumarate (DMF) are oral treatments used as first-line treatment for MS (100–105). Second- and third-line antibody treatments are rituximab, alemtuzumab, ocrelizumab, ofatumumab, and antibodies that target BAFF and APRIL. Modulating B cell

TABLE 1 | Overview of first-line MS treatments.

Name	Target	Primary mode of action	MS type	Important clinical observations	Reference
IFN-β1a *Avonex®*, IFN-β1a *Rebif®*, IFN-β1b *Betaferon®*	/	• Increases the expression of anti-inflammatory agents while downregulating pro-inflammatory cytokines • Shifts the immune response from a T-helper (Th) 1 phenotype to Th2 • Reduces trafficking of inflammatory cells toward the BBB	RRMS	• Reduction in relapse rate, magnetic resonance imaging (MRI) lesion activity, brain atrophy, risk of sustained disability progression • Increase in time to reach clinically definite MS after the onset of neurological symptoms	(100, 107, 152–157)
Glatiramer acetate *Copaxone®*	/	Induces tolerogenic T cell immune responses and $CD4^+$ and $CD8^+$ regulatory T cells due to mimicry of MBP	RRMS	• Reduction in relapse rate • Improvement of disability measured using Expanded Disability Status Scale (EDSS)	(79, 105, 158–162)
Teriflunomide *Aubagio®*	Dihydroorotate dehydrogenase	Inhibits *de novo* pyrimidine synthesis by blocking the enzyme dihydroorotate dehydrogenase	RRMS	• Reduction in exacerbation rate, annualized relapse rate, risk of sustained accumulation of disability	(163–168)
Dimethyl fumarate, BG-12 *Tecfidera®*	/	• Interferes in the citric acid cycle • Activates the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway	RRMS	• Reduction in annual relapse rate • Reduction in disability progression	(102, 108, 109, 168)

TABLE 2 | Overview of second-line MS treatments.

Name	Target	Primary mode of action	MS type	Important clinical observations	References
Natalizumab *Tysabri®*	VLA-4 (α4-integrin)	Inhibits migration of lymphocytes to the CNS	RRMS	• Reduction in exacerbation rate, annual relapse rate and disability rate	(169–171)
FTY720 *Fingolimod®*	Sphingosine-1-phosphate receptor (S1PR)	• Downregulates S1PR on lymphocytes • Inhibits egression from lymphoid organs into the circulation	RRMS	• Reduction in relapse rate, disability progression and total number of gadolinium-enhancing lesions	(172–180)

TABLE 3 | Overview of third-line MS treatments.

Name	Target	Primary mode of action	MS type	Important clinical observations	References
Monoclonal anti-CD20 antibody rituximab *rituxan®*, *mabThera®*, *zytux®*	CD20	Depletes $CD20^+$ B cells	RRMS PPMS SPMS	• Reduction of new brain lesions and clinical relapses	(85, 87, 181–185)
Monoclonal anti-CD20 antibody *ocrelizumab®*	CD20	Depletes $CD20^+$ B cells	RRMS PPMS	• Reduction in gadolinium-enhancing (Gd) T1 lesions, in total number of new and persisting Gd-enhancing lesions and in annualized relapse rate • Improved efficacy compared with rituximab with lesser infusion-related reactions	(181, 186, 187)
Monoclonal anti-CD20 antibody *ofatumumab®*	CD20	Depletes $CD20^+$ B cells	/	• Reduction in cumulative number of new Gd-enhancing lesions and new and enlarging T2 lesions	(188–191)
Alemtuzumab *campath®, lemtrada®*	CD52	Depletes $CD52^+$ B and T cells	RRMS	• Reduction in rate of sustained accumulation of disability, disability progression, and the annualized rate of relapse • Improvement of disability scores	(120, 192–194)
Anti-BAFF; anti-APRIL *atacicept®,* belimumab *benlysta®*, tabalumab, blisibimod	BAFF and/or APRIL	Blocks activation of B cells via inhibition of BAFF and APRIL or the BAFF receptor	RRMS	• Increase in inflammatory disease activity and annualized relapse rate (atacicept®)	(70, 149–151, 195, 196)

functions is an important tool for treating MS patients, although information on the effects of therapy on B cell functions is limited. Investigating the effects of treatment on B cell functions is of potential relevance to the efficacy of such treatments and it will help to increase our insight into the involvement of PB B cells in MS pathogenesis.

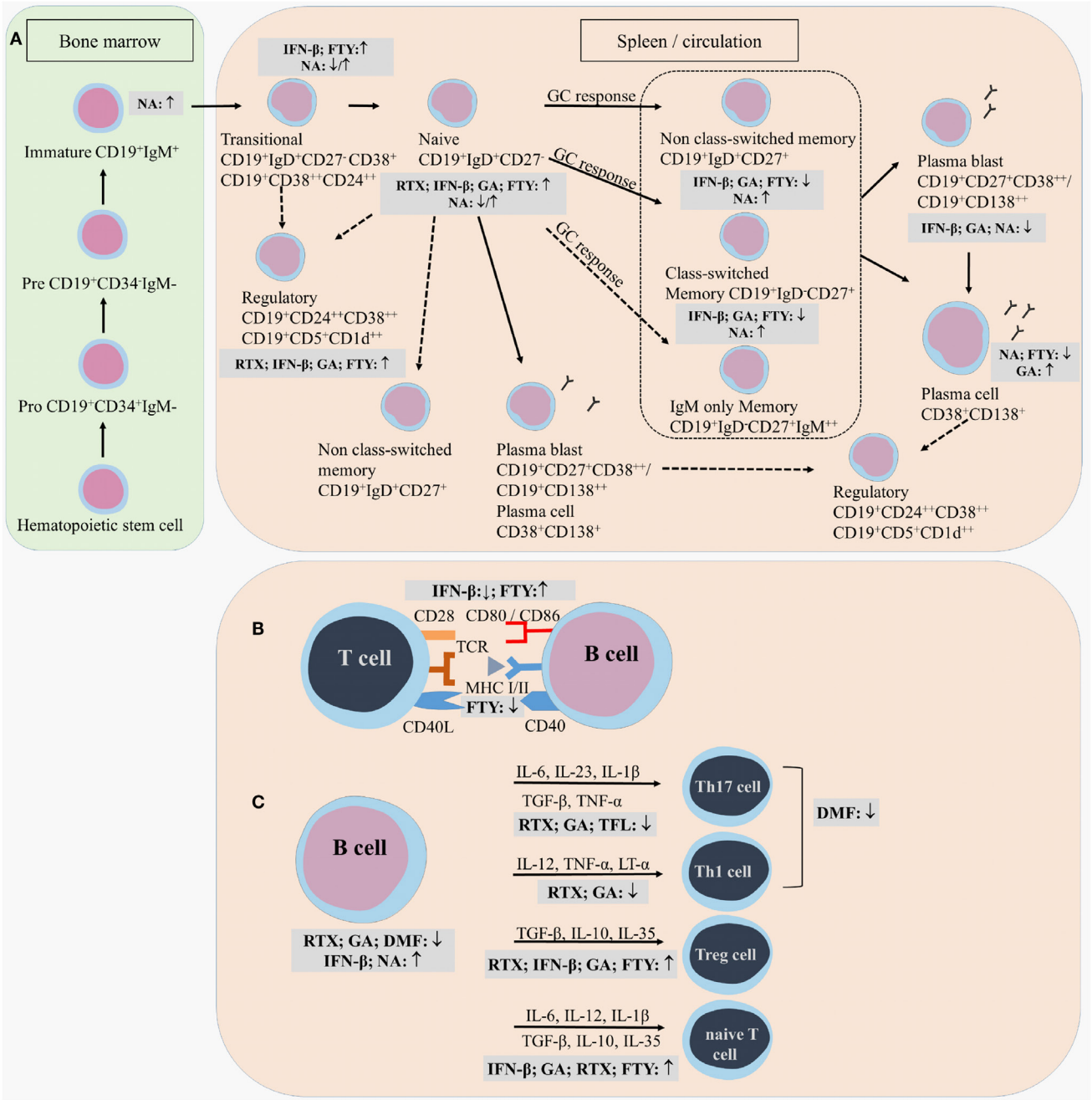

FIGURE 3 | Effects of immunomodulatory therapy on B cell subtype distribution and function. B cell development in the bone marrow and periphery **(A)**, antigen presentation and costimulatory molecules expressed on the B cell surface **(B)** and B cell cytokine production **(C)** are shown together with the effects of treatment on the different B cell subtypes and functions. CD, cluster of differentiation; IFN-β, interferon-β; FTY, fingolimod; GA, glatiramer acetate; NA, natalizumab; DMF, dimethyl fumarate; TFL, teriflunomide; RTX, rituximab; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor; Th, T helper.

EFFECTS OF TREATMENT ON TOTAL B CELL NUMBERS

Total B cell numbers and percentages in the PB were changed during treatment, both in cross-sectional and longitudinal studies, with an increase in the frequency of CD19$^+$ B cells in IFN-β-treated MS patients and a decrease in GA- and DMF-treated MS patients (**Figure 3**) (79, 106–110). Different studies indicated that the percentage of B cells was increased in the PB and decreased in the CSF of natalizumab-treated MS patients, due to the inhibition of lymphocyte migration into the CNS (106, 111–118). This increase in PB B cells was observed up to

30 months after start of the treatment (112). Opposite effects were observed in fingolimod-treated MS patients where total B cell numbers in the PB were diminished because of the lymphocyte entrapment within secondary lymphoid organs. No changes were observed in CSF B cell numbers under fingolimod treatment (118, 119). In a study with 69 RRMS patients treated with rituximab, a decrease of 95% in the percentage of $CD20^+$ B cells was evidenced from 2 weeks after treatment until 24 weeks (87). By week 48, B cells returned to 31% of baseline values. Alemtuzumab treatment caused a general depletion of both T and B cells in the PB of treated patients (120).

EFFECTS OF TREATMENT ON B CELL SUBTYPE DISTRIBUTION

Different effects on B cell subtype distribution were demonstrated using different MS treatments. An increased frequency of immature and transitional B cells was generally evidenced under different treatments, including IFN-β, natalizumab, fingolimod, and during repopulation following rituximab or alemtuzumab treatment (106, 107, 111, 113, 120–124). These reports all point toward an increased output of B cells from the bone marrow under immunomodulatory treatment. In this regard, an increased release of lymphoid committed progenitor cells was shown during natalizumab therapy in MS (112). However, in a cross-sectional study with 8 natalizumab-treated RRMS patients, a significant decrease in the percentage of transitional B cells was evidenced (106). Also in fingolimod treated MS patients, the output of newly produced B cells or immature B cells from the bone marrow was decreased (121).

Therapeutic effects on frequencies of Bregs have only been described in relation to the use of fingolimod, rituximab and alemtuzumab therapy. In 48 fingolimod treated MS patients, a proportional increase of Bregs was recently described compared to 74 untreated MS patients and 70 healthy controls (125). During repopulation after B cell depletion by rituximab or alemtuzumab, naive B cells with an increased expression of CD38 and CD5, which are described as Bregs, were predominantly present, both in MS and other autoimmune diseases (31, 122, 126).

Peripheral blood naive B cells were increased in IFN-β, GA, natalizumab, and fingolimod-treated MS patients in comparison with treatment-naive MS patients in different cross-sectional and longitudinal studies (79, 106, 115, 119, 127). This indicates that the B cell population shifts toward a less disease promoting B cell pool after different MS treatments. For GA and natalizumab, this could not be reproduced in other studies where a decreased frequency of naive B cells was observed or no change in B cell subtype distribution at all (106, 107, 127). However, no information was available about the treatment duration, which makes it difficult to compare the studies.

Also contributing to a less disease promoting B cell phenotype is the significant decrease in the frequency of non-class-switched, class-switched memory B cells, and plasma blasts in both cross-sectional and longitudinal studies of IFN-β-, GA-, and fingolimod-treated MS patients, even when using different B cell classifications (36, 79, 106, 107, 119, 125, 127–129). Although a decrease in the proportion of plasma blasts was observed in natalizumab-treated MS patients, a higher percentage of memory and marginal zone B cells was reported (112, 114, 115, 127, 130). This memory B cell increase is probably due to the reduced retention of memory B cells in the spleen (112). In the CSF, natalizumab treatment particularly depleted $CD5^+$ B cells and plasma blasts (131).

Data on B cell subtype distribution are missing for DMF and teriflunomide-treated MS patients. *In vitro* studies have shown that teriflunomide induces cell cycle arrest in B cells without inducing apoptotic cell death (101, 132, 133). Moreover, the effects of different third-line treatments on B cell subtype distribution is poorly investigated in MS as not all treatments are FDA approved and clinical trials are ongoing. To our knowledge, no data are available on the repopulation of B cells after discontinuation of the B cell-depleting therapies ocrelizumab and ofatumumab. Further research is warranted to increase the understanding of the exact mechanism of action and to investigate restoration of the immune balance following depletion therapies.

From this overview, we can conclude that immunomodulatory treatment of MS patients induces a shift in the distribution of B cell subtypes toward a more regulatory or anti-inflammatory phenotype. This is of high clinical importance as a disturbed balance between the different B cell subtypes is observed in MS. For different MS treatments, the effects on B cell subtype distribution have already been investigated to some extent, still conflicting data are present. This is probably due to variation in measurement time points and B cell characterization strategies. Furthermore, as each treatment requires a different time to reach a steady state of immunological parameters and treatment efficiency, it is difficult to compare study results. Therefore, it is essential to use a longitudinal design of the study and take into account the pharmacodynamical properties of the treatment, since some treatment effects could get lost when only measuring in a cross-sectional manner. B cell subtype analysis can also be highly relevant in the search for new markers for progressive multifocal leukoencephalopathy (PML) in natalizumab-treated patients, as B cells were described as potential carriers of the John Cunningham (JC) virus into the CNS (134). Other research is focused on finding risk factors for the development of PML during natalizumab treatment (112, 130, 135, 136).

EFFECTS OF TREATMENT ON B CELL EFFECTOR FUNCTION

Here, we present the available data on the effect of immunomodulatory treatment on antibody-dependent and -independent B cell functions. These include antibody production, antigen presentation, costimulation, migration, and cytokine production (**Figure 3**).

Effects of Treatment on Antibody Production

Glatiramer acetate treatment did not change serum levels of total IgG and IgM in MS patients, but *in vitro* levels of IgG and IgM antibodies were increased after stimulation of PB B cells from these patients (79). Natalizumab-treated MS patients showed lower levels of IgM in both serum and CSF and lower anti-neurofilament light antibodies in the serum than non-natalizumab-treated

MS patients (116, 137). Longitudinal data of 24 MS patients confirmed these results with a decrease in neurofilament light antibody levels, a decline in total IgG levels in the PB and CSF, and a decline in total IgM in the PB (116, 137). Further, the IgG index, which reflects intrathecal IgG production, was decreased during natalizumab treatment, resulting in the disappearance of OCB in some of the treated MS patients (138). Whether a decline in the anti-neurofilament light antibodies is a consequence of a decrease in total antibody levels is not stated. Additionally, vaccination studies in fingolimod-treated healthy volunteers have demonstrated a mild to moderate decrease in IgG and IgM antibody levels toward some antigens, suggesting that fingolimod could reduce autoantibody production in MS as well (139). Teriflunomide, in contrast, did not influence immune responses toward influenza vaccines, indicating that the protective immune responses are preserved in these patients (140).

The anti-BAFF antibody atacicept® did not show beneficial results in clinical trials for MS and even led to worsening of the disease. More patients with optic neuritis who received atacicept® progressed to clinical definite MS (141). The efficacy of this therapy was proven in a clinical trial for RA wherein circulating IgG and IgA rheumatoid factor (RF) and total IgM, IgA, and IgG levels were reduced (142, 143). These observations indicate that, although MS and RA are both autoimmune diseases in which B cells are involved, different effector mechanisms of B cells are involved in both diseases. Since atacicept® affects antibody-producing plasma cells and clinical efficacy of atacicept® is shown in RA, one can speculate that in RA pathogenesis autoantibody production is more important than in MS pathogenesis. This underlines the multifactorial functions of B cells in autoimmunity.

Effects of Treatment on B Cell Antigen Presentation, Costimulation, Migration

Most information on effects of treatment on B cell antigen presentation, costimulation, and migration is available for IFN-β. *Ex vivo* analysis of PB B cells from 15 IFN-β-treated MS patients showed a decreased percentage of CD80, CD86, and CCR5 positive total and $CD27^-$ naive B cells compared to untreated MS patients (36). This pointed toward a less migratory and costimulatory phenotype of these B cells in the PB under treatment, which was confirmed *in vitro* (36, 144, 145). Furthermore, the increase in CD80 positive cells during relapses in MS patients was shown to be counteracted by IFN-β treatment (68). Since CD80 expression is associated with a Th1 phenotype and CD86 expression is associated with a Th2 response, these findings could indicate a shift from Th1 to Th2 in IFN-β treated MS patients (146). Within the $CD27^+$ memory B cell compartment, the percentage of CD86 positive B cells was increased while the percentage of CXCR3 positive cells was decreased in the IFN-β group compared to healthy controls, indicating that memory B cells were less able to migrate to the CNS (36). IFN-β pretreated B cells were less able to induce proliferation of anti-CD3 and anti-CD28 stimulated $CD4^+$ T cells than untreated B cells, further proving the immunomodulatory capacity of IFN-β therapy (144).

In a longitudinal study, B cell expression of the adhesion marker intracellular adhesion molecule (ICAM)-3 was reduced during GA treatment, indicating a potential role for GA in controlling the migration of B cells toward the CNS (147). Other longitudinal data showed a decrease in B cell expression of the antigen-presenting molecule human leukocyte antigen (HLA)-DR/DP/DQ and an increase in CD80 and CD86 costimulatory molecules on PB B cells in fingolimod treated MS patients (119). In contrast, a decreased expression of CD80 and stable CD86 expression was evidenced on B cells from fingolimod treated MS patients when compared to untreated MS patients in another study (128).

No data are present, to our knowledge, concerning the effects of DMF, teriflunomide, natalizumab and the CD20-depleting antibodies like rituximab, ocrelizumab, and ofatumumab on B cell surface expression of antigen presentation, costimulation, and migration markers. Natalizumab treatment could indirectly have an effect on these B cell functions due to the observed B cell subtype redistribution and general immune modulation. Because DMF and teriflunomide are recent FDA approved drugs, more research is warranted to investigate the effects of these treatments on B cell functions. Still, it can be concluded that different MS therapies can influence the interaction of B cells with T cells or other immune cells. As a consequence, inflammatory responses that are detrimental for the CNS are tempered, which is reflected in the clinical outcome of the treated MS patients.

Effects of Treatment on Cytokine Production by B Cells

In a cross-sectional study of IFN-β treated RRMS patients, increased serum levels of BAFF were observed compared to healthy controls, untreated, and GA-treated RRMS patients (107, 148). Twelve months after discontinuation of alemtuzumab treatment, increased serum BAFF levels were also observed (122). The BAFF-depleting antibody atacicept® exacerbated MS, which could be due to the decreased functionality of Bregs, as BAFF and APRIL signaling is highly implicated in the survival of Bregs. Still, the exact reason for the observed increased disease activity needs to be elucidated (149–151).

In terms of changes in cytokine production, IFN-β treatment caused induction of IL-10 production by B cells *in vitro* (144). Although GA did not directly modulate B cell proliferation or cytokine secretion *in vitro* (9), *ex vivo* analysis showed an increased secretion of IL-10 by B cells of 22 RRMS patients treated with GA (79). Intracellular flow cytometric analysis of B cells isolated from GA treated MS patients showed no increased frequency of IL-10 positive B cells in the PB of MS patients, indicating that GA does not influence the number of cytokine producing cells but rather the secretion of the cytokines (79). Further, a decreased capacity to secrete LT-α and IL-6 was indicated after B cell stimulation via CD40 and CD40L interaction or via Toll-like receptor triggering (79). An elevated IL-10 production was also evidenced for PB B cells from fingolimod-treated MS patients and repopulated B cells after rituximab treatment (31, 80, 125, 128). In fingolimod treated MS patients, the increased IL-10 production was accompanied by a decreased TNF-α production, while B cells following rituximab treatment secreted less pro-inflammatory cytokines IL-6, LT-α, and TNF-α (31, 80, 125, 128). Limited data is present of the effects of DMF and teriflunomide on the immune function in

MS patients. In psoriasis patients, it was shown that DMF altered the immune and T cell cytokine profile (102, 110). Teriflunomide limits the secretion of pro-inflammatory molecules by immune cells, including IL-6 and IL-8 (101).

Thus, similar effects have been observed for all studied treatments on the cytokine production by B cells, correcting the imbalance between regulatory and disease promoting B cell functions in MS. We have to keep in mind that since different B cell subtypes produce different cytokines, by changing B cell subtype distribution, cytokine balances are changed as a secondary effect of the treatment. Data are missing on the effects of treatment on cytokine production by B cells for some FDA approved treatments such as natalizumab and for some treatments in clinical trials such as anti-CD20 monoclonal antibodies. It can be speculated that a potential mode of action by which these treatments contribute to the improvement of MS pathogenesis can be by influencing B cell cytokine production from a pro-inflammatory phenotype toward a more regulatory phenotype, still this needs to be further investigated.

CONCLUSION

It is eminent that B cells are major players in MS pathogenesis and contribute to the disease via both antibody-dependent and -independent mechanisms. B cells are essential for antigen presentation and costimulation of T cells, for the production of cytokines and to produce antibodies that will target components of the CNS. Thus, focusing on effects of treatment on these cells will help in our understanding of MS pathogenesis. Although initially not designed for that purpose, many MS modifying treatments influence both antibody-dependent and -independent B cell functions. Research on effects of therapy on B cell phenotype and function has demonstrated a shift from pro-inflammatory B cell functions toward more anti-inflammatory and regulatory functions. Still, each treatment influences this balance in its own manner. IFN-β, natalizumab, fingolimod, BAFF and APRIL targeting monoclonal antibodies, rituximab and alemtuzumab, induce compositional changes of the B cells, resulting in a less disease promoting distribution of B cells in both the PB and CSF of MS patients. GA, DMF, and teriflunomide work primarily via modulating B cell cytokine production. Still, all these effector mechanisms of B cells are interconnected and cannot be separated from each other. Investigating the mechanism of action of different treatments in different autoimmune diseases leads to new insights into that specific disease. For example, atacicept® has different clinical effects in RA and MS, indicating different roles of B cells in these diseases. More research is needed since inconsistencies between studies are present due to differences in B cell subtype definition and time point of measurement. Consensus in B cell subtype characterization will have added value in future research. Further, researchers should take into account pharmacodynamics of the compounds in order to decide on the specific time point for measuring B cell characteristics. Analysis of treatment effects on B cell subtype distribution and function can alternatively lead to prognostic knowledge for determining therapy efficiency. Finally, research should focus on finding specific therapies for the treatment of SPMS although initial efforts have been made. Further analysis of B cell functions in MS pathogenesis and the effects of treatment on these functions is hereby important in order to increase insight into the role of B cells in the disease process. This could lead to the development of novel and more specific therapies.

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REVIEW
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Hypersensitivity responses in the central nervous system

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Immune-mediated tissue damage or hypersensitivity can be mediated by autospecific IgG antibodies. Pathology results from activation of complement, and antibody-dependent cellular cytotoxicity, mediated by inflammatory effector leukocytes include macrophages, natural killer cells, and granulocytes. Antibodies and complement have been associated to demyelinating pathology in multiple sclerosis (MS) lesions, where macrophages predominate among infiltrating myeloid cells. Serum-derived autoantibodies with predominant specificity for the astrocyte water channel aquaporin-4 (AQP4) are implicated as inducers of pathology in neuromyelitis optica (NMO), a central nervous system (CNS) demyelinating disease where activated neutrophils infiltrate, unlike in MS. The most widely used model for MS, experimental autoimmune encephalomyelitis, is an autoantigen-immunized disease that can be transferred to naive animals with $CD4^+$ T cells, but not with antibodies. By contrast, NMO-like astrocyte and myelin pathology can be transferred to mice with AQP4–IgG from NMO patients. This is dependent on complement, and does not require T cells. Consistent with clinical observations that interferon-beta is ineffective as a therapy for NMO, NMO-like pathology is significantly reduced in mice lacking the Type I IFN receptor. In MS, there is evidence for intrathecal synthesis of antibodies as well as blood–brain barrier (BBB) breakdown, whereas in NMO, IgG accesses the CNS from blood. Transfer models involve either direct injection of antibody and complement to the CNS, or experimental manipulations to induce BBB breakdown. We here review studies in MS and NMO that elucidate roles for IgG and complement in the induction of BBB breakdown, astrocytopathy, and demyelinating pathology. These studies point to significance of T-independent effector mechanisms in neuroinflammation.

Keywords: antibody, complement, neuroinflammation, multiple sclerosis, neuromyelitis optica, autoantibody, central nervous system

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Introduction

Evolution and function of the immune system in mammals are driven by the need for protection against pathogenic infection. The balance between the conflicting requirements for capacity to recognize a universe of continually evolving microorganisms while avoiding deleterious responses to self poses a challenge. Hypersensitivity responses are defined as disorders that are caused by the immune response and include autoimmune responses. Three of the four classically described types of hypersensitivity involve antibodies. Type I hypersensitivity involves IgE antibodies and atopy and will not be discussed further. Types II and III hypersensitivity involve IgG antibodies and are

implicated in immune pathology, especially the Type II hypersensitivity response, which explicitly involves autospecific antibodies. Type IV hypersensitivity involves T cell response, particularly CD4 T cell responses.

Generation of the self-specific antibodies that underpin the Type II hypersensitivity response occurs during B cell development when IgH V, J, and D segments recombine with junctional diversity, as well as IgL V–J recombination, resulting in over 10^{11} potential specificities. Selection against self-recognition occurs via IgL receptor editing as well as deletion via apoptosis. Nonetheless, B cell receptors with specificity for autoantigens persist in the adult repertoire. Protection against autoimmunity relies on a number of regulatory mechanisms, including the requirement for T cell help to generate a high affinity isotype-switched antibody response and that T cell activation is under separate and complex control. Autoantibodies may contribute to clearance of debris and effete cells as part of physiologically normal function, and so may not always be intrinsically deleterious. The T cell response that is required for IgG isotype switching does not itself need to be autospecific, since B cells may present innocuous or protective cross-reactive epitopes for T cell help (e.g., Molecular Mimicry). Nevertheless, it is clear from the fact of antibody-mediated autoimmune diseases that self-specific B cell clones can become activated and undergo isotype switching, with deleterious consequences. The degree to which this plays a role in neurological disease is of interest here.

Multiple sclerosis (MS) and neuromyelitis optica (NMO) are both autoimmune inflammatory demyelinating diseases in the central nervous system (CNS). The cause of MS is unknown, but multiple factors are considered to be involved in pathogenesis of MS. These include antibody-dependent mechanisms that contribute to the demyelination observed in Pattern II lesion pathology (1). Key features of Type II hypersensitivity that are relevant to discussion of their role in MS are specificity for tissue antigens (therefore autospecificity), recruitment of effector leukocyte responses, and activation of complement. In NMO, autoantibody binding to aquaporin-4 (AQP4) causes inflammation, astrocyte damage, cytokine release, and demyelination (2).

This review will deal with the role of Type II hypersensitivity autoantibody-driven responses in inflammatory demyelinating disease, with particular relevance to MS and NMO.

Autoantibody in MS

Detection of IgG oligoclonal bands (OCB) in the cerebrospinal fluid (CSF) is one of the clinical criteria supporting the diagnosis of MS (3). CSF OCB occur in more than 90% of MS patients (4). Other isotypes, such as IgM and IgA, can also be found in CSF OCB (5, 6). Intrathecal IgM synthesis, presumed to be T cell independent, has been detected in 55% of MS patients (7, 8). OCB and polyspecific production of antibodies against measles, rubella, and varicella zoster virus, the so-called "MRZ reaction," is associated with increased risk of converting from clinically isolated syndrome to MS (9). Possible involvement of antibodies in MS pathogenesis is suggested by beneficial response to therapeutic plasma exchange in MS patients retrospectively identified as having Pattern II lesions (10). However, it is important to note, that treatment with CD20-directed B cell-depleting therapeutics reduced the relapse rate in MS patients without affecting the presence of antibodies in the CSF (11).

Multiple sclerosis lesions have been classified on the basis of pathological patterns. Pattern II lesions are defined by presence of antibodies and activated complement product deposition. These lesions have been described in over 50% of actively demyelinating MS lesions (1). The specificity of the autoantibodies in MS remains largely unknown. MS lesions are mainly found in the CNS white matter, so one might expect candidate autoantibodies to be directed against antigen structures within this region. In MS lesions, autoantibodies against the potassium channel KIR4.1, myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) have been identified (12–15). However, consensus is lacking whether these autoantibodies are of pathogenic significance in patients with MS. A number of studies report failure to detect KIR4.1-specific IgG in serum or CSF from all but a fraction of patients with MS (16). Antibodies to MBP, although detectable, are not considered a meaningful biomarker for MS, since they have also been shown to be increased in response to neuronal damage (17).

The occurrence of pathogenic anti-MOG Ab is very rare in adult MS patients (18–20). Recent studies have described that anti-MOG Ab is detected in pediatric MS, ADEM (21, 22), and now in AQP4 seronegative NMO patients (23–27). During the progression of pediatric MS, epitope spreading can increase the number of CNS-reactive antibodies (28). This process of epitope spreading can be driven by antigen-presenting cells that present products of antibody-mediated breakdown of myelin and axonal specific antigens to T cells in the CNS (28).

Some earlier confusion about anti-MOG IgG in MS derived from use of assay techniques, such as ELISA and Western Blot, which detected antibodies that recognize incorrectly folded and denatured MOG, and therefore did not necessarily recognize MOG expressed in the CNS. Implementation of techniques, such as cell-based and tetramer assays (29), has improved discrimination of pathogenic antibodies and B cells, and, for example, allowed demonstration that axopathic and/or demyelinating autoantibody responses can occur in some patients with MS (30). However, anti-MOG antibodies are not considered to play a major role in adult MS, and at this time, no serum antibody specificity in adult MS is considered to be of diagnostic value. This leaves unanswered the question of what are the antigen specificities in OCB and what is their role in MS. Lipids have been identified among the autoantigens for OCB antibodies (31) and one study showed that lipid-specific oligoclonal IgM antibodies, especially for phosphatidylcholine, were prognostic for aggressive evolution of MS (32).

The animal model experimental autoimmune encephalomyelitis (EAE) can be induced by immunization with different myelin peptides, e.g., from MBP and MOG. This model is generally considered to be a T cell-mediated disease and cannot be transferred with antibodies (33). Nevertheless, co-transfer of IgG specific for MOG converted a non-demyelinating uniphasic EAE in Lewis rats to a relapsing–remitting demyelinating disease (34, 35).

Choice of antigen is highly influential when inducing EAE in mice (36). MOG–peptide-induced EAE has been shown to

have no requirement for B cells, as it can be induced in animals without B cells (36, 37). Depletion of B cells exacerbated the clinical score in p35–55 MOG-induced EAE (38) indicating a regulatory role for B cells in EAE. On the other hand, MOG-specific TCR-transgenic mice that also expressed autoantibodies against MOG showed an accelerated and exacerbated course of EAE (39). Immunization of these or non-transgenic mice with human recombinant MOG extracellular domain or a fusion protein of MBP and proteolipid protein (MP4) both induced EAE, where activated B cells and antigen-specific antibodies played a pathogenic role in association with T cell-mediated inflammation (36, 40–43). Antigen-independent B cell infiltration and ectopic germinal center formation have been shown in mice with EAE induced by immunization with either a fusion protein incorporating the extracellular domain of mouse MOG or p35–55 peptide, both being T cell dependent (44). Thus, antibodies and B cells have a role to play in the animal model for MS, though the specific role is dependent on immunization strategy.

Autoantibody in NMO

In NMO, disease-specific NMO–IgG (primarily of the IgG_1 subclass) is a biomarker. The predominant NMO-associated antibody specificity is for the water channel AQP4 (45). AQP4 is densely localized in membranes of ependymal cells and astrocytes, to form the glia limitans of blood–brain barrier (BBB) and the CSF–parenchymal barrier (46). NMO–IgG/AQP4–IgG is thought to mediate pathogenesis by binding selectively to AQP4 on CNS astrocytes, causing complement fixation, generation of chemotactic signals (e.g., C3a, C5a), immune cell infiltration, and subsequent loss of AQP4 and glial fibrillary acidic protein (GFAP) on the astrocytes (2). Lesions in NMO are frequently found in the optic nerve and the spinal cord central gray matter as optic neuritis and transverse myelitis; however, brain lesions are also found at other sites of high AQP4 expression, such as the circumventricular organs (47–50). NMO–IgG is pathogenic only when reaching the CNS parenchyma as demonstrated in experimental animal studies where direct administration of NMO–IgG into the CNS or into the blood in mice with pre-established CNS inflammation-induced NMO-like histopathology, whereas peripheral administration into naïve animals had no effect (47, 51). In line with this observation, AQP4–IgG may exist for years after the first NMO attack without a relapse (52).

Other reported autoantibodies in NMO include anti-MOG as mentioned above (23–27), NMDA-type glutamate receptor (e.g., CV2/CRMP5), and glycine receptor antibodies (53–55). These and other autoantibodies may be useful biomarkers for NMO. However, their pathogenic importance has not been clarified. Future studies are required to establish this.

Leukocytes in MS and NMO

Although the distribution of actively demyelinating lesions differs between MS patients, they are predominantly found within the optic nerves, spinal cord, brainstem, and periventricular white matter of the cerebral hemispheres (56). It has become clear in recent years that gray matter is not spared, even during the earliest phases of MS. Gray matter lesions show demyelination, neuronal loss, and atrophy (57–59). Gray matter lesions can be localized in or around the cortical and subcortical gray matter (60).

Inflammation is seen in both white and gray matter lesions at different stages of disease. It consists mainly of T-lymphocytes with a dominance of $CD8^+$ T cells. However, B cells and plasma cells are also found in lesions. Macrophages are mainly found in white matter lesions, where they phagocytose myelin (56). The infiltration of T and B cells in CNS lesions was more profound in relapsing MS compared to progressive MS (61). Although the global composition of inflammatory cells is similar between relapsing-remitting and progressive disease (61), the relative number of plasma cells is higher in the progressive phase (61, 62). Clonally expanded B cells are detected in the CSF (63), in the meningeal lymphoid follicles, as well as in the parenchymal infiltrates in MS patients (64–66). A high ratio of B cells to monocytes in the CSF determined by flow cytometry correlated with rapid MS progression (67). Furthermore, lesion activity on MRI correlated with the numbers of plasmablasts in the CSF (68). These findings support a role for B cells in MS pathology.

Comparing the inflammation in MS lesions with NMO lesions, several studies have found that while the infiltrating cells in MS mostly consist of mononuclear cells, such as macrophages and T cells, inflammation in NMO include neutrophils, eosinophils, and mononuclear cells (2, 69, 70). These infiltrating cells, in particular macrophages, are implicated in Type II hypersensitivity through antibody-dependent cell-mediated cytotoxicity (ADCC).

The role of neutrophils and eosinophils in NMO pathology has been studied in animal models where NMO patient autoantibodies have been transferred to the CNS of mice to induce such pathology (51, 70, 71). When mice were made neutropenic, neuroinflammation was greatly reduced at 24 h and 7 days following intracerebral injection of patient autoantibodies (70). The fact that this had no effect on complement activation identified distinct modes of antibody effect. Neutropenic mice did not show loss of AQP4 or myelin, whereas intracerebral injection into neutrophil-enriched mice increased the areas of AQP4 and myelin loss and the number of inflamed cerebral vessels, thereby showing a role for granulocytes in tissue damage (70). Consistent with this, other studies showed that administration of a neutrophil protease inhibitor decreased the loss of AQP4 and myelin (70). Note that these studies would exclude either microglia or macrophages as mediators of pathology, since those cells should not have been affected by manipulations leading to neutropenia.

Eosinophils and neutrophils infiltrated NMO lesions in mice, after continuous infusion of patient autoantibodies (72). These granulocytes correlated to increased lesion size and both ADCC and complement-dependent cell-mediated cytotoxicity (CDCC) were involved (72). In addition, inhibition of eosinophil degranulation protected against ADCC and CDCC (72). Organotypic slice cultures were used to analyze synergy between antibody and leukocytes in induction of pathology. These transwell-based vibratome tissue slices from spinal cord, optic nerve, or hippocampus allowed analysis of an intact neuronal–glial network *in vitro*, and of effects of complement or leukocytes independently of infiltrating blood-derived cells or mediators. Pathology was complement dependent and under circumstances of suboptimal NMO–IgG, could be enhanced by addition of leukocytes, or pro-inflammatory cytokines (73), or eosinophils or their granule

toxins (72). These studies also indicated that granulocytes play a role in formation of NMO lesions through both ADCC and CDCC. Addition of macrophages to slice cultures exacerbated pathology, dependent on complement, whereas natural killer (NK) cells caused loss of GFAP, AQP4, and myelin loss independent of complement (74). However, no evidence was found to support a role for NK cells in pathology in biopsy material from either MS or NMO patients (69), and granulocytes are the more likely effector cell.

Mechanism of action of pathogenic IgG in conjunction with leukocytes, or ADCC, involves Fc receptors (FcR). These are membrane glycoproteins expressed by leukocytes that have specific affinity for the Fc portions of immunoglobulin molecules, and thus link leukocytes via IgG to specific targets while signaling via the FcR. These are essential for a wide spectrum of biological activities, including transport of antibodies across cell membranes, induction of phagocytosis, and regulation of leukocyte function. Cross-linked FcR-bound antibody can initiate a signal transduction cascade that induces immune cell activation, resulting in cytokine production, immune cell proliferation, and degranulation of neutrophils, eosinophils, and mast cells (75, 76).

All of the effector mechanisms thus far described are components of the peripheral immune response. There is thus interest in the extent to which antibody entry from blood contributes to demyelinating pathology.

BBB Integrity in Hypersensitivity Autoimmune Diseases in the CNS

Inflammation during disease activity in MS and NMO is frequently associated with BBB leakage, suggesting infiltration of the brain by inflammatory cells or immunoglobulin entering the CNS from the circulation (77). Studies of lesion pathology suggest that inflammation drives demyelination and neurodegeneration in MS patients (78). The BBB disruption in MS is primarily caused by infiltration of T cells responding to augmented expression chemokines and adhesion molecules at the luminal vascular endothelium, leading to migration of macrophages and dendritic cells, further increase of BBB permeability and leakage of inflammatory cytokines in the CNS to amplify the cascade of events (61). Complement components generated via the complement cascade are implicated in altered BBB permeability, further promoting inflammatory cell recruitment and Ig extravasation. Importantly, there can also be a role for antibody in BBB breakdown.

The BBB may be impaired before the occurrence of demyelinating foci and T-cell infiltration around small vessels (78). Disturbance of the BBB can be visualized by magnetic resonance imaging (MRI) through leakage of the magnetic marker gadolinium (Gd) diethylenetriamine pentaacetic acid (contrast enhancement) (79–81). An abnormal intra-BBB IgG synthesis rate was reported to correlate to the total area of MRI abnormality in the cerebrum (82). Elevated CSF/serum albumin ratio is evidence of BBB damage (83, 84). MS lesions are characterized by centrally placed inflamed veins, and fingerlike extensions of periventricular lesions (so-called Dawson's fingers) (78). Collectively, the diagnostic implications of intra-BBB IgG synthesis and formation of OCB are well-established in MS, but how intra-BBB IgG production influences BBB integrity is not known.

A particular case in point that may help answer this question is provided by studies in NMO. Intrathecal AQP4–IgG is detectable in the CSF of the majority of AQP4–IgG seropositive NMO patients who have acute disease relapse with AQP4–IgG serum titers >1:250 (85, 86). The AQP4–IgG present in the CSF has been correlated with astrocyte damage, a primary pathological process in NMO (87, 88). Intrathecal IgG synthesis in NMO only occurs rarely and does not persist over time, and serum-derived AQP4–IgG is probably of major pathogenic importance (89). Taken together, these findings suggest entry of serum-derived AQP4–IgG to CNS during disease activity in NMO, which may further be deposited on astrocytic foot processes at the BBB, subpial, and subependymal regions. Thus, the destruction of the BBB may be an important step in the development of NMO because circulating AQP4–IgG has to pass through the BBB to reach the astrocytic endfeet, where AQP4 is localized. Astrocytes interact with endothelial cells to maintain the CNS BBB. We have very recently evaluated the pathogenic impact of AQP4–IgG in the CSF and find that intrathecal injection of AQP4–IgG together with human complement into the CSF of mice results in pronounced deposition of AQP4–IgG along subarachnoid space and subpial spaces, which initiated perivascular astrocyte-destructive lesions and consequently BBB breakdown (**Figure 1**) (90). These data suggest a model whereby a small amount of AQP4–IgG initially is spilled over to the CSF, and then initiates a pathogenic process, giving the characteristic CSF data and radiological features of human NMO. Thus, AQP4–IgG in CSF is a significant element in NMO pathogenicity and can be a critical element, which promotes perivascular astrocyte pathology and consequently BBB disruption. Whether these principles can apply to other antibody specificities, such as MOG-IgG, and to MS where there are intrathecal antibodies as well as BBB disruptions now become important questions.

Factors indicative of BBB integrity may serve as surrogate markers of NMO disease activity. Matrix metalloproteinase-9 (MMP-9) participates in the degradation of collagen IV, a major component of the cerebral vascular endothelial basement membrane (91), and of dystroglycan that anchors astrocyte endfeet to the basement membrane (92). MMP-9 is upregulated in MS lesions (93) and elevated serum levels of MMP-9 were reported in NMO and MS patients (91), interestingly higher in NMO than in MS (94), and likely increase BBB permeability in both diseases via effect on CNS microvascular endothelial cells. Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) play important roles in lymphocyte migration into the CNS. Higher levels of ICAM-1 and VCAM-1 have been reported in relapsing NMO patients and in MS compared to patients with non-inflammatory neurological disorders (95, 96). Furthermore, levels in NMO were higher than in MS and correlated with CSF albumin quotient (96). Another NMO marker of BBB breakdown, vascular endothelial growth factor-A (VEGF-A), has been implicated in promoting BBB breakdown in demyelinating disorders (97). Interestingly, an *in vitro* study demonstrated that AQP4–IgG binding to astrocytes alters AQP4 polarized expression leading to increased permeability of the astrocyte/endothelial barrier,

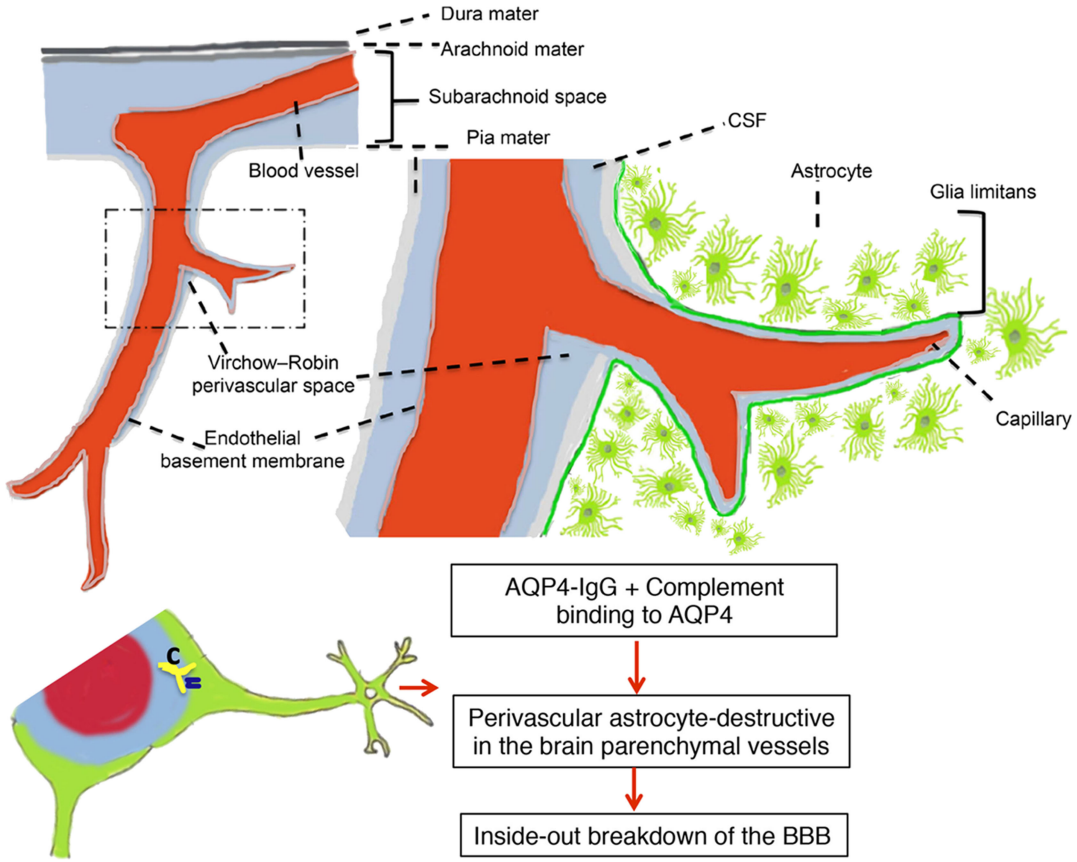

FIGURE 1 | Perivascular astrocyte-destructive lesions in the brain parenchymal vessels associated with breakdown of the blood–brain barrier. Schematic presentation of subpial vasculature in relation to subarachnoid space and brain parenchyma showing relevant anatomical structures, including the pial vessel, subarachnoid space, the Virchow–Robin space, and the subpial glia limitans surrounding penetrating vessels into the brain. The intrathecal distribution pattern of aquaporin-4-immunoglobulin G from cerebrospinal fluid (CSF) into the brain parenchyma via a paravascular route leads to perivascular astrocyte-destructive lesions and blood–brain barrier breakdown [modified after Asgari et al. (90)].

reversed by application of an anti-VEGF-A blocking antibody, suggesting the potential role of VEGF-A in NMO pathology (98). Studies in AQP4 knock-out mice have highlighted important functional roles for AQP4 in the maintenance of BBB integrity as indicated by tight junction opening in brain microvessels, swelling of perivascular astrocytic processes, and BBB hyperpermeability (99). These data suggest that the pathogenic significance of serum-derived AQP4–IgG in NMO include BBB dysfunction. Whether astrocyte specificity of antibodies is required for analogous effects, in NMO as well as MS, is not known.

Central nervous system proteins are detected in sera and CSF of NMO patients, likely as part of compromised BBB and tissue damage. Neurofilament (NF) heavy chain levels have been implicated in optic neuritis associated with NMO, with high serum NF levels correlating with poor clinical outcome (100). In addition, astrocytic markers, including GFAP and S100B, are detected in the CSF in several inflammatory CNS disorders, including MS and NMO, and both are elevated in AQP4 IgG seropositive patients. CSF and serum levels of S100B correlated with active NMO disease, suggesting S100B may be a potential biomarker of acute relapse in seropositive NMO (87, 101).

Blood–brain barrier breakdown is thus a potentially important pathogenic element in inflammatory demyelinating diseases, and may be driven by antibodies as part of hypersensitivity processes in the CNS.

Cytokines and Chemokines in Hypersensitivity Disorders in CNS

Cytokines and chemokines are involved in the control of inflammatory processes associated with demyelinating diseases in the CNS (102). They can be protective, but may also have deleterious effects. Changes in the microenvironment of the CNS following injury trigger an innate immune response, which involves germline-encoded pattern recognition receptors, such as toll-like receptors (103). These receptors recognize endogenous agonists released from damaged tissue as well as molecular patterns expressed by pathogens. This innate immune response includes induction of soluble products such as cytokines and chemokines that are critical for priming the antigen-specific adaptive immune response (104). Infiltrating cells and glial cells are both sources of cytokines and chemokines in the CNS.

Recruitment of leukocytes to tissue in hypersensitivity responses is driven by chemokines and by some cytokines. A number of studies support their involvement in NMO and MS, including that their levels in serum and CSF change dramatically compared to in healthy individuals. The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of MS and in EAE has been broadly studied. Many of them have pathological and clinical significance in the context of autoantibody-mediated demyelination, although this has received less attention. Similarly, although the list of studies that have focused on cytokine and chemokine profiles in NMO is growing (105–110), there is still limited information about their functional significance in the pathogenesis of NMO.

Cytokines and chemokines that are classically implicated in recruitment and activation of B cells and leukocytes in a Type II hypersensitivity response would potentially include B-cell activating factor (BAFF), IL-1β, IL-6, TNFα, type I IFN, CXCL1/CXCL2 (and other CXCR2-binding chemokines), CXCL10 (IFN-induced protein-10), CXCL13 (B lymphocyte chemoattractant), CCL2 (macrophage chemotactic protein-1), and CCL11 (eotaxin). This is by no means a complete list but represents the principal candidate mediators that would be important in antibody-mediated pathology in MS and NMO. Evidence for their involvement is summarized in **Table 1**. Additionally, the role of selected entities, such as IL-1, IL-6, type I IFN, and certain chemokines, are separately discussed.

IL-1

Increased levels of IL-1β have been reported in serum and CSF from MS and NMO patients (110, 139, 140). Increased expression of IL-1β by microglia/macrophages was detected in NMO patients with active lesions (characterized by AQP4 loss, astrocyte injury, immunoglobulin and complement deposition, and granulocyte infiltration). This likely depended on complement activation and granulocyte infiltration, since it was not shown in MS lesions or in advanced NMO lesions, which lacked complement activation and granulocyte infiltration (114). It was also shown that IL-1 enhanced formation of NMO lesions in spinal cord slice cultures treated with NMO–IgG and complement, but not in culture without NMO–IgG (73).

IL-6

IL-6 levels in the CNS are normally undetectable, but increase during neuroinflammation, indicating their involvement in CNS diseases (141). Astrocytes and microglia are both sources of IL-6 (119, 141, 142). Elevated levels of IL-6 in the serum and CSF of NMO patients have also been reported (106, 140, 143). The severity of NMO–IgG and complement-induced lesions was increased when spinal cord slice cultures were treated with IL-6 (73). In another study, IL-6 was injected into the CNS of rats, and at the same time NMO–IgG was administered intraperitoneally. The results showed that IL-6 did not trigger formation of perivascular lesions with AQP4 loss distant from the needle track (114). Such findings suggest that IL-6 contributes to the pathogenesis of NMO as a secondary factor by facilitating the formation of NMO lesions. IL-6 also induces plasmablasts to produce autoantibody (144). IL-6 may also affect BBB integrity and has been implicated in BBB disruption (145, 146). All of these activities would potentially contribute to antibody-mediated pathology in MS and NMO.

TABLE 1 | Cytokines, chemokines, and soluble mediators in CNS hypersensitivity.

Mediator	Cell source	Role in hypersensitivity related process in CNS
BAFF	Astrocytes, leukocytes (111, 112)	Survival and maturation of B cells (111, 112)
IL-1	Microglia, astrocytes, neutrophils (113, 114)	Recruitment of leukocytes (115) Enhance C3 expression by astrocytes (116–118) T cell survival and effector functions (113)
IL-6	Microglia and astrocytes; virtually all immune cells (110, 119, 120)	Recruitment of leukocytes (120) Survival of plasmablasts, production of antibody (110)
TNFα	Microglia, astrocytes, and ependymal cells (121, 122)	Possible role in recruitment of leukocytes (122) Enhance C3 expression by astrocytes (117, 123) Cytotoxic for oligodendrocytes via TNFR1 cells (122)
Type I IFN	Glial cells, neurons, and leukocytes (124)	Proposed to reduce leukocyte migration across the BBB (124) Possible influence on complement induction (125, 126)
CXCL1	Astrocytes (127, 128)	Recruitment of neutrophils and T cells (129)
CXCL10	Astrocytes (121)	Recruitment of macrophages, neutrophils, and B cells (130)
ROS/RNS	Activated macrophages, granulocytes (131)	Influences leukocyte recruitment by affecting BBB permeability, and causing vasodilation (131) Cytotoxic to oligodendrocytes (131)
CXCL13	Microglia (132); follicular dendritic cells (133)	B cell recruitment (133) IgG affinity maturation (133)
CCL2	Glial cells, especially astrocytes (119)	Recruit monocytes through CCR2 (134) Promotes cytotoxic granule release by NK cells (135)
CCL11	Lymphocytes, macrophages, endothelial cells, and eosinophils (136–138)	Recruit eosinophils through CCR3 (137) Activation of basophils and T lymphocytes (136)

Type I IFN

Type I IFNs, including IFN-α and IFN-β, are known to play a crucial role in immune responses by activating JAK/STAT signals through their common receptor (IFNAR) (124). Unlike MS, IFN-β therapy has been reported to have very poor efficacy or to even exacerbate NMO (147) [reviewed in Ref. (124)]. IFN-β treatment in a NMO patient was associated with increased relapses and AQP4 antibody titers (147). Type I IFN signaling via the IFNAR receptor is required for NMO-like pathology in a mouse model (148). IFN-β therapy induced elevated serum levels of BAFF (111), which may facilitate autoantibody production in NMO (149). Elevated levels of IL-17, IFN-β, and neutrophil elastase were reported in serum from NMO patients, and the same study showed that IFN-β increased the formation of neutrophil extracellular traps (NETs) (150). Together, these findings suggested the severe exacerbation and increased relapses in NMO might be associated with IFN-β induced BAFF as well as degranulation and NETs formation by granulocytes (151). The fact that IFN-β had no effect on the development of NMO lesion in spinal cord slice culture, when it is treated with NMO–IgG and complement may reflect lack of neutrophil involvement (73). Lack of effect of IFNAR1-deficiency on cuprizone-induced de- and remyelination or glial cell response (152) may also reflect lack of neutrophil involvement.

Cytokine Regulation of Complement in CNS

The complement system is an essential part of innate immunity and is important for protection against pathogens. The complement system is implicated in the pathogenesis of both MS and NMO (153, 154). Complement is activated by classical, alternative, and lectin pathways. All three pathways lead to activation of C3 convertases, release of C3b opsonin, C5 conversion, and, finally, membrane attack complex (MAC) formation. The activation of the complement pathway yields also C3a and C5a anaphylatoxins, potent inflammatory mediators, which target a broad spectrum of immune and non-immune cells. C3a and C5a are strong leukocyte chemoattractants, including neutrophils and B cells (75, 155). The classical pathway plays a major role in antibody-mediated pathology, and is activated when IgG or IgM antibodies bind to cell surface antigens.

Biosynthesis of complement in the human brain is reported to be generally low or non-detectable under normal health conditions (153). Complement activity presents a potent threat to the body's own cells that are tightly protected by complement regulatory proteins, including decay-accelerating factor (DAF) and CD59. These complement regulatory proteins exist to protect the body's own cells from damage caused by the activation of the complement pathway by blocking the formation of the C3 convertase and the MAC, respectively. DAF prevents the formation of C3 convertase by accelerating dissociation of C4b2a and C3bBb (classical and alternative C3 convertase). The complement regulator CD59 blocks the formation of the MAC by binding to C8, and thereby preventing further assembly of MAC. Therefore, the regulation of the expression of CD59 is a potentially important factor in protecting against MAC-mediated cytopathology (153). It has been shown that NMO–IgG and complement caused more severe longitudinally extensive spinal cord pathology in mice that lacked the complement regulator protein CD59 (156). However, the mechanism responsible for regulation of CD59 remains largely unknown.

Complement binding receptors are expressed on the surface of leukocytes and contribute to their response. The complement receptor 1 (CR1) is expressed on both neutrophils and B cells. It blocks the formation of C3 convertase by preventing its association with C2a. In addition, complement receptor 2 (CR2) participates with the B cell co-receptor complex in B cell activation. Complement receptors 3 and 4 (CR3 and CR4) are expressed on neutrophils and stimulate phagocytosis of bacteria and other particles that have complement components bound to their surface. CR3 is also important for leukocyte adhesion and migration processes (75, 153).

Complement was suggested to play a role in IL-6-induced CNS pathology (123). However, in contrast to IL-1β, IL-6 had no effect on the induction of complement by astrocytes in cell culture (157). The induction of complement seen in GFAP–IL-6 transgenic mice (123, 158), therefore, might not reflect the action of IL-6 alone, but rather of IL-6 acting in concert with other cytokines, including IL-1β. IL-1 is involved in regulation of complement component C3 in astrocytes (157, 159). Whether and how IL-1 influences complement-mediated astrocyte damage remains to be addressed.

Type I IFN can also influence complement in the CNS. The level of terminal complement complex, C1-inh, C4, and C3bc increased in IFN-α2a-treated MS patients during the initial part of the treatment (125, 126). It was shown that IFN-α and IFN-β, in a dose-dependent manner, stimulated the synthesis of C2, C1-inh, and factor B, but not C3 in human monocytes *in vitro* (160). It was earlier noted that IFN-α/β selectively stimulated the synthesis of factor B and C1 inh, but reduced C3, and had no effect on C2 (161). The results from these studies suggest the involvement of type I IFN in the induction of selective complement components, but how the increased complement level is directly mediated by IFNAR signaling was not determined. In antibody-mediated pathology, such as in NMO, where the complement system is known to play a significant role and there is evidence for the involvement of type I IFN, it is tempting to speculate that the induction of complement by type I IFN is one of the underlying mechanisms that facilitate the formation of NMO lesion.

Regulatory Role for Microglia in Antibody-Mediated Pathology

Microglia are considered to play a critical role in regulation of inflammatory processes within the CNS. In this regard, IL-6 also exerts a protective function and has anti-inflammatory activities (162, 163). Administration of human rIL-6 dramatically reduced demyelination and inflammation, which was induced by TMEV in the spinal cord of mice (164). A fusion protein of the soluble IL-6 receptor to IL-6 (IL6RIL6) prevented neuronal

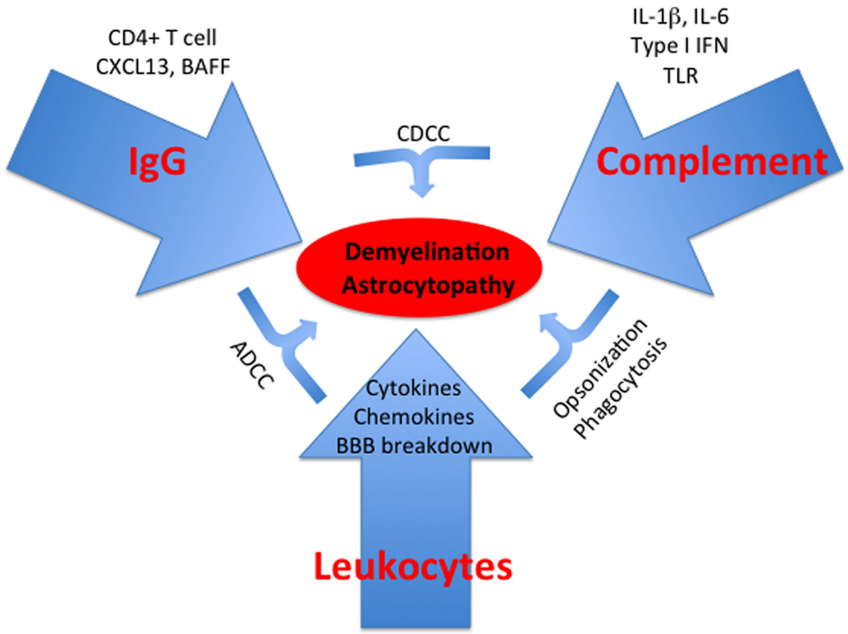

FIGURE 2 | Type II hypersensitivity responses in the CNS. Schematic summarizing key aspects discussed in the text. TLR, toll-like receptor.

and oligodendrocyte degeneration in organotypic hippocampal slices (165). This is in line with *in vivo* results that showed administration of IL6RIL6 to rats after sciatic nerve transection-stimulated remyelination (166) as well as -accelerated regeneration of axotomized peripheral nerve in transgenic mice expressing both IL-6 and IL-6R (167). However, IL-6-activated microglia produced NO, resulting in neural injury *in vitro* (168).

Chemokine and Cytokine Involvement in Leukocyte Recruitment

The cytokine IL-6 is implicated in extravasation of leukocytes into the CNS (168–172). Injection of IL-1β into CNS caused the formation of perivascular lesions with granulocytic infiltration and AQP4 loss distant from the injection site (114), also suggesting a role for IL-1β in leukocyte extravasation. The level of CXCL10, a downstream chemokine of type I IFN signaling (173–175), is elevated in NMO (105, 109). Astrocytes (119) and neutrophils (176) both produce CXCL10. Although CXCL10 is primarily associated with recruitment of T cells, it can also induce neutrophil recruitment (177, 178). One mechanism by which type I IFN signaling exacerbates NMO may involve induction of CXCL10 and thereby recruitment of neutrophils.

Concluding Remarks

We have here reviewed evidence for a role for antibody-mediated hypersensitivity mechanisms in MS and NMO. It must be emphasized that these mechanisms do not normally occur in isolation from effector T cell-mediated responses, whether $CD4^+$ or $CD8^+$. Also, direct pathology mediated by activated leukocytes may also contribute along with the ADCC mechanisms that we have highlighted. Nevertheless, the studies that we have reviewed demonstrate that antibody and especially IgG are powerful mediators in neuroinflammation and that they must be given equal weight in consideration of design of therapies for MS and NMO (**Figure 2**).

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REVIEW
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The role of latently infected B cells in CNS autoimmunity

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The onset of multiple sclerosis (MS) is caused by both genetic and environmental factors. Among the environmental factors, it is believed that previous infection with Epstein–Barr virus (EBV) may contribute in the development of MS. EBV has been associated with other autoimmune diseases, such as systemic lupus erythematous, and cancers like Burkitt's lymphoma. EBV establishes a life-long latency in B cells with occasional reactivation of the virus throughout the individual's life. The role played by B cells in MS pathology has been largely studied, yet is not clearly understood. In MS patients, Rituximab, a novel treatment that targets $CD20^+$ B cells, has proven to have successful results in diminishing the number of relapses in remitting relapsing MS; however, the mechanism of how this drug acts has not been clearly established. In this review, we analyze the evidence of how B cells latently infected with EBV might be altering the immune system response and helping in the development of MS. We will also discuss how animal models, such as experimental autoimmune encephalomyelitis (EAE) and murine gammaherpesvirus-68 (γHV-68), can be used as powerful tools in the study of the relationship between EBV, MS, and B cells.

Keywords: multiple sclerosis, EBV, B cells, γHV-68, EAE

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INTRODUCTION: MS AND ENVIRONMENTAL FACTORS

MS is a neurodegenerative disease that affects the central nervous system (CNS). Largely accepted as an autoimmune disease, the mechanism of how MS develops is still not clear. However, thanks to studies using experimental autoimmune encephalomyelitis (EAE), the animal model for MS, we now know that MS lesions are caused primarily by myelin-specific T cells and macrophages that infiltrate the brain and cause myelin degradation and axonal degeneration (1). The primary T cells that infiltrate the CNS are $CD4^+$, Th1, and Th17 cells. These cells initiate acute lesions that are characterized by the breakdown of the blood–brain barrier (BBB), which drives the inflammatory process of MS (2). In addition, $CD8^+$ T cells that recognize myelin proteins can also can be found in the perivascular regions (3). These regions also contain other immune cells, such as dendritic cells (DCs), B cells, microglia, astrocytes, macrophages, and natural killer T cells (NKT) (4).

While the etiology of MS is still unknown, several genetic and environmental factors have been identified as possible elements that increase the risk of developing MS. Among the specific genetic markers related to the development of MS is the presence of genes related to alleles in the human leukocyte antigen (HLA) class II region [which is part of the major histocompatibility complex (MHC)], especially genes containing HLA-DRB1*15.01 (5, 6). While genome-wide association studies (GWAS) have identified several non-MHC associations with MS in Caucasian populations,

these appear to have a modest impact in the overall risk of disease, making MHC the main susceptibility locus (6, 7).

In addition to genetic research, studies in migration, discordancy among identical twins, and geographical gradients strongly suggest that environmental factors influence susceptibility to MS. Several environmental factors have been linked to increased risks of developing MS, including vitamin D deficiency (8), cigarette smoking (9), and infection by viruses, such as Epstein–Barr virus (EBV) (10). Among these factors, the relationship between EBV and MS is one that provides the strongest evidence of association. Though studies involving MS patients, together with investigation using EAE and other animal models of MS have yielded high quantities of data, the extent of the contribution of environmental factors in the onset of autoimmunity is still widely unknown. In the following sections, we explore some of the proposed mechanisms for how previous infection with EBV can contribute to MS, discuss the importance of B cells on MS pathology, and finally, propose an animal model that will help to further explore the relationship between EBV, memory B cells, and the pathology of MS.

EPSTEIN–BARR VIRUS AND MULTIPLE SCLEROSIS

Epstein–Barr virus is a γ-herpesvirus that infects both epithelial cells and B cells (11). Infected B cells are activated and differentiate to memory B cells, which then are released to peripheral circulation where they are recognized by T lymphocytes (12). Although the immune system is able to control the EBV infection, the provirus remains latent in the host's B-lymphocytes for the rest of his/her life. During latency, the main reservoir for EBV is long-lived memory B cells that have gone through somatic hypermutation and immunoglobulin class-switch recombination (13). The host cell expresses EBV gene products including six nuclear proteins (EBNA-1/2/3A/3B/3C/LP), three membrane proteins (LMP-1/2A/2B), and EBV-encoded small RNAs (EBER-1 and EBER-2). These products can control the host's cell cycle and prevent apoptosis. The virus reactivates again at intervals during the host's life (14). Primary infection with EBV is transmitted through saliva and, when it occurs during childhood, is asymptomatic (15). In contrast, if the infection occurs during puberty or early adulthood, it can cause infectious mononucleosis (IM), which is characterized by vague malaise followed by fever, sore throat, swollen posterior cervical lymph nodes, and fatigue (14).

Historically, EBV infection has been associated with the development of several autoimmune diseases and cancers. Some of these include Burkitt's lymphoma, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and MS.

A connection between MS and EBV was first suggested when it was recognized that there are similarities in the demographic distribution of MS and IM (10), whereby both IM and MS occur at higher incidences in developed countries. Subsequent studies found that although 90% of the general population has circulating anti-EBV antibodies, these antibodies are found in almost 100% of MS patients (16), and that people with a history of IM have a two to three times higher risk of developing MS (17). Contrastingly, in developing countries, where infection with EBV occurs early in life, individuals show a low incidence of IM and, consequently, the risk of developing MS is much lower (18, 19). This so-called "paradox" reveals that the relationship between MS and EBV is related to the stage in life when the infection with EBV occurs (16), together with the associated development, or not, of IM. The factors that determine the relationship between EBV, IM, and MS, however, have not yet been clearly established.

In support of the epidemiological data, it has been described that MS patients show increased levels of serum or plasma IgG antibodies against the EBNA family in general, and in particular against EBNA 1, EBNA2 (20), EBNA3 (EBNA3A), EBNA4 (EBNA3B), EBNA6 (EBNA3C), LMP1, EBV capsid protein VP26 (21), early antigen complex (20, 22) EBV viral capsid antigen (23), and the EBV lytic protein BRRF2 (24). In addition, patients with MS also have elevated levels of these antibodies in the cerebrospinal fluid (CSF), including IgG antibodies to EBNA1, viral capsid antigen, EBV early antigen, Epstein–Barr virions and BRRF2 (24). Furthermore, increased antibody titers have been observed in adults more than 10 years before the development of the first MS symptoms (25).

Several hypotheses have been proposed to explain the relationship between EBV and MS. Among these, the most studied are molecular mimicry, bystander damage and mistaken self, and the EBV-infected autoreactive B cell hypothesis.

Molecular Mimicry

This hypothesis postulates that T cells specific for EBV antigens (such as EBNA-1) are structurally related to CNS antigens like myelin basic protein (MBP). In this way, a TCR would be able to recognize more than one peptide and lead to recognition of autoantigens (26, 27). Additionally, it has been shown that anti-EBV antibodies, such as anti-EBNA-1, are cross-reactive for epitopes of neuroglial cells (28) and transaldolase, a protein expressed selectively in oligodendrocytes (29). Although this theory explains the development of autoreactive immune cells, it is not likely to be the sole cause of the onset of the disease, as the development of autoreactive cells and antibodies still requires leakage past the BBB and some targeting or inflammation at site of damage. Further, the presence of latently infected B cells alone does not necessarily influence cross-reactivity. Though the presence of latently infected B cells in the brains of MS patients (30) remains controversial (31, 32), B cells and plasma cells are commonly found in MS lesions, appear in large numbers in chronic MS plaques, and are present in areas of active myelin breakdown (33). Moreover, lymphoid B cell follicle-like structures that feature characteristics similar to germinal centers have been observed in the cerebral meninges of MS patients with secondary progressive MS and are usually associated with cortical neuronal loss and demyelination (34).

The Bystander Damage Hypothesis

This hypothesis establishes that the activation of $CD8^+$ or $CD4^+$ T cells directed against EBV antigens, particularly lytic antigens, can result in bystander damage to the CNS. However, in order for this hypothesis to be possible, it would be necessary for infected B cells to be present in the CNS, which has been rather hard to prove. Serafini et al. showed that meningeal B cell follicles and acute white matter lesions express EBV nuclear transcripts (EBERs)

(30); however, further attempts to detect EBV in MS brains have been futile (31, 32, 35). Under the bystander damage hypothesis, MS would not be an autoimmune disease, *although* secondary autoimmune responses could occur as a result of sensitization to CNS antigens released after virus-targeted bystander damage (30). A caveat to this hypothesis would be that overall and relative to other viruses, EBV does not directly damage the cells that it infects, leaves little bystander inflammation and is not likely to induce disease through this type of mechanism preferentially in the CNS.

Mistaken Self Hypothesis

In this hypothesis, the stress protein αB-crystallin that is expressed *de novo* in infected lymphoid cells is recognized by T-cells that are activated by microbial antigens, hence the accumulation of the αB-crystallin self antigen in oligodendrocytes provokes a CD4 T cell response with resultant demyelination (36). To date, little to no data exists to fully support this scenario.

The EBV-Infected Autoreactive B Cell Hypothesis

Pender has proposed a new theory, where EBV specific $CD8^+$ T cells do not effectively eliminate EBV-infected B cells, leading to the accumulation of autoreactive B cells infected with EBV in the CNS (37). If this theory proves true, it is possible that boosting the immune system with $CD8^+$ T cells specific for EBV epitopes could be a successful treatment for MS patients.

In support of this theory, Pender et al. recently performed a trial where they treated a patient with secondary progressive MS using AdE-1-LMPpoly, a recombinant adenovirus vector that encodes multiple $CD8^+$ T-cell epitopes from the latent EBV proteins EBNA1, LMP1, and LMP2A (38). The patient was treated with EBV specific CD8 T cells expanded with AdE-1-LMPpoly and IL-2. The results showed an improvement in symptoms including reduction in fatigue and pain. More studies are needed in order to determine if this regimen could be effective in treating secondary progressive MS. In addition, more research is needed to investigate the treatment's mechanism of action, which is believed to occur through the elimination of EBV-infected B cells in the CNS (39). Nonetheless, since this treatment depletes B cells in general, it may block a number of putative autoimmune mechanisms and does not specifically demonstrate Pender's hypothesis.

In summary, these four hypotheses explain some of the potential scenarios that contribute to the development of autoimmunity by EBV. However, each of them fails to explain key characteristics of MS pathogenesis. Since sample collection from MS patients is limited, the development of animal models to help understand and explain these hypotheses is imperative and will eventually help us to understand the role of latently infected B cells in this relationship.

As an alternative to these hypotheses, we propose that EBV infection and latency establishes a precondition to the immune response where subsequent challenges show acceleration and/or enhanced Th1 outcomes that eventually will lead to the onset of MS (**Figure 1**). In this scenario, the latently infected B cell is not an initiator but instead acts as a necessary co-factor in disease progression.

THE IMPORTANCE OF B CELLS IN MULTIPLE SCLEROSIS

B cells found in the CNS and CSF of MS patients are clonally expanded and have gone through IgG class-switch and somatic hypermutation (40–42). In MS patients, more than 90% of B cells in the CSF express the memory B cell marker CD27 and a fraction of CSF B cells express CD138 and/or CD38, suggesting stimulation of the maturation of clonal activated memory B cells into antibody producing plasma blast. On the other hand, naïve B cells expressing CD27 IgD^+ naive B cells are significantly lower in the CSF compared to blood (43, 44). The memory B cells that can be found in the CSF have an upregulation of co-stimulatory molecules, which suggests an active B and T cell interaction (45).

Until recently, it was believed that the only role B cells played in MS pathogenesis was the production of autoantibodies; however, with the realization that B cell depleting drugs, such as Rituximab, ocrelizumab, and ofatumumab, had an important effect in diminishing relapses in patients with relapsing-remitting MS (RRMS), it has became more evident that B cells may be acting as antigen-presenting cells (APCs) during MS. In fact, patients treated with B cell depleted therapy show a rapid response to the treatment, and since these antibodies do not affect plasma cells, it is now believed that autoantibodies are not as important in the pathogenesis of MS as B cells functioning as APCs or immunomodulators (46–49). In their role as APCs, it has been suggested that B cells and DCs interact via cytokine-dependent feedback loops to shape the T cell response to viral infections. When B cells are stimulated with cytokines, TLR ligands, or antibodies, these cells release diverse cytokines including IL-10, TGFβ, IL-6, or IL-17, which have a suggested modulatory effect in DCs (50–52). One of these effects is the suppression of Ag presentation by IL-10. It has also been seen that high levels of TGFβ are produced by B cells stimulated with LPS, which regulates Th1 response in NOD mice, induces the apoptosis of T cells, and impairs the ability of APCs to present auto-Ags. In addition, IL-6 promotes the differentiation of B cells into Ab secreting plasma cells in mice and humans, and IL-17 has been seen to control DC maturation in mice infected with *Trypanosoma cruzi* (53). Alternatively, IL-12 production on DCs inhibits T cell derived IFNγ, as well as the production of pro inflammatory cytokines through its actions on DCs (54).

In autoimmunity, the APC role of B cells has been primarily studied in EAE, which has long been accepted as the best *in vivo* model of MS. In active EAE, mice are immunized with myelin peptides, most often derived from either MBP or myelin oligodendrocyte glycoprotein (MOG) that are emulsified in complete Freund's adjuvant (CFA, which is composed of mineral oil and desiccated *Mycobacterium tuberculosis*) (55). In addition, two injections of pertussis toxin (PTX) might be needed, depending on the strain of mouse used. EAE leads to an ascending paralysis in 10–12 days after induction and is characterized by a CD4-mediated autoimmune reaction. SJL mice injected with PLP generally develop a relapsing-remitting disease course. In C57Bl/6 mice, EAE induction with MOG results in a chronic progressive disease (55). Alternatively, passive EAE can be induced if MOG-specific T cells are transferred to naïve mice.

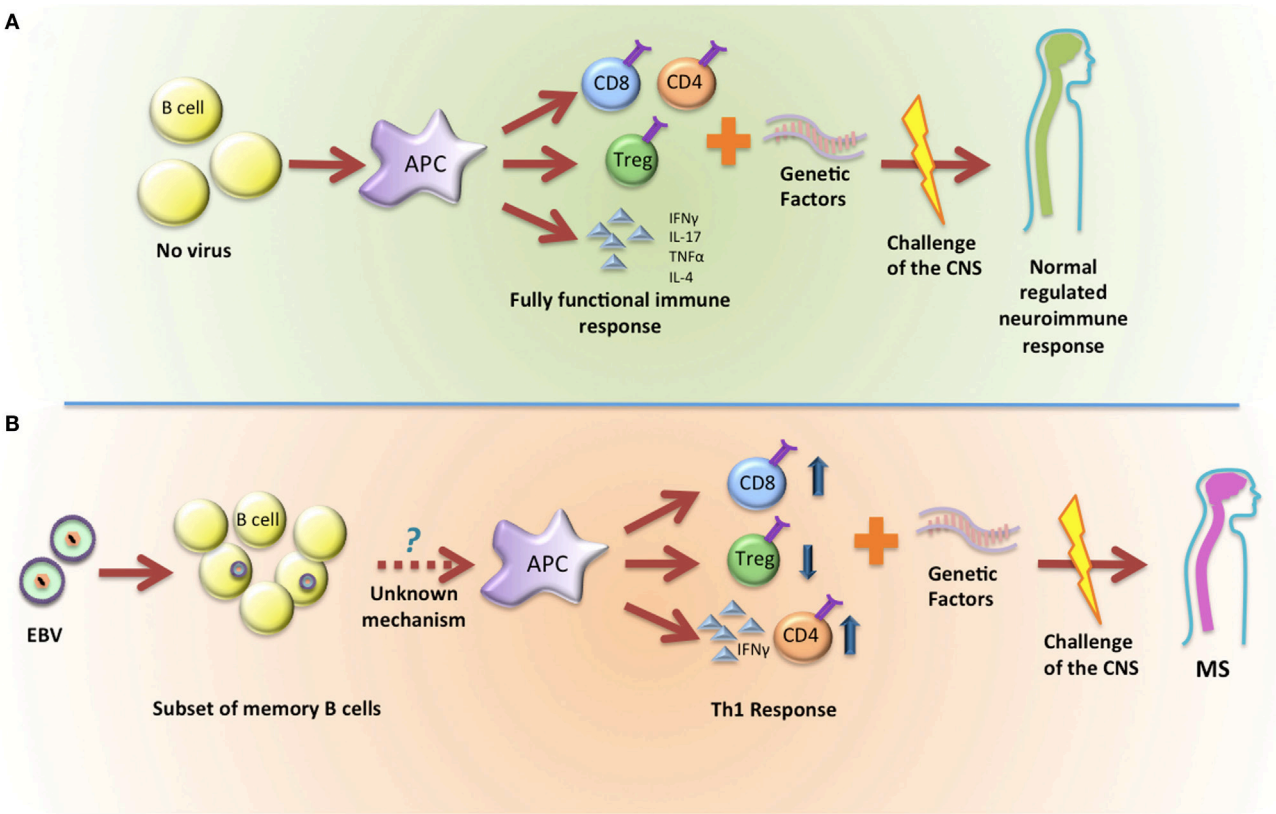

FIGURE 1 | Latent infection with EBV establishes a precondition that leads to the development of MS. **(A)** In normal conditions, B cells will activate APCs and, depending on the stimulus they receive, they can promote a fully functional immune response that will not impact the development of MS. **(B)** EBV latently infected B cells will activate APCs and promote a skewed Th1 response that, when combined with genetic factors, will lead to the development of MS.

It has been observed that B cell antigen presentation plays a critical role in the initiation of EAE (56, 57). Mice with a BCR specific for MOG but that cannot secrete antibodies are susceptible to EAE, while mice deficient in MHC II on B cells are resistant to EAE (58). This is further confirmed in patients since it has been described that contrary to other autoimmune diseases, such as RA, central tolerance of B cells is not affected in MS. Instead, only peripheral tolerance seems to be defective in MS, which can be the result of defective Treg function (59, 60). Patients with RRMS show memory B cells in peripheral blood, which are able to respond to MBP. Finally, it has been described in patients with MS that a pool of IgG-expressing B cells is capable of bidirectional exchange through the BBB (7).

One of the important characteristics of B cells in MS patients is cytokine production. In EAE mice, B cell depletion seems to deplete B cells that are producing IL-6, which helps to ameliorate symptoms of the disease. In MS patients, B cells produce higher levels of IL-6 compared to healthy controls. After depletion of B cells with anti-CD20, and after B cell reconstitution, the new cells do not seem to produce the same level of IL-6 than before depletion, which might help to understand the ameliorating effect in patients. All of this is accompanied by reduced levels of IL-17 secreted by peripheral T cells (61). In contrast, B cells that show a regulatory phenotype, commonly referred as Bregs or B10 cells, due to their ability to secrete IL-10, a cytokine known to be immunoregulatory, are able to modulate the autoimmune response in EAE (62). In RRMS, it has been shown that during relapses, patients have reduced levels of Bregs as well as memory B cells in peripheral blood compared to healthy donors (63, 64).

ANIMAL MODELS TO STUDY MULTIPLE SCLEROSIS AND EPSTEIN–BARR VIRUS INFECTION

While numerous lines of evidence point toward a relationship between MS and EBV, the study of this interaction is limited since EBV only infects humans and, while most patients become infected with the virus during childhood or adolescents, the onset of MS does not occur until years later.

Despite these limitations, a current murine virus can be used to study γ-herpesviruses. Murine γ-herpesvirus 68 (γHV-68), is a γ-herpesvirus that has provided a widely used model to study human γ-herpesviruses, in particular EBV and Kaposi's sarcoma-associated herpesvirus (KSHV) (65). γHV-68 shares most of its genomes with these two viruses, and, importantly, genes that are associated with EBV cell tropism – latency and transformation – are present in γHV-68 (66).

Mice are inoculated with γHV-68 usually via intraperitoneal or intranasal methods. Despite of the route of infection, the virus main reservoir will be the spleen and it will be cleared 14–16 days post infection, at that point, it will establish a life-long latency in primarily isotype-switched B cells $CD19^+$ IgD^-, which are considered as memory B cells (67). During early stages of latency, the virus also establishes itself in macrophages and splenic DCs, although to a much lesser extent. In these other APCs, γHV-68 latency decreases considerably with time (68, 69).

In γHV-68 infection, the virus is able to modify the expression of different genes in the cells that harbor the latent virus. Many of these genes are inflammatory cytokines, such as IFNγ, IL-18 receptor, SOCS3, and a wide array of known stimulated IFNαβ genes (50–52). But also, once the virus has established latency in B cells, it continues expressing latency genes that are able to regulate the expression of genes in B cells. In the same way, B cells will express other genes in order to control γHV-68 reactivation. All of this will bring different outcomes that will differentiate latently infected B cells from uninfected B cells.

Among the viral genes expressed during latency, we can find M2, a protein that can suppress STAT1/2 expression and that as a consequence, leads to the inhibition of the interferon response (70), as well as being able to induce the expression of IL-10 in primary B cells. Despite M2 being unique for γHV-68, EBV also is able to modulate the immune response by producing its own viral IL-10 (vIL-10) (71). In addition, M1 a secreted protein with a superantigen-like activity might play an important role in maintaining latency (72). Additionally, EBV encodes for 25 pre-miRNAs that may play a role in immune response whose target transcripts are immune recognition, apoptosis, and cell cycle pathways. γHV68 can generate 15 mature miRNAs; however, their function is less understood than in EBV (73, 74). However, it is known that micro RNAs are not necessary for acute replication, but that they are important in the establishment of latency in germinal center and memory B cells (75).

Infection of γHV-68 is able to increase Heparin sulfate (HS) in the surface of B cells. HS is a co-factor for cytokines, chemokines, and growth factors, and its upregulation is dependent on the expression of type I IFNs that increase responsiveness to APRIL, a cytokine important for B cell survival and T cell-independent B cell responses (76). It is well known that IFN α/β are important to direct γHV-68 into latency, and that they are also important in maintaining latency (77). Moreover, Latency Membrane Protein (LMP-1) is a virus protein that has been shown to control EBV's latent life cycle. LMP-1 is upregulated in the presence of Type I IFN, in particular IFN α (78), and this unique feedback maintains the latent life cycle and as well as promotes host IFN production (79). Intriguingly, it is important to remark that IFN α and IFN β present functional differences (80) that are in a unique balance with each other. While not completely understood, Type I IFNs have been largely used in the clinic with different purposes, while IFN α is used to treat chronic hepatitis C infection, IFN β has been effective for the treatment of MS. Addition of either IFN α or IFN β generally resolves in diminishment of the other. Based on the effectiveness of Betaferon in the clinic and its putative role in upsetting the balance between LMP-1 and IFN α, a better understanding of the roles and functions of IFN α and IFN β should be explored in the context of EBV infection and MS. In particular, it would be interesting to explore whether IFN α/β produced by infected B cells for the maintenance of latency are able to promote APC maturation.

There is even stronger evidence that γHV-68 a successful model to help understand the relationship between EBV and MS. Peacock et al. describe that EAE induced mice infected with γHV-68 show exacerbated symptoms of EAE compared to non-infected mice (81). Moreover, similar to what is observed in MS patients, it has been described that γHV-68 is capable of inducing the expression of αB crystallin in mice infected with the virus. These mice develop a strong immune response against heat shock protein (82). These experiments, however, do not address the changes in the pathology of EAE or MS.

Combining EAE and γHV-68 models, our research has focused on determining the relationship between EBV infection and the onset of MS. Recently, we demonstrated that mice that were latently infected with γHV-68 before the induction of EAE showed increased ascending paralysis, as well as augmented neurological symptoms and brain inflammation. This was the result of a stronger Th1 response in infected mice, characterized by higher levels of IFNγ and diminished IL-17 levels. CD8 infiltration into the CNS was also noted in these latently infected mice. This is remarkable, given that EAE pathology generally lacks the presence of CD8 T cell infiltration and has a predominant Th17 response. Conversely in MS, CD8 T cells infiltration and a combined Th1/Th17 response are hallmarks of disease pathology. Another important aspect is the upregulation of the co-stimulatory molecule CD40 on APCs during EAE induction in latently infected mice (83). Recently, we showed that the enhanced disease observed in γHV-68 latently infected mice depends on maintaining the latent life cycle of the virus, and this is strongly associated with pSTAT1 and CD40 upregulation on uninfected $CD11b^+CD11c^+$ cells. This CD40 upregulation leads to a decrease in the frequency of regulatory T cell (84). CD40 signaling is important in the activation and suppression of Tregs and that its upregulation is associated with an enhanced Th1 response and fewer Tregs. Further, it has been associated with the development of autoimmunity (85, 86). Moreover, the decrease in peripheral Treg frequencies observed in latent γHV-68 infection is also well described in MS patients (87, 88). It is highly likely that the mechanisms in place that maintain latency also modulate a pro-Th1 response and reduce Treg control. This results in prevention of virus reactivation and may not always be in the best interest of the virus. IFN α/β are required for the maintenance of latency and are likely candidates for the Th1 modulation. Further research is needed to determine if factors, such as IFN α/β, are involved in the enhancement of EAE symptoms, and in particular, to understand potential differences between uninfected and latently infected B cells.

Finally, studies performed on non-human primates would be an important tool in the study of EBV and MS. In marmosets, for example, EAE is effectively inhibited when marmosets are treated with anti-CD20; however, treatment with anti-BlyS or anti-APRIL, which mainly depletes peripheral B cells, but not $CD40^{high}$ B cells, only delays the onset of EAE (89, 90). It has been proposed that the difference in the effectiveness of the treatments resides in the fact that cells infected with CalHV3 are among the B

cells depleted by anti-CD20; CalHV3 is the marmoset equivalent of human EBV, and is a B-cell transforming lymphocryptovirus (91). Moreover, it has been described that a small percentage of Japanese macaques which are naturally infected with a gamma 2-herpesvirus, named JM radhinovirus, isolated from CNS lesions, spontaneously develop an encephalomyelitis that is similar to MS (92). In addition, since EBV has not just been associated with MS but with other autoimmune diseases like lupus and inflammatory bowel disease, it is possible that the mechanism of action is similar in these diseases, making γHV-68 even more important in the study of the development of autoimmunity.

It is our contention that EBV acts a co-factor that sets up a precondition in which any subsequent environmental stress runs the risk of an overly responsive, under regulated Th1 response. Specificity toward the CNS, myelin sheath, and oligodendrocyte is dictated by the secondary stress event and not EBV latency. While EAE is an acceptable model that mimics many of the characteristics of MS, it does not represent how MS is induced; given that not every person infected with EBV develops MS, genetic predispositions, as well as other environmental factors must be involved in the expression of the disease. With that in mind, other environmental events and stresses that target the myelin sheath or oligodendrocytes, such as a secondary virus infection or toxin, likely act to initiate the disease in the presence of latently infected B cells. For example, agents like cuprizone, a copper-chelating agent, that is known to cause demyelination in the CNS through oligodendrocyte apoptosis (93, 94), may well be active MS inducers.

By studying these models in the context of latently infected B cells, we will be able to better investigate the role of latent virus infection in the initiation and progression of MS.

CONCLUSION

Determining the mechanism that describes how environmental factors, such as EBV and IM, are related to the onset and development of MS is vital to understanding how MS pathogenesis is developed. The efficacy of treatments, such as Rituximab and Betaferon that indirectly act to inhibit EBV latency in B cells by depleting B cells or upsetting the IFN balance, serves to demonstrate the important role that EBV latent infection plays in MS progression. It is also important to remember that neither B cell depletion nor IFN I addition are successful therapies for EAE and were instead chosen because of their efficacy in other autoimmune diseases. With the aid of new animal models that consider the role of latent infection, it is expected that these complicated causal mechanisms can be more easily studied and new and more effective treatments for MS patients will more closely at hand.

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Cytokine-Defined B Cell Responses as Therapeutic Targets in Multiple Sclerosis

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Important antibody-independent pathogenic roles of B cells are emerging in autoimmune diseases, including multiple sclerosis (MS). The contrasting results of different treatments targeting B cells in patients (in spite of predictions of therapeutic benefits from animal models) call for a better understanding of the multiple roles that distinct human B cell responses likely play in MS. In recent years, both murine and human B cells have been identified with distinct functional properties related to their expression of particular cytokines. These have included regulatory (Breg) B cells (secreting interleukin (IL)-10 or IL-35) and pro-inflammatory B cells (secreting tumor necrosis factor α, LTα, IL-6, and granulocyte macrophage colony-stimulating factor). Better understanding of human cytokine-defined B cell responses is necessary in both health and diseases, such as MS. Investigation of their surface phenotype, distinct functions, and the mechanisms of regulation (both cell intrinsic and cell extrinsic) may help develop effective treatments that are more selective and safe. In this review, we focus on mechanisms by which cytokine-defined B cells contribute to the peripheral immune cascades that are thought to underlie MS relapses, and the impact of B cell-directed therapies on these mechanisms.

Keywords: multiple sclerosis, B-lymphocytes, cytokine-defined responses, immune modulation, B-cell depletion, B cell modulation

INTRODUCTION

In addition to their potential to differentiate into antibody producing plasma cells, B cells can efficiently present antigens to T cells and modulate local immune responses through secretion of soluble products, such as pro-inflammatory or anti-inflammatory cytokines (**Figure 1**) (1). Historically, B-cell implication in multiple sclerosis (MS) pathogenesis was based on the common finding of abnormally increased immunoglobulin levels in the cerebrospinal fluid (CSF) of patients as well as antibody deposition noted in brain lesions (2–5). However, the success of B-cell-depleting therapy to limit new MS relapses without obviously impacting abnormal CSF antibody levels underscores antibody-independent contributions of B cells to relapsing disease activity (6–11). In this review, we focus on implication of pro-inflammatory or anti-inflammatory cytokine-defined B cell responses in MS and the impact of B-cell-directed therapies on their functions.

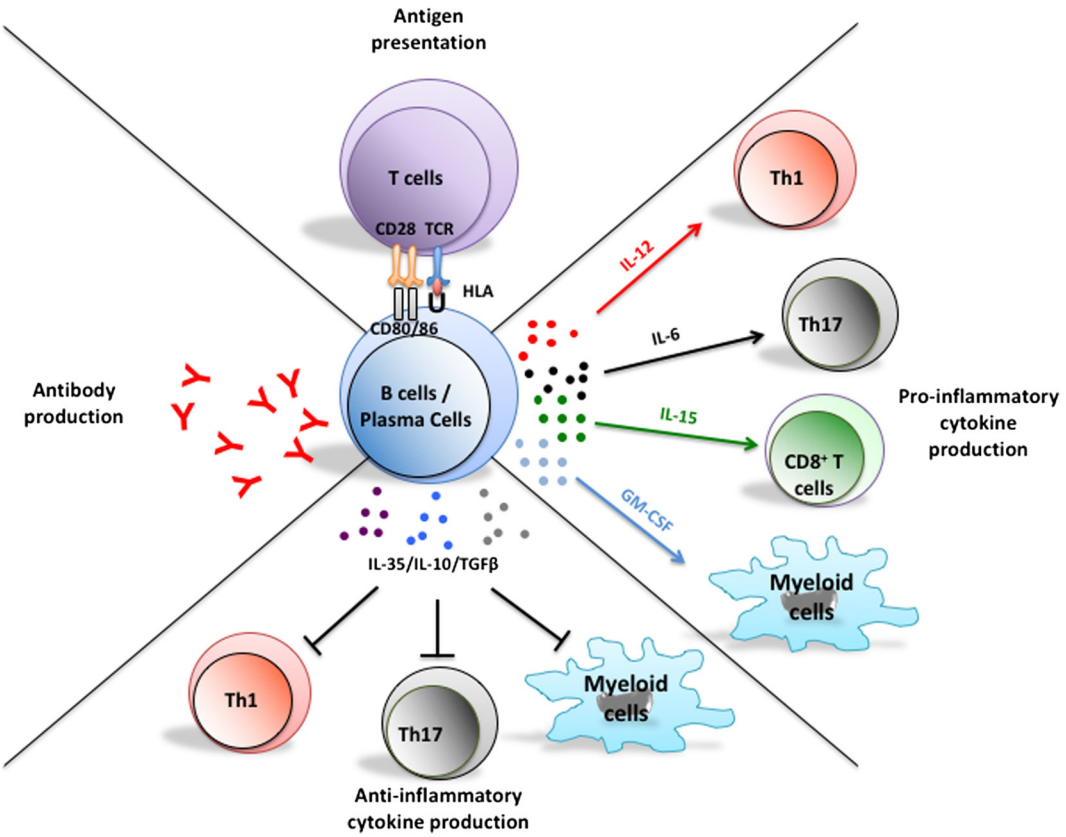

FIGURE 1 | Multifaceted functions of B cells and their implications in the pathogenesis of MS. In addition to their potential to differentiate into antibody-secreting plasmablasts and plasma cells, B cells can present antigen to T cells, as well as up- or down-regulate local immune responses through elaboration of pro- or anti-inflammatory cytokine, respectively. Plasma cells can also secrete pro- and anti-inflammatory cytokines that could modulate T cell and myeloid-cell responses. Abnormal B cell responses of potential relevance to MS include aberrant production of autoreactive antibodies, exaggerated activation of T cells through antigen presentation, and induction of pro-inflammatory T cell and myeloid-cell responses through abnormal secretion of pro-inflammatory B cell cytokines and/or insufficient secretion of B cell regulatory cytokines.

CYTOKINE-DEFINED B CELL RESPONSES IN MS AND EAE

Several functionally distinct cytokine-defined B cell responses have been implicated in the pathophysiology of MS and its commonly used animal model, experimental autoimmune encephalomyelitis (EAE). Nonetheless, translation of B-cell-related findings from mouse to human has not always been straightforward. Characterizing and defining the roles of distinct human B cell subsets in health and disease are important requisites for rational development of more selective and effective B cell-targeting therapies.

INTERLEUKIN-10-PRODUCING B CELLS

Interleukin (IL)-10 is a cytokine with pleiotropic effects in immunoregulation and inflammation (12). In mice, knock-out (KO) of IL-10 selectively from B cells results in more severe EAE (13), and adoptive transfer of *in vitro*-induced IL-10-producing B cells suppresses EAE in an IL-10-dependent manner (14–16). Inducing EAE in IL-10 reporter mice implicated the draining lymph nodes (rather than spleen or spinal cord) as the sites where IL-10$^+$ B cells regulate disease-relevant immune responses (17). The IL-10$^+$ B cells in this study exhibited plasma cell/plasmablast markers, consistent with an earlier report showing that CD138$^+$ plasma cells are able to produce IL-10 (17), thus also highlighting the previously unappreciated antibody-independent functions of plasma cells. Although IL-10 production from B cells can be induced by both innate [toll-like receptors (TLRs)] and adaptive (cognate interaction) stimuli (14, 16, 18), the targets of regulation of innate- and adaptive-induced IL-10-producing B cells may differ depending on context. For example, innate signal-induced IL-10-producing B cells are able to down-regulate pathogenic T-cell responses indirectly through dendritic cells (14), whereas adaptive signal-induced IL-10-producing B cells directly down-regulate antigen-specific T-cell responses (15, 16).

In humans, both naïve and memory B cells are capable of producing IL-10 in a context-dependent manner (19–22). Human CD27$^-$ (naïve) B cells, but not CD27$^+$ (memory) B cells,

are able to produce IL-10 upon CD40-ligand stimulation (11, 23–25), a response found to be abnormally deficient in B cells of MS patients (24). By contrast, IL-10$^+$ B10 cells are induced within the CD27$^+$ memory pool by stimulation through TLR4 and TLR9 and can suppress tumor necrosis factor α (TNFα) production by monocytes through an IL-10-dependent mechanism. Unexpectedly, B10 cells were reportedly increased in several human autoimmune diseases, including MS, upon stimulation (21). A better understanding of these cells, including defining surface markers and master transcriptional regulators, could facilitate future cell-based therapies for MS.

IL-35 PRODUCING B CELLS

Interleukin-35 is an anti-inflammatory cytokine of the IL-12 family (26). Although the EBI3 subunit of IL-35 was first identified in EBV-infected B cells (27), functions of IL-35 were initially described in regulatory T cells (28–30). More recently, IL-35-producing B cells were found to play important roles in recovery from EAE and experimental autoimmune uveitis (31, 32). In these contexts, IL-35-producing B cells inhibited pro-inflammatory immune responses either directly through IL-35 (31) or indirectly through induction of IL-10-producing B cells (32). These IL-35-producing B cells also exhibited plasma cell phenotypic markers (31). Besides IL-10 and IL-35, B cells can also produce Transforming-growth factor β or Granzyme B that may down-regulate immune responses (33–39); their relevance to MS (or EAE) is yet to be determined.

TUMOR NECROSIS FACTOR α AND LYMPHOTOXIN-α PRODUCING B CELLS

Tumor necrosis factor α and Lymphotoxin-α (LTα) are actively involved in promoting pro-inflammatory immune responses to protect against pathogen invasion (40). In addition, TNFα is also known to play a pathogenic role in several autoimmune diseases, including rheumatoid arthritis (41) and inflammatory bowel disease (42), in which TNFα-blocking therapies have been successful (41). In MS, however, TNFα blockade increased disease activity (43) highlighting the challenge of broadly targeting individual cytokines (versus targeting particular cytokine-expressing cells). Stimulation through CD40 and the B-cell receptor (BCR) significantly increases TNFα and LTα secretion from human B cells, compared to either stimulation alone (19). B cells of MS patients produce abnormally higher levels of both TNFα and LTα upon such dual stimulation (11, 23, 24). A microRNA (miR)-132:SIRT1 axis controls expression of TNFα and LTα by human B cells (23). Abnormally increased expression of miR-132 by MS B cells inhibited their SIRT1 expression, resulting in enhanced pro-inflammatory cytokine production. *In vitro* addition of the SIRT1-agonist resveratrol normalized the exaggerated pro-inflammatory cytokine expression of MS B cells (23).

IL-6 PRODUCING B CELLS

Interleukin-6, a cytokine with both pro-inflammatory and anti-inflammatory properties, can be produced by both immune and non-immune cells (44). IL-6 can induce Th17-cell differentiation from naïve T cells (45) and inhibit regulatory T cells (46–48). By contrast, IL-6 may induce IL-10-producing regulatory B cells and myeloid cells (18, 49). B cells of MS patients secrete abnormally high levels of IL-6 (50) and IL-6 knock-out selectively from B cells resulted in decreased Th17 responses and diminished EAE severity (50, 51). How B cell-derived IL-6 is regulated, and whether B-cell IL-6 also contributes to Th17 differentiation and regulatory T-cell dysfunction in MS, remains unknown.

IL-15 PRODUCING B CELLS

Interleukin-15 belongs to the four α-helix bundle family of cytokines and can be produced by multiple cell types (52). IL-15 knock-out mice develop more severe EAE (53), in part attributed to IL-15's ability to inhibit pathogenic Th17-cell differentiation (54), and to induce regulatory CD8$^+$ CD122$^+$ T cells (55). In patients with MS, however, IL-15 is abnormally increased in both serum and CSF (56, 57), where it may have disease-promoting (rather than disease-inhibiting) potential (58, 59). B cells from MS patients reportedly produce more IL-15 than controls, and activation of B cells through CD40 and the BCR induces IL-15 secretion that enhanced both the migratory capacity of CD8$^+$ T cells across a model of the blood–brain barrier and CD8$^+$ T cell cytotoxicity toward oligodentrocytes (59).

GRANULOCYTE MACROPHAGE COLONY-STIMULATING FACTOR-PRODUCING B CELLS

Granulocyte macrophage colony-stimulating factor (GM-CSF) is an important growth factor for myeloid lineage cell development and function, which is secreted by both immune and non-immune cells during infection and autoimmune disease (60). GM-CSF KO is resistant to active EAE induction (61), and GM-CSF KO Th17 cells fail to induce passive EAE (62–64). Since GM-CSF-producing T cells are reportedly increased in the circulation of MS patients (65–67), T cells have been thought to be the main source of GM-CSF of relevance to MS and EAE (65–68). A murine B-cell population generated from B1a cells, termed "innate response activator (IRA)" B cells (69), was described to produce GM-CSF and found to play a GM-CSF-mediated protective role during infections (69, 70), as well as a GM-CSF-mediated pathogenic role in atherosclerosis (71). In contrast to the murine IRA cells, a recently described human GM-CSF producing B cell subset belonged to the memory pool, and co-expressed high levels of TNFα and IL-6 (72). The human GM-CSF-producing B cells enhanced myeloid-cell pro-inflammatory responses in a GM-CSF-dependent manner and were abnormally increased in MS patients. B cell depletion in patients with MS resulted in a B cell–GM-CSF-dependent decrease of pro-inflammatory myeloid-cell responses, highlighting the potential pathogenic role of this B cell population *in vivo* and revealing a novel disease-implicated axis involving B cell:myeloid-cell interactions (72).

B CELL-TARGETING THERAPIES AND EFFECTS IN MS

The use of B cell-depleting agents in MS was initially driven by the long-standing recognition of abnormal antibody presence in both the CSF and brain lesions of MS patients (2–4, 73). Therapies directed against B cells include agents that impact their survival (rituximab, ocrelizumab, ofatumumab, alemtuzumab, and atacicept), and their trafficking to the CNS (natalizumab and fingolimod). In this section, we will highlight the mechanisms of action of these and other MS-related therapies that may impact B cells, with a focus on how such therapies may influence MS disease-relevant cytokine-defined B cells responses.

ANTI-CD20 MONOCLONAL ANTIBODIES

CD20 is a transmembrane protein with incompletely understood function, expressed on immature, transitional, naïve, and memory B cells, but not on stem cells, pro-B cells, and plasma cells (74). Rituximab, ocrelizumab, and ofatumumab are anti-CD20 monoclonal antibodies that induce B cell lysis via different combinations of antibody-dependent cell cytotoxicity, complement-dependent cytotoxicity, or apoptosis (75, 76). In MS, anti-CD20 antibodies rapidly and significantly reduced the number of new gadolinium-enhancing brain lesions and significantly reduced relapse rates (6–10, 77). Treatment reduced circulating B cell counts by >90% of baseline values, while serum and CSF immunoglobulin G levels remained largely unchanged (77–79), pointing to an important antibody-independent contribution of B cells to MS relapsing disease activity. An attractive hypothesis that has emerged is that pro-inflammatory B cells in untreated patients abnormally activate disease-relevant responses of other immune cells – hence removal of such B cells diminishes disease activity. In support of this view, anti-CD20-mediated B-cell depletion decreases both Th1 and Th17 T cell responses (11, 50) and pro-inflammatory myeloid-cell responses (that in turn could drive Th1 and Th17 responses) in the periphery of treated patients (72). In addition to cognate interactions in which B cells may serve as efficient antigen-presenting cells (APC) to activate T cells that recognize the same antigen, abnormal B-cell secretion of pro-inflammatory cytokines (including IL-6, TNFα, LTα, and GM-CSF) has now been implicated in abnormal T-cell and myeloid-cell responses of MS patients and may involve "bystander activation" (i.e., not be predicated by cognate antigen-specific interactions). Rituximab treatment could also diminish T cells within the CSF (79), providing further support that, when present, B cells may contribute to disease activity by enhancing peripheral T-cell activation and trafficking, and/or by CNS resident B cells promoting chemotaxis of T cells into the CNS. Alternative mechanisms proposed include an increased frequency of circulating regulatory T cells following B cell depletion (80) and in addition to depleting circulating B cells, anti-CD20 treatment also removes a small population of $CD20^{dim}$ T cells (81, 82). Initial studies of this T cell subset point to their potential to produce pro-inflammatory cytokines (81), though their significance in relation to MS disease activity warrants further investigation.

Elegant work using somatic mutation analysis of the Ig gene in B cells derived from both CNS and peripheral compartments of the same MS patients indicates that bi-directional trafficking of B cells occurs between the CNS and periphery and that much of the activation and maturation that results in clonal enrichment of B cells within the CNS may actually occur in the periphery (presumably through cognate–antigen interactions with T cells) (83–86). Hence, efforts to deplete or modulate the profile and functions of B cells in peripheral compartments of MS patients may meaningfully influence the profile and activities of B cells within the CNS, even if the treatment itself does not efficiently penetrate the CNS (as is generally the case for monoclonal antibodies). There have also been efforts to selectively eliminate B cells in the CNS using intrathecally administered rituximab (87). However, a complication in interpreting this result has been the finding that even small doses of rituximab infused into the CSF results in rapid and substantial peripheral B cell depletion (87). Data regarding effects of anti-CD20-mediated peripheral B-cell depletion on inflammation within the CNS compartment remain limited. Early work suggested that rituximab may be more effective at depleting CSF B cells in patients with relapsing compared to progressive forms of MS (77, 78), possibly due to differences in Blood-brain barrier permeability. In the earlier OLYMPUS trial (88), rituximab failed to limit progression of disability in PPMS patients compared to placebo treatment, though *post hoc* sub-group analysis suggested that patients who had gadolinium-enhancing lesions at baseline, and particularly younger patients, did benefit (88). The ORATORIO study, focusing on earlier disease, and using ocrelizumab, demonstrated that anti-CD20 therapy could limit disease progression in PPMS patients (89). The mechanisms underlying this benefit of B cell depletion in patients with progressive MS remain to be elucidated (see Michel et al., in this issue).

In addition to the decreased MS disease activity observed following B cell depletion with anti-CD20 antibodies, there is a suggestion that the benefit of B cell depletion may persist in at least some patients even as reconstitution of B cells occurs (6, 7). This would imply that the re-emerging B cells differ importantly from the B cells present prior to depletion. Indeed, the B cells that reconstitute following anti-CD20 depletion have been shown to be largely naïve B cells which, when activated, express more IL-10 and less pro-inflammatory cytokines, including TNFα, IL-6, and GM-CSF, compared to pre-treatment B cells (72).

ATACICEPT

B-cell activating factor of the TNF family (BAFF) and a proliferation-inducing ligand (APRIL) are expressed by a variety of immune and non-immune cells (90, 91). Both cytokines signal through transmembrane activator and cyclophilin ligand interactor (TACI) and B cell maturation antigen (BCMA), while only BAFF binds to BAFF-R (90, 91). Both play important roles in the survival, maturation, and function of B cells and plasma cells (92–94). BAFF can also promote differentiation and expansion of Th17 cells in models of infectious and autoimmune diseases (95). BAFF and APRIL levels are reportedly elevated in MS patients (96, 97), where they are highly expressed by peripheral blood monocytes and T cells. BAFF is also abnormally expressed

TABLE 1 | Selected therapies approved for (or under investigation for) multiple sclerosis, and their *in vivo* effects on the profiles and cytokine responses of B cells.

Drug name	Main drug target(s)	Effects on peripheral B cell subsets	Changes in expression of B cell cytokines
IFN-β	IFN-βR	⇑ $CD19^+$ B cells (108) ⇑ $CD19^+CD24^{++}CD38^{++}$ B cells (108) ⇓ % $CD19^+CD38^-IgM^-IgD^-$ (108) ⇓ % $CD80^+$ B cells (107, 109) ⇓ % $CD40^+$ B cells (109)	⇑ IL-10, TGF-β, IL-12p70, IL-27p28 (108, 109) ⇓ IL-1β, IL-23p19/40 (108, 109)
Glatiramer acetate	MHC class II (126)	⇓ $CD19^+$ B cells (127) ⇓ % $CD27^-$ B cells (128)	⇑ IL-10, IL-6 (127) ⇓ LTα (127)
Natalizumab	Alpha-4-integrin	⇑ % $CD19^+$ B cells (114–116, 129) ⇑ $CD19^+CD10^-CD138^-$ B cells (130) ⇑ $CD19^+CD10^+$ pre-B cells (130) ⇑ % $CD27^+IgD^+$ B cells (114) ⇓% $CD27^-IgD^+$ B cells (114) ⇑ % $CD27^+IgD^-$ B cells (114)	Unknown
Mitoxantrone	Type II topoisomerase (121, 131)	⇓ $CD19^+$ B cells (132) ⇓ % $CD27^+$ B cells (24)	⇑ IL-10 (24) ⇓ LTα (24)
Fingolimod	S1P1R	⇓ $CD19^+$ B cells (116–119) ⇓ % $CD27^+$ $CD38^{int\text{-}low}$ B cells (117, 120) ⇑ % $CD27^-$ B cells (117, 119, 120) ⇑ % $CD38^+CD27^-CD24^+CD5^+$ B cells (120) ⇑ % $CD10^+CD38^{hi}CD24^{hi}$ B cells (117)	⇑ IL-10, ⇓TNFα (117, 120)
Dimethyl-fumarate	Nrf2 (133)	⇓ $CD19^+$ B cells (124, 125)	Unknown
Teriflunomide	Mitonchondrial enzyme dihydroorotate dehydrogenase (DHODH) (134)	⇓ Proliferation of T cells and B cells ⇓ Antibody titers against neoantigen but not recall antigens (135, 136)	Unknown
Alemtuzumab	CD52	⇓ $CD19^+$ B cells (123, 137, 138) ⇑ $CD19^+CD23^-CD27^-$ (after 1 month) (137) ⇑ $CD19^+CD23^+CD27^-$ (after 3–12 months) (137) Partial reconstitution of $CD19^+CD23^+CD27^+$ B cells (after 12 month) (137) ⇑ $CD19^+CD24^{hi}CD38^{hi}$ (at 6 months) (123)	May result in shift in the balance between pro- and anti-inflammatory cytokine networks in B cells
Rituximab Ocrelizumab Ofatumumab	CD20	⇓ $CD19^+$ B cells (but not plasma cells) Early reconstitution of $CD27^-$B cells and $CD19^+IgD^+CD38^{hi}CD10^{lo}CD24^{hi}$ B cells (6–10, 79, 98)	⇓ IL-6, TNFα, LTα ⇓ GM-CSF ⇑ IL-10 (11, 24, 50)
Daclizumab	IL-2R-α	⇓ $CD19^+$ B cells (139) No change in $CD19^+$ B cells (140)	Unknown
Atacicept[a]	BAFF/APRIL	⇓ % mature B cells and plasma cells (not memory B cells) (101, 103, 105)	Unknown but may result in ⇓ IL-10 and IL-35

[a]*Clinical trial program of atacicept in MS was discontinued when early studies indicated treatment resulted in increased disease activity (103).*

by astrocytes within MS lesions (77–79, 98). Atacicept, a soluble, recombinant fusion protein containing the extracellular ligand-binding portion of TACI receptor and a modified Fc portion of human IgG, prevents binding of BAFF and APRIL to their receptors (99). Atacicept thereby limits survival of mature and activated B cells as well as antibody-secreting plasma cells but does not appear to target pro- or memory B cells (100, 101). Treatment with atacicept reduces circulating B cell counts (by 60–70%) and substantially reduces serum IgM and IgA (but to a lesser extent IgG) levels (100, 102–104). While emerging as beneficial in systemic lupus erythematosus, development of atacicept in MS was halted due to increased relapsing disease activity (100, 103–105). Why atacicept induced rather than limited new MS disease activity remains unknown but may reflect differential effects on functionally distinct B cell or plasma cell responses. Indeed, BAFF can induce IL-10-producing B cells and suppress the generation of IL-15^+ B cells (106). Hence, the dysregulated cytokine balance in B cells of untreated MS patients may actually be aggravated by atacicept, leading to aberrant responses of disease-relevant immune cells, such as pathogenic T cells and myeloid cells.

APPROVED MS THERAPIES THAT MAY IMPACT B CELL RESPONSES

While most approved MS therapies were developed based on their presumed ability to target T cells, many of them are now understood to also impact B cells in potentially disease-relevant ways (**Table 1**). For example, interferon (IFN)-β decreases the frequency of CD80-expressing B cells in treated MS patients, which could in turn limit peripheral T cell activation (107). IFN-β also enhances the numbers of circulating transitional B cells (108) and, such as glatiramer acetate (GA), may result in an

anti-inflammatory shift of B cell cytokine responses (109, 110). Both treatment with IFN-β and GA unexpectedly increased serum BAFF (111, 112), which would not only support certain aspects of B cells and plasma cells but may also shape the balance between cytokine-defined B cells, as described above. While natalizumab modifies frequencies of circulating B cells and plasma cells (**Table 1**) its effects, if any, on B cell cytokine-response profiles are largely unknown (113–115). It is noteworthy that *in vivo* treatment with natalizumab not only limits trafficking but may also modify T cell activation, which may reflect a role for VLA-4 during T cell interaction with APC, including B cells (113). Treatment with fingolimod (FTY720), an S1P receptor targeting agent, not only reduces the overall number of circulating B cells but can also modulate the cytokine-response profile of B cells in treated patients (116–120). Indeed, the proportion of peripheral blood memory B cells was reduced in fingolimod-treated patients, while the proportion of regulatory and transitional B cells was increased (117–120). The shift in circulating B-cell subsets was paralleled by changes in B-cell cytokine production, with reduced TNFα and enhanced IL-10 expression in B cells from fingolimod-treated patients (117, 120). Fingolimod treatment may also enhance migration capacity of regulatory B cells based on *in vitro* modeling of blood–brain barrier trafficking (120). Mitoxantrone, a chemotherapeutic agent that targets type II topoisomerase (121), preferentially reduces memory B cell counts and leads to a profile of circulating B cells with a less pro-inflammatory profile (24). Alemtuzumab, a humanized monoclonal antibody-targeting CD52, rapidly depletes multiple immune cell subsets (including B cells) followed by distinct kinetics of reconstitution (122). B cells reconstitute faster than T cells (within 3–6 months post-treatment), with naïve B cells dominating the re-emerging B cell pool. Such a treatment effect would be expected to induce a favorable shift in the balance between pro- and anti-inflammatory B cell responses (122, 123). Further studies are required to elucidate potential MS-relevant effects of dimethyl-fumarate (BG12) and teriflunomide on B-cell profiles, including cytokine responses (124, 125).

CONCLUSION

The success of anti-CD20 therapy in MS establishes that B cells contribute to relapsing disease activity. Though unwelcome, the observation that treatment with atacicept (**Table 1**) exacerbates MS, serves to reinforce the concept that targeting B cells can change the face of CNS disease activity, while also underscoring the importance of elucidating the functional heterogeneity that exists within the B cell pool. Emerging studies indicate that responses of cytokine-secreting B cells in the periphery may influence new MS disease activity, potentially through aberrant peripheral activation of other immune cells. B cells may play additional roles in propagating disease activity within the CNS (see Michel et al., in this issue). Success of B cell targeting therapies may lie in restoring and maintaining a favorable balance between pro- and anti-inflammatory B cell activities in patients.

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MINI REVIEW
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The ins and outs of B cells in multiple sclerosis

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B cells play a central role in multiple sclerosis (MS) pathology. B and plasma cells may contribute to disease activity through multiple mechanisms: antigen presentation, cytokine secretion, or antibody production. Molecular analyses of B cell populations in MS patients have revealed significant overlaps between peripheral lymphoid and clonally expanded central nervous system (CNS) B cell populations, indicating that B cell trafficking may play a critical role in driving MS exacerbations. In this review, we will assess our current knowledge of the mechanisms and pathways governing B cell migration into the CNS and examine evidence for and against a compartmentalized B cell response driving progressive MS pathology.

Keywords: multiple sclerosis, B cells, lymphocyte trafficking, chemokines, blood–brain barrier

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INTRODUCTION

In recent decades, accumulating evidence has brought B cells into focus as critical players in multiple sclerosis (MS) pathogenesis. B cells are present at elevated levels in inflamed MS central nervous system (CNS) tissue and are significantly increased in MS cerebrospinal fluid (CSF) (1, 2). Furthermore, IgG is synthesized intrathecally in MS patients (3), and IgG and complement are characteristic features of both type 2 and active MS lesions (4–6). In the CSF, the presence of oligoclonal IgG bands (OCBs) are a long-standing hallmark of MS diagnosis, and in the meninges, B cell-predominant lymphoid aggregations [germinal center (GC)-like structures] are observed in some relapsing and secondary progressive patients (7, 8). Finally, clinical trials of the anti-CD20 monoclonal antibodies rituximab (9), and ocrelizumab (10), have demonstrated beneficial effects on MRI lesion load and relapse activity in MS patients.

Many questions about the role of B and plasma cells in MS remain unanswered. What factors drive B cells into the CNS, through which pathways do they travel, and are these cells persistent or transient? When during the course of disease do B cells populate the CNS and are there particular CNS niches in which B cells thrive? How may (GC)-like structures contribute to MS pathology? In this review, we will examine the chemotactic cues, migratory pathways, and CNS factors that facilitate B cells trafficking and survival in the inflamed CNS, and evaluate evidence supporting a compartmentalized B cell response in MS pathogenesis.

B CELL MIGRATION INTO THE CNS IN HEALTH AND DISEASE

B Cells are Directed into the CNS by Chemokine Signaling

B cells may be observed in the healthy brain but are sparse in number, and increase drastically during neuroinflammation (11, 12). B cells express a robust array of chemokine receptors that largely dictate their movement, and the B cell chemokine receptor profile is dependent upon their state of

TABLE 1 | B cell chemokines in multiple sclerosis.

Chemokine	Levels in MS	Chemokine receptor	Reference
CCL2	Expressed by astrocytes and macrophages in acutely demyelinating lesions and active chronic lesions, at lesion edge, and in reactive astrocytes surrounding lesions Decreased in CSF	CCR2, CCR3	(19–22)
CCL3	Unchanged in CSF	CCR1, CCR5	(19)
CCL20	Undetectable in CSF Decreased in serum during relapse	CCR6	(23)
CXCL10	Increased in CSF Upregulated in MS lesions	CXCR3	(19, 24)
CXCL12	Upregulated in chronic active and inactive MS lesions on astrocytes and blood vessels	CXCR4, CXCR7	(14)
CXCL13	Increased in actively demyelinating MS lesions, secreted by macrophages in the perivascular cuffs. Not present in chronic inactive lesions. Shown to be the most important determinant for B cell recruitment into the CNS. Increased in CSF during relapse and remission	CXCR5	(14, 16, 25)
CX3CL1	Increased in CSF	CX3CR1	(26, 27)

differentiation and external microenvironment. The local milieu of cytokines in the inflamed CNS may also promote B cell migration by enhancing B cell chemoattraction and lymphoid organization. For instance, lymphotoxin-α expressed along the outer layer of inflamed vessel walls may facilitate lymphoid organogenesis and the formation of meningeal B cell GC-like structures (13).

Several chemokines and their receptors (in parentheses) have been shown to influence CNS B cell trafficking: CCL2 (CCR2, CCR3), CCL3 (CCR1, CCR5), CCL20 (CCR6), CXCL10 (CXCR3), CXCL12 (CXCR4, CXCR7), and CXCL13 (CXCR5) (**Table 1**). Among these factors, CXCL13 may play a central role. The CSF concentration of CXCL13 is elevated in MS patients (14), correlates with conversion from clinically isolated syndrome (CIS) to definite MS (15), and shows a strong correlation with B cell numbers in the CSF of MS and other neuroinflammatory diseases (14, 16). Indeed, nearly all CD19+ CSF B cells express the CXCL13 receptor, CXCR5 (14). Elevated CSF CXCL13 correlates strongly with the CNS accumulation of class-switched CD27+ memory B cells, CD27−IgD− B cells, and unswitched CD27+ memory B cells, but bears no relationship to the numbers of CD138 + CD38+ antibody-secreting plasmablasts and plasma cells (17). The ability of CXCL13 blockade to disrupt the formation of GC-like structures in the pancreatic islets of NOD mice suggests that meningeal B cell aggregates in MS patients may also develop from migrating memory B cells that differentiate intrathecally to plasmablasts and plasma cells (18).

As short-lived plasmablasts comprise a significant proportion of the CSF B cell population in MS (28), the chemokines CXCL10 and CXCL12 may also act as chemoattractants for CXCR3+ and CXCR4+ plasmablasts and additionally regulate the dynamics of CNS B cell trafficking in disease. Since CXCL10 is constitutively expressed by a subset of cells in the CNS subventricular zone, the gradient of CXCL10 may be a potent chemo-attractant signal for both activated T cells and antibody-secreting cells (29).

Adhesion Molecules, B Cells, and the Blood–Brain Barrier

B cells follow chemokine gradients into the CNS via one of several anatomical pathways: (1) through the choroid plexus into the CSF; (2) through parenchymal vessels into the perivascular space; or (3) or through the post-capillary venules into the subarachnoid and Virchow–Robin spaces (30). B cells entering into the CNS through the choroid plexus must traverse apical tight junctions between epithelial cells composing the blood–CSF barrier, whereas B cells trafficking through parenchymal vessels or stromal venules ultimately need to traverse the tight junctions of the microvascular endothelial cells composing the blood–brain barrier (BBB). While the stages of lymphocyte transmigration across the blood–CSF barrier have yet to be described in detail, the sequence of leukocyte rolling, activation, arrest, crawling, and migration has been defined in great detail for blood–brain barrier trafficking (30). Basic adhesion molecule interactions important for T cell transmigration across the BBB include selectins during rolling (31), leukocyte very late antigen-4 (VLA-4) and endothelial vascular cell adhesion molecule-1 (VCAM-1) during the rolling and arrest, leukocyte lymphocyte function associated antigen-1 (LFA-1), and endothelial intercellular adhesion molecule-1 (ICAM-1) during arrest and migration (32, 33), as well as activated leukocyte cell adhesion molecule (ALCAM), and CD6 in migration (34).

The specific molecules required for B cell transmigration, however, are less clearly understood. Similar to requirements for T cell BBB transit, *ex vivo* studies using human adult brain-derived endothelial cells (HBECs) show that blockade of VLA-4, but not VCAM-1, inhibits B cell transmigration (35). Consistent with these findings, mice lacking the VLA-4 α-4 subunit specifically on B cells but not on other lymphocyte populations reduced disease severity significantly, and inhibited the recruitment of B cells into the CNS in an experimental autoimmune encephalitis model (36). In natalizumab-treated MS patients, CSF B and plasma cells are decreased in concert with the reduction in intrathecal CD4+ and CD8+ T cells (37). Complete (55%) or partial (27%) loss of CSF OCBs was observed in a natatlizumab-treated patient cohort following 2 years of therapy, suggesting that continuous trafficking of B cells to the CNS may be required to maintain the plasma cell niches producing intrathecal oligoclonal IgG (38). Antibody blockade of ICAM-1 and ALCAM also result in reduced migration of CD19+ B cells in *ex vivo* transmigration assays using HBECs as an artificial BBB (34, 35). The exact roles of ICAM-1

and ALCAM in CNS B cell trafficking *in vivo*, however, remain to be determined.

Recently, CNS meningeal lymphatic vessels containing T lymphocytes were discovered running parallel to the dural sinuses (39). These vessels drain to the deep cervical lymph nodes and may provide a novel route for trafficking B and T cells into or out of the CNS. This pathway may involve similar or distinct chemokine and adhesion molecules in the transit of various B cell populations that may infiltrate into the brain parenchyma, circulate in the CSF, populate GC-like structures, and transit back to the peripheral lymphoid compartment (39).

BIDIRECTIONAL B CELL TRAFFICKING IN MS

In general, lymphocytic surveillance of the healthy CNS is significantly lower than that of other peripheral organs (40). The majority of data, particularly in humans and mice, indicate that activated antigen-experienced T and B cells constitute almost the entirety (41) or the vast majority (17, 42) of the infiltrating lymphocytes. Whether activated lymphocytes return from the CNS compartment to the peripheral circulation has remained uncertain.

Recently, the ability of B cells to exit the CNS compartment and re-enter the peripheral circulation and, potentially germinal center responses, has been investigated by deep sequencing (43). Deep, or next-generation sequencing, allows for high-throughput recovery of B cell IgG heavy-chain variable region (VH) repertoires from patient fluids and tissues. When compared to single-cell methods, the large number of VH sequences analyzed by deep sequencing provides a more complete representation of the B cell Ig repertoire contained in a biological sample and substantially increase the likelihood of observing identical or related VH sequences between samples. This enhanced sensitivity likely accounts for the frequent identification of common peripheral and CNS B cell clones with deep sequencing (43–45) and the rare identification of those with single-cell analyses (46, 47).

Using diverse strategies, patient populations, and methods, the VH repertoire from the peripheral blood, cervical lymph nodes, meninges, parenchyma, and CSF have been compared within the same MS patient (43–45). A common finding of each investigation was overlapping clonal B cell populations common to both the peripheral and CNS compartments. Overlapping peripheral blood and CSF B cell clones were observed among multiple subsets of Ig class-switched and post-germinal center B cells: CD27(+)IgD(−) memory B cells, CD27(hi)CD38(hi) plasma cells/plasmablasts, and CD27(−)IgD(−) negative memory B cells (44, 48, 49). While the number of overlapping sequences observed in each study varied due to technique and disease activity, lineage analysis of bi-compartmental B cell clones demonstrated patterns of somatic hypermutation consistent with bidirectional exchange (43–45). Some lineages showed a balanced distribution of peripheral and CNS compartment clones; while other lineages exhibited isolated CNS clones that were closely related to germline sequences. The pattern of overlapping B cell clones in these lineage trees suggest that B cells may travel back and forth across the BBB and re-enter germinal centers to undergo further somatic hypermutation (43–45) (**Figure 1**). In-depth analysis of the relationship between overlapping B cell clones in the cervical lymph nodes and CNS compartment of the same patient revealed that most of the shared VH clones were less mature sequences that originated, more often, in the periphery (45). More mature B cell clones tended to be restricted to either the peripheral lymph node or CNS compartment. Permutation testing supported a model in which B cell maturation into antibody-secreting cells occurs in both the periphery and CNS with antigen-specific maturation occurring in the periphery.

COMPARTMENTALIZATION OF THE CNS B CELL RESPONSE IN MS

A key question related to MS pathogenesis is whether B cell-mediated antigen-driven responses are generated, supported, and sustained within the CNS (43)? CNS B cells show evidence of clonal expansion (50, 51), and express somatically mutated, class-switched Ig transcripts (46, 52–55). As noted previously, B cells with clonally related VH sequences are recovered on both sides of the BBB; however, CNS B cells may eventually form a compartmentalized population that is independent of the peripheral B cell pool as disease progresses. Interestingly, compartmentalized CNS inflammation has been hypothesized to drive treatment-resistant progressive disease (56).

Oligoclonal CSF IgG (OCBs) are observed in over 95% of MS patients. The CSF Ig proteome and B cell Ig transcriptome show strong overlap, indicating that CSF B cell clones are a major contributor to MS intrathecal IgG (57). In a subsequent study, peptide sequences from the CSF Ig proteome were also found to match heavy- (VH) and light-chain (VL) transcriptome sequences recovered from the CNS parenchyma and CSF of the same individual (58). The CSF Ig proteome covered high percentages of VH (CNS-77%; CSF-84%) and VL (CNS-39%; CSF-60%) transcriptome sequences in one patient and were somewhat limited in a second due to low CSF Ig quotient (58). The results indicate that B cells and IgG in MS CSF accurately mirror the humoral immunity present at the site of brain tissue damage (**Figure 1**). Indeed, 39–62% of the B cell transcriptome sequences recovered from the meninges, demyelinating plaques, normal appearing white matter, and CSF of the same MS patient were shared between intrathecal compartments, indicating that a significant fraction of intrathecal B cells trafficked through the CNS (59). Some expanded B cells clones, however, appear restricted to regions of MS plaque and meninges, suggesting some potential for localized tissue injury (59).

Interestingly, recent studies have questioned whether the CSF-restricted OCBs identified by isoelectric focusing are truly exclusive to the CNS (60). While the majority of CSF OCBs matched IgG-VH transcripts only recovered from the CSF B cell transcriptome, several OCB peptides matched bi-compartmental peripheral blood and CSF VH sequences. Although the type of MS and disease therapies were not reported, lineage tree analysis of bi-compartmental B cell populations suggested that these B cell groups underwent immune stimulation on both sides of the BBB (**Figure 1**). As a result, there remains the possibility that CNS immune populations may maintain molecular links with the periphery despite contrary data from isoelectric focusing.

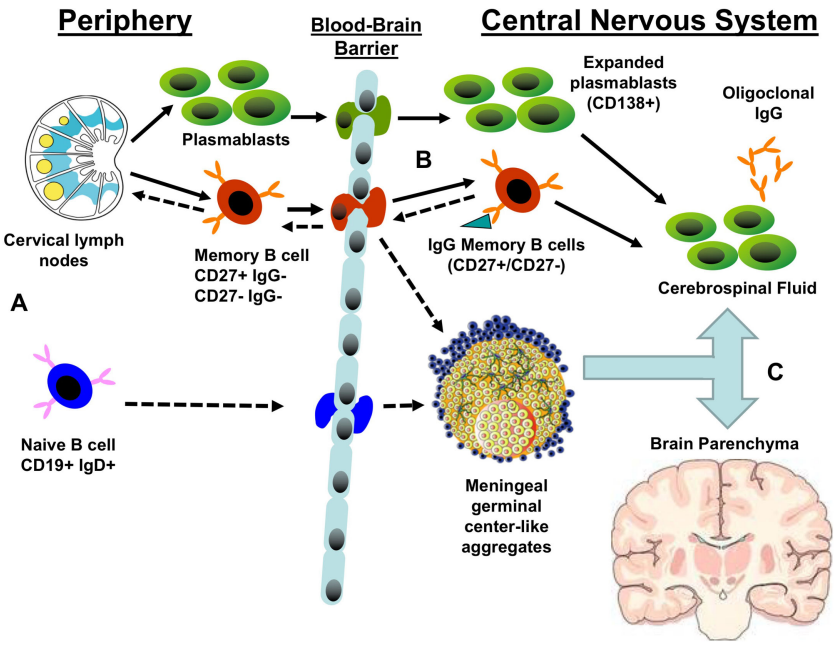

FIGURE 1 | Potential patterns of B cell trafficking in multiple sclerosis. **(A)** The predominant stream of migratory B cells from the periphery to the CNS are likely to consist of either memory B cells or plasmablasts produced in the germinal centers of cervical lymph nodes. The presence of CSF B cell clones closely related to germline sequences suggests that naïve B cells may transit the blood–brain barrier to populate meningeal germinal center-like structures and produce CNS-restricted memory B cells. **(B)** Both migratory plasmablasts and memory B cells may contribute to the pool of central nervous system (CNS) antibody-secreting cells that produce the oligoclonal bands. Memory B cells may also enter germinal centers in meningeal lymphoid aggregates or draining cervical lymph nodes, resulting in further clonal expansion and affinity maturation. **(C)** A significant fraction of expanded B cell clones circulates between CNS compartments: cerebrospinal fluid, meningeal lymphoid aggregates, parenchymal lesions, and normal white matter. Solid arrows represent established pathways; dashed arrows represent putative pathways.

Ig VH gene usage from the periphery and CNS provides additional data supporting compartmentalization of the humoral immune response in MS patients. The analysis of Ig VH sequences from demyelinating plaques and CSF of affected individuals reveal substantial VH4 family bias compared to normal VH4 prevalence (61, 62). Similar to patients with viral meningitis, CNS VH4 germline sequences displayed evidence of clonal expansion and extensive somatic mutations consistent with antigen selection (53, 54). MS patient with the longest disease course had the largest number of distinct IgG clonal populations, while the patients with recent diagnoses had limited clonal populations. CSF B cells from patients with a single demyelinating event (clinically isolated syndrome) also showed clonally expanded, somatically hypermutated VH genes (63, 64). Interestingly, both the overrepresentation of VH4 family sequences (65) and a unique pattern of somatic hypermutation "antibody gene signature" (66) within the CSF Ig VH transcriptome predicted transition to clinically definite MS. Recent deep sequencing of MS CSF VH repertoires from six MS patients has also revealed an overrepresentation of VH4-39, VH4-59, and VH4-61 heavy-chain sequences. The bias of MS CSF B cell heavy chains to VH4 germline sequences suggests that their basic structure may define an antigen-binding pocket that favors interaction with target antigen(s). As a result, a compartmental CNS humoral immune response may be able to drive CNS injury independent of peripheral immune activity.

Lastly, the GC-like structures or lymphoid infiltration have been noted in a large proportion of meningeal tissue from secondary progressive early stage cortical biopsies (7, 8, 67, 68). These CNS-specific immune infiltrates correlate with the severity of disease progression (8) and are associated with cortical neuronal loss in adjacent gray matter (69). The composition of these infiltrates included B cells, T cells, and dendritic cells, whose organization may resemble lymphoid follicles (7, 67). In addition, the presence of IgG and CXCL13 (7, 67) provide additional information, suggesting the active attraction and maintenance of B cells in MS meninges. The identification of CD19 + CD38hiCD77 + Ki67 + Bcl2− centroblasts in the CSF but not the peripheral blood of MS patients suggests that a compartmental humoral immune response in the MS CNS recapitulates all stages of B cell differentiation and may create a self-sufficient CNS response that is independent of the immune activity in the periphery (13). Additional data, however, are required to establish the relationship between the generation and maintenance of meningeal GC-like structures, intrathecal B cell clonal populations, and progressive disease (**Figure 1**). Peripheral B cell depletion, effective in early phase clinical trials in relapsing MS (9, 10), has not delivered similar efficacy for the treatment of primary progressive disease (70). This could be directly related to the inefficient depletion of the intrathecal B cell population in progressive (71) versus relapsing MS (72) due to compartmentalization of the B cell response in progressive disease and inefficient transit

of anti-CD20 monoclonal antibody across the BBB. Interestingly, intrathecal administration of anti-CD20 monoclonal antibody rapidly depleted both peripheral and CD19+ B cells within days of delivery (73). Therefore, intrathecal anti-CD20 therapy may offer a novel avenue to evaluate the role of intrathecal B cell inflammation in progressive disease. The recent development of novel MRI techniques to identify meningeal follicles may offer a non-invasive tool to correlate therapeutic response with changes in meningeal inflammation (74).

CONCLUSION

Molecular analysis of the B cell response in MS has demonstrated that antigen-experienced B cells are shared between multiple CNS compartments and the peripheral immune response. Several features of CNS clonal B cell populations suggest that B cell subsets may not be shared between the CNS and periphery as disease progresses and that meningeal GC-like structures may support an independent, compartmentalized immune response that is correlative with measures of CNS injury. The data supporting the trafficking of B cells back and forth across the BBB are undermined by the technical constraints of single-cell PCR, deep sequencing, and sampling errors. For instance, the VH sequences defining the bi-compartmental B cell clones may be skewed by errors in PCR sequencing, multiple cDNA copies from the same cell, errors in flow cytometry, or limited blood and CSF sampling. Future studies are needed to confirm present data using defined MS cohorts at multiple stages of disease. The influence of current MS therapeutics on B cell trafficking and survival may be critical for understanding MS pathogenesis and establishing biomarkers of disease activity and therapeutic efficacy.

AUTHOR CONTRIBUTIONS

Drs. KB, GO, and JB participated in the analysis, interpretation, writing, and critical review of the manuscript for important intellectual content.

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B Cells in the Multiple Sclerosis Central Nervous System: Trafficking and Contribution to CNS-Compartmentalized Inflammation

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Clinical trial results of peripheral B cell depletion indicate abnormal proinflammatory B cell properties, and particularly antibody-independent functions, contribute to relapsing MS disease activity. However, potential roles of B cells in progressive forms of disease continue to be debated. Prior work indicates that presence of B cells is fostered within the inflamed MS central nervous system (CNS) environment, and that B cell-rich immune cell collections may be present within the meninges of patients. A potential association is reported between such meningeal immune cell collections and the subpial pattern of cortical injury that is now considered important in progressive disease. Elucidating the characteristics of B cells that populate the MS CNS, how they traffic into the CNS and how they may contribute to progressive forms of the disease has become of considerable interest. Here, we will review characteristics of human B cells identified within distinct CNS subcompartments of patients with MS, including the cerebrospinal fluid, parenchymal lesions, and meninges, as well as the relationship between B cell populations identified in these subcompartments and the periphery. We will further describe the different barriers of the CNS and the possible mechanisms of migration of B cells across these barriers. Finally, we will consider the range of human B cell responses (including potential for antibody production, cytokine secretion, and antigen presentation) that may contribute to propagating inflammation and injury cascades thought to underlie MS progression.

Keywords: B cells, multiple sclerosis, central nervous system, meningeal inflammation, trafficking

INTRODUCTION

Roles of B cells in central nervous system (CNS) inflammatory diseases have been investigated in patients and through elegant animal model studies. Here, we will focus on studies carried out in human, with animal work described in more detail elsewhere in this issue. B cell responses have long since been recognized in MS with variable degrees of evidence implicating them in both early/

TABLE 1 | Strength of evidence implicating B cells in early/relapsing and later/progressive MS.

	Early/relapsing MS	Later/progressive MS
Clinical arguments	Anti-CD20 therapy robustly limits new focal inflammatory brain lesions and MS relapses (34–36) PLEX may improve resolution of steroid refractory relapses (38)	Anti-CD20 therapy may limit worsening of disability in (*post hoc*) subgroup analysis of PPMS patients (younger patients; those exhibiting gadolinium enhancing lesions) (37)
Biological arguments	CSF OCB already present early in relapsing MS course in many patients; IgG levels (39) and presence of IgM OCBs have been associated with MS activity (39–41)	CSF OCB present in majority of patients later in MS course; some implication that their presence is associated with more aggressive or progressive course (39, 40)
	Abnormal autoantibodies against MOG (27–30) and KIR4.1 (31, 32) reported in some patients with MS; clinical significance remains unclear IgG transfer from MS patients can induce complement-mediated demyelination in animals (27, 42)	
	Dynamic exchange of B cell clones found in MS CNS and periphery, and evidence that activation/maturation may occur in the periphery (13, 43)	Shared B cell/PC clones within different CNS subcompartments including parenchymal lesions, CSF as well as meninges (10)
Pathological arguments	Common lesion type in pathologic classification of demyelinating lesions notable for deposition of immunoglobulin (Ig) and complement (8) Antibodies and myelin fragments have been identified within phagocytic cells in MS lesions (6, 44)	
	Meningeal inflammation including presence of B cells, as well as subpial cortical demyelinating lesions can be features of early MS (9)	B cell-rich meningeal aggregates associated with subpial cortical lesions reported as more common in progressive forms of MS (10–12)

PLEX, plasma exchange; OCB, oligoclonal bands; MOG, myelin oligodendrocyte glycoprotein; PC, plasma cell.

relapsing and later/progressive disease (**Table 1**). The abnormal presence of antibodies in the CNS continues to represent the most consistent immunodiagnostic feature in patients with MS. Cerebrospinal fluid (CSF)-restricted oligoclonal bands (OCBs) are reported in the CSF of over 90% of MS patients throughout disease stages (1–5). Over the years, pathological implication of B cells has included the demonstration of antimyelin antibodies inside phagocytic cells within MS lesions (6, 7), the observation that the most common demyelinating lesion pattern (Pattern II) is characterized by prominent deposition of antibodies and complement (8), and the more recent descriptions of meningeal immune cell collections that can be B cell rich (9–12). The latter were first described in a proportion of patients with progressive forms of MS and subsequently also within meninges of patients considerably earlier in their disease course (9–12). Molecular analyses of the Immunoglobulin (Ig) variable gene region of B cells and plasma cells from active parenchymal lesions, the CSF, or meninges of MS patients have revealed the persistence of (presumably antigen driven) clonotypes that are shared between these three different CNS subcompartments (10, 13–18). Antibodies generated from clonally expanded plasma cells derived from the CSF of MS patients were capable of both binding human and mouse CNS tissue, and causing complement-mediated demyelination and astrocyte activation in spinal cord explants (19). In spite of the long-standing implication of clonally expanded B cell populations and abnormal antibodies in the MS CNS, the antigens recognized by these antibodies are still subject of debate and different targets have been suggested such as viruses, axoglial proteins, and glycolipids (20–25). The more recent work-deriving antibodies from CSF-expanded B cell clones of MS patients suggest that they may preferentially target neurons and astrocytes (19, 26). The significance of serum antibodies to molecules, such as MOG and KIR4.1, also continues to be investigated (27–33).

The observation that B cell depletion with anti-CD20 monoclonal antibodies substantially limits new relapsing MS disease activity (34–36, 45, 46) has made it clear that B cells play important roles in the immune cascades underlying CNS inflammation and has reinvigorated research efforts to elucidate mechanisms underlying such B cell roles. Of interest in this regard, is the observation that while anti-CD20 therapy rapidly reduces new relapsing MS disease activity, the abnormalities in CSF antibody measures seem to persist in the face of the therapeutic benefit (47). This indicates that the therapeutic mechanisms of action by which B cell depletion limits new MS relapses reflect at least in part antibody-independent roles of B cells. Indeed, B cells are now recognized to have multiple functions that may contribute to MS pathogenesis, in addition to their capacity to differentiate into antibody-secreting cells (plasmablasts/plasma cells) (**Figure 1**). B cells can be highly efficient antigen-presenting cells (APC) to T cells when presenting antigens that they initially recognize with their surface B cell receptor (BCR) (48). In this context, Harp et al. reported that memory B cells in MS patients can efficiently present neuro-antigens to T cells (49, 50). Moreover, activated B cells can modulate the local inflammatory response of both T cells and myeloid cells through secretion of proinflammatory or anti-inflammatory cytokines (described in detail in Li et al., in this issue). Some B cells support proinflammatory functions of other cells through production of TNFα, IL-6, GM-CSF, and Lymphotoxin-alpha (51–55), while IL-10 and IL-35 producing B cells possess anti-inflammatory (regulatory) roles (53, 56–58). In MS, B cells seem to be abnormally polarized toward a more proinflammatory phenotype (54, 55, 59, 60), and defects in their regulatory function have also been suggested by some but not all authors (55, 59–62).

Anti-CD20 therapy has also been studied in Phase III trials of patients with primary progressive MS (PPMS). Treatment in the initial trial using rituximab, failed to limit disease progression though a benefit was suggested in the subgroup of younger patients, and those with evidence of focal inflammatory brain lesions (37). The follow-up ORATORIO study of anti-CD20 (ocrelizumab) focused on younger patients who were closer to clinical PPMS disease-onset, and demonstrated a modest

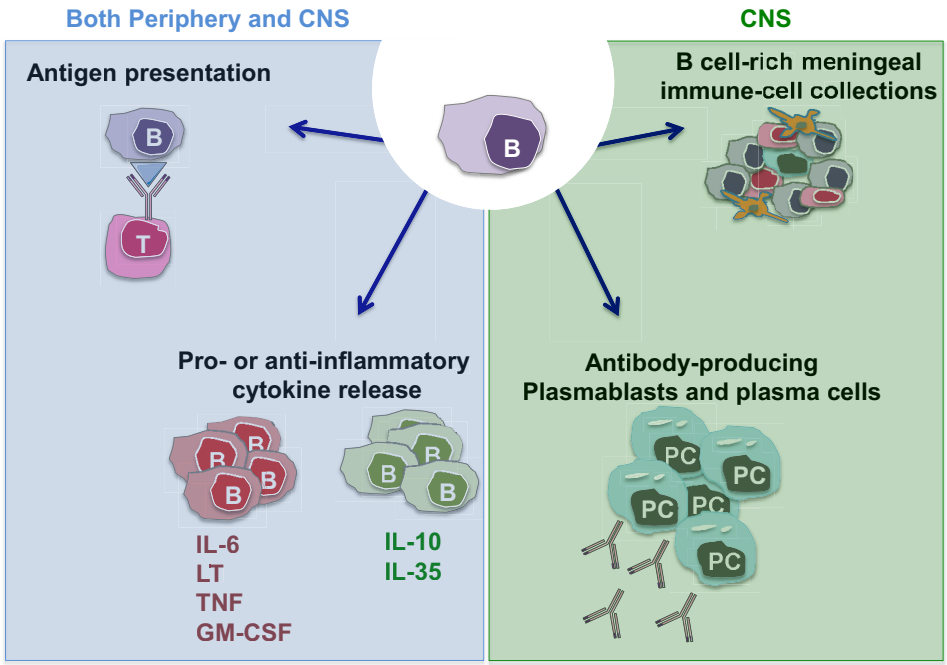

FIGURE 1 | Potential MS-relevant B cell responses. (1) B cells can function as efficient antigen-presenting cells (APC) especially in context of cognate B cell:T cell interactions. This may activate pathogenic T cells that in turn contribute to disease propagation. (2) B cells and/or plasma cells have the potential to produce anti-inflammatory cytokines (such as IL-10 and IL-35) but also proinflammatory cytokines (IL-6, LTα, TNFα, and GM-CSF). A lack of balance involving over-propensity of B cells to produce proinflammatory cytokines and their deficient production of anti-inflammatory cytokines has been demonstrated in patients with MS. Such B cell responses within the CNS may contribute to propagating CNS-compartmentalized inflammation. (3) B cells can differentiate into plasmablasts and plasma cells, which can elaborate pathogenic autoantibodies (and possibly also cytokines). (4) Similar to their established roles in normal lymphoid architecture formation, B cells may release factors that contribute to the formation and/or maintenance of persisting immune cell aggregates in the meninges of MS patients.

treatment benefit in limiting the rate of progression of neurological disability (63, 64). A number of factors may limit a more robust effect of peripheral B cell depletion on progressive MS biology. Meningeal immune cell aggregates in which B cells can be a prominent feature may not be as efficiently targeted by anti-CD20 antibodies that only weakly penetrate the CNS. It is also possible that long-lived plasma cells (that do not express CD20) and the antibodies they generate may play a more important role in progressive forms of MS compared to relapsing MS. B cells are known to play important roles in the formation of normal lymphoid follicle architecture (65, 66). Observations of B cell-rich immune cell collections in the meninges of MS patients [some of which recapitulate lymphoid follicle-like features (9, 11, 12) and reviewed by Pikor and Gommerman, in this issue] raise the intriguing possibility that B cells contribute to the formation and/or maintenance of such structures. In doing so, the B cells may contribute to propagation of inflammation within the MS CNS. Some or all of the diverse functions of B cells which are now thought to contribute to inflammatory responses in the periphery of MS patients may also be relevant within the CNS.

It now appears likely that functionally distinct B cells contribute to the MS disease process through diverse mechanisms within the distinct disease compartments and throughout different stages of the disease. Peripheral proinflammatory B cells play an important role in relapsing disease mechanisms (see Li et al., in this issue), whereas meningeal collections of B cells potentially participate in the maintenance and propagation of CNS-compartmentalized disease. This review will focus on studies that implicate human B cells within the CNS of MS patients. We will highlight available findings form human studies that (I) consider the sites and characteristics of B cells within the MS CNS subcompartments, including CSF, parenchyma, and meninges; (II) how B cells might get there (barriers/trafficking); and (III) what they might do there (responses that may be relevant to CNS injury processes).

Where Are B Cells Within the MS CNS?

B cells, plasmablasts, and/or plasma cells have been described in several subcompartments of the CNS of patients with MS, including the CSF, parenchyma, and meninges (**Figure 2A**). Emerging studies are adding to our understanding of the profiles of such cells as well as the relationship between such cells in the different CNS compartments.

Cerebrospinal Fluid B Cells in MS

Early studies investigating CSF cytology suggested that the number and the relationship of B cells to other CSF immune cells (principally monocytes) may be associated with MS disease severity and progression (67). In particular, a high ratio (predominance of B cells) was associated with more rapid disease progression, whereas a low ratio (predominance of monocytes) was found in patients with slower progression (67). Subsequent

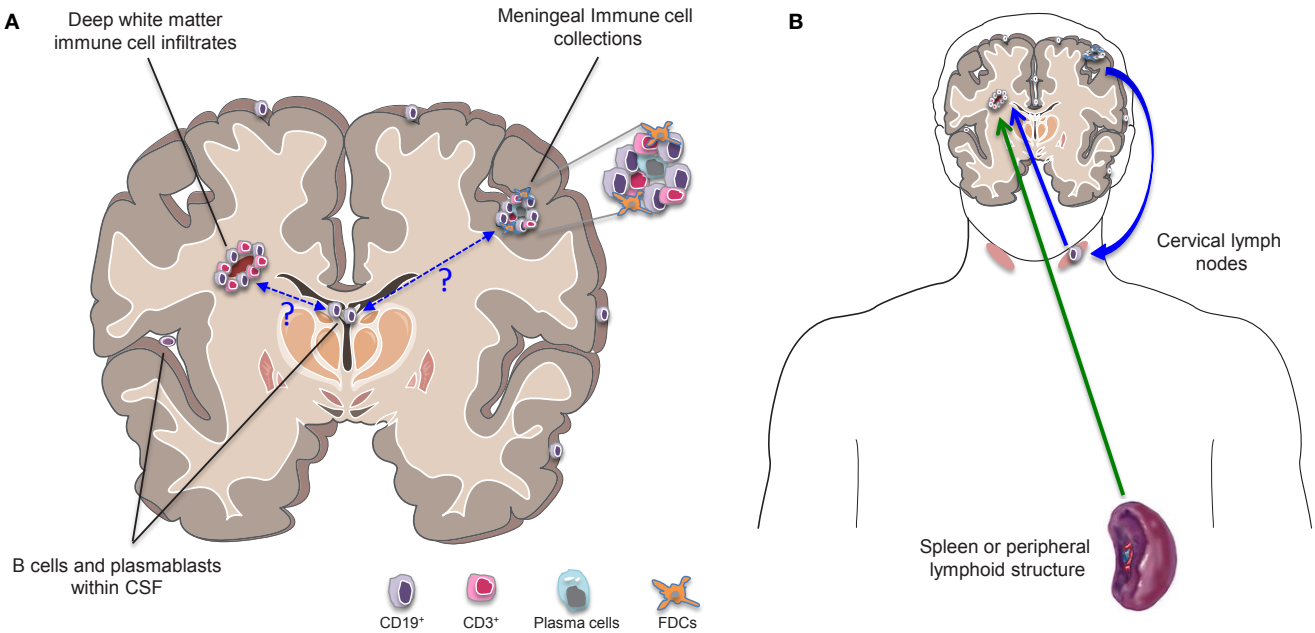

FIGURE 2 | B cells in different compartments and implication for MS disease activity. **(A)** Cells of the B cell lineage (including primarily memory B cells, plasmablasts, and plasma cells) are found to persist in the inflamed MS CNS and occupy multiple subcompartments. These include the cerebrospinal fluid (CSF), parenchymal white matter lesions, and collections of immune cells within the meninges, which can be B cell rich. Somatic mutation analysis has demonstrated that the same B cell clones may occupy all three CNS subcompartments. Exactly how and where such clones initially access the CNS and how they communicate across these CNS subcompartments remains largely unknown. **(B)** The traditional view has held that new MS disease activity is triggered by activation of immune cells in the periphery (possibly triggered by pathogen-associated molecules recognized by cross-reactive T cells; referred to as molecular mimicry) and subsequent trafficking of the activated cells into the CNS (green arrow). However, the demonstration that the CNS clearly has lymphatics that drain into cervical lymph nodes and evidence from somatic mutation analysis indicating bidirectional trafficking of B cells between the CNS and the periphery (with much of the activation and clonal expansion apparently occurring in the periphery) suggests that relapses may also be "invited" from within the CNS (blue arrows). This might occur if proinflammatory B cells exit the chronically inflamed CNS carrying CNS antigens, which they may then present to T cells in the draining cervical lymph nodes with subsequent trafficking of the activated T cells into the CNS.

work demonstrated that CSF B cells in MS CSF are largely class-switched $IgD^{-}IgM^{-}$ memory B cells (68) and that the main B cell effector subset are short-lived plasmablasts (69, 70). Over the years, several groups carried out somatic hypermutation analysis of the variable region of the heavy chain immunoglobulin (Ig V_H) in B cells obtained from CSF of MS patients in comparison to circulating blood B cells obtained from the patients at the same time (14–18). A consistent finding was that MS CSF harbored increased frequencies of clonally expanded B cells (with post-germinal center memory characteristics) compared to the blood. The mutations appeared to be highly concentrated within the CDR3 region, which has been taken to indicate an antigen-driven selection process of B cells accumulating in the CSF of patients. Presorting and amplifying the variable region of the IgG gene from both total $CD19^{+}$ B cells and $CD138^{+}$ plasma cell/plasmablasts purified from the CSF of MS patients revealed that both sorted subsets harbored somatically mutated expanded clones (71). The repertoire within the $CD138^{+}$ subset was more restricted though little sequence overlap was observed between the $CD19^{+}$ and $CD138^{+}$ repertoires (71). More recently, analysis of genes for IgM-chains in CSF B cells of MS patients revealed extensive accumulation of somatic hypermutation and clonal expansion in IgM-producing B cells (72). Whether or not these cells initially trafficked into the CNS as naïve B cells, their coexpression of activation-induced cytidine deaminase (AID, an enzyme crucial for somatic hypermutation and class switch recombination of antibodies, that is normally expressed during activation of B cells in germinal centers) provides further support that the intrathecal milieu in patients with MS sustains accumulation of germinal center-like experienced B cells that can produce both IgM and IgG antibodies. Additional elegant work examining both CSF B cell IgG-H and Ig-κ chains transcriptomes, as well as the oligoclonal Ig proteomes derived from the same CSF of patients with relapsing-remitting MS, showed a correspondence between CSF Ig proteomes and B cell Ig transcriptomes, providing the most direct evidence that expanded CSF B cell clones are responsible for producing the abnormal Ig that comprises the CSF OCB in MS patients (73).

Parenchymal B Cells in MS Brains

Most histopathologic studies of MS are based on autopsy tissues, which tend to be obtained relatively late in the disease course. When patients undergo biopsy earlier in the course of disease, such tissues may not be representative of typical MS pathology as biopsies tend to be done only when the lesion and/or clinical presentation are sufficiently atypical. Thus, available insights into

the distribution and magnitude of B cell infiltrates within the MS CNS (whether in the parenchyma or meninges) largely reflect longer-standing disease states. With respect to the parenchyma, studies have generally indicated that the classical deep white matter perivascular demyelinating lesions of MS typically exhibit relatively few B cells and plasma cells compared to the greater abundance of myeloid cells and T cells (8, 74, 75). At the same time, demyelinating lesion classification has identified "Type II" lesions (that exhibit considerable Ig and complement deposition) as the most common demyelinating lesion type in MS (8, 74). It is noteworthy that in a study of 26 active lesions from 11 patients diagnosed with relatively early MS, demyelinated lesions reportedly exhibited considerable numbers of B cells as well as IgG-positive plasma cells, in addition to T cells and myeloid cells (75, 76). This raises the possibility that B cells and plasma cells may be a more common feature of early, as compared to later, MS parenchymal lesions. Somatic mutation analysis of B cells and plasma cells isolated from both parenchymal lesions and CSF of the same MS patients at autopsy demonstrated clonally expanded and somatically hypermutated populations within the tissue samples, as well as shared clones populating the tissue and CSF (77). Shared clones were subsequently demonstrated in parenchyma and meninges of the patients (10). These observations point to relatedness of expanded B cell and plasma cell clones in both parenchymal and extraparenchymal subcompartments of the MS CNS, at least later in disease.

B Cells Within Meningeal Immune Cell Collections in MS

Cellular immune aggregates have been reported in the meninges of patients with MS, some of which were found to be rich in B cells, and have been referred to as "ectopic follicles" or "follicle-like structures" based on their resemblance to tertiary lymphoid tissues (TLT) (11, 12). Early studies described these structures mainly in subsets of relatively late-phase SPMS and PPMS patients (11, 12, 78, 79). Presence of these meningeal immune cell collections was associated with more aggressive clinical disease and a greater extent of tissue injury in the subjacent cortical regions. The pattern of the demyelinating subpial cortical injury associated with meningeal inflammation involved a gradient of microglial activation, reduced numbers of oligodendrocytes, and neuronal loss, such that the most severe injury was present in the most superficial cortical layers. Meningeal immune cell aggregates were most commonly found in the deep sulci of the temporal, cingulate, insula, and frontal cortex (79). Based on these initial reports, the prevailing concept was that meningeal inflammation in MS is a feature of a subset of patients with late/progressive disease. However, recent imaging studies demonstrate leptomeningeal contrast enhancement in the brain of individuals with RRMS (80) and substantial meningeal inflammation has also been described in biopsy material obtained from patients relatively early in their MS disease course (9). While these biopsies were obtained for diagnostic purposes of atypical deep white matter lesions, the biopsy trajectories captured meningeal and cortical tissue that exhibited the typical features of cortical MS injury, and demonstrated considerable meningeal inflammation. The cortical tissue underlying the meningeal immune cell collections in these early cases depicted a similar demyelinating injury pattern (with enhanced microglial activation, reduced number of oligodendrocytes, and neuronal/neuritic loss) as was described in the more chronic cases (11, 78). Further characterization of meningeal immune cell aggregates in MS indicated that at least some are enriched in proliferating ($Ki67^+$) $CD20^+$ B cells. Presence of some plasma cells/plasmablasts, $CD8^+$ and $CD4^+$ T cells, and CXCL13-producing $CD35^+$ cells with follicular dendritic cell characteristics were also described (12, 79, 81). As noted, somatic hypermutation analysis of the BCR in B cells and plasma cells isolated from meningeal immune cell aggregates, as well as parenchymal lesions and CSF from the same MS patients, demonstrated that related B cell clones populate all three compartments (10), again underscoring the relatedness of clonally expanded B cells found in the MS CNS. CNS-infiltrating B cells are also clonally related to peripheral B cells (13, 14, 18) raising intriguing questions about the dynamics involved in trafficking of B cells into the CNS and among these distinct CNS subcompartments as discussed below. It is still not clear whether formation of meningeal immune cell collections occurs commonly and throughout the different (early and late) phases of disease, and whether their presence contributes to, or is merely the consequence of underlying tissue injury. Indeed, some groups did not identify the presence of meningeal immune cell aggregates or a relation between such aggregates and cortical injury (82). These discrepancies may reflect true biological heterogeneity across patients, transient presence of meningeal inflammation (for example, during periods of more active CNS inflammation), or technical reasons as these structures tend to be very small ($\leq$100 μm in thickness) and may be lost depending on the approach to tissue processing.

What Routes Might B Cells Use to Infiltrate the CNS?

Anatomical Routes to Cross the Blood/CNS Barriers

The CNS (comprising the brain and spinal cord) which was historically referred to as immune privileged is now referred to as immune specialized with the understanding that peripherally derived immune cells do patrol the CNS as part of normal physiologic immune surveillance (83–85). This immune specialization is conferred by the presence of barriers that restrict the passage of large molecules and limit broader cell infiltration. While the most known of these specialized barriers is the blood–brain barrier (BBB), two less well studied but nonetheless important other barriers are the blood–meningeal barrier (BMB) and the blood CSF barrier (BCB).

The Blood–Brain Barrier

The BBB is a structure formed by specialized endothelial cells (ECs) that separates the CNS from systemic circulation. CNS blood vessels are made of two main cell types: the ECs themselves and the mural cells that sit on the abluminal surface of the EC layer (i.e., pericytes and astrocytes). CNS ECs are characterized by the presence of tight junctions that limit the paracellular flux of solutes and by a very low rate of transcytosis. These properties

permit a tight control of the exchange between the brain and the blood (86). Maintenance of the BBB is governed by both cellular and non-cellular elements that interact with the ECs. Astrocytes, pericytes, and the extracellular matrix together provide structural and functional support to the BBB (87–89). The term "neurovascular unit" (NVU) additionally refers to neurons and microglia cells that also contribute to this barrier. At the level of the postcapillary venule, two distinct basement membranes (endothelial and parenchymal) define the inner and outer border of the perivascular space. Basement membranes keep members of the NVU in place and regulate their intercellular cross-talk.

The Blood–Meningeal Barrier

The meninges are composed of three layers that surround the CNS (Dura mater, Arachnoid mater, and Pia mater) and contain the CSF located within the subarachnoid space. In the brain, the gray matter is directly adjacent to the meninges. While the meninges were initially considered as a mere physical barrier preventing entry of infections and toxins into the CNS, more recent findings have established this tissue as a site of active immunity in both health and disease (84, 85, 90). Similar to barrier membranes in the gut and lungs, the meninges can house a wide variety of immune-competent cells such as macrophages, dendritic cells, mast cells, innate lymphoid cells, and fibroblasts that can provide effective protection against microbes (91–93). As is the case with the other immunologically competent barriers, the meninges can also be the site of chronic inflammation in pathologic states. The description of meningeal immune cell collections associated with MS has reinforced the concept that the BMB could be an important pathway in immune cell CNS trafficking. Indeed, live imaging studies in animals demonstrate that lymphocytes cross the BMB prior to onset of CNS inflammation and appear to become reactivated in the subarachnoid space as part of disease instigation (94, 95). Relatively, little is known about the properties of ECs located in the meninges, which appear to differ in important ways from parenchymal ECs associated with the BBB. For example, meningeal microvessels lack the rich astrocytic ensheathment, which characterizes the microvessels in the CNS parenchyma (96).

The Blood CSF Barrier

Another port of entry into the CNS is the choroid plexus (CP) forming a barrier between the blood and CSF. This villous structure extends into the ventricular organs and is also responsible for producing the CSF. The CP is made up of a layer of epithelial cells surrounding a core of fenestrated capillaries and connective tissue, allowing the free diffusion of solutes from the blood toward the parenchyma through inter-endothelial gaps. The monolayer of epithelial cells has tight gap junctions that prevent the flux of macromolecules and cells and acts as a blood:CSF barrier (97). It has been shown that ICAM-1 and VCAM-1 are constitutively expressed by CP epithelial cells (98), and this barrier may be a port of entry of pathogenic TH17 cells during the commonly used animal model of CNS inflammation, experimental autoimmune encephalomyelitis (EAE), an influx mediated at least in part *via* CCR6/CCL20 interactions (99).

Molecular Mechanisms Underlying Cell Trafficking into the CNS

The Multistep Process of Leukocyte Extravasation

In healthy individuals, there is a very low rate of ongoing immune surveillance of the CNS. Immune cell migration across barriers is normally tightly regulated and involves a multistep process. These different steps include rolling, firm adhesion, crawling, and extravasation (97, 100–104). The initial contact between leukocytes and the endothelium is usually mediated by adhesion molecules of the selectin family. This first step allows the reduction of the leukocyte velocity in the bloodstream, hence allowing them to detect the chemokine factors secreted by, or bound to ECs. The binding of chemokines to their cognate receptors expressed on the surface of leukocytes leads to an increased avidity/affinity of interaction between cellular adhesion molecules (immunoglobulin family members such as VCAM1, ICAM1, ALCAM, and MCAM) and adhesion molecule receptors such as those of the integrin family, which contributes to firm adhesion of the cells to the endothelium. Subsequent leukocyte polarization and crawling (typically against the direction of blood flow) to sites permissive for diapedesis, requires the expression of ICAM1 and 2 (but not VCAM1) by ECs and is a prerequisite for immune cell diapedesis across the BBB (94).

Leukocytes can then migrate through inter-endothelial regions (diapedesis) or directly through the ECs themselves. Expression of several of these adhesion molecules has been found to be highly increased in MS tissue and is thought to contribute to the extravasation of leukocytes into the CNS parenchyma of patients (100–106). Different preferential pathways and molecular mechanisms of trafficking across the BBB have already been identified for T cells and monocytes [for review, see Ref. (97)]. Less is known concerning B cell migration into the CNS.

Molecules Implicated in B Cell Migration into the CNS

Natalizumab, which binds VLA-4, is one of the most potent therapies in RRMS. Studies have mainly focused on its impact on T cells migration across the BBB, but B cells express also high levels of VLA-4 (107, 108). A major role of VLA-4 in B cells migration across human adult brain-derived ECs has been shown *in vitro*, with a prominent role also identified for ICAM-1 (108). A recent study has reported that the selective inhibition of VLA-4 expression on B cells reduces the susceptibility to EAE by decreasing B cell accumulation inside the CNS but also by interfering with TH17/macrophage recruitment (109). Finally, another adhesion molecule named ALCAM (activated leukocyte cell adhesion molecule) seems to promote B cell trafficking into the CNS across the BBB (103). Nonetheless, little is known about whether distinct B cell subsets that have been implicated in MS utilize particular molecular pathways to get across the BBB, and whether and how B cells traffic across the other CNS barriers (BMB and CP), are among key questions that have not yet been elucidated.

Dynamics of B Cell Infiltration into the MS CNS

Until recently, the documentation of clonally expanded B cells in the MS CNS including CSF, lesions, and meninges, has been taken as evidence that B cell clonal expansion is driven (by one or more unknown antigens) within the CNS of patients (10, 13–18). More recent evidence points to the potential for more dynamic, bidirectional exchange of B cells between the CNS and periphery (**Figure 2B**), including clonal expansion that occurs in both compartments (13, 14). Since the initial study implicating active diversification of B cells on both sides of the BBB (18), two additional complementary studies confirmed that particular B cells found outside the CNS (in both peripheral blood and draining cervical lymph nodes), share clonality with B cells populating the brain (13, 14) and exhibit evidence of presumably antigen-driven expansion on both sides of the BBB. In one of these studies, using paired CNS tissue and draining cervical lymph nodes from the same patient source, not only were shared B cell clones identified in the two compartments, but the founding clones and much of the subsequent maturation involved in the bidirectional exchange, appeared to take place in the cervical lymph nodes rather than the CNS (13, 14). This could provide a mechanism for "epitope spread," a phenomenon well described in animal studies whereby the antigenic target of the CNS inflammatory attack shifts over time as injury exposes additional epitopes (110). Supporting a role for B cells in such "epitope spread" in patients with MS are observations from antigen array studies indicating that the circulating repertoire of serum anti-CNS antibodies appears to expand in children with MS, yet constrict in children with monophasic CNS inflammatory disease, over time (111).

It is now also apparent that the CNS is not as "immune privileged" as previously thought, with organized lymphatic draining that allows CNS antigens and potential APC to exit from the CNS including the meninges and to access the periphery (84, 85). Based on these observations, one can challenge the prevailing view that MS relapses are invariably triggered by some external stimulus (e.g., pathogen exposure) resulting in peripheral immune cell activation and trafficking into the CNS. Instead, cells capable of antigen presentation, such as B cells, may drain from the CNS into the draining lymph nodes, and present CNS antigens to T cells with subsequent T cell activation and trafficking involved in new relapsing disease activity.

How Might B Cells Within the CNS Contribute to MS Disease Mechanisms?

While the capacity of B cells to mediate aberrant T cell activation in the periphery could explain the substantial contribution of B cells to relapsing MS biology (evidenced by robust relapse-reduction following B cell depletion with anti-CD20 therapy), whether and how B cells may also contribute to progressive (non-relapsing) disease remains to be elucidated. The biology underlying CNS injury in progressive MS is now thought to involve a combination of degeneration and ongoing inflammation that is compartmentalized within the CNS (112). Such compartmentalized inflammation involves astrocyte and microglial activation, though the molecular mechanisms driving such chronic activation remain largely unknown. Since B cells are recognized to persist in the chronically inflamed MS CNS (10, 13, 14, 16), and evidence has mounted that B cells of patients with MS exhibit abnormal proinflammatory response profiles (54, 55, 59, 60), it has been tempting to consider whether B cells chronically residing in the CNS may contribute to propagating local injury processes even independent of B cell roles in relapsing disease biology. This concept is reinforced by reports of meningeal immune cell infiltrates which can be rich in B cells and that have now been identified in both early and late (9, 11, 12, 79) stages of MS. One potential mechanism by which B cells could contribute to ongoing injury is through secretion of CNS-directed autoantibodies (5, 6, 8). As noted, somatic mutation analysis has indicated that clonally expanded B cells and plasma cells are shared between the different CNS subcompartments (CSF, parenchyma, and meninges) (10). Moreover, CSF-derived B cell clones can produce antibody that binds CNS cells (including neurons and astrocytes) and can be shown to cause complement-mediated injury to such structures in CNS explants (19, 26). Antibody-independent contributions (e.g., **Figure 1**) of B cells to propagating inflammation in the MS CNS should also be considered. The great majority of B cells identified in the MS CNS (regardless of subcompartment) appear to be preferentially memory rather than naïve B cells (14, 15), and it is now recognized that memory B cells of MS patients may have particular proinflammatory propensities including the capacity to express exaggerated levels of immune activating molecules and proinflammatory cytokines (55, 59). This may be particularly relevant when considering meningeal B cell-rich immune collections and the subpial cortical demyelinating injury, which is now thought to importantly contribute to progressive loss of neurological function in patients with MS. These subpial demyelinating lesions are notable for microglial activation, astrogliosis, and neuronal loss, and their location may be associated with regions subjacent to areas of meningeal immune cell collections (81). It is intriguing to speculate whether particular B cell subsets persisting within such immune cell collections may impact the underlying glial neural cells through the release of specific soluble factors. In turn, what factors within the inflamed CNS milieu may sustain B cells in that environment? Does CNS persistence of particular B cell clones relate to the antigenic specificity of the B cells? In the case of primary CNS lymphoma, there is some evidence that specific recognition by tumoral B cells of CNS antigens contribute to fostering local tumor survival and proliferation (113), and such a mechanism may also contribute to persistence of B cells in the MS CNS. A number of features of the inflamed MS CNS may support B cells unrelated to their antigenic specificity. These include soluble factors known to support B cell survival that are produced by activated astrocytes and microglia – such as BAFF, IL-6, IL-10, and IL-15 – all reportedly found at increased levels within the CSF of MS patients (114–116). Some of these factors (BAFF and IL-6) also support the survival of plasma cells. In the context of EAE, plasmablasts and plasma cells have been implicated in regulating neuroinflammation through their production of cytokines such as IL-10 and IL-35, although it is

BOX 1 | B cells in the MS CNS: what remains to be elucidated?

What are preferential routes of migration of B cells into the CNS?

Which molecules are involved in migration of distinct B cell subsets?

What are the molecular mechanisms that favor B cell persistence in the MS CNS?

What are the antigenic specificities of abnormal CSF immunoglobulins (Ig) in MS?

Which Ig are disease-relevant vs. an epiphenomenon of chronic activation?

Which response profiles (proinflammatory/anti-inflammatory) characterize CNS B cells?

What are the different roles of B cells within immune cell aggregates in the meninges?

What are the interactions between B cells and other cells in the inflamed MS CNS?

- Do they have direct effects on oligodendrocytes and neurons?
- Do they present antigen/s to T cells? Do they modulate T cell activation/polarization?
- Do they influence astrocyte/microglia activation/polarization and *vice versa*?

BOX 2 | The case of Neuromyelitis Optica (NMO).

For years, neuromyelitis optica (NMO) was largely considered a variant of MS until the discovery of serum antibodies to the water channel aquaporin (AQP)-4, which distinguished patients with NMO from those with MS (118, 119). A growing range of clinical syndromes found to harbor such antibodies has since lead to the characterization of "NMO spectrum disorders" (NMOSD) as a pathophysiologic spectrum that should be considered distinct from multiple sclerosis (120). Unlike MS (in which no particular antibody has been firmly linked to pathophysiology), a convergence of pathologic (121–125) and clinical (126–131) observations supports a pathophysiologic role of anti-AQP-4 antibodies in NMOSD [reviewed by Ref. (132, 133)]. While anti-AQP-4 antibodies are thought to be pathogenic in NMOSD, the observation that decreased NMO relapses seen following anti-CD20-mediated B-cell depletion do not correlate well with changes in anti-AQP4 antibody titers (134–136), indicates that the role of B cells in NMO may extend beyond antibody production. Such antibody-independent roles may include the capacity of B cells activate T cells and/or myeloid cells, as also implicated in MS. The observation that anti-AQP-4 antibodies are more readily detectable in serum rather than CSF of NMOSD patients has raised the question whether pathogenic antibodies are exclusively generated in the periphery and subsequently access the CNS, or whether plasmablasts and plasma cells that secrete such antibodies can be induced and fostered within the CNS. A recent study indicates that during NMO exacerbations, a substantial fraction of the intrathecal Ig proteome is generated by B cells of both peripheral and central origin (137). This suggests that in order for NMO therapies aiming to target the source of anti-AQP-4 antibodies to be most effective, they will need to access both the periphery and the CNS. Pathologically, NMO is characterized by an astrocytopathy with vasculocentric deposition of complement, vascular fibrosis, and eosinophilic infiltration, with associated white matter and gray matter injury. Meningeal immune cell collections with follicle-like features and cortical demyelination do not appear to be features of NMO pathology (138).

unclear if this is occurring exclusively in the periphery (lymph nodes) or also in the CNS itself (56, 117). Thus, identifying the particular B cell subsets that preferentially migrate into, and are then fostered within, the MS CNS, and elucidating how they may contribute to propagating local injury responses are of considerable interest for future studies.

Perspectives

The success of anti-CD20 therapies has made it clear that B cells contribute substantially to the initiation of MS relapses. Growing evidence suggests this largely reflects non-antibody-dependent proinflammatory roles of B cells in the periphery, where they can aberrantly activate disease-relevant T cells, which in turn traffic to the CNS and mediate relapses. The inflamed MS CNS appears to foster persistence of B cells and plasma cells and the same clonally expanded populations can be found within different CNS subcompartments (CSF, parenchyma, and meninges). There is an early appreciation that multiple distinct barriers separate the CNS from the periphery, including the BBB, meningeal, and choroidal interfaces. Elegant studies now underscore the bidirectional trafficking of B cells between the CNS and the periphery and reveal that maturation of expanded clones that populate the CNS of patients may be peripherally rather than centrally driven. Despite key advances, little is known about B cell contributions to the chronic non-relapsing CNS-compartmentalized inflammation that may underlie progressive tissue injury and worsening of disability in MS. A number of observations make such contributions (through both antibody-dependent and antibody-independent mechanisms) plausible and worthy of further study. Key observations reviewed here include the known persistence of B cells in the inflamed MS CNS of patients; the demonstration that CSF-derived B cell clones isolated from MS patients can bind CNS (including neurons and astrocytes) and cause complement-mediated injury; the now recognized abnormal proinflammatory response propensity of MS B cells; potential cross-talk between B cells and activated CNS glial cells; and the reported association between B cell-containing meningeal immune cell infiltrates and presence of the subpial cortical injury increasingly thought to underlie progressive decline of functions in patients with progressive MS. Future work should aim to address key remaining questions (**Box 1**) thereby shedding light on which functionally distinct B cell subsets are present in the different anatomical subcompartments in the CNS, which molecular mechanisms and barriers are involved in their trafficking into those sites, what their antigenic specificities are, how are they fostered in the local environment, how they interact with glial and neural cells and ultimately how they contribute to disease propagation in the MS CNS as compared to the case of NMO (**Box 2**). These insights will hopefully help guide novel therapeutic options that may prove as useful for limiting progressive disease biology as peripheral B cell depletion has been for limiting relapses.

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MINI REVIEW
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Meningeal Tertiary Lymphoid Tissues and Multiple Sclerosis: A Gathering Place for Diverse Types of Immune Cells during CNS Autoimmunity

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Collections of leukocytes in the meningeal space have been documented in Multiple Sclerosis (MS). These meningeal aggregates, which in the context of other autoimmune diseases have often been termed tertiary lymphoid tissues (TLT), have been associated with sub-pial cortical damage and disease progression. However, the key molecular and cellular signals required for their formation and maintenance remain unclear. Herein, we review TLT structures in other disease states in order to provide a framework for understanding these structures in the MS meninges. We then assess the evidence that the meningeal compartment serves as an important nexus for immune cells as well as a location for drainage of antigen into cervical lymph nodes. Extrapolating what is known about the molecular and cellular cues that initiate the formation of leukocyte aggregates in non-lymphoid tissues, we speculate on what signals lead to the formation and maintenance of meningeal TLT structures. Referring to the animal model of MS [experimental autoimmune encephalomyelitis (EAE)], we also explore what is known about these structures in supporting B cell and T cell responses during neuroinflammation. Last, we examine the evidence that connects these structures to ongoing neuropathology. Collectively, our review points to the meningeal compartment as an important player in neuroinflammatory processes. Moreover, we hypothesize that in order to gain insights into pro- and anti-inflammatory properties of lymphocytes in MS, one must understand the cellular scaffolds that support lymphocyte retention within the meninges, thus highlighting the importance of non-immune cells (stromal cells) in the neuroinflammatory process.

Keywords: meninges, multiple sclerosis, experimental autoimmune encephalomyelitis, tertiary lymphoid tissues, stromal cells

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OVERVIEW

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) resulting in demyelination and axonal loss with consequential clinical impairment. MS is most commonly diagnosed as relapsing-remitting MS (RRMS), which is thought to reflect the waxing and waning of underlying CNS-targeted immune responses. Most individuals with RRMS go on to develop a

progressive form of MS, termed secondary progressive MS. The relative absence of new gadolinium-enhancing lesions in the CNS of individuals with progressive MS has for a long-time supported the hypothesis that there is limited infiltration of peripheral immune cells into the CNS at this stage of disease (1). This concept has been recently challenged following the observation of meningeal leukocytic infiltrates (consisting of T cells, B cells, plasma cells, monocytes and macrophages) in both SPMS [in as many as 40% of cases; (2–4)] and Primary Progressive MS (5), correlating with proximal neuropathology (6). However, meningeal inflammation may also contribute to neuroinflammatory processes early on in MS, and recent MRI studies demonstrate leptomeningeal contrast enhancement in RRMS [~19% of cases; (7)].

Meningeal leukocytic aggregates have been referred to as tertiary lymphoid tissues (TLT), provoking the hypothesis that such aggregates support disease-relevant immune responses locally within the CNS. Although there is some discrepancy as to whether meningeal follicle-like structures recapitulate all of the features of TLT, for the purposes of this review, we will refer to them as TLT (8–10).

TERTIARY LYMPHOID TISSUES

Tertiary lymphoid tissues (TLT) are locally inducible leukocyte aggregates that form in chronically inflamed non-lymphoid tissues and share cellular and organizational similarities with secondary lymphoid organs (SLO). TLT arise within the target tissues of many autoimmune diseases and certain sites of chronic infection, including in the synovial membrane of the joints (rheumatoid arthritis), salivary glands (Sjögren's syndrome), thymus (Mysasthenia gravis and Grave disease), meninges (MS), the liver (hepatitis C viral infection), the lung (Influenza A viral infection), as well as at sites of chronic graft rejection, atherosclerosis, and cancer (8, 10). The majority of information we have on TLT structure and formation is from disease settings that do not involve the CNS. This may be due to the limitations in studying TLT in post-mortem tissue (brain tissue from autopsies tends to be obtained very late in the disease process when inflammation may be less pronounced), the relatively smaller size of meningeal TLT, and variability in histology and dissection protocols for assessing meningeal TLT. As such, this section will focus on what we have learned about TLT in other disease settings that may be more amenable to study, and we will then apply these findings to CNS autoimmunity in a subsequent section.

TLT Structure

Lymphoid architecture is orchestrated by specialized stromal cell subsets. Follicular dendritic cells (FDC; $CD45^{-}CD31^{-}Pdpn^{\pm}$) and recently defined *Cxcl12*-expressing reticular cells (CRC; $CD45^{-}CD31^{-}Pdpn^{\pm}$) (11) secrete B cell chemoattractants CXCL13 and CXCL12, respectively. FDC further upregulate molecules involved in trapping and presenting antigen (CD35, FDC-M1, and FDC-M2) to support germinal center responses. The T cell zone is supported by fibroblastic reticular cells (FRC; $CD45^{-}Pdpn^{+}CD31^{-}$) that secrete T cell chemoattractants (CCL19, CCL21) as well as T and B cell survival factors (IL-7, BAFF) and form long reticular channels supporting the passage of antigen through the lymphoid organ (12). TLT encompass a spectrum of lymphoid tissue-like organization depending on the target tissue. TLT are primarily described as B cell-rich infiltrates, with a varying degree of T cell infiltration and sometimes segregation into distinct B and T cell compartments resembling SLO architecture. Although FDC markers have been detected within TLT in various chronic inflammatory conditions, not all TLT demonstrate germinal centers or reticular conduits reminiscent of FRC (10, 13, 14). Mature TLT can also contain high endothelial venules (HEV) and lymphatic vessels, suggesting an avenue for entry of naive lymphocytes (via the HEV) and egress of antigen and activated or memory lymphocytes via lymphatic vessels (15).

TLT Formation

Lymphorganogenesis requires the well-defined interaction of embryonic/neonatal lymphoid tissue inducer cells (LTi; $CD45^{+}CD4^{+}CD3^{-}$) with stromal lymphoid tissue organizer cells (LTo) to promote LTo maturation into specialized lymphoid stromal cells that in turn form an immune-competent niche [reviewed by Ref. (12, 16–20)]. In the context of lymphoneogenesis in the adult host, distinct cell types may substitute as TLT inducer and organizer cells. LTi equivalent cell types implicated in TLT formation include: innate lymphoid cells (21–23), T cells (24–27), NKT cells (28), as well a myeloid cells (28, 29). Although the exact combination of molecular cues may differ, a unifying feature of TLT-inducing leukocytes is their production of cytokines, especially IL-17 [reviewed by Ref. (30)] and/or IL-22 (21, 22, 27), and their ability to engage receptors of the Tumor Necrosis Factor (TNF) superfamily (LTβR, TNFR) by virtue of their expression of cognate ligands LTαβ and TNFα.

The origin and phenotype of TLT organizer cells remains more elusive. Seminal studies have demonstrated that stromal precursor cells reside quiescently throughout the periphery, and mature to acquire phenotypic and functional capacities consistent with lymphoid tissue stromal cells in response to inflammation (29, 31). A population of $CD45^{-}CD31^{-}Pdpn^{-}$ cells, which transcriptionally most closely resemble FRCs (32), has also been demonstrated to differentiate into *de novo* FRCs in the inflamed LN (33, 34), illustrating that even within adult SLOs, mesenchymal precursors can recapitulate an LTo-like function. Nevertheless, further studies are needed in order to elucidate to what extent mesenchymal precursor cells in the adult host resemble embryonic LTos and how such LTo-like cells differentiate in order to support emerging TLT.

Several markers have been useful for assessing the phenotype of tissue-resident stromal cells, including: podoplanin (Pdpn or gp38); the endothelial cell marker, CD31; EPCAM, a marker of epithelial cells; as well as the expression of homeostatic chemokines. For example, in several models of TLT formation, peripheral $Pdpn^{+}$ stromal cells express CXCL13 (26, 27, 29, 31), while in a model of atherosclerosis, vascular smooth muscle cells were found to express both CXCL13 and CCL21 within aortic TLT (35). Both $Pdpn^{+}CD31^{-}$ stromal cells and $EPCAM^{+}$ epithelial cells express CXCL12 within TLT in the lungs (26) and salivary glands (27), respectively. Nevertheless, without a consistent panel of mesenchymal/lymphoid stromal cell markers applied to different models of TLT formation, it is difficult to

interpret whether different inflammatory insults instigate distinct maturation protocols from ubiquitous precursor cells, or whether tissue-specific differences exist.

In summary, our current understanding is that precursors of lymphoid-like stromal cells reside quiescently throughout peripheral non-lymphoid tissues and are poised to mature into lymphoid stromal cells at sites of persistent inflammation. In response to local inflammatory cues, tissue-resident stromal cells acquire phenotypic and functional capacities consistent with lymphoid tissue stromal cells.

STRUCTURE AND FUNCTION OF MENINGEAL TLT DURING CNS AUTOIMMUNITY

Meningeal aggregates in the MS CNS are often referred to as TLT; however, as is true for ectopic lymphoid tissues in general, this term captures a range of lymphoid tissue-like organization. Animal studies characterizing meningeal inflammation in EAE demonstrate TLT formation in mice with different genetic backgrounds and disease-induction protocols (24, 36–39). These meningeal infiltrates often resemble mature TLT, with the presence of lymphoid-like stromal cells, elaboration of an extra-cellular matrix (ECM) network, and expression of cytokines and homeostatic chemokines. Below, we will review our current understanding about the structure and capacity for the meninges to support TLT formation, as well as the clinical and neuropathological correlates of meningeal TLT in both MS and EAE (see also **Table 1**).

Anatomical Structure of the Meninges

The meninges are a series of membranes that envelope the brain and spinal cord, serving as a canal for circulating cerebrospinal fluid (CSF). The outermost membrane is the dura, which cocoons the CNS and is attached to the skull and spinal column. The leptomeninges that envelope the entire CNS consist of the arachnoid and pia mater and are separated by the subarachnoid space. Large conducting blood vessels transecting the leptomeninges are embedded within the pia mater**,** which is lined by the glial limitans, a barrier comprising astrocytic end-foot processes (55). Cells of the pia mater continue to line intracerebral arteries but gradually become less dense as the arteries penetrate the CNS parenchyma (56–58). The meningeal space is depicted in **Figure 1**. Cells that reside in the meningeal compartment include fibroblasts and peri-endothelial cells (myofibroblasts, pericytes, and vascular smooth muscle cells), as well as CNS-resident macrophages and dendritic cells. A recent study by Louveau and colleagues has revealed the presence of lymphatic vessels within the dural sinuses, implying there is direct communication between the meningeal environment and the draining cervical LNs (cLN) (59).

The Meninges – A Portal of Leukocyte Entry and Accumulation in the Inflamed CNS

The blood endothelium transecting the subarachnoid space (the blood–CSF barrier) represents an important route of leukocyte entry into the meninges. In the steady state, the subarachnoid space represents an avenue for immune-surveying lymphocytes to scan the CNS (60). Studies examining the kinetics of meningeal infiltration in EAE report an influx of immune cells prior to clinical onset (39). In addition, using specialized fluorescent reporter mice and two-photon live imaging, myelin-specific T cells have been shown to first cross the blood–CSF barrier in the subarachnoid space, where antigen-specific re-priming must occur in order to gain access to the parenchyma and instigate clinical symptoms of EAE (47). With respect to MS, biopsies from early stage MS patients identified a subset of patients with evidence of cortical demyelinating lesions associated with meningeal inflammation (53). Moreover, a recent study has estimated that almost 20% of individuals with RRMS demonstrate meningeal contrast enhancement (7).

EAE studies, post-mortem histological analyses, and CSF samplings all demonstrate that accumulation of proliferating,

TABLE 1 | Association of immune cell phenotypes and pathology with TLT.

Feature	Evidence	Reference
B cell responses – EAE	B cell-rich meningeal aggregates during EAE	(36) (40) (24)
	FDC-M1- and CD35-immunoreactive cells (FDC-like cells) and CXCL13 transcripts within meningeal TLT	(36)
B cell responses – MS	B cell- and plasma cell-rich meningeal TLT	(2) (41)
	CD35- and CXCL13-immunoreactive cells (FDC-like cells) within meningeal TLT	(2)
	Activated B cells (clonal expansion, somatic hypermutation, Ig class switching) within meningeal aggregates	(42) (43) (44) (45)
T cell responses – EAE	T cells infiltrate the meninges and are reactivated in the subarachnoid compartment	(46) (47) (48) (49) (50)
	T cell epitope spreading concurrent with presence of meningeal TLT	(37)
	Th17 cells contribute to meningeal TLT formation	(51) (24) (52)
T cell responses – MS	T cell accumulation within meningeal TLT	(4) (53) (5)
Neuropathology – MS	Cortical demyelination	(54) (3) (6) (53) (4) (5)
	Glial limitans damage, increased microglial activation	(3) (6) (4) (41)
	Cortical astrocyte and oligodendrocyte loss	(6)
	Neuronal loss	(6) (5)
Clinical correlates	Earlier age of clinical onset, faster time of disease progression, earlier age at death	(3) (4)

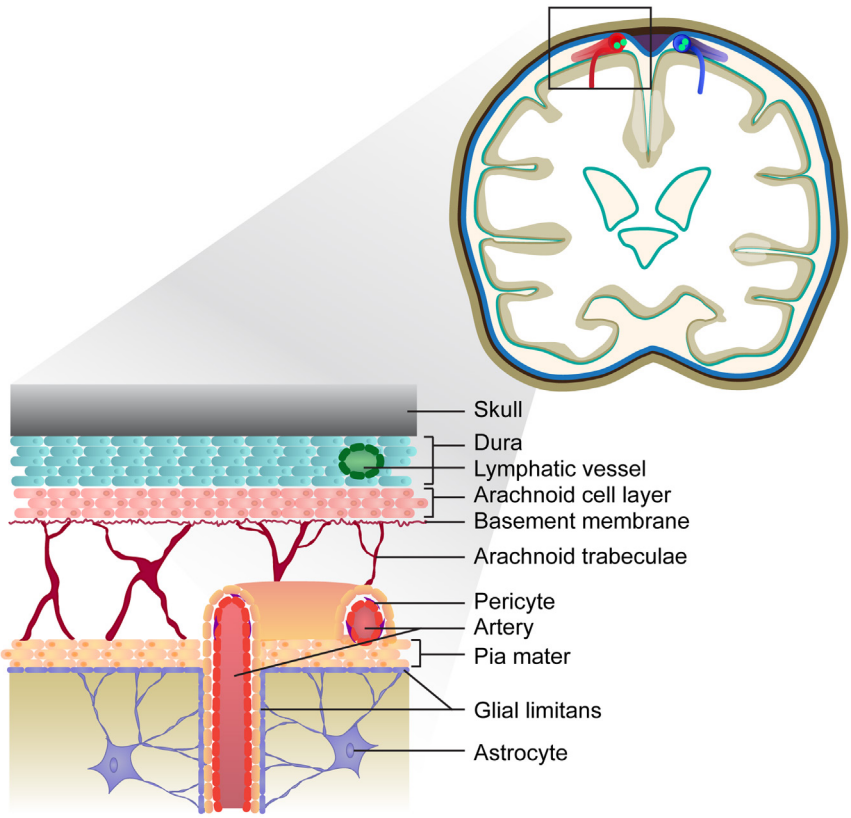

FIGURE 1 | Depiction of the meninges in the healthy brain. The meninges consist of three layers: the dura is the outermost layer, followed by the arachnoid and pia, which form the leptomeninges. Lymphatic vessels embedded within the dura drain the sagittal sinus (not depicted). The vasculature transecting the meninges is embedded within the pial cell layer, and represents a route of leukocyte entry into the CNS.

antigen-experienced T cells (48, 61, 62), and B cells (3, 4, 42–45) can occur within the meningeal compartment itself during CNS autoimmunity. In terms of T cell responses, epitope spreading of myelin-reactive T cells is suggested to occur within the meninges (37). While antigen-presenting phagocytic cells have been shown to productively interact with myelin-specific T cells in the subarachnoid space (46, 48–50), recent studies also demonstrate that meningeal stromal cells may be important for propagating encephalitogenic T cell responses within the CNS (52). With respect to meningeal B cell responses, class-switched memory B cells and plasmablasts/plasma cells have long been detected in the CSF of individuals with MS, and contribute to intrathecal production of antibodies, a hallmark of MS (43, 63). While antigen-experienced B cells populate the meningeal compartment, clonally related B cells are also located in parenchymal lesions, the normal-appearing white matter (42), and in the periphery (44, 45, 64), making it unclear where B cells are first primed. The presence of myelin antigens in the cLN of individuals with MS, but not healthy controls (65), and the discovery of lymphatics draining from the meningeal space to cLN (59) suggest that antigen-dependent B cell responses can be initiated in the cLN. Recently, deep sequencing analyses of the B cell receptor variable heavy chain (VH) between matched CNS and peripheral samples demonstrated that upwards of 90% of founder B cell clones were localized within cervical lymph node tissues (44). However, it remains possible that the spatial–temporal distribution of founder clones differs at disease onset. In summary, the blood–CSF barrier is an important portal of entry for leukocytes into the CNS. The subarachnoid space represents an important site of accumulation for activated lymphocytes, as well as dendritic cells (46), neutrophils, and mast cells (39, 66) within the inflamed CNS.

How Do TLT Form within the Meninges?

The presence of immune cells in the meninges and CSF of individuals with MS does not in and of itself confirm that the meninges constitute an immune-competent niche. To endow "immune competence," such an environment would need to be populated by stromal support cells that secrete lymphocyte chemoattractants (such as CXCL13, CCL19, CCL21) and possibly also survival/differentiation factors (cytokines). Indeed, meningeal stromal cells have the capacity to secrete mediators such as TNFα, iNOS, IL-6, TGF-β, and IL-23 under inflammatory conditions (52, 67). Pro-inflammatory cytokines themselves can induce lymphocytic accumulation within the meningeal compartment, as demonstrated following injection of TNFα and IFNγ directly into the subarachnoid space (68) or intra-cortically (69) in rodents immunized sub-clinically with MOG. While these studies did

not examine whether cytokine-induced TLT-like aggregates are supported by an underlying stromal cell and ECM network, TLT surrounded by a reticular network have been observed upon adoptive transfer of IL-17 producing encephalitogenic T cells in mice (24).

Expression of both T cell and B cell chemoattractants and lymphoid-like FRCs and FDCs has been demonstrated in the inflamed CNS. In both EAE and MS, CXCL13 expression and FDC-like cells ($CD35^+$ and FDC-$M1^+$) are reported within B cell-rich meningeal aggregates (2, 36, 40), while CCL19 and CCL21 transcripts have been detected within parenchymal and sub-meningeal lesions in the brains of SWR/J × SJL/J F1 mice with EAE (38). In the context of MS, over three decades ago, Prineas and colleagues described the presence of reticular-like cells embedded within lymphoid-like structures and lymphatic capillaries within old plaques in the MS CNS (70). While the phenotype of these reticular cells has not since been explored in the MS CNS, the elaboration and maturation of a reticular lymphoid-like stromal cell network were recently described in the brain meninges of mice with Th17 adoptive transfer EAE (52). FRC-like stromal cells were found to secrete pro-inflammatory cytokines, homeostatic chemokines (CXCL13, CCL21), as well as CXCL1 and BAFF, forming an immune-competent microenvironment. IL-17- and IL-22-derived signals were shown to promote the physical elaboration of the reticular network while acquisition of lymphoid-like stromal cell properties was in part lymphotoxin dependent, suggesting that multiple pathways culminate in the elaboration of an immune-regulatory stromal cell scaffold in the inflamed CNS. In summary, the meninges are a CNS environment poised to establish an inflammatory niche that is capable of supporting immune-responses within the CNS.

Meningeal Inflammation and Cortical Pathology in MS

Cortical lesions characterized by demyelination, axonal atrophy, and microglial activation in the sub-pial mater have been shown to underly meningeal lymphocytic infiltrates in the progressive MS CNS (3, 5, 54), although another study failed to see a correlation between meningeal inflammation and sub-pial demyelination (71). The presence of meningeal TLT in SPMS correlates with accelerated clinical disease (earlier age of clinical onset, faster time of disease progression and earlier age of death) compared to SPMS cases without meningeal TLT (4). It is postulated that soluble factors emanating from meningeal lymphocytic aggregates degrade the glial limitans, promoting a gradient of demyelination and neuronal injury (3, 6). While B cells, plasma cells (IgA^+, IgG^+, IgM^+), $CD4^+$ and $CD8^+$ T cells, monocytes/macrophages infiltrate the subarachnoid space, a recent study revealed that only the accumulation of plasma cells and macrophages was significantly elevated in meningeal TLT compared to region-matched controls, and the accumulation of these particular cell types was associated with underlying cerebellar gray matter demyelination (41). In addition, the accumulation of meningeal $CD3^+$ T cells correlates with axonal loss and microglial activation in the underlying normal-appearing white matter in the spinal cord in progressive MS (72). Taken together, these observations may reflect distinct susceptibility of different regions of the CNS to immune cell-mediated injury.

CONCLUSIONS

The presence and inducible formation of an immune-competent niche in the meninges suggests that these structures may support disease-relevant immune responses in the CNS. While TLT-associated immune responses are proposed to contribute to ongoing neuropathology and disease exacerbation, these structures likely evolve to support cell types regulating the balance of pro-inflammatory and anti-inflammatory responses in the meninges. Indeed, the accumulation of regulatory T cells within chronic aortic TLT is associated with clinical benefit in a rodent model of atherosclerosis (35). On the other hand, the presence of TLT in cases of enteropathic infection or cancer is associated with clinical benefit (21, 22, 73, 74). Therefore, one must consider that the cellular constituents of meningeal TLT may change over time, implicating altered neuropathological and clinical consequences within the inflamed CNS. We propose that an understanding of the cellular scaffolds that support lymphocyte retention within the meninges (i.e., specialized non-immune stromal cells) will lead to a better understanding of the meningeal compartment in the context of MS/EAE.

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REVIEW
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Involvement of CD8+ T Cells in Multiple Sclerosis

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Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system characterized by focal demyelination patches associated with inflammatory infiltrates containing T lymphocytes. For decades, CD4+ T cells have been recognized as playing a major role in the disease, especially in animal models, which has led to the development of several therapies. However, interest has recently developed in the involvement of CD8+ T cells in MS following the analysis of infiltrating T cells in human brain lesions. A broad range of evidence now suggests that the pathological role of this T cell subset in MS may have been underestimated. In this review, we summarize the literature implicating CD8+ T cells in the pathophysiology of MS. We present data from studies in the fields of genetics, anatomopathology and immunology, mainly in humans but also in animal models of MS. Altogether, this strongly suggests that CD8+ T cells may be major effectors in the disease process, and that the development of treatments specifically targeting this subset would be germane.

Keywords: multiple sclerosis, autoimmunity, CD8+ T cells

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INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), resulting in disability. The clinical manifestations are very variable, and include motor, sensory, visual, and cognitive symptoms, none of them being disease specific (1). The disease is thought to be a result of both genetic and environmental factors, including infectious agents, vitamin D deficiency, obesity, and smoking (2). Neuropathological studies show disseminated patches of demyelination among the brain and the spinal cord, resulting in altered nerve conduction. Axonal loss can also be observed. Demyelinated patches are characterized by immune cell infiltration, which is absent in normal brain tissue. The infiltrate is mainly composed of macrophages, and, to a lesser extent, T and B lymphocytes (1, 3, 4).

In addition to the immune cell infiltrates, a wide range of evidence points to the pivotal role of the immune system in the development of the disease. Indeed, the genetic variants conferring a higher susceptibility to MS are associated to immune mechanisms, the animal models used for disease characterization and comprehension are autoimmune ones, and current treatments

Abbreviations: BBB, blood–brain barrier; CNS, central nervous system; CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalomyelitis; EBV, Epstein–Barr virus; HLA, human leukocyte antigen; HV, healthy volunteers; MAIT, mucosal-associated invariant T; MBP, myelin basic protein; MHC, major histocompatibility complex; MRI, magnetic resonance imaging; MS, multiple sclerosis; NAWM, normal-appearing white matter; TCR, T-cell receptor.

regulate or modulate the immune system (1). For decades, MS has been considered as being driven by CD4+ T cells, especially in animal models of the disease (1). However, treatment with monoclonal anti-CD4 antibody in phase II trials failed to reduce MS activity [measured by magnetic resonance imaging (MRI)] (5). In recent years, numerous other immune populations have been shown to be important in MS development. Among these, CD8+ T cells have appeared as potential major effectors within the CNS, especially in studies using human samples. In this review, we will focus on the various results that seem pivotal in understanding the involvement of CD8+ T cells in the pathophysiology of MS. The potential regulatory role of CD8+ T cells in the disease will be described elsewhere in this issue [for other reviews, see Ref. (6, 7)].

MHC CLASS I GENES' IMPACT ON MS RISK

Numerous alleles associated with immune response have been found to be linked with increased MS risk (8–12). The strongest association, with an odds ratio of 3, was seen for human leukocyte antigen (HLA)-DRB1*15:01 in European and United States populations, which identifies CD4+ T cells as potent effectors in the disease (13). Major histocompatibility complex (MHC) class I alleles are also associated with MS, though to a lesser extent. HLA-A3 and B7 alleles were the first to be described as associated with a higher risk of developing the disease (14, 15). More recently, HLA A*0301 was associated with a higher risk (2×) and HLA A*0201 with a protective effect (50%) (16, 17). These associations were confirmed by Friese and colleagues in "humanized" transgenic mice for HLA*0301 and/or HLA-A*0201. These mice either developed (HLA*0301) or were protected from (HLA*0201) the disease after proteolipid protein (PLP) injection (18). In addition, these alleles are known to work in synergy with MHC class-II alleles, such as DRB1*1501, resulting in an increased risk when both are present (16). These data strongly suggest that some CD8+ T cells may have a beneficial or pathogenic effect, depending on the genetic background (**Table 1**). On top of this genetic evidence, the presence of CD8+ T cells in MS lesions, as well as their cytotoxic profile, evinces their involvement in the disease.

TABLE 1 | MHC-I alleles and their additive effect on MS risk with MHC-II alleles.

MHC allele	Odds ratio
HLA-DRB1*1501	2.9–3.6
HLA-A*0201	0.52–0.7
HLA-DRB1*1501 + HLA-A*0201	1.5
HLA-A*0301	1.9–2.1
HLA-DRB1*1501 + HLA-A*0301	3.7–5.2
HLA-A*0201 + HLA-A*0301	1
HLA-B*0702	1.6–2.2
HLA-DRB1*1501 + HLA-B*0702	2.9

MHC alleles described as implicated in MS risk are listed with their corresponding risk factor expressed by odds ratio. These data are from Fogdell-Hahn et al. and Harbo et al. (16, 17).

CD8+ T CELLS ARE PRESENT AND HAVE A PATHOGENIC PROFILE IN THE MS CNS

CD8+ T Cell Infiltration in CNS Lesions

One of the major indicators pointing toward an implication of CD8+ T cells in the pathophysiology of MS is the presence of these cells, in a greater number than CD4+ T cells, in the brain lesions of MS patients. The fact that CD8+ T cells outnumber CD4+ T cells in MS lesions was first observed in the 80s, in particular in the parenchyma, and was regardless of differing clinical parameters, such as disease duration, disease evolution, and therapy (19). In 1986, Hauser et al. studied 16 cases of progressive MS, and observed up to 50 times more CD8+ T cells in both the parenchyma and in perivascular cuffs of active lesions, with no case of more CD4+ than CD8+ T cells. CD8+ T cells also predominated in normal-appearing white matter (NAWM) (20). Recently, our study of 22 lesions in three MS patients found the same, CD8+ T cells being predominant regardless of the lesion type studied (21). Other studies have reached the same conclusion by single cell analysis in different MS patients (22–24). Although not all the studies concur with the above (25), others put forward the hypothesis that CD8+ T cells might be more prevalent in the parenchyma while CD4+ T cells would stay in the perivascular areas (26). Recently, in tissue block section of MS patients, CD8+ T cells were described as being often present in cortical plaques (54 of 70 cortical plaques analyzed). This type of plaque has been found to be associated with disease progression and cognitive impairment in the early stages of MS (27).

MHC-I Expression and CNS Damage

MHC-I expression and presentation is necessary for CD8+ T cells to carry out their cytotoxic functions. In 2004, a study including 30 MS patients and 21 controls quantified the expression of MHC-I on various cell subtypes within the CNS by immunohistochemistry and fluorescence methods (28). While constitutive expression of MHC-I on macrophages/microglia and endothelial cells was observed, MHC-I expression was gradually upregulated on astrocytes, oligodendrocytes, neurons, and axons depending on the disease type (inactive, chronic active, and active MS) and lesion activity (inactive, periplaque white matter, and active), making these cells potential targets for CD8+ T cells in the context of the disease. Consistent with this, CD8+ T cells have been shown to be able to mediate axonal transection *in vitro* (29). In this study, murine neurons induced to express MHC-I were pulsed with a dominant peptide of the lymphochoriomeningitis virus envelope glycoprotein (GP33). Five to 30 min after culture with antigen-specific cytotoxic CD8+ T cells, neurite breakage appeared in contact zones between CD8+ T cells and neurites. Confocal live imaging gave a clear image of this process. Axonal transection has also been suggested in MS (30). Indeed, axonal injury, in 88 brain biopsy samples from 42 patients, correlated with the number of CD8+ T cells, but not CD3+ T cells, found in the lesions (31). Variable proportions of lesion-infiltrating CD8+ T cells express granzyme B [**Figure 1**, personal results from Ref. (21)] and interferon γ (IFNγ), further evincing the ability of these cells to damage the CNS (21, 25, 32). In conclusion, CD8+

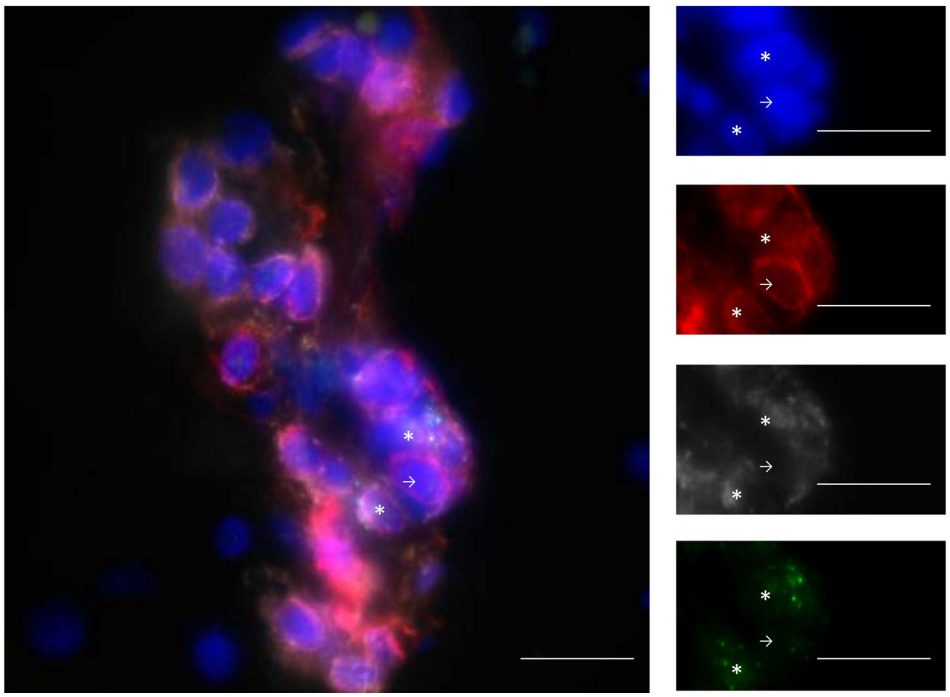

FIGURE 1 | Infiltrating T cells are mainly CD8+ T cells and express GZM-B. Example of staining with DAPI (blue), CD3 (red), CD8 (gray), and GZM-B (green). The line in the pictures indicates 20 μm. Stars show $CD3^+CD8^+GZM\text{-}B^+$ and arrows show $CD3^+CD8^-GZM\text{-}B^-$ cells. GZM-B: granzyme-B. From personal data.

T cells seem more likely than $CD4^+$ T cells to mediate CNS damage, in particular through their cytotoxic and proinflammatory properties.

Pathogenic $CD8^+$ T Cells in the CSF

Deciphering the mechanisms involved in MS development is made difficult by the limited access to the CNS compartment. As such, a lot of studies focus on the cerebrospinal fluid (CSF) as a surrogate compartment for understanding the T cell processes occurring *in situ*. In 2007, an enrichment of effector memory $CD8^+$ T cells in the CSF of 52 relapsing–remitting MS patients was observed at the beginning of the disease (33). This, together with an enrichment of granzyme B-expressing $CD8^+$ T cells, has been confirmed in 17 other relapsing-remitting patients (25). Interestingly, the effector memory $CD8^+$ T cells had increased *in vitro* migration through a model of the blood–brain barrier, especially those producing granzyme B, perforin, IFNγ, and interleukin 17 (IL-17). This was further confirmed in a mouse experimental autoimmune encephalomyelitis (EAE) model (25). Another study found that granzyme A and B levels were higher in the CSF of patients in flare up, compared to those in clinical remission and control patients (34). Altogether, these results suggest a specific enrichment of effector memory $CD8^+$ T cells in the CNS compartment in MS and place them as disease effectors.

$CD8^+$ T Cell Migration into the Brain

Studying the mechanisms leading to $CD8^+$ T cell transmigration into the CNS further highlights their involvement in the disease process. Blockade of α4 integrin in EAE mice immunized with myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ yields a decreased number of infiltrating $CD8^+$ T cells, together with a reduced EAE score. However, a similar effect has been described for $CD4^+$ T cells (25). More recently, melanoma cell adhesion molecule (MCAM), expressed by a subset of human effector $CD8^+$ T cells, was reported to be upregulated during MS relapse compared to controls (35). Interestingly, MCAM blockade prevents the transmigration of human $CD8^+$ T cells across a blood–brain barrier (BBB) model and decreases the EAE score in active, transfer and spontaneous models (36–38). As MCAM binds itself and laminin 411 (37), which are both expressed by endothelial cells, the mode of action of MCAM blockade is not yet known (35). P-glycoprotein (also known as multidrug resistance protein 1), a transporter involved in drug efflux (39) and in cytokine/chemokine secretion (40), has also been shown to be important for the trafficking of $CD8^+$ T cells into the brain during the disease. Indeed, Mdr1a/b KO mice show significantly reduced EAE (40). In another study, P-glycoprotein silencing led to decreased CD8 infiltration into the brain, with no effect on $CD4^+$ T cells (41). P-glycoprotein control of endothelial C–C chemokine ligand 2 (CCL2) secretion was responsible for this result. Indeed, EAE mice lacking this protein or CCL2 show significantly reduced $CD8^+$ migration into the brain. More significantly, CCL2 transcript has also been found to be elevated in six MS lesions compared to six controls (41).

In conclusion, various studies on brain, spinal cord, and CSF, as well as on the mechanisms allowing T cell entry into the

brain highlight CD8+ T cells' potential role in the development of MS.

CD8+ T CELL REPERTOIRE AND REACTIVITY IN MS

A number of studies have focused on the T cell pools that arise with MS in order to understand their role in its pathophysiology. Different CNS and non-CNS antigens have been used to search for autoreactive T cells (3, 42), but to date the triggering antigen(s) is unknown. The search for a specific antigen is made more difficult because of the mechanisms of molecular mimicry, epitope spreading, bystander activation, and/or dual T cell receptor (TCR) (43–48). Numerous studies have analyzed the T cell repertoire, allowing the identification of reactive T cells without the need to know the recognized antigen. Indeed, after antigen recognition, T cells undergo clonal proliferation, and this expanded T cell population can be identified within the total T cell pool.

CD8+ T Cell Repertoire in Lesions and in CSF

Two different groups, including our own, have studied the TCR repertoire of lesion-infiltrating T cells, either by single cell analysis (22, 23) or by whole TCR analysis using high-throughput sequencing, in comparison with sorted populations from the blood (21). These studies show that infiltrating CD8+ T cells are oligoclonal, i.e., only clones bearing particular TCRs specific to each patient are represented within the lesion, which is less the case for CD4+ T cells. These oligoclonal CD8+ T cells are also found in different locations in the brains of the same patients. In addition, some of these clones harbored silent mutations (23). These data strongly suggest antigen-driven selection or activation processes, and identify these infiltrating CD8+ T cells as disease effectors.

CD8+ T Cell Repertoire Alteration in CSF and Blood

Different studies have observed that the blood of MS patients exhibits more clonal expansions than that of controls (49–53). In 35 patients, our group showed that the repertoire was more skewed in the CD8 than in the CD4 compartment, further suggesting the involvement of CD8+ T cells (50). The repertoire was even more skewed in the CSF than the blood of MS patients, especially in CD8+ T cells which interestingly expressed memory T cell markers (54–56). Of note, clonal expansions were correlated to the clinical activity of the disease. We observed more blood expansions in MS patients with an active form of the disease, assessed by MRI (57). Muraro et al. published a case study that extensively studied the TCR Vβ repertoire in the CSF of one MS patient, and described more expansions in that compartment during relapse than during remission (58). Altogether, these data strongly suggest an implication of oligoclonal T cells, especially CD8+ T cells, in the pathophysiology of MS.

However, whether expanded T cells in blood and CSF correlates with T cell expansion in the CNS, which is likely to participate in disease development, was unknown until recently. Indeed, only one study had suggested that some expanded CNS CD8+ T cell clones could persist in the blood and in the CSF for several years, seen in two MS patients (24). Very recently, we confirmed and extended this result by comparing the TCR repertoires in the blood, CSF, and CNS (22 lesions with various locations and activities) of three MS patients (21). Using CDR3 spectratyping and high-throughput sequencing, we showed for the first time that the CSF repertoire mirrors that of the CNS, which is, to a lesser extent, also similar to that of blood CD8+ T cells. This further evinces the involvement of CSF and blood expanded CD8+ T cells in MS and further identifies CSF expanded T cells as good surrogates for infiltrating T cells.

CD8+ T Cell Autoreactivity

A number of studies have been looking at autoreactive T cells in MS, using different methods and antigens, but the results are hardly comparable between the studies and no consensus has been found as to the presence of CNS-specific CD4+ or CD8+ T cells in MS patients [reviewed in Ref. (42)]. In 2004, Crawford et al. adapted a flow cytometry approach to analyze antigen-specific CD4+ and CD8+ T cell proliferative response in MS patients. They showed that relapsing–remitting MS patients have a higher proportion of CD8+ T cell responses against CNS peptides compared to healthy volunteers (HV) or primary progressive MS patients and that this is not the case for CD4+ T cells (59). In another study, CD8+ T cells specific to apoptotic epitopes have been shown to be overrepresented in MS patients (in a cohort of 26 compared to 27 HV), and to be able to produce IFNγ and/or IL-17 when stimulated with apoptotic epitopes (60). Interestingly, these cells have been found also in the CSF and correlate with the level of disease disability, which strongly suggests their involvement in the immunopathology (60). Finally, Zang et al. tested the proliferation of CD8+ T cells in contact with irradiated PBMC incubated with degenerated myelin basic proteins (MBP), showing that autoreactive CD8+ T cells recognizing MBP proteins were increased in the blood (15 MS patients compared to 15 HV) (61). Recently, high-throughput sequencing on paired blood and CSF samples of MS patients and control gave further evidence of the implication of a particular subset of CD8+ T cells in MS (62), a specific enrichment of Epstein–Barr virus (EBV)-specific CD8+ T cells being observed in the CSF of MS patients.

These results evince the existence of a pool of CD8+ T cells able to damage the CNS, however the triggering events as well as the antigens recognized remain unclear, necessitating the development of other methods in order to isolate and study the relevant T cells.

ANIMAL MODELS IDENTIFY CD8+ T CELLS AS POTENT EFFECTORS IN THE DISEASE

Prior to 2001, most models focused on the involvement of CD4+ T cells in the development of EAE. Two groups, developed, in parallel, EAE models based on specific CD8+ T cell adoptive transfer. First, in mice C57BL/6, Sun et al. observed that the adoptive

transfer of $pMOG_{35–55}$ $CD8^+$ T cells led to the development of an EAE that was both longer and more severe than the disease actively induced by the injection of the $MOG_{35–55}$ peptide (63). EAE did not develop in mice lacking β2 microglobulin, further supporting the involvement of $CD8^+$ T cells in the development of the disease (63). These data were confirmed in 2005, and the authors further identified the $pMOG_{37–46}$ peptide as the minimal peptide recognized by the $pMOG_{35–55}$-specific $CD8^+$ T cells that both led to IFNγ production by these cells and to the development of severe EAE (64). In another 2001 study, Huseby et al. showed that a rapid and severe CNS-autoimmune disease could be induced in C3H mice after adoptive transfer of $CD8^+$ T cells lines reacting against the $MBP_{79–87}$ peptide (65). The severity of the disease was reduced when coinjecting anti-IFNγ with the CD8 MBP-specific cell lines, supporting a pivotal role for this cytokine in the development of the disease. Interestingly, the clinical symptoms, as well as the CNS lesion types and distributions observed, were different from those induced after $CD4^+$ T cell transfer, better reflecting the clinical features of the human disease (65). The authors then proposed that the different MS clinical manifestations could be linked to the activation of either $CD4^+$ or $CD8^+$ T cells. Since then, $CD8^+$ specific models have mainly been developed by transfer of T cells bearing TCR recognizing transgenic epitopes expressed on oligodendrocytes (66). In 2008, Saxena et al. developed a double knock-in transgenic mouse in which the influenza hemagglutinin (HA) expression was restricted to oligodendrocytes (67). The injection of *in vitro* activated $CD8^+$ T cells bearing a TCR specific for HA led to CNS inflammation and demyelination. Of note, the activated $CD8^+$ T cells produced granzyme B and IFNγ and exhibited cytotoxicity against cells loaded with HA *in vitro*. The authors were able to track these cells in the lesions, in close proximity to oligodendrocytes and in association with microglia activation. In another study, Na et al. showed that double transgenic mice with ovalbumin (OVA) expression in oligodendrocytes and OVA-specific TCR $CD8^+$ T cells (OT-I) – but not OVA-specific TCR $CD4^+$ T cells (OT-II) – develop a spontaneous EAE with demylinated and infiltrated lesions (68). The disease developed during the first 10–12 days of life, when $CD8^+$ T cells still have access to the CNS, and was amplified by IFNγ. Of note, blocking the recognition of the OT-I $CD8^+$ T cells with an antibody specific for the OVA-peptide/MHC-I complex prevents disease development (69). Interestingly, most of these models develop either clinical manifestations and/or infiltration characteristics that have a greater resemblance to MS symptoms and infiltrates than CD4-mediated EAE models (65, 67, 68).

Another model has been used to study the mechanisms by which $CD8^+$ T cell infiltrate and damage the CNS. In Theiler's murine encephalomyelitis virus (TMEV)-infected mice, the viral model of MS, $CD8^+$ T cells secreting perforin were shown to be involved in BBB disruption and astrocyte activation (70). $CD8^+$ T cells have also been shown to be able to enter the CNS in a naïve CL4 mice model without peripheral activation ($CD8^+$ T cells specific for HA). Even if they remained inactivated *in situ*, they were able to proliferate when HA was injected intracerebrally. Interestingly, blocking MHC-I led to the reduction by 76% of the trafficking (71). Recently, Sobottka et al. further described mechanisms of presentation into the CNS. Using living brain slices preincubated with IFNγ (to mimic CNS inflammation) and OVA, they showed that OT-I $CD8^+$ T cells (OVA-specific) were able to mediate axonal damage. Moreover, they obtained the same results using transgenic oligodendrocytes-expressing OVA in their living brain slices, making them potential target cells for $CD8^+$ T cell pathogenicity (72).

Animal models depending on $CD8^+$ T cells are, thus, relatively recent, and their study may shed new light on the mechanisms involving these cells in disease development (**Table 2**).

IL-17-PRODUCING $CD8^+$ T CELLS AS POTENT EFFECTORS IN MS

Similarly to $CD4^+$ T cells, the implication of IL-17-producing $CD8^+$ T cells in MS has been recently suggested. The first study suggesting this was performed on 18 frozen CNS samples from 14 MS patients (26). Seventy to 80% of the infiltrating T cells, both $CD4^+$ and $CD8^+$ T cells, expressed IL-17 in active and chronic active lesions, shown through double immunofluorescent staining. This percentage was dramatically lower, at 20%, in chronic inactive lesions and NAWM, suggesting significant involvement of these IL-17-producing CD4 and $CD8^+$ T cells in MS pathogenesis. After *in vitro* stimulation of blood samples, another study observed more IL-17 producing $CD8^+$ T cells in the 20 MS patients than in the 16 controls (74). $CD8^+$ T cells secreting IL-17 after *in vitro* stimulation were present in greater frequency in the CSF than in the blood of 17 MS patients in the early stages of the disease (75). Finally, in EAE, IL-17-producing $CD8^+$ T cells were found to be necessary for IL-17-producing $CD4^+$ T cell accumulation in the CNS and for disease development (75).

In human, the *in vitro* production of IL-17 is restricted to CD161-expressing cells (76). These cells have been shown to be present in CNS lesions of MS patients and the majority of them produce IFNγ (IL-17 staining was not performed on these samples) (32). In addition, an enrichment of $CD8^+CD161^{hi}$ in the blood of MS patients has also been evidenced, suggesting a specific involvement in the disease (32).

Recently, it has been shown that more than 80% of these cells are mucosal-associated invariant T (MAIT) cells. MAIT cells are a subset of innate effector memory T cells bearing a semi-invariant TCR (Vα7.2-Jα33/12/20) in humans (77–82). They are restricted to the MHC class-I related protein I (MR1) and have antimicrobial properties both *in vitro* and *in vivo* (83–86). Although they have been correlated with various autoimmune diseases (87–90), their implication, especially in MS (32, 91, 92), remain elusive. MAIT cells are present in the CNS of MS patients, but at very low frequencies compared to in the blood (92–94). This argues against a particular implication of this IL-17-producing $CD8^+$ T cell subset in the pathophysiology of MS (92, 95), similar to what has been described for psoriasis, where conventional IL-17-producing $CD8^+$ T cells might be more pathogenic than MAIT cells (87).

Other IL-17-producing $CD8^+$ T cell subsets have been described (96), with different markers, such as MCAM (97), but further research is necessary to decipher their involvement in MS pathogenesis.

TABLE 2 | Summary of EAE models used to study CD8+ T cells.

Model	Mode	Background	Results	Publication
WT	MOG_{35-55} $CD8^+$ injection	WT (C57BL/6)	Severe and permanent EAE	(63)
	MBP_{79-87} $CD8^+$ injection	WT (C3H)	Severe EAE with similarities with MS features	(65)
	MOG_{35-55} $CD8^+$ injection	WT (C57BL/6)	Severe and long-term EAE	(64)
Transgenic	HA $CD8^+$ injection	Oligodendrocytes Tg for HA expression (CL4)	CNS inflammation and demyelination	(67)
	OVA $CD8^+$ injection	Oligodendrocytes Tg for OVA expression (C57BL/6)	Severe/lethal EAE	(68)
"Humanized" transgenic	MOG_{35-55} + MOG_{181} injection PLP_{45-53} injection	HLA-A*0201 Tg (C57BL/6) HLA-A*0201-2D1 TCR (specific for PLP_{45-53}) double Tg (CBA/c × C57BL/6)	More severe EAE than MOG_{35-55} alone MS-like disease (relapsing–remitting)	(73) (18)

HA, hemagglutinin; OVA, ovalbumin; Tg, transgenic; MOG, myelin oligodendrocyte glycoprotein; MBP, myelin basic protein; PLP, proteolipid protein; EAE, experimental autoimmune encephalomyelitis; WT, wild type; CNS, central nervous system; MS, multiple sclerosis; TCR, T cell receptor.

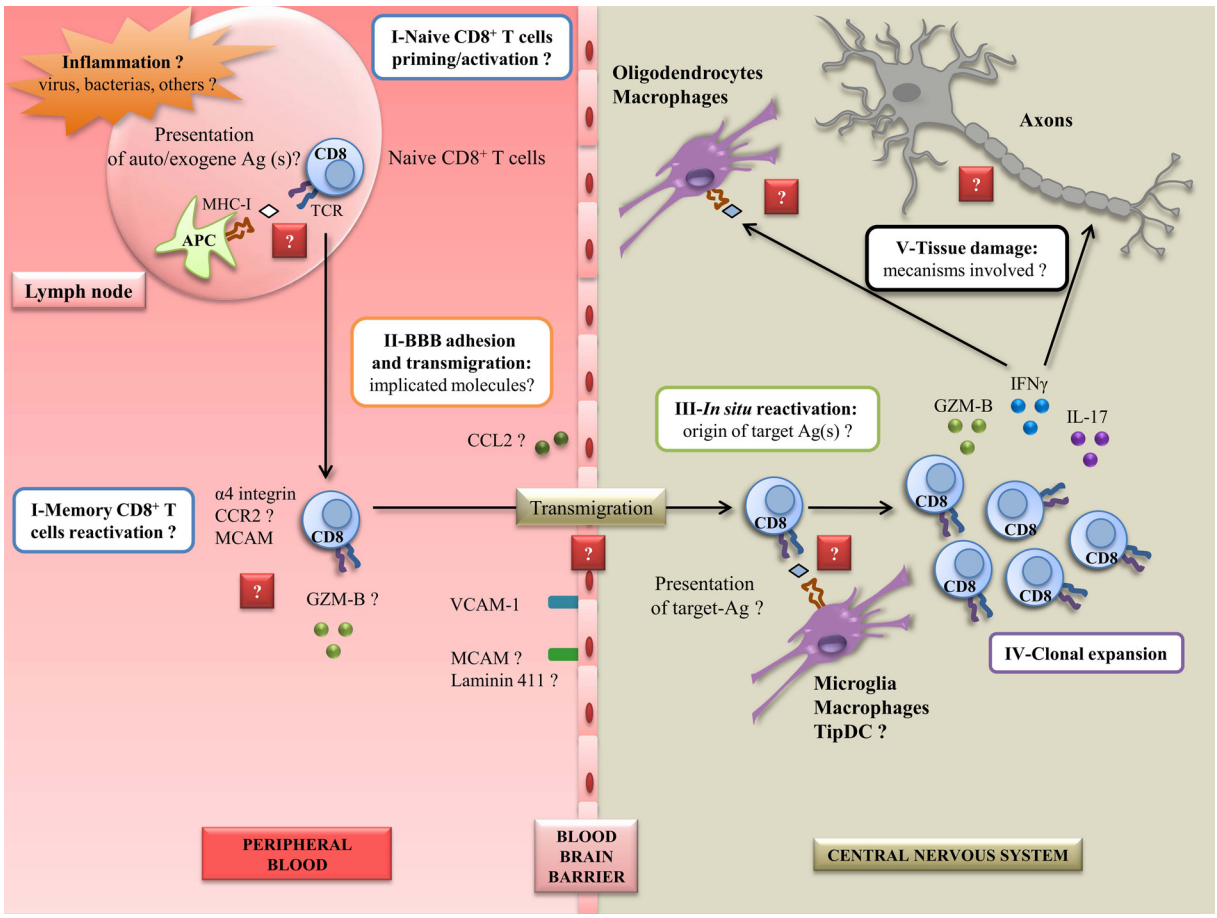

FIGURE 2 | Steps to elucidate to better understand CD8+ autoreactivity mechanisms in MS disease. A peripheral inflammation induced by pathogens (such as EBV) could occur in case of uncontrolled infection. This can lead to the activation or reactivation of $CD8^+$ T cells, and the expression of several molecules implicated in adhesion, migration, and cytotoxicity, currently not well characterized. In this inflamed state, the BBB could overexpress adhesion and chimoattractant molecules, leading to the entry of $CD8^+$ T cells into the CNS. *In situ*, $CD8^+$ T cells could be reactivated by resident APC presenting target Ag(s), unknown for now. This could lead to the clonal expansion of $CD8^+$ T cells along with the secretion of proinflammatory molecules. Finally, in this step, $CD8^+$ T cells could be able to mediate damage to resident cells and axons potentially by the recognition of CNS derived peptides. Ag: antigen; GZM-B: granzyme-B; BBB: blood–brain barrier; CNS: central nervous system; MS: multiple sclerosis; EBV: Epstein–Barr virus; APC: antigen-presenting cell; TCR: T-cell receptor; MCAM: melanoma cell adhesion molecule; VCAM-1: vascular cell adhesion molecule 1; DC: dendritic cell; MHC-I: major histocompatibility complex I; CCR2: C–C chemokine receptor type 2; CCL2: C–C chemokine ligand 2; IFNγ: interferon γ; IL-17: interleukin-17.

MULTIPLE SCLEROSIS: A CLOSE COLLABORATION BETWEEN CD4+ AND CD8+ T CELLS

Even though we are convinced that CD8+ T cells play a pivotal role in MS pathophysiology, it seems obvious that they interact with other subsets – especially CD4+ T cells – in mediating MS development. Indeed, CD8+ T cells may migrate first into the CNS, further allowing the infiltration of CD4+ T cells. This proposition is supported by the fact that a small infiltrate of CD4+ T cells was found in the CNS of a CD8-specific mouse model of EAE (67). Moreover, that fact that both CD8+ and CD4+ T cells produce IL-17 at the same frequency in brain lesions gives evidence for the involvement of both cell types. However, whether the damage caused by these cells is direct or bystander is still unclear. Indeed, the initiating event, which is still unknown, leads to an *in situ* inflammatory context. Amplifying inflammatory loops are likely to develop, leading to BBB disruption and to the infiltration of other immune cell subsets. The inflammatory climate and the subsequent destruction of CNS cell types result in the release of self-antigens. These antigens, usually sequestered in a compartment that is poorly accessible for immune cells, are then available for recognition and further activation of the immune compartment *in situ*. This phenomenon is well described in the EAE model. Ji et al. showed that in their EAE model, induced by the transfer of MOG-specific CD4+ T cells, particular dentritic cells (DC) derived from inflammatory monocytes (Tip-DC) lead to epitope spreading to MBP-specific CD8+ T cells *in situ* (98).

To conclude, it appears clear to us that CD8+ T cells are involved in the pathophysiology of MS, in particular as potent effectors for CNS damage. However, other cell subsets, including CD4+ T cells, are likely to act in synergy to trigger the disease, probably by giving aid to pathogenic CD8+ T cells. More studies are needed to decipher the exact steps involving CD8+ T cells in the disease. Indeed, numerous questions remain unanswered: how and why CD8+ T cells get activated/reactivated in MS patients; how they cross the BBB; what the target antigen(s) is (are); how they mediate damage *in situ*. Focusing on these different questions and mechanisms is essential in order to develop effective therapeutic approaches (**Figure 2**).

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REVIEW
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CD8+ T-Cells as Immune Regulators of Multiple Sclerosis

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The vast majority of studies regarding the immune basis of MS (and its animal model, EAE) have largely focused on CD4+ T-cells as mediators and regulators of disease. Interestingly, CD8+ T-cells represent the predominant T-cell population in human MS lesions and are oligoclonally expanded at the site of pathology. However, their role in the autoimmune pathologic process has been both understudied and controversial. Several animal models and MS patient studies support a pathogenic role for CNS-specific CD8+ T-cells, whereas we and others have demonstrated a regulatory role for these cells in disease. In this review, we describe studies that have investigated the role of CD8+ T-cells in MS and EAE, presenting evidence for both pathogenic and regulatory functions. In our studies, we have shown that cytotoxic/suppressor CD8+ T-cells are CNS antigen-specific, MHC class I-restricted, IFNγ- and perforin-dependent, and are able to inhibit disease. The clinical relevance for CD8+ T-cell suppressive function is best described by a lack of their function during MS relapse, and importantly, restoration of their suppressive function during quiescence. Furthermore, CD8+ T-cells with immunosuppressive functions can be therapeutically induced in MS patients by glatiramer acetate (GA) treatment. Unlike CNS-specific CD8+ T-cells, these immunosuppressive GA-induced CD8+ T-cells appear to be HLA-E restricted. These studies have provided greater fundamental insight into the role of autoreactive as well as therapeutically induced CD8+ T-cells in disease amelioration. The clinical implications for these findings are immense and we propose that this natural process can be harnessed toward the development of an effective immunotherapeutic strategy.

Keywords: CD8, multiple sclerosis, EAE, T-cells, immune regulation

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INTRODUCTION

Studies addressing the immunobiology of multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE) have focused on CD4+ T-cells as the main orchestrators of pathogenesis and regulation. CD8+ T-cells are the most abundant T-cells in CNS lesions of MS patients (1) and exhibit oligoclonal expansion (2–4). This indicates an important role for these cells in the target organ. However, the functional nature of these cells during disease and its treatment is unclear and somewhat controversial. There are abundant CNS-specific (5, 6) and therapeutically induced CD8+ T-cell responses in MS patients (5–8). Recent studies suggest that certain MHC class I alleles can be associated with genetic risk or protection in MS (9–11). Functional roles for some of these MHC class I molecules have been tested in the EAE models. 2D1-TCR humanized transgenic

mice, expressing MS risk variant HLA-A3 together with TCR that recognizes myelin proteolipid protein (PLP), develope spontaneous EAE in only 4% of mice and mild EAE early on when immunized with PLP peptide. A quarter of these mice went on to develop a severe disease course with 2D1$^+$-TCR$^+$–CD8$^+$ T-cells present in the CNS of these mice, suggesting a pathogenic role for HLA-A3-restricted myelin-specific CD8$^+$ T-cells (12). However, introduction of HLA-A2 alleles in the same model completely abrogates spontaneous and induced EAE, providing evidence for the protective role for HLA-A2-restricted CD8$^+$ T-cells (12). We are only beginning to understand these responses and here attempt to provide an overview of such studies. We will summarize the evidence for both pathogenic and regulatory functions of CD8$^+$ T-cells in MS and EAE. We will provide an overview of the various cellular and molecular interactions that mediate the role of these cells and develop a model for such functions during disease.

PATHOGENIC ROLE FOR CD8$^+$ T-CELLS IN EAE

Much of the focus regarding the pathogenesis of EAE has revolved primarily around myelin-specific CD4$^+$ T-cells. Adoptive transfer of CD4$^+$ T-cells isolated from myelin antigen-primed animals is sufficient to induce disease. This observation partly facilitated the overall ignorance surrounding CD8$^+$ T-cells and their potential contribution to disease. A pathogenic role first became evident when a CD8$^+$ T-cell-mediated model of EAE was developed using the self-protein myelin basic protein (MBP) (13). In attempts to prime an MHC class I-restricted T-cell response, C3H.Fej, and C3H MBP-deficient shiverer mice were infected with MBP-expressing vaccinia. CD8$^+$ T-cell lines specific for $MBP_{79–87}$ drove pathogenesis and demyelination when transferred into wildtype (WT) C3H recipients. Mice developed neurological symptoms including ataxia, spasticity, and lost weight when compared to control animals that received vaccinia-specific CD8$^+$ T-cells. Histologically, perivascular cuffs composed primarily of lymphocytes and macrophages were detected in the brain but not in the spinal cord. IFNγ was found to play an important role in mediating MBP-specific CD8$^+$ T-cell-driven disease, as its neutralization reduced severity. The break of peripheral tolerance following viral infection was also shown to induce CD8$^+$ T-cell-mediated CNS autoimmunity (14). In this report, dual TCR-expressing CD8$^+$ T-cells recognizing both viral antigen and MBP triggered disease. Following viral infection, CD8$^+$ T-cells, macrophages, and activated microglia infiltrated both the brain and spinal cord. Clinically, mice lost weight and exhibited symptoms of ataxia, impaired mobility, and tail weakness.

CD8$^+$ T-cell-mediated EAE has also been induced in C57BL/6 (B6) mice through transfer of myelin oligodendrocyte glycoprotein (MOG)-specific CD8$^+$ T-cells (15). MOG-specific CD8$^+$ T-cells isolated from mice immunized with $MOG_{35–55}$ peptide were encephalitogenic, and transferred severe paralytic disease to B6 mice. One caveat to this study is that cells were nylon wool-enriched, calling purity into question. Disease was transferred using <1e6 $MOG_{35–55}$ CD8$^+$ T-cells and resulted in more severe EAE compared to active immunization. Transferred cells could be re-isolated 6–8 months later, possibly due to additional IL-2 stimulus. How these cells induced pathology was not investigated.

A separate group identified $MOG_{37–46}$-specific CD8$^+$ T-cells as autoaggressive effectors (16). In this system, MOG-specific CD8$^+$ T-cells were generated following immunization. Restimulation with antigen and IL-2 readily yielded IFNγ from these cells, but not TGFβ or IL-10. These cells, which were found to be H-2D^b-restricted, could induce EAE when transferred into SCID or naïve WT B6 recipients. $MOG_{37–46}$ elicited the best IFNγ response from $MOG_{35–55}$-primed lymph node cells, although bound MHC poorly. When used to induce active EAE in B6 mice, $MOG_{37–46}$ led to similar disease as $MOG_{35–55}$-immunized mice. Using $MOG_{37–50}$/H-2D^b tetramers, MOG-specific CD8$^+$ T-cells were found to persist within the CNS.

While these studies utilized myelin-components to examine the potential pathogenic role of CD8$^+$ T-cells in EAE, non-myelin antigen-driven systems have been used as well. One report describes CD8$^+$ TCR transgenic mice recognizing glial fibrillary acidic protein (GFAP), an intermediate filament protein expressed in the CNS by astrocytes and in various peripheral tissues (17). BG1 transgenic mice are reactive to the $GFAP_{264–274}$ peptide presented on H-2K^b, and develop spontaneous inflammatory CNS disease by 6–12 months of age. Interestingly, GFAP-expressing vaccinia induced distinct disease pathology compared to spontaneous disease. Lesion localization and clinical manifestations of disease was dependent upon how CNS-reactive CD8$^+$ T-cells were activated. CD8$^+$ T-cells isolated from brains of WT BG1 mice were poor secretors of IFNγ, IL-17A, and granzyme B, suggesting alternative effector mechanisms.

Efforts to study the role of Src homology 2 domain-containing protein tyrosine phosphatase (SHP-2) in EAE demonstrated that disease could be ameliorated through phosphatase inhibition (18). The competitive inhibitor, NSC-87877 led to reduced demyelination and blocked CD8$^+$ but not CD4$^+$ T-cell migration into the CNS, suggesting a pathogenic role for CD8$^+$ T-cells in this model.

A study of engineered transgenic NOD mice expressing a $MOG_{35–55}$-reactive TCR (1C6) lends further support for pathogenic CD8$^+$ T-cells in EAE (19). 1C6 mice spontaneously generated MOG-specific CD4$^+$ and CD8$^+$ T-cells that secrete pro-inflammatory cytokines. 1C6 CD8$^+$ T-cells could recognize $MOG_{35–55}$ in the context of MHC class I and II, and when adoptively transferred into NOD. Scid recipients, induced optic neuritis and mild EAE, while 1C6 CD4$^+$ T-cells induced severe EAE.

CD8$^+$ T-cells' ability to target CNS components has also been evaluated in several viral models (20–22). LCMV GP33 peptide-specific CD8$^+$ T-cells can induce lesions in cultured murine neurons presenting GP33 in MHC class I. While this report relies on peptide pulsing and artificial upregulation of MHC class I, viral infection-induced upregulation of class I has been demonstrated in Borna disease virus-infected rat neuronal cultures, which could be targeted by antiviral CD8$^+$ T-cells, eventually leading to apoptosis of neurons (23). Although electrical signals were not initially disrupted in this model and longer incubation times were needed for neuronal apoptosis, another study has demonstrated impaired murine neuronal signaling following neuron/CD8$^+$

T-cell interactions along with eventual apoptosis which interestingly occurred independent of perforin/granzymes (24). To this end, IFNγ-production from CNS CD8+ T-cells and subsequent IFNγ signaling in neurons has been shown to be significant for intracranial LCMV disease in mice (25). In another study, OT-I CD8+ T-cells formed immune synapses with MHC class I (H-2K^b)-expressing axons presenting SIINFEKL peptide, and loss of axon integrity was observed. Additionally, axonal injury was dependent upon antigen-specific TCR recognition and granzyme B (26).

Another report also described mice expressing neo-self antigen in oligodendrocytes (ODCs) targeted by transgenic CD8+ T-cells (27). In this model, ovalbumin was expressed exclusively in the cytosol of ODCs and therefore ignored by CD4+ T-cells and B-cells. Following immunization, mild EAE was observed in some ODC–OVA mice. Studies using double transgenic ODC–OVA/OT-I mice demonstrate treatment with D1 mAb (specific for H-2K^b/OVA) prevented the lethal EAE normally observed in these animals (28). Double transgenic mice were also given D1 prophylactically, which in certain instances led to spontaneous disease remission.

CD8+ T-cells have also been shown to indirectly influence CNS autoimmunity. Tc17 cells, coined for their ability to produce IL-17A, were detected in the lymph nodes and CNS of $MOG_{37–50}$ EAE mice (29). Tc17s differ from conventional CD8+ T-cells regarding granzyme B and IFNγ expression, and thus are impaired in their cytotoxic capacity. In a separate study implementing CD4+ and CD8+ T-cell co-transfer, Tc17 cells were found to help CD4+ Th17 cells accumulate in the CNS and induce EAE (30). Furthermore, their ability to produce IL-17A was required to render CD4+ T-cells encephalitogenic.

REGULATORY ROLE FOR CD8+ T-CELLS IN EAE

While evidence exists to suggest a pathogenic role for CD8+ T-cells in MS and EAE (reviewed in Ref. (31) and discussed above), there is a growing body of evidence supporting the opposite conclusion – CD8+ T-cells play an important regulatory role in the pathogenesis of MS and MS-like disease. Ultimately, CD8+ T-cell subsets likely perform varying effector functions in the context of MS/EAE. However, the seeming discrepancy is in part due to a lack of concrete *in vivo* evidence demonstrating a cytotoxic effect of CD8+ T-cells in MS lesions. Furthermore, it has been demonstrated that depletion of CD8+ T-cells prior to EAE induction results in exacerbated disease (32). Similar results are seen in mice lacking MHC class I (although a role for NK cells can be argued) (33) and in CD8-deficient mice (32, 34, 35). This is in addition to work from our lab, which clearly demonstrated – in marked contrast to their CD4+ counterparts – neuroantigen-specific CD8+ T-cells failed to adoptively transfer EAE disease to naïve recipient mice (36). We have seen this protective CD8+ T-cells phenotype very robustly in several models of EAE (37).

The notion of a regulatory CD8+ T-cell subset (CD8+ Tregs) in MS is not a new idea. Studies spanning several decades point to the suppressive potential of CD8+ T-cells in MS patients (5–8, 38–41). In lieu of these examples, T-cell-mediated tolerance studies have largely focused on CD4+CD25+Foxp3+ T-cells. Although full appreciation of CD8+ Treg function and significance in MS and EAE is lacking, the last 15 years have seen a steady growth toward this understanding.

CD8+ T-cells' suppressive ability has been described in many mouse models, including cancer (42), diabetes (43), colitis (44), SLE-like disease (45), Grave's disease (46), and transplant tolerance (47). Inhibitory CD8+ T-cell subsets involved in autoimmunity in both mice and humans have been exhaustively reviewed in Ref. (48). These regulatory CD8+ T-cells have been extensively studied in T1D where it has been shown that low-avidity autoreactive CD8+ T-cells convert into memory-like autoregulatory cells and blunt diabetes progression (49, 50). However, CD8+ Treg participation in EAE is less-widely studied. Moreover, unlike murine CD4+Foxp3+ Tregs, a universal CD8+ Treg phenotype has yet to be described. For example, in EAE, CD8+CD28− T-cells have been shown to play an inhibitory role (32) while others show CD8+CD122+ T-cells to be protective (51–53). Little is known concerning the induction of these cells in MS-like disease, though the involvement of one subtype versus another surely is influenced by disease setting and may depend on the cell's antigen specificity/MHC-restriction. Studies of anterior chamber-associated immune deviation (ACAID) represent some of the best efforts to understand antigen-specific CD8+ Tregs, which appear to be Qa-1-restricted (54–56). Several ACAID studies further complicate the CD8+ Treg phenotyping picture (e.g., Foxp3+, CD94+, CD103+, TGFβ-producing, etc.) (56–60). Interestingly, immune deviation can be elicited against myelin antigens (61, 62), pointing to the potential role for Qa-1-restricted CD8+ T-cells in EAE disease. Qa-1-restricted CD8+ T-cells have been described as being important for protection in MBP-driven EAE (63). We have demonstrated that Qa-1-restricted CD8+ T-cells suppress EAE. We have also demonstrated that GA treatment induces CD8+ Treg in mice, and that these CD8+ T-cells are required for GA to be therapeutically effective in ameliorating EAE disease (64).

While little is still known about Qa-1-restricted CD8+ Tregs, even less was understood about CNS-specific CD8+ T-cells until very recently. We observed the surprising result that neuroantigen-specific CD8+ T-cells could suppress EAE induction and even ameliorate established EAE disease (36). To our knowledge, this was the first documentation of neuroantigen-specific CD8+ Tregs in mice. In our recently published and unpublished results, adoptive transfer of both $MOG_{35–55}$- and $PLP_{178–191}$-specific CD8+ T-cells can suppress EAE (34, 65). Due to mechanistic studies, we will elaborate upon later that these cells are quite distinct from previously described Qa-1-restricted CD8+ Tregs (37).

Recent work has suggested a role of IL-10-producing CD8+ T-cells in diminishing disease pathology in virus-induced encephalitis models. These IL-10-producing CD8+ T-cells display a more functional profile including increased expression of pro-inflammatory cytokines and chemokines, are immunosuppressive, and their presence in the CNS following Coronavirus infection reduces tissue destruction and morbidity in these mice (66).

INTERACTIONS BETWEEN CD8+ Tregs AND OTHER CELL TYPES IN EAE/MS

Advancement in therapy for MS patients, particularly cellular immunotherapy, necessitates the full understanding of regulatory immune cell interplay. Studies concerning the functional interactions between CD8+ Tregs and other cells in the context of MS and MS-like disease are therefore of paramount interest. The next several sections will provide mechanistic insights into CD8+ T-cell-mediated modulation of other immune cells including CD4+ T-cells and antigen presenting cell (APC) populations.

Influence of CD8+ T Regulatory Cells on CD4+ T-Cells

Qa-1-restricted CD8+ T-cells have been shown to modulate EAE disease through action on CD4+ T-cells. It has been demonstrated in a model of MBP-driven EAE that CD4+ T-cell vaccination protocol-mediated protection against EAE disease is dependent on the presence of Qa-1-restricted CD8+ T-cells that recognize specific TCRVβ molecules on MBP-reactive CD4+ T-cells (63). In this particular example, CD8+ T-cells mediated their control by preferentially suppressing Th1 CD4+ T-cells during EAE. While this report did not directly test cytotoxic killing as a means of suppression, the group had previously established this capability in T-cell vaccination scenarios. Data from another group later confirmed a cytotoxic effect by demonstrating that CD8αα+TCRαβ+ T-cells from lines that recognize TCRVβ8.2+ (MBP-reactive) CD4+ T-cells could protect against EAE disease in recipient mice by the targeted killing of these pathogenic cells via Qa-1-recognition (67).

We have showed that the disease-ameliorating effect of GA-therapy in EAE is dependent upon Qa-1-restricted CD8+ Tregs (64). In this report, we demonstrated that the protective ability of CD8+ T-cells was completely lost or diminished when unable to produce IFNγ or perforin, respectively. These CD8+ T-cells could kill GA-loaded target T-cells and even limited the proliferation of *ex vivo* neuroantigen-specific CD4+ T-cells (64). Furthermore, the GA-induced Qa-1-restricted CD8+ T-cells in this study were important for generation of CD4+ Tregs (64). These GA-specific CD8+ T-cells have the potential to kill GA-expressing CD4+ T-cells and limit proliferation of neuroantigen-specific and anti-CD3-stimulated CD4+ T-cells (8, 40). We have also demonstrated that GA therapy, whose effects require CD8+ T-cells in mice (64), was able to increase the induction of CD4+CD25+ Tregs from the CD4+CD25− T-cell population in MS patient blood (40).

Distinct from the non-classical HLA-E-like Qa-1-restricted murine CD8+ Tregs, we have also demonstrated the existence of neuroantigen-specific CD8+ Tregs in MS and EAE. Neuroantigen-specific, MHC class Ia-restricted CD8+ T-cells can kill MOG-loaded CD4+ T-cells in mice (34, 36) and mediate their disease-ameliorating effects via the targeting of encephalitogenic CD4+ T-cells during EAE disease (34). We have also demonstrated an ability of neuroantigen-specific CD8+ Tregs to induce anti-inflammatory profiles in CD4+ T-cells during EAE (65). Importantly, we have also shown that neuroantigen-specific CD8+ T-cells are detectable in MS patient blood, and possess capacity to suppress CD4+ T-cell proliferation (5, 68).

Influence of CD8+ Tregs on Dendritic Cells

The potential for CD8+ T-cells to alter CD4+ T-cell priming through direct effects on DCs is worth investigation. CD8+CD28− T-cells have been implicated as regulators of EAE disease. It has been demonstrated that DCs have reduced costimulatory molecule (CD80, CD86, and CD40) expression after culture with CD8+CD28− T regulatory cells, rendering these DCs as substandard APCs (32). It has been similarly demonstrated that DCs cultured with CD8+CD122+ T-cells had a reduction in CD80/86 and MHC molecules and showed inferior antigen-presentation ability compared to DCs cultured with CD8+CD122− T-cells (52).

While it remains unclear whether Qa-1-restricted CD8+ Tregs have a direct effect on DCs, we have shown that neuroantigen-specific CD8+ Tregs can both kill and suppress antigen presentation of MOG-loaded bulk APCs (contains DCs) (36). Interestingly, we have demonstrated that neuroantigen-specific CD8+ Tregs have little effect on DC surface expression of MHC or costimulatory molecules, but rather shift the inflammatory profiles of CD11c+ DCs from IL-12 to IL-10 (65). Early human MS work from our lab points to the potential of GA therapy-induced CD8+ Treg-mediated killing of APCs, as CD4+ T-cells were only a part of the larger target pool (8).

Influence of CD8+ Tregs on Monocytes/Macrophages

Another potential mechanism of suppression is CD8+ T-cell-mediated regulation of monocytes or macrophages, which are present in MS lesions and important for pathology in the CNS of EAE mice. Interestingly, GA treatment has been demonstrated to affect monocyte populations in EAE. For example, anti-inflammatory type II monocytes are induced in GA-treated mice, which can shift inflammatory cytokine profiles toward immunosuppressive IL-10, expand Th2 cells, and induce CD4+ Tregs capable of ameliorating EAE (69). We have observed similar results and have further demonstrated that the action of GA on monocytes elicits CD8+ Tregs and actually requires CD8+ T-cells for its ameliorative effects in EAE (64). This GA-induced monocyte-CD8+ T-cell interaction is largely unknown in MS, as is the effect of GA-induced CD8+ T-cell targeting of other macrophage populations. While a direct link to CD8+ T-cells has yet to be confirmed, studies from us and others have shown modulation of monocytes following GA therapy in humans (40, 70, 71). As mentioned in the section above, GA-induced CD8+ T-cell-mediated killing of APC populations like dendritic cells and monocytes/macrophages while unconfirmed, cannot be ruled out, as CD4+ T cells were only a portion of a larger affected target pool (8). Refining these assays for direct detection of killed targets is needed going forward.

Beyond GA-induced CD8+ Tregs, neuroantigen-specific CD8+ Tregs could conceivably modulate monocytes/macrophages in EAE. We have demonstrated that these cells can kill MOG-loaded bulk APCs, which may contain monocytes/macrophages, and can suppress their antigen presentation (36). However, we did not

observe a substantial neuroantigen-specific CD8+ Treg effect on monocytes during EAE (65). Furthermore, neuroantigen-specific CD8+ Tregs from MS patients do not appear to specifically target monocytes. More work is needed to understand the potential functional interactions between CD8+ T-cells and monocytes/macrophages during MS and MS-like disease, and may ultimately be a GA treatment-specific phenomenon.

Potential CD8+ T-Cell: B-Cell Interactions in MS/EAE?

In light of depletion therapy success, more focus is now being given to B-cells and their role in MS. The literature supports both a pathogenic (72–82) and regulatory (76, 83–94) role for B-cells in MS/EAE, and it is intriguing to speculate about the potential immune cell interplay between CD4+ T-cells, B-cells, and CD8+ T-cells therein. There is evidence in the literature to support a B-cell effect on CD8+ T-cells (54, 55, 82, 95–100). Many of these reports point to B-cell antigen presentation to CD8+ T-cells and even a B-cell requirement for CD8+ Treg function in some models. There is also literature supporting a role for Bregs in controlling CD8+ T-cell responses (17, 101–105). Additionally, CD8+ T-cells can be detected in follicles and modulate B-cell biology, such as germinal centers and antibody production (45, 106–111). The significance of these CD8+ T cell and B-cell subset interactions in the context of MS/EAE remains to be seen.

PATHOGENIC ROLE FOR CD8+ T-CELLS IN MS

Due to the inherent complexity of studying CD8+ T-cell function in the human brain, only circumstantial evidence exists regarding a pathogenic role for CD8+ T-cells in MS.

CD8+ T-cells are the most abundant T-cells found in the CNS lesions of MS patients, far outnumbering CD4+ T-cells (1). In patients with active disease, CD8+ T-cells were detected in increasing amounts from the center to the edge of the lesions studied (112). The CD8/CD4 ratio is shown to have been as high as 50/1 in the lymphocytic perivascular cuffs at the edge of active plaques (113). CD8+ T-cells displaying activated and memory phenotypes (suggesting previous interaction with local antigens) have also been detected in the CNS and CSF of MS patients (3, 114). CD8+ T-cell clones have also been shown to move throughout the affected CNS and into normal appearing white matter (NAWM) (112). One study demonstrated that there is diffuse infiltration by CD8+ T-cells combined with microglial activation and meningeal inflammation in the NAWM of MS patients (115).

Unfortunately, assigning function to these CD8+ T-cells remains a challenging task, although speculations have been made that CD8+ T-cells present in the CNS lesions of MS patients may be cytotoxic toward CNS cells including glia and axons. CD8+ MHC class I-restricted myelin peptide-specific T-cells have been shown to cause injury to human ODCs *in vitro* (116). Similarly, an MBP-specific memory phenotype CD8+ T-cell line generated from the peripheral blood of MS patients, in addition to secreting IFNγ and TNFα, was able to lyse COS-MBP/HLA-A2-transfected cells that were presenting endogenous MBP (114).

CD8+ T-cells have also been detected near or attached to ODCs and demyelinated axons in MS patients (117–119). Importantly, MHC class I molecules are present on astrocytes, ODCs, neurons, and endothelial cells (120, 121). Furthermore, MHC class I molecules are upregulated – depending on disease severity – and can be induced by IFNγ (121). CNS blood vessel endothelium as well as several APCs also express MHC class I molecules, which can cross-present exogenous peptides (122). Thus, it is not surprising that CD8+ T-cells have been demonstrated to interact with APCs at CNS plaque margins (119). The potentially detrimental nature of this interaction is supported by a study that showed that the amount of CD8+ T-cells and macrophages present in an MS lesion is proportional to the amount of acute axonal damage present (123).

Effector cytokines from CD8+ T-cells can also enhance their cytotoxic function and activate other immune cells to amplify inflammatory cascades in the CNS. For example, neuroantigen-specific CD8+ T-cells present in the peripheral blood express IFNγ and TNFα in response to their cognate antigen *ex vivo* (6, 124, 125). IFNγ- and IL-17-producing CD8+ T-cells can be recruited into the CNS when responding to apoptotic T-cell-associated self-epitopes (126). One report demonstrated that CD8+ but not CD4+ T-cells from patients with acute RRMS had increased ability to be recruited in inflamed CNS venules (127). Additionally, CD8+ IL-17-secreting T-cell numbers have been shown to be significantly elevated in acute CNS lesions of MS patients (128). IFNγ- and IL-17-secreting CD8+CD161+ T-cells were also found to be elevated in the peripheral blood of MS patients (129). Higher frequency of CD8+ T-cells expressing cytotoxic molecules like perforin has been shown to be present in MS patients, particularly during a relapse (130).

REGULATORY ROLE FOR CD8+ T-CELLS IN MS

In light of the present literature, it can be appreciated that CD8+ T-cells in MS and other autoimmune diseases are phenotypically and functionally diverse, and can potentially regulate the pathogenic immune processes. Besides cytolytic molecules like perforin and granzyme, CD8+ T-cells are armed with immunosuppressive cytokines, such as IL-10, that can dampen the inflammatory response.

The evidence for CD8+ T-cell regulatory function in MS has existed for a long time and has been largely ignored by the field. CD8+ T-cells from the peripheral blood of MS patients displaying reduced levels of suppressor function was the first report that suggested a regulatory function for CD8+ T-cells in MS (131). This was followed by another study that demonstrated a similar defect in CD8+ T-cell-mediated suppression in patients with chronic progressive MS (132). Since then, mounting evidence has accumulated in the field of MS disease and others that collectively points toward a regulatory role for CD8+ T-cells in autoimmune diseases (50, 133, 134). More recently, our lab has provided direct evidence for CD8+ T-cell regulatory function in MS and has established clinical correlations with the disease activity (31).

As in the mouse, phenotypic identification of human CD8+ Tregs has been challenging. Human CD8+CD28− T-cells have been shown to possess suppressor activity and are the most extensively studied population of CD8+ Tregs. In MS, they were found to be present at significantly reduced frequency in the blood of RRMS patients as compared to healthy donors (135). Although it is not a marker for CD8+ Tregs, FoxP3-expressing CD8+ T-cells are present in human blood. They possess regulatory activity (136), which is Foxp3-dependent (137), and are associated with autoimmune diseases such as IBD and MS (133, 138). CD8+FoxP3+ cells are present at reduced levels in the CSF of MS patients during acute exacerbation (137). CD8+CXCR3+ T-cells are human counterparts of the well-known regulatory CD8+CD122+ T-cells found in mouse. Human CD8+CXCR3+ T-cells are suppressive in nature and their function is IL-10-dependent (139). Although all of these CD8+ Treg subsets have potent immunosuppressive functions, so far their antigen specificity remains unknown.

Our lab showed for the first time that CNS-specific CD8+ T-cells have potent suppressor activity toward myelin antigen-specific CD4+ T-cells (5). These CNS-specific CD8+ T-cells were reactive to several myelin antigens including MOG, PLP, MBP, MAG and others and are present in the peripheral blood of healthy donors and MS patients (6). Mechanistically, these CNS-specific CD8+ T-cells are MHC class I-restricted, and their suppressive function is IFNγ-and perforin-dependent (5, 68). Our findings lend credence to the hypothesis that CNS-specific CD8+ T-cells in the CNS would function to dampen the inflammatory response by targeting pathogenic CD4+ T-cells and APCs, rather than causing damage themselves. Phenotypically, these cells are CD8+CD27−CD28−CD45RO−CD62L−CD57+ or a terminally differentiated subset of CD8+ T-cells (68).

Similar to Qa1-restricted CD8+ T in murine models, HLA-E-restricted CD8+ T-cells in humans perform a regulatory function and are involved in the maintenance of self-tolerance (140). The nature of NKG2 receptors present on CD8+ T-cells determines the functional outcome of their interaction with Qa1-expressing T-cell targets. For example, NKG2C-expressing CD8+ T-cells suppress Qa1-expressing target T-cells while NKG2A-expressing CD8+ T-cells get suppressed by these targets, and therefore cannot perform regulatory functions. A recent study showed reduced expression of FoxP3 and CD122 in NKG2C-expressing CD8+ T-cells from MS patients compared to healthy controls, suggesting a reduced regulatory potential of these cells in MS patients (41).

Although, there are only a handful of studies that report the phenotypic and functional significance of CD8+ T-cells in MS patients, one prominent feature that emerges from these studies is an underlying defect in the CD8+ Treg component. Of note, this defect is found specifically during MS relapses. Since a relapse represents the active phase of the disease, any significant differences in the phenotype and functions of immune cells between relapse and remission may be directly correlated with the immunopathogenesis of MS. Interestingly, frequency of circulating CD8+FoxP3+ T-cells was found to be significantly lower in the peripheral blood of MS patients during relapse as compared to remission (138). Another study showed that CD8+CD25+CD28− T-cells harbored potent suppressive activity and were lower in MS patients during relapse when compared to healthy controls (141). Importantly, treatment with glucocorticoids leads to a significant increase in the frequency of these CD8+ Tregs in the blood of MS patients. This was an interesting observation, suggesting that recovery from relapse under glucocorticoid treatment might be mediated by the regulatory function of CD8+ T-cells. Furthermore, deficiency in CD8+ Treg function is not limited to the blood, as evidenced by the significantly reduced CD8+ T-cell cloning frequency in the CSF during MS relapse as compared to remission, suggesting loss of CD8+ Tregs in the CSF during relapse (142).

Our own studies show that the terminally differentiated CD8+ T-cell pool, which harbors the CNS-specific CD8+ Tregs, is significantly reduced during MS relapse as compared to remission (68). Furthermore, relapses in MS are associated with significantly lower CNS-specific CD8+ T-cell suppressor ability, while this potential in MS patients during quiescence is similar to healthy donors, suggesting a role with disease activity (5). Of clinical significance, we showed that the CNS-specific CD8+ Treg suppressive function is restored in MS patients during remission and this recovery in CD8+ Treg-mediated suppression correlated with the distance in time from an acute clinical episode. This suggests that the correction of the neuroantigen-specific CD8+ suppressor deficit would correlate with recovery from an acute relapse (5). One caveat to the study is that the quiescence samples could still potentially have pseudo relapses in the CNS in the absence of any clinical signs. Nonetheless, these findings raise the possibility that reduction in CNS-specific CD8+ T-cell suppression might be used as a marker to predict relapses in MS patients.

Although etiology of MS remains unknown, epidemiological studies suggest an association between Epstein–Barr virus (EBV) and MS (143). EBV-reactive CD8+ T-cells are present in the peripheral blood of MS patients (144). By using high throughput sequencing, a recent study demonstrated intrathecal enrichment of EBV-reactive CD8+ T-cells in MS patients (145). However, the function of these CD8+ T-cells in the CNS remains speculative. Interestingly, adoptive immunotherapy with *in vitro*-expanded autologous EBV-specific CD8+ T-cells in secondary progressive MS had no adverse effects and was associated with clinical improvement and reduced disease activity on MRI (146). This study suggests that the EBV-specific CD8+ T-cells in the CNS of MS patients might be playing a regulatory role by limiting EBV-infected B-cells and antibody production.

The pathogenic function of CD8+ T-cells in MS is believed to be largely derived from its cytotoxic potential toward CNS tissues including glial cells and axons. However, there is a clear lack of evidence in this area in human MS. Interestingly, a recent study demonstrated that CD4+ but not CD8+ T-cells from peripheral blood of MS patients expressed NKG2C and had elevated levels of cytotoxic molecules FasL, granzyme B, and perforin. Intriguingly, these CD4+ T-cells were cytotoxic toward HLA-E-positive human ODCs *in vitro* (147). This study suggested a novel mechanism for CNS damage in MS which is, in contrast to the widely held view, potentially mediated by CD4+ T-cells.

Although the pathogenic role of CD8+ T-cells in MS remains largely speculative, the studies discussed above strongly suggest that there is now ample evidence for the regulatory role for CD8+ T-cell subsets in the disease process. Lack of their regulatory function specifically during relapses should be probed further, as this could be a major underlying factor leading to relapse in MS.

THERAPEUTIC INDUCTION OF CD8+ Tregs

The majority of drugs used for the long-term management of MS are immunomodulatory in nature. The precise mechanisms by which these drugs act are under constant investigation. We have convincingly demonstrated that CD8+ Tregs not only exist physiologically but can also be induced therapeutically by GA treatment. Both, CD4+ and CD8+ T-cells reactive to GA are present in the peripheral blood of healthy donors and MS patients (7). Although CD4+ T-cell responses are comparable between the two groups, untreated MS patients have reduced GA-induced CD8+ T-cell responses and this deficiency is corrected after GA therapy (7). Functionally, these GA-reactive CD8+ T-cells are HLA-E-restricted and have a strong suppressive potential against CD4+ T-cells (8). Interestingly, GA-reactive CD8+ T-cells obtained from untreated MS patients have reduced suppressor ability and GA therapy restores the CD8+ T-cell suppressive potential in MS patients (8). These were the pioneering findings that linked the regulatory function of CD8+ T-cells with the therapeutic action of the drug. The proof of principle came from our EAE studies discussed above where we showed that GA does not work in the absence of CD8+ T-cells in mice (64), suggesting that CD8+ T-cells are absolutely required for GA action and all the other reported immunomodulatory effects of GA might lie downstream to the induction of CD8+ Tregs by the drug. The idea is also supported by our surprising observation that GA reverses the CD4/CD8 T-cell ratio and increases CD8+ T-cell-mediated suppression as early as 12 h after GA therapy initiation in humans (40). Similar to our findings, a 1-year follow-up study after IFNβ treatment showed expansion of regulatory CD8+ T-cell subsets (CD8+CD25+ and CD8+CD25+CD28−) in the responder cohort (148). Another study found a higher frequency of regulatory CXCR3+CD8+ T-cells 6 months after IFNβ therapy (149). Collectively, these studies suggest that therapeutic induction of CD8+ Tregs might be the underlying factor in other MS therapies as well. Natalizumab treatment results in a decreased CD4+/CD8+ ratio in the CSF and peripheral blood of MS patients (150). Fingolimod therapy is associated with altering the cytokine status of CD8+ T-cells in peripheral blood (151). However, detailed dissection of the role of CD8+ T-cells has not been performed in the setting of these treatments.

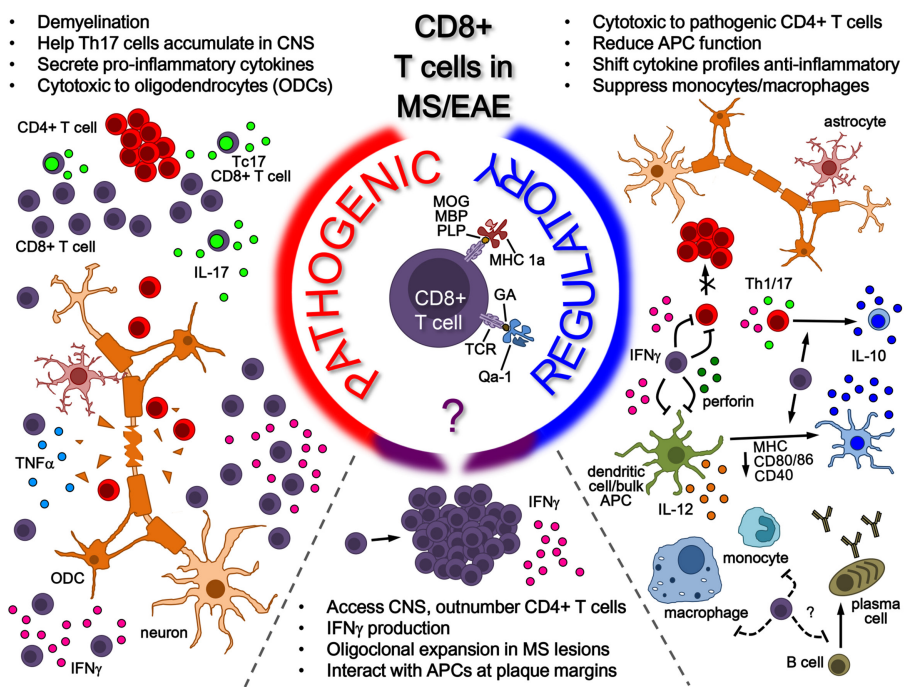

FIGURE 1 | Role of CD8+ T-cells in MS/EAE. While the antigenic specificity of pathogenic CD8+ T-cells remains unknown, their pathogenic function is mainly attributed to pro-inflammatory cytokine secretion (in the peripheral immune system and potentially in the CNS) as well as cytotoxicity toward oligodendrocytes in the CNS. On the other side, several lines of evidence indicate a regulatory role for CD8+ T-cells in both MS and EAE. Neuroantigen-specific autoregulatory T-cells are classically MHC Class I restricted, whereas there are also examples of HLA-E/Qa1-restricted regulatory T-cells that may be naturally occurring or induced through therapy. Mechanisms for CD8+ T-cell-mediated regulation include secretion of cytokines such as IL-10 and IFNγ, cytotoxicity toward pathogenic immune cells and modulation of APC functions, both in the periphery and possibly in the CNS.

SUMMARY AND MODEL

The potential roles of CD8+ T-cells in MS is summarized in the model shown in **Figure 1**, where the various pieces of evidence supporting the potential of CD8+ T-cells for both pathogenic and regulatory roles in MS/EAE disease are depicted. On the pathogenic side of the model, CD8+ T-cells, whose antigenic specificity has yet to be fully elucidated, have been shown to be involved in several disease-driving mechanisms, ranging from cytotoxicity and demyelination to pro-inflammatory cytokine production. This is in addition to hallmark activation behavior in disease lesions, such as oligoclonal expansion and IFNγ production. Interestingly, this fails to rule out the activation of a regulatory population, as indicated in the bottom portion of the model. As illustrated on the regulatory side – to which our lab has made several novel contributions – several lines of evidence exist demonstrating the regulatory mechanisms performed by CD8+ T-cells in the context of MS/EAE, which can either be neuroantigen specific (MHC class 1a-restricted) or GA/Copaxone® specific (HLA-E/Qa-1-restricted). Their protective functions, which seem to depend on IFNγ and perforin production, range from direct cytotoxicity to pathogenic CD4+ T-cells to modulation of pro-inflammatory cytokine profiles to inhibition of APC function. It is still unclear to what extent CD8+ T-cells affect other cell populations such as B-cells, but some evidence demonstrates a suppressive effect on monocytes and macrophages. These all serve to suppress CNS auto-inflammation and protect myelinated axons – effectively limiting EAE disease pathogenesis. The potential role for these CD8+ Tregs in ultimately modulating MS disease is of high interest.

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PERSPECTIVE
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The non-obese diabetic mouse strain as a model to study CD8+ T cell function in relapsing and progressive multiple sclerosis

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Multiple sclerosis (MS) is a neurodegenerative disease resulting from an autoimmune attack on central nervous system (CNS) myelin. Although CD4+ T cell function in MS pathology has been extensively studied, there is also strong evidence that CD8+ T lymphocytes play a key role. Intriguingly, CD8+ T cells accumulate in great numbers in the CNS in progressive MS, a form of the disease that is refractory to current disease-modifying therapies that target the CD4+ T cell response. Here, we discuss the function of CD8+ T cells in experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. In particular, we describe EAE in non-obese diabetic (NOD) background mice, which develop a pattern of disease characterized by multiple attacks and remissions followed by a progressively worsening phase. This is highly reminiscent of the pattern of disease observed in nearly half of MS patients. Particular attention is paid to a newly described transgenic mouse strain (1C6) on the NOD background whose CD4+ and CD8+ T cells are directed against the encephalitogenic peptide $MOG_{[35–55]}$. Use of this model will give us a more complete picture of the role(s) played by distinct T cell subsets in CNS autoimmunity.

Keywords: multiple sclerosis, relapsing–remitting multiple sclerosis, progressive multiple sclerosis, experimental autoimmune encephalomyelitis, CD8+ T cell, CD4+ T cell, non-obese diabetic mouse, 1C6

Abbreviations: B6, C57BL6/J; CNS, central nervous system; CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalomyelitis; HLA, human leukocyte antigen; MBP, myelin oligodendrocyte glycoprotein; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NOD, non-obese diabetic; PLP, proteolipid protein; RR, relapsing-remitting; SCID, severe combined immunodeficient; SP, secondary progressive; T1D, type 1 diabetes; TcR, T cell receptor; Tg, transgenic; Th, T helper; WT, wild-type.

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INTRODUCTION

Multiple sclerosis (MS) is a complex chronic neurological disease that results from an immune-mediated attack against central nervous system (CNS) myelin. It is characterized by demyelination, axon damage, white matter inflammation, and glial scarring (1). Approximately 2.5 million people in the world are affected by MS (2) and it is therefore important that we better understand its causes, with the goal of developing treatments that can improve disease outcomes. The four known subtypes of MS are relapsing–remitting (RR), secondary progressive (SP), primary progressive, and progressive relapsing. Approximately 85% of MS patients display an RR disease course in which repeated periods of inflammatory response are followed by remission. Nearly 50% of RR patients eventually transition to a chronic SP phase marked by slow and steady increase in disability, and more than half of all MS patients will exhibit some form of progressive disease (3). Although $CD4^+$ T helper cells have been thought to be the main players in the pathogenesis of MS, the evidence suggests that disease processes in MS involve other immune cell types that include, among others, $CD8^+$ T cells, B cells, macrophages, microglia, and neutrophils (4). Our goal here is to discuss the role of $CD8^+$ T cells in the pathology of MS with a particular focus on findings from studies of experimental autoimmune encephalomyelitis (EAE), a mouse model of MS.

T CELLS IN MS PATHOGENESIS

Autoimmunity results from (a) the escape of self tissue antigen-specific T cells from negative selection and deletion in the thymus and (b) the breakdown of peripheral immune tolerance mechanisms, such as inhibitory receptor signaling and regulatory T cell-mediated inhibition. MS specifically results from an autoreactive T cell inflammatory reaction against myelin-producing CNS oligodendrocytes (1). The role of T cells in MS pathogenesis is strongly supported by genetic analysis demonstrating that polymorphisms in the human leukocyte antigen (HLA) gene region (5) and in genes encoding T cell-related signaling molecules and cytokines (6) which are strongly linked to MS susceptibility. Furthermore, immunohistochemical analysis of acute and recent MS lesions reveal extensive perivascular infiltration of T lymphocytes (7).

Myelin-reactive T cells proliferate and differentiate into effector subpopulations in peripheral lymphoid tissues, where they recognize myelin-derived antigenic epitopes presented by specialized antigen-presenting cells. The $CD4^+$ IFN-γ-secreting Th1 and IL-17-secreting Th17 subsets have been well described in both MS (8, 9) and EAE (10). However, $CD8^+$ T cells can also respond vigorously to myelin antigen and induce neuroinflammatory damage (11). Activated T cells express cell adhesion molecules (e.g., LFA-1, VLA-4, and PSGL-1) and chemokine receptors (e.g., CCR5 and CXCR3) that permit them to cross the blood brain barrier (12). Once in the CNS, they are reactivated by local antigen-presenting cells, such as microglia and dendritic cells (13), which themselves produce proinflammatory cytokines (e.g., IFN-γ, IL-23, TNF-α, and lymphotoxin-α) and chemokines (e.g., RANTES, CXCL10, and IL-8). This attracts other immune effector cells from the circulation (12–14).

It has been more than 20 years since the first effective disease-modifying therapies were introduced for RR–MS. Both IFN-β and glatiramer acetate appear to modulate the function of inflammatory T cells (15, 16). More recently developed drugs, such as natalizumab (17) and rituximab (18), also target lymphocyte function, thus emphasizing the critical role played by dysregulated adaptive immune responses in MS. However, there are no currently available treatments for progressive MS, which has led some to argue that this form of the disease is driven by neurodegenerative rather than inflammatory mechanisms (19). On the other hand, lymphocytes accumulate in the meninges (20–22) and $CD3^+$ T cells are detected in normal-appearing white matter of the spinal cord during progressive disease (20). Intriguingly, $CD8^+$ T cells are detected in normal-appearing white matter, active lesions, and inactive lesions in progressive MS (23), and can directly induce demyelination (24). Thus, $CD8^+$ T cell function may present an attractive target for the treatment of progressive MS.

$CD8^+$ T CELLS IN MS

A number of current MS drugs, such as interferon-β (15), glatiramer acetate (16), and natalizumab (17), appear to modulate $CD4^+$ T cell responses, indicating the relevance of these cells to pathogenesis. However, multiple lines of evidence suggest that $CD8^+$ T cells also play a key role in MS pathology. On the genetic level, positivity for the HLA class I allele A3 increases one's risk of developing MS (25, 26); as the human $CD8^+$ T cell repertoire is restricted by class I molecules, this implies that $CD8^+$ T cell reactivity to specific myelin antigens can predispose an individual to developing MS. Myelin-specific $CD8^+$ T cells show oligoclonal expansion in plaques, cerebrospinal fluid (CSF), and blood of MS patients, and the frequency of $CD8^+$ T cells greatly exceeds that of $CD4^+$ T cells in acute MS lesions (27). Interestingly, $CD8^+$ T cells are detected in NAWM in MS brains, suggesting that they are among the first lymphocytes on the scene in the earliest stages of disease (23). In addition, during MS, $CD8^+$ T cells upregulate cell adhesion molecules involved in immune trafficking into the CNS; increased frequency of $CCR5^+$ and $CXCR3^+$ $CD8^+$ T cells in peripheral blood correlates with increased annualized MS lesion load (28) and increased $PSGL\text{-}1^+CD8^+$ T cell frequency is observed in active MS (29).

$CD8^+$ T cells can execute inflammatory damage in the CNS via two distinct mechanisms: a direct mechanism by which they attack MHC class I-expressing axons, or an indirect one by which they attack oligodendrocytes, thereby exposing axons to further damage (30). Granzyme B-positive $CD8^+$ T cells are found in close proximity to demyelinated axons in MS lesions and their cytolytic granules appear to be polarized toward the site of injury (24). CSF levels of granzymes A and B are elevated during active MS (31) and highly differentiated $CD8^+$ T cells are enriched in the CSF during early MS (32). Importantly, various cells of the CNS, including neurons, astrocytes, and oligodendrocytes, can be induced to express MHC class I on their surface in the context of an inflammatory response. These cells are, thus, susceptible to

$CD8^+$ T cell-mediated killing (24). Indeed, abundance of $CD8^+$ T cells in MS brain tissue positively correlates with the extent of axonal damage (33). Taken together, these data suggest that $CD8^+$ T cells proliferate in response to myelin antigens, and traffic to the CNS, where they can help to initiate and maintain tissue inflammation and damage.

ANIMAL MODELS OF MS

Experimental autoimmune encephalomyelitis is a murine disease that recapitulates the immunopathogenesis of MS. It can be induced (a) by active immunization with encephalitogenic peptides derived from myelin component proteins, such as myelin basic protein (MBP), proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG) or (b) by passive ("adoptive") transfer of myelin-reactive lymphocytes to genetically susceptible recipient mice (34). Classic adoptive transfer approaches entail the isolation and re-stimulation of myelin-specific lymphocytes from actively immunized donor mice, followed by transfer to genetically susceptible recipient mice. As will be described below, EAE can also be induced by the adoptive transfer of transgenic, myelin antigen-reactive, T cells.

Immunization of C57BL/6J (B6) mice with $MOG_{[35–55]}$ is a popular model of EAE induction, in part because of the ready availability of genetically modified strains on the B6 background (34). These mice typically develop an ascending paralysis that is either monophasic or chronically non-remitting (35), which does not reflect the full clinical course of MS. By contrast, immunization of SJL/J mice with $PLP_{[139–151]}$ induces a RR disease pattern characterized by epitope spreading of the immune response to secondary myelin antigens (36). Furthermore, as we will discuss, immunization of non-obese diabetic (NOD) background mice with $MOG_{[35–55]}$ induces a RR → SP disease course characterized by extensive demyelination and axonal loss (37). This disease course is reminiscent of that seen in close to half of MS patients (3).

In recent years, the field has welcomed the introduction of T cell receptor (TcR) transgenic (Tg) mouse strains, such as MBP-reactive Ac1-11 (38) and T/R (39), PLP-reactive 5B6 (40), and MOG-reactive 2D2 (41) and 1C6 (42), in which >90% of an animal's T cells are directed against a defined myelin epitope, thus providing a readily available source of myelin antigen-specific T cells for adoptive transfer. Although EAE develops spontaneously on the T/R Tg strain (39) and can be observed at a low spontaneous frequency in 2D2 mice, the disease typically requires robust induction of T cell activation, either via the use of adjuvants that stimulate the innate immune system or by the *ex vivo* triggering of TcR and costimulatory receptors on myelin-reactive T cells (10, 43). No single model of EAE can recapitulate the complex clinical presentation of MS in its entirety. However, depending on the immunogen used and the method of induction, one can observe paralysis of the extremities (44), ataxia (10), optic nerve inflammation (45), and weight loss (46).

Although EAE models have yielded invaluable insights into the role of autoreactive $CD4^+$ T cells in pathogenesis, they have been less forthcoming with respect to the role of $CD8^+$ T cells. This is in part because 21–23mer peptides, such as $MOG_{[35–55]}$ or $PLP_{[139–151]}$, are optimally presented by MHC class II molecules. Nevertheless, Ford et al. demonstrated that adoptive transfer of $CD8^+$ T cells from $MOG_{[35–55]}$-immunized B6 mice into severe combined immunodeficient (SCID) recipients resulted in severe EAE, and found that the pathogenic $CD8^+$ T cells recognize a minimal core epitope $MOG_{[37–46]}$ in the context of class I $H\text{-}2D^b$ (47). Notably, $CD8^+$ T cells from $MOG_{[35–55]}$-immunized NOD mice elicited reactivity toward a core epitope $MOG_{[39–47]}$ (48).

Early models, involving the transfer of ectopic Ag-specific T $CD8^+$ T cells to Tg mice expressing the ectopic Ag in CNS tissue, supported a role for $CD8^+$ T cells in CNS inflammation (49). More recently, models with $CD8^+$ T cells directed at myelin Ag have been described, such as Tg mice (strain 8.8) that express a MHC class I-restricted TcR specific for $MBP_{[79–87]}$ (46). Infection of these mice with a recombinant vaccinia virus encoding MBP efficiently induced EAE. Intriguingly, wild-type (WT) vaccinia virus induced EAE in 8.8 mice as well. The effect appeared to be due to the expression of endogenous TcR chains, as WT virus did not cause EAE in 8.8 × $Rag1^{-/-}$ mice and as $CD8^+$ T cells co-expressing 2 distinct β-chains (Vβ8 and Vβ6) were detected in WT virus-infected mice. Their findings suggest a cellular mechanism by which dual antigen-specific $CD8^+$ T cells can be initially activated in response to virus and then induce damage to CNS tissue, and thus reveal a potential mechanism by which viral infection can trigger $CD8^+$ responses directed against myelin. More recently, a Tg strain (BG1) was described that carries $CD8^+$ T cells specific for astrocytic glial fibrillary acidic protein (50). These mice spontaneously develop a RR disease course characterized by lesions in both CNS white and gray matter. Intriguingly, B6 mice adoptively transferred with BG1 $CD8^+$ T cells develop atypical EAE and suffer from weight loss upon inoculation with Vac-GFAP, in a manner reminiscent of Vac-MBP-inoculated 8.8 mice (46). However, WT Vac does not induce symptoms in BG1 mice, indicating that this phenotype was not caused by the expression of endogenous TcR β-chains (50). The relevance of $CD8^+$ T cells to virally induced CNS autoimmune pathology is further supported by Pirko et al., who infected B6 mice with Theiler's murine encephalomyelitis virus and found that the presence of T1 "black holes" on MRI – a classic sign of chronic and irreversibly damaged lesions – correlated with the accumulation of $CD8^+$ T cells (51). As epitopes 35–55 of MOG are located in its extracellular domain, in the future it would be interesting to examine whether MHC class I-restricted peptides derived from myelin-associated glycoprotein (MAG) or PLP, both of which have extracellular domains (52), could induce $CD8^+$ T cell-driven EAE.

NOD MICE AS A MODEL FOR AUTOIMMUNE DISEASE

Type 1 diabetes (T1D) is an autoimmune disease initiated by the infiltration of mononuclear cells into the pancreatic islets of Langerhans (insulitis). This is followed by autoreactive T cell-mediated destruction of insulin-producing pancreatic β-cells (53). T1D and MS have been linked epidemiologically. A large-scale Danish study found that T1D patients had a threefold-greater risk of developing MS (54), and a study of Sardinian MS

patients found that they were approximately threefold more likely to have T1D than their healthy siblings (55). Indeed, MS and T1D share common etiological factors, such as increased incidence at northern latitudes and among individuals born in springtime (56). Furthermore, MS patients have islet-antigen-reactive T cells in their repertoire, whereas T1D patients have T cells that respond to myelin-derived epitopes (57). Taken together, these findings suggest that MS and T1D may share common pathogenic mechanisms.

The NOD strain has been used as a model for T1D for over three decades (58). NOD mice spontaneously develop T cell responses to the β-cell antigen GAD65, resulting in insulitis and subsequent β-cell destruction (59). NOD mice can also develop self-reactive inflammatory responses to a variety of tissues, and EAE can be induced in these animals upon active immunization with $MOG_{[35–55]}$ (60, 61). More than 20 non-MHC genetic loci (termed *Idd*) have been identified that can contribute to T1D pathogenesis on the NOD strain (62). In an elegant series of experiments, Encinas et al. immunized a series of congenic NOD mice carrying B6-derived *Idd* loci with $MOG_{[35–55]}$ (60). They found that NOD mice carrying B6-origin *Idd3* developed milder EAE than WT NOD mice. In contrast to WT NOD, B6-*Idd3* mice did not develop chronic disease, suggesting that differences in this genetic region were potentially responsible for the development of progressive EAE in NOD mice. Thus, the immunoregulatory factor(s) encoded in the *Idd3* locus have been the subject of intense interest. The gene encoding the T cell growth factor IL-2 is located in *Idd3*, and there is a coding polymorphism between the B6 and NOD *Il2* genes (60). NOD.*Idd3* lymphocytes transcribe twofold more *Il2* than WT NOD mice. This seemingly paradoxical observation is explained by the findings that excess IL-2 in NOD.*Idd3* mice increases the function of IL-2-dependent FoxP3$^+$ regulatory T cells, which suppress pancreatic inflammation (63), and that IL-2 responsive macrophages can suppress inflammatory Th17 responses (64). IL-21 is also encoded in the Idd3 locus (65), and NOD.*Idd3* T cells produce less IL-21 than their WT NOD counterparts. This reduction in IL-21 results in defective NOD.*Idd3* Th17 responses due to both T cell-intrinsic and -extrinsic mechanisms (66). Furthermore, IL-21 plays a crucial role in CD8$^+$ T cell-driven T1D (67). Thus, the *Idd3* locus can likely restrict tissue inflammation in both the pancreas and CNS through multiple regulatory mechanisms.

EAE IN NOD MICE

Upon immunization with $MOG_{[35–55]}$, NOD mice develop RR–EAE that progresses to a chronic SP disease course characterized by loss of both axons and myelin as measured by magnetic resonance imaging (37). During the initiation phase, inflammatory cells infiltrate the perivascular and arachnoid space, and splenocyte production of IFN-γ, TNF-α, and IL-6 is enhanced. By contrast, IL-17 is upregulated in re-stimulated splenocytes during relapses that follow the initiation phase. These relapses are characterized histologically by mononuclear cell infiltration into the white matter and demyelination (68). However, CNS-resident cells, such as microglia and astrocytes, also participate in the transition from RR to SP phase in NOD–EAE, with reactive gliosis being present from the early stage of the disease (69).

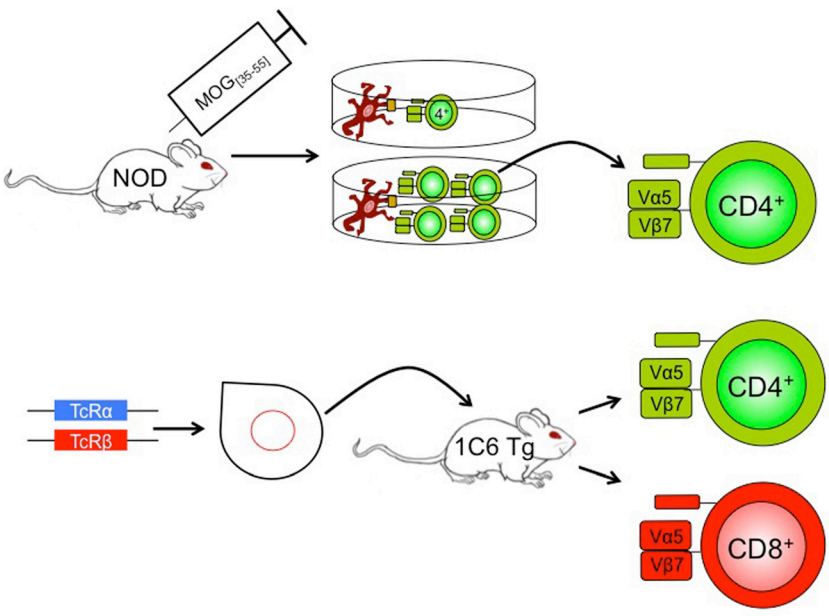

FIGURE 1 | Generation of 1C6 mice. A NOD mouse was actively immunized with $MOG_{[35–55]}$. CD4$^+$ T cells were isolated from the immunized mouse and were re-stimulated at one T cell per well with antigen-presenting cells plus $MOG_{[35–55]}$. The T cell receptor of an expanding clone was sequenced (Vα5Jα22; Vβ7Dβ2Jβ2.7), cloned, and injected into NOD pronuclei to derive 1C6 transgenic mice. The resulting mice possessed both CD4$^+$ and CD8$^+$ T cells that were $MOG_{[35–55]}$ reactive. Tg, transgenic; TcR, T cell receptor.

Both CD8$^+$ and CD4$^+$ T cells appear to play important roles in mediating EAE on the NOD background. NOD mice deficient in key regulators of CD4$^+$ T cell function show alterations in their susceptibility to EAE (70, 71). Immunization with MOG$_{[35–55]}$ causes the expansion of an IFN-γ$^+$ CD8$^+$ T cell population in NOD mice that recognize the core epitope MOG$_{[39–47]}$ (48). Our preliminary data reveal the infiltration of inflammatory effector CD44^{hi} CD8$^+$ T cells into the CNS upon active immunization with MOG$_{[35–55]}$. These CD8$^+$ T cells display a Tc1 phenotype with the production of proinflammatory cytokines, such as IFN-γ, TNF-α, and IL-2 (unpublished).

1C6 TCR TRANSGENIC MICE

Recently, Anderson et al. generated a novel TcR transgenic mouse model on the NOD background, using the TCRα and TCRβ chains of a MOG$_{[35–55]}$ CD4$^+$ clone (42). Surprisingly, the resulting strain (named 1C6) possesses both CD4$^+$ and CD8$^+$ T cells with specificity for MOG$_{[35–55]}$ (**Figure 1**), with the proportion of CD4$^+$/CD8$^+$ T cells in peripheral lymphoid tissues being similar to that seen in WT animals. The 1C6 transgenic strain is thus the first to have both myelin-reactive CD4$^+$ and CD8$^+$ T cells, allowing us to interrogate the relative contribution of both cell types to EAE. Active immunization of 1C6 mice with MOG$_{[35–55]}$ induces a RR to SP disease as seen in NOD mice. Furthermore, 1C6 mice develop spontaneous EAE and optic neuritis at a frequency of about 2%. Adoptive transfer of 1C6 CD4$^+$ T cells, CD8$^+$ T cells, or both together, to lymphocyte deficient NOD. Scid mice, followed by immunization with MOG$_{[35–55]}$, results in the development of EAE. Both the incidence and severity of EAE were lower in 1C6 CD8$^+$ T cell transfer recipients when compared to mice receiving 1C6 CD4$^+$ T cells alone or 1C6 CD4$^+$ plus CD8$^+$ T cells. It remains to be seen whether immunization with the class I-restricted MOG$_{[39–47]}$ peptide (48) could induce disease of greater incidence and/or severity in NOD.Scid mice reconstituted with 1C6 CD8$^+$ T cells given that the 21-mer MOG$_{[35–55]}$ peptide is optimally presented by MHC class II molecules. 1C6 CD8$^+$ T cells did have the capacity to induce optic neuritis; furthermore, they produced IFN-γ and granzyme B, and degranulated, in response to MOG$_{[35–55]}$ (42).

Importantly, the 1C6 model will enable us to distinguish between the molecular pathogenesis of CD4$^+$ versus CD8$^+$ T cell-driven CNS autoimmunity. CD4$^+$ and CD8$^+$ T cells express many of the same effector molecules; it is therefore difficult to draw conclusions on the relative contribution of the two cell types based on active immunization of whole-animal gene knockouts. Furthermore, T-cell-specific transgenic deleter strains, such as *Lck*-Cre and *CD4*-Cre, target gene expression in both CD4$^+$ and CD8$^+$ T cells (72, 73). To address this gap in the field, we have developed an EAE induction protocol in which T cells are isolated from 1C6 mice, are stimulated and differentiated *ex vivo* into defined effector subsets (Th1 or Th17 CD4$^+$; Tc1 or Tc17 CD8$^+$), and are then adoptively transfer to NOD.Scid mice (unpublished data). Using retrovirally mediated gene transduction, we can now manipulate the expression of a target gene in 1C6 CD4$^+$ T cells, CD8$^+$ T cells, or both concomitantly, to assess in which cell type the molecule of interest exerts its effects (**Figure 2**). This will allow us to ascertain in which T cell compartment a given immune regulatory molecule exerts its function, in a mouse model that can recapitulate both the relapsing/remitting and progressive phases of MS.

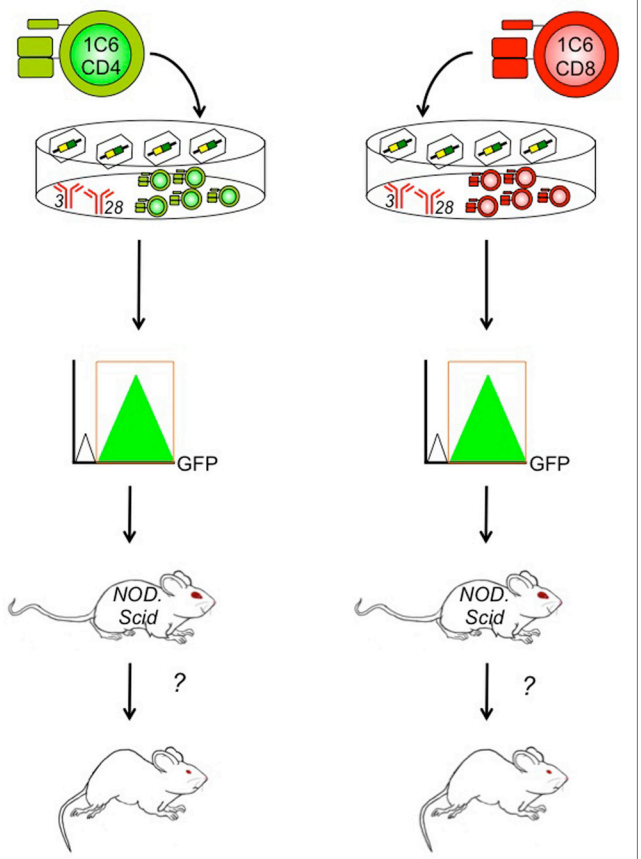

FIGURE 2 | Determining the contribution of specific genes to CD4$^+$ and/or CD8$^+$ T cell-driven EAE using the 1C6 model. CD4$^+$ (left) and/or CD8$^+$ T cells (right) are isolated from the peripheral lymphoid tissue of 1C6 mice. They are stimulated *ex vivo* using plate-bound anti-CD3 and anti-CD28 monoclonal antibodies that mimic the physiological signals required for T cell proliferation. They are coincidentally transduced with gene-specific retroviruses that can augment or knock down expression of the candidate molecule. Transduced 1C6 T cells are purified using high-speed cell sorting based on expression of retrovirally encoded bicistronic reporters, such as GFP. They are then transferred to NOD.Scid mice that are assessed for the development of EAE. This design can help us isolate the function of candidate T cell regulatory molecules to CD4$^+$ T cells, CD8$^+$ T cells, or both, in the context of MS-like disease.

CONCLUSION

Several decades' worth of evidence from animal models have supported the idea that CD4$^+$ T cells are the chief drivers of inflammation in MS. However, histopathological and clinical findings from human patients indicate that CD8$^+$ T cells are key players as well. Indeed, several popular therapeutic reagents that are believed to subvert CD4$^+$ T cell function in MS – namely, interferon-β, natalizumab, and alemtezumab – could also

exert effects on $CD8^+$ T cells. Furthermore, to date, no effective treatments exist for progressive MS, and there are indications that $CD8^+$ T cells play a role in tissue damage during this disease state (23). Thus, targeting autoreactive $CD8^+$ T cells could present an attractive mechanism by which to treat progressive MS.

The 1C6 mouse model will allow us to examine this possibility. As these mice possess both $CD8^+$ and $CD4^+$ myelin-reactive T cells, we can address both the role of $CD8^+$ T cells in CNS autoimmunity and the pathophysiology of progressive MS. In particular, genetic or pharmacological manipulation of 1C6 T cells permits us to interrogate the function of candidate genes or signaling pathways in both $CD4^+$ and $CD8^+$ T cell-driven EAE, simultaneously or separately, in the same animal. Such an approach could ultimately help us to design and validate novel therapeutics for the betterment of MS patients.

AUTHOR CONTRIBUTIONS

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Evidence from human and animal studies: pathological roles of CD8+ T cells in autoimmune peripheral neuropathies

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Autoimmune peripheral neuropathies such as Guillain-Barre Syndrome (GBS) and chronic inflammatory demyelinating polyneuropathy (CIDP) affect millions of people worldwide. Despite significant advances in understanding the pathology, the molecular and cellular mechanisms of immune-mediated neuropathies remain elusive. T lymphocytes definitely play an important role in disease pathogenesis and CD4+ T cells have been the main area of research for decades. This is partly due to the fact that the most frequent animal model to study autoimmune peripheral neuropathy is experimental allergic neuritis (EAN). As it is induced commonly by immunization with peripheral nerve proteins, EAN is driven mainly by CD4+ T cells. However, similarly to what has been reported for patients suffering from multiple sclerosis, a significant body of evidence indicates that CD8+ T cells may play a pathogenic role in GBS and CIDP disease development and/or progression. Here, we summarize clinical studies pertaining to the presence and potential role of CD8+ T cells in autoimmune peripheral neuropathies. We also discuss the findings from our most recent studies using a transgenic mouse line (L31 mice) in which the T cell co-stimulator molecule B7.2 (CD86) is constitutively expressed in antigen presenting cells of the nervous tissues. L31 mice spontaneously develop peripheral neuropathy, and CD8+ T cells are found accumulating in peripheral nerves of symptomatic animals. Interestingly, depletion of CD4+ T cells accelerates disease onset and increases disease prevalence. Finally, we point out some unanswered questions for future research to dissect the critical roles of CD8+ T cells in autoimmune peripheral neuropathies.

Keywords: Guillain-Barre syndrome, chronic inflammatory demyelinating polyneuropathy, CD8 T cells, macrophages, cytokines, co-stimulatory molecules, animal models

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INTRODUCTION

Autoimmune peripheral neuropathy, in the broadest sense, refers to a range of clinical syndromes mediated by aberrant immune response against self-antigens derived from peripheral nervous tissues including motor, sensory, and autonomic nerves. Guillain-Barré Syndrome (GBS) and chronic inflammatory demyelinating polyneuropathy (CIDP) are prototypical autoimmune peripheral

neuropathies. The common incidence is about 1.6/100,000/year with a prevalence of 6–8.9/100,000 (1, 2). Clinically, GBS and CIDP patients present weakness, areflexia, and sensory deficits. Characterized by an acute and sudden onset, GBS is the most common cause of acute flaccid paralysis and represents a serious neurological emergency with 4% of GBS patients dying within the first year (3). CIDP is characterized by either chronic progressive, stepwise progressive, or relapsing weakness (1, 4). Although the majority of CIDP patients initially improve with immunosuppressive treatment, the relapse rate is about 50% (5). Over the last three decades, even with improved therapeutic options, both diseases still carry a severe prognosis as they are associated with significant mortality and sustained disability with 28% of patients requiring an assistive device to ambulate (6). The pathological changes in GBS and CIDP patients are characterized by inflammatory infiltration of both T cells and macrophages into peripheral nerves, as well as areas of demyelination, with or without axonal damage in peripheral nervous system (PNS) (4, 7).

Both humoral and cellular immune responses against antigen epitopes of Schwann cells, myelin and/or axons have been postulated to be responsible for autoimmune peripheral neuropathy. However, the extent and the details of the cascade leading to the peripheral nervous system damage are incompletely defined. Apart from the humoral immune response, T cells play a decisive role in the pathogenic sequence of immune-mediated nerve damage. Activated $CD4^+$ T cells may operate by recruiting macrophages to exert damage on peripheral nerve tissue or may help B cells to produce antibodies against peripheral nerve components, thereby inducing complement activation. In addition to mediating local inflammatory response as $CD4^+$ T cells do, activated $CD8^+$ T cells, acting as cytotoxic effector cells, can contribute directly to the damage of both myelin and axons.

From recent research, $CD8^+$ T cells emerged as important players in multiple sclerosis (MS) pathogenesis. Increasing evidence indicates that $CD8^+$ T cells predominate and outnumber $CD4^+$ T cells in all MS lesions, regardless of disease stages (8). Furthermore, depletion of $CD4^+$ T cells did not show any therapeutic effect in MS patients (9), but when all T cells were targeted, a significant reduction in MS relapse was observed (10). In fact, infiltration of nervous tissues by $CD8^+$ T cells has been observed in various classical neurodegenerative disorders, such Amyotrophic Lateral Sclerosis, Alzheimer disease, and Parkinson's disease (11–13). However, due to the rare incidence of the diseases and the shortage of appropriate animal models, our understanding on the relative contribution of $CD4^+$ and $CD8^+$ T cells in the pathogenesis of GBS and CIDP is very much limited. Because experimental allergic neuritis (EAN), the most used animal model for the study of autoimmune peripheral neuropathy, is driven mainly by $CD4^+$ T cells (14, 15), $CD4^+$ T cells have been the main area of research for decades. In this perspective article, we will summarize clinical studies pertaining to the presence and potential role of $CD8^+$ T cells in autoimmune peripheral neuropathy. We will also discuss the findings from various current available animal models. Emphasis will be given to our most recent studies using B7.2 transgenic (L31) mice where animals develop spontaneous autoimmune peripheral neuropathy and $CD8^+$ T cells are the major players (16). L31 mice provide a unique opportunity to investigate underlying mechanisms of $CD8^+$ T cell-mediated autoimmune peripheral neuropathy.

PUTATIVE RELEVANCE OF $CD8^+$ T CELLS IN AUTOIMMUNE PERIPHERAL NEUROPATHY: EVIDENCE FROM GBS AND CIDP PATIENTS

In humans, two-thirds of GBS are preceded by infections with viruses or bacteria especially *cytomegalovirus* (CMV) and *Campylobacter jejuni* (17, 18). Although not as frequent as in GBS, the onset and the relapse of CIDP can also be triggered by infections or immunization (5, 19). Biochemical and histopathological evidence suggests the potential involvement of T cells in the pathogenesis of these autoimmune peripheral neuropathies. The levels of soluble interleukin-2 receptors (20) and the frequencies of activated T cells were elevated in the serum of GBS (21) and CIDP (22) patients. Multifocal infiltration of lymphocytes were also found in post mortem and biopsy specimens of most GBS and CIDP cases (23). However, the specific targets and actors ($CD4^+$ and/or $CD8^+$ T cells) of the immune response remain uncertain. Although some discrepancies exist, several data imply putative relevance of $CD8^+$ T cells in the pathogenesis of autoimmune peripheral neuropathies. For instance, the mean proportion of $CD8^+$ T cells significanly increased in the blood GBS patients compared to the control group of healthy donors (24); $CD8^+$ T cells were found to outnumber $CD4^+$ T cells at the lesion sites of CIDP (25) and GBS (26) patients. Interestingly, Sindern et al. (27) revealed that the composition of the T cell subpopulations in the blood of GBS patients depends in particular on the nature of the proceeding infection. They found that in GBS patients with evidence of recent CMV infection, the proportion of $CD8^+$ T cells were abnormally high whereas the proportion of $CD4^+$ T cells were abnormally low; in contrast, $CD8^+$ T cells were abnormally low in GBS patients with evidence of *C. jejuni* infection. Furthermore, they reported an increase of activated cytotoxic/suppressor T cells ($CD8^+CD38^+$) in progressive and plateau phases of GBS, which was normalized in the recovery phase. More direct evidence in supporting pathogenic contribution of $CD8^+$ T cells in CIDP was provided by two recent studies. Mausberg et al. (28) reported that $CD8^+$ T cells exhibited a much broader clonal activation pattern than $CD4^+$ T cells in the blood of CIDP patients. In addition, IVIg treatment, which was beneficial to patients, normalized the distorted $CD8^+$ T cell repertoire and reduced the number of highly activated Vβ elements within the $CD8^+$ T cell population. Another study by Schneider-Hohendorf et al. (29) reported that T cells in CIDP biopsies showed strong monoclonal and oligoclonal restrictions in their T cell repertoire, which were reflected in the patients' blood $CD8^+$ T cell pool. Taken together, these data support the hypothesis of an antigen driven, $CD8^+$ T cell-mediated attack against nerve tissues, even if the target (antigen) of this immune response still remains to be identified.

CD4+ AND CD8+ T CELLS IN AUTOIMMUNE PERIPHERAL NEUROPATHY: INSIGHTS FROM ANIMAL MODELS

CD4+ T Cells in EAN and NOD B7.2KO Mice

First described in 1955, EAN can be induced either by immunization with myelin peptide or by active transfer of antigen sensitized T cells in rats, mice, rabbits, and guinea pigs (14, 15). Many of our current knowledge of immune-mediated mechanisms of demyelination were primarily based on studying EAN, the animal model for human GBS and CIDP (30). EAN resembles many of the clinical and electrophysiological aspects of human GBS/CIDP. The pathological hallmark of EAN consists of infiltration of peripheral nerves by lymphocytes, predominantly CD4+ T cells, and macrophages with segmental demyelination and some axonal damage. Previous studies have shown that EAN belongs to the group of CD4+ T cell-mediated autoimmune diseases that can be transferred to naïve animals by CD4+ P2-reactive T cells (31). While EAN has provided valuable information regarding immunopathogenic mechanisms, it has been criticized for its artificial manipulation resulting in the bias towards CD4+ T cells. Development of spontaneous autoimmune peripheral neuropathy in B7.2 deficient NOD mice (32) introduced another tool for mechanistic studies. The individual role of CD4+ T cells vs. CD8+ T cells in the pathogenesis of the disease has been carefully investigated. While transfer of purified CD4+ T cells isolated from affected animals induced the disease in NOD·SCID mice, the transfer using preparation of CD4+ T-depleted cells failed in triggering the disease (32). On the other hand, spontaneous autoimmune neuropathy was rapidly induced in NOD·SCID mice after transfer of CD8+-depleted preparation from affected mice (32). These results highlight the necessary and sufficient role of CD4+ T cells in the effector phase of this autoimmune disease model.

CD8+ T Cells in B7.2 Transgenic L31 Mice

We recently established a novel and clinically relevant animal model of spontaneous autoimmune peripheral polyneuropathy in which CD8+ T cells play a critical role (16). Transgenic mice with constitutive expression of the co-stimulator B7.2 were originally generated by placing the murine B7.2 cDNA under the transcriptional control of a MHC-I promoter and an immunoglobulin enhancer (33). Among multiple transgenic lines, Line 31 (L31) mice spontaneously developed neurological symptoms at the age of 4–6 months (34). Massive infiltration of CD8+ T cells and B7.2 high expression macrophages were found in inflamed nerves (16). Deficiency in CD4+ T cell generation accelerated disease onset and increased disease prevalence (35).

L31 mice and L31mice deficient in CD4+ T cells (L31/CD4KO) exhibit motor and sensory deficits, including weakness and paresis of limbs, numbness to mechanical stimuli, and hypersensitivity to thermal stimulation. Stereotypic pathological changes, including demyelination, axonal damage, and infiltration of CD8+ T cells and macrophages were found not only in sciatic nerve of symptomatic L31 mice (**Figures 1A,B**) but also in cranial nerves, e.g., facial (**Figure 1C**) and trigeminal (**Figure 1D**) nerves. However, it is worth noting that only limited inflammatory reaction and demyelination were observed in the spinal cords of diseased transgenic mice (16), which is consistent with reports from GBS patients (36). In addition, there was no tissue destruction or immune cell infiltration in other organs (34). The mechanism by which the autoimmune cascade is initiated in L31 mice remains elusive. We hypothesize that PNS selectivity could be determined, in one hand, by the distribution of B7.2 expression. In this model, B7.2 expressed constitutively only on resident microglia and macrophages of the nervous system, but not on APCs of any other tissues. We have shown that this B7.2 expression on the nervous tissues is an absolute requirement for susceptibility to disease development (34). On the other hand, as we detected immune infiltrates in pre-symptomatic animals in the DRG and spinal roots where the blood nerve barrier is fenestrated under physiological conditions (16), it implies that the virtual absence of the barrier in these peripheral nerve structures allows CD8+ T cells, during immune patrolling, to encounter resident macrophages overexpressing B7.2. The CD8+ T cells are reactivated by PNS self-antigens presented by resident macrophages. They are responsible to initiate PNS antigen specific autoimmune response. Although molecular targets for the CD8+ T cells are not fully defined, MHC class I is expressed constitutively on rodent Schwann's cells and they are able to activate CD8 T cells (37). MHC class I upregulation has been observed on Schwann's cells in sural nerves of GBS patients (26, 38). We are currently assessing MHC I expression by the cellular elements of the peripheral nerves of L31 mice. The demonstration of direct contact between T cells and MHC class 1–expressing target cells (neurons and/or Schwann's cells) would be a strong evidence for a CD8+ T cell–mediated attack.

In the blood of diseased L31 mice, the ratio of CD8+/CD4+ T cells was about five times higher than that in wild type (WT) mice, which is mainly due to the significant increase of absolute CD8+ T cell numbers in the circulation (**Figure 2A**). Furthermore, these CD8+ T cells exhibited activated phenotypes, as the majority of these CD8+ T cells expressed high levels of CD44. A greater part circulating $CD8^+CD44^{hi}$ T cells exhibited CD62-L^{lo} phenotype (**Figure 2B**) that is different from the small amount of central memory CD8+ T cells ($CD44^{hi}CD62\text{-}L^{hi}$) in the blood of WT littermates, suggesting that CD8+ T cells in symptomatic L31 mice had already encountered antigens. The increase of $CD8^{+/}CD44^{hi}/CD122^{hi}$ population in the blood (**Figure 2B**) entailed a group of effecter CD8+ T cells, while the increase of $CD8^+/CD44^{hi}/CD122^{lo}$ cell population (**Figure 2B**) suggested the existence of atypical memory CD8+ T cells such as those found during chronic viral infections which also exhibit low levels of CD122 expression (39).

In affected sciatic nerves of L31 mice, T cell infiltration was skewed strikingly towards CD8+ T cells (**Figure 2C**). These infiltrated CD8+ T cells displayed activated phenotypes characterized by CD62-$L^{lo}/CD43^{hi}$ expression, but did not exhibit high levels of CD25, which is usually considered as an early T cell activation marker (**Figure 2D**). Furthermore, the data from the diseased nerves of L31/CD4KO revealed a significant increase of cytokines (IFNγ, TNF) and chemokines (CXCL9, CXCL10) (**Figure 2E**). These cytokines/chemokines released by CD8+ T cells and/or macrophages are essential in promoting recruitment

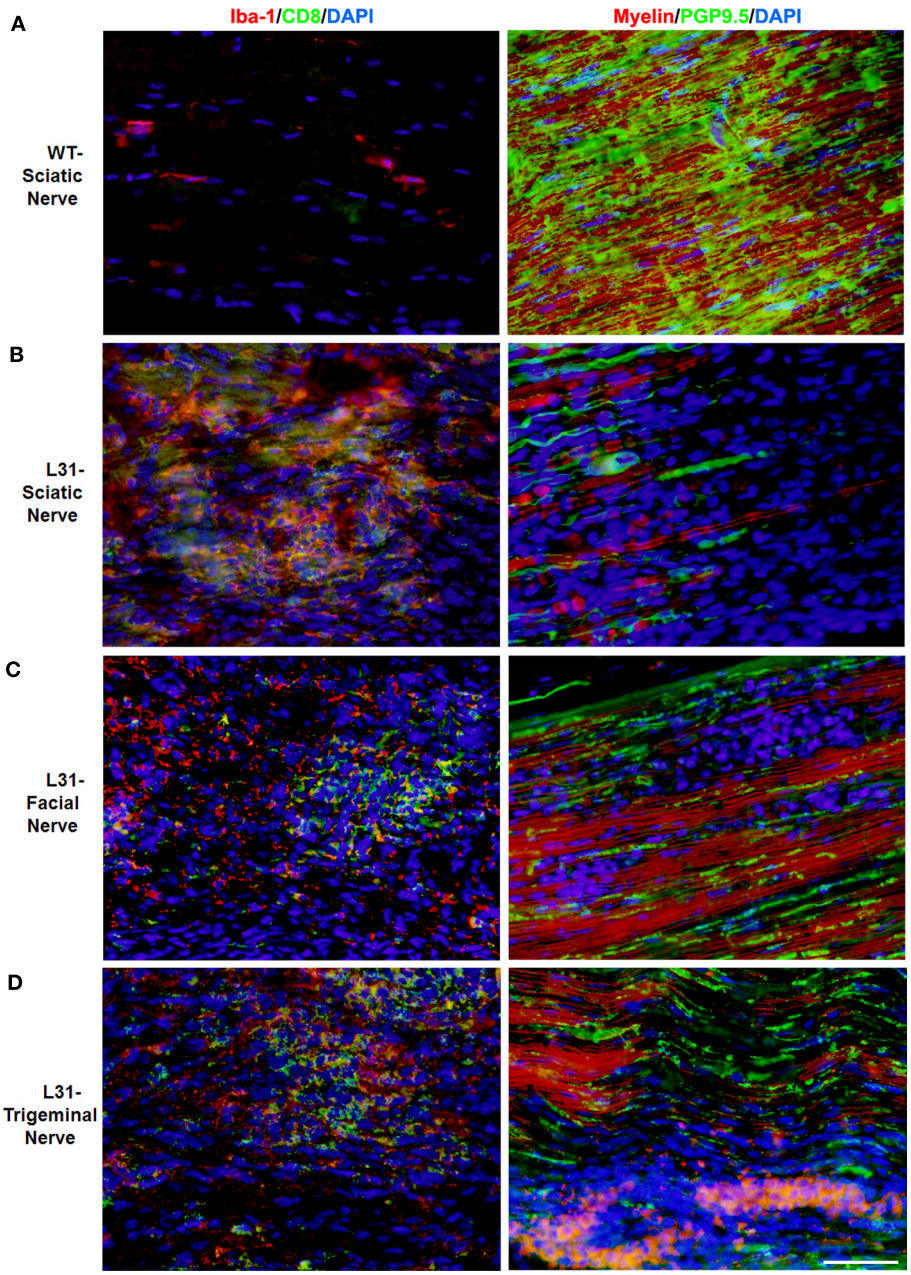

FIGURE 1 | Pathological changes in peripheral nerves of L31 mice after disease onset. Immune cell infiltration was revealed by immunohistochemistry analysis using antibodies against CD8 and Iba-1 (macrophages), while demyelination and axonal damage were detected with antibodies against PGP9.5 and fluoromyelin, respectively. **(A)** Only few resident macrophages and no CD8+ T cells were detected in sciatic nerves of wild type (WT); there were massive infiltration of Iba-1+ macrophages and CD8+ T cells as well as severe demyelination and axonal damage in sciatic **(B)**, facial **(C)**, and trigeminal nerves **(D)** of symptomatic L31 mice (L31). Scale bar: 50 μm.

and interactions between different subsets of immune cells. The fact that L31 mice deficient in IFNγ receptors were completely resistant to disease development (35) supports the requirement of IFN-γ, mainly derived from CD8+ T cells in disease pathogenesis.

Macrophages are also abundantly accumulating in peripheral nerves of symptomatic L31 mice. It remains to determine whether and to what extent these cells are required for disease pathogenesis. We are particularly interested in assessing the critical role of macrophage phagocytosis in Ag presentation, disease initiation and macrophage-associated oxidative burst-mediated damage of the nervous tissue. Other mechanisms involving macrophages, such as antibody-dependent cell-mediated-cytotoxicity could also be of relevance.

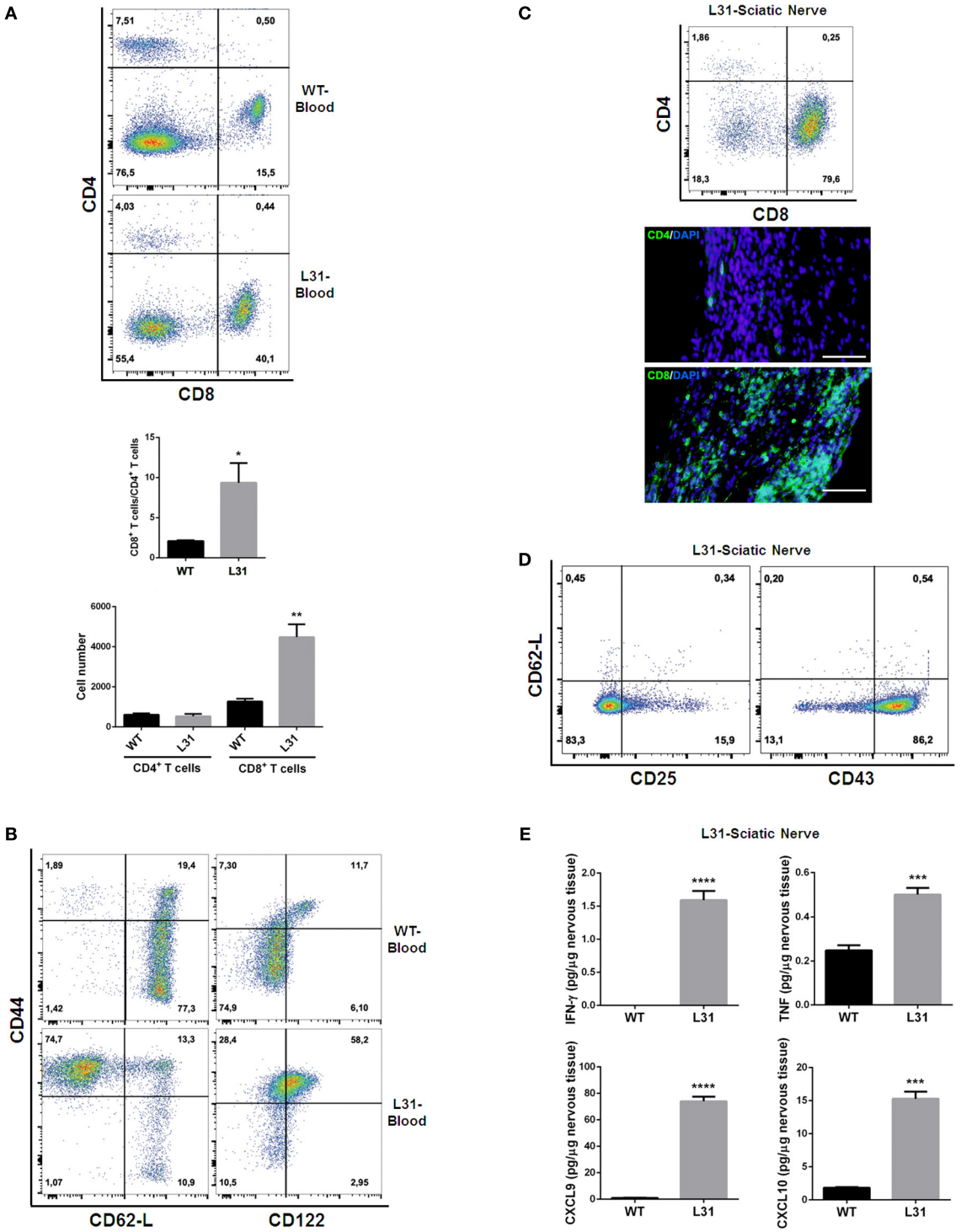

FIGURE 2 | T cell phenotypes in symptomatic L31 mice. Flow cytometry analysis revealed that the ratio of CD8+ T cells vs. CD4+ T cells was significantly higher in the circulating system of symptomatic L31 mice (L31) by comparing with that in wild type (WT) mice, this enhancement was derived from the dramatically increase of CD8+ T cell absolute numbers in the blood of L31 mice **(A)**. CD8+ T cells from the blood of symptomatic L31 mice exhibited memory/effector phenotypes, which were determined by $CD44^{hi}CD62\text{-}L^{lo}CD8^{+}$, as well as the expression levels of CD122 in $CD44^{hi}CD8^{+}$ population **(B)**. In parallel, both flow cytometry and immunohistochemistry analyses demonstrated that infiltration of T cells in affected sciatic nerves was predominated by CD8+ T cells **(C)**, Scale bar: 50 μm. The majority of infiltrated CD8+ T cells have low expression of CD62-L and high expression of CD43, but without the high levels of CD25 expression, which is a typical phenotype of activated T cells **(D)**. Cytokine/chemokine expression was analyzed using LUMINEX assay. The results indicated a significantly increase of both pro-inflammatory (IFNγ and TNF) and chemokines (CXCL9 and CXCL10) in affected sciatic nerves of symptomatic L31 mice **(E)**. $n = 3$–4/group, $^{***}p < 0.001$; $^{***}p < 0.0001$.

To summarize, the massive infiltration of T cells in diseased nervous tissues of L31 mice is $CD8^+$, not $CD4^+$ T cells. $CD8^+$ T cells in the blood and in the nerves of diseased L31 display memory/effector phenotypes. Lack of T cells prevents disease development in L31 mice (34). L31 mice deficient in $CD4^+$ T cells have an accelerated disease onset and an increased disease penetrance (35). Interestingly, L31 mice have an increased number of Foxp3-expressing $CD4^+$ T cells in their lymphoid organs, which display an activated phenotype (unpublished data). Initiation of disease requires Ag specific $CD8^+$ T cell activation since L31 mice with an almost unique TCR specificity expressed by $CD8^+$ T cells (L31/OT-1 mice) never developed disease (35). Hence, $CD8^+$ T cells arise as key players in disease pathogenesis in L31 B7.2 transgenic mice. Different from EAN where $CD4^+$ T cells are the main culprits in mediating neuropathy, L31 mice provide a unique opportunity to investigate the involvement of $CD8^+$ T cells in autoimmune peripheral neuropathy. In L31 mice, $CD4^+$ T cells may function as regulators in the disease pathogenesis.

LESSONS FROM ANIMAL MODELS FOR HUMAN AUTOIMMUNE PERIPHERAL NEUROPATHY

Whether induced or spontaneous, no single animal model perfectly mimics a human disease. These models represent only particular aspects of a complex human autoimmune disease, and not all animal response patterns are replicated in the human immune system. Among the most frequently used animal models of autoimmune peripheral neuropathy that we discussed above, EAN and NOD B7.2KO mice have provided valuable information in the principles of $CD4^+$ T cell-mediated autoimmunity and in the development of clinically applicable immunotherapies. However, caution should be taken in the interpretation of results, especially, EAN, which is induced using complete Freund's adjuvant. L31 mice offer a unique and excellent tool to investigate $CD8^+$ T cell-mediated spontaneous autoimmmune neuropathy. As epidemiological studies have provided strong evidence on the involvement of viral infection in the onset of GBS and CIDP, this model might be essential in elucidating their viral etiology. However, L31 mice do not recover without intervention, suggesting this model is not appropriate for examining the mechanism of self-recovery in GBS patients. On the whole, autoimmune peripheral neuropathy is neither a single disease nor a disease stemming from one etiology; instead these are syndromes with multiple variants and very complex mechanisms. Thus, while recognizing the limitations of extrapolating findings from each animal model to human disease, what is important is the integration of the diverse data generated from different models into coherent framework for understanding the entirety of autoimmune peripheral neuropathy.

FUTURE DIRECTIONS: QUESTIONS NEED TO BE ADDRESSED

Although the potential contribution of $CD8^+$ T cells in autoimmune neuropathy, including MS, GBS, and CIDP, has brought considerable attention in recent years, our knowledge on $CD8^+$ T cells is still very much limited. Why do so many people have viral or bacterial infections, but only a small population develop GBS/CIDP? Where is the site of the abnormality that initiates the disease process? What are the key molecules or cells that determine the fate of the disease? Why do some patients recover spontaneously, while others do not? Whether and to what extent $CD8^+$ T cells are engaged in different stages of the diseases, and whether $CD8^+$ T cells can be targeted for disease prevention and effective treatment? Use of appropriate animal models should help answer these questions and decipher the role of $CD8^+$ T cells in human autoimmune peripheral neuropathy.

STATEMENT ON ANIMAL ETHICS

All experiments were in accordance with the guidelines of the Canadian Council on Animal Care, and approved by the animal care committee of McGill University (Permit #5533).

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OPINION
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γδ T cells and NK cells – distinct pathogenic roles as innate-like immune cells in CNS autoimmunity

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Introduction

Multiple sclerosis (MS) is a chronic inflammatory, demyelinating disease that affects the central nervous system (CNS) resulting in progressive cognitive decline and physical disability. Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS that has been used to understand the cellular and molecular mechanisms underlying CNS inflammation and autoimmunity. Since the discovery of IL-17-sereting $CD4^+$ T cells (Th17 cells) over 10 years ago, these cells have been the main focus of attention as mediators of pathology in MS and EAE (1, 2). However, in recent years evidence has emerged that lymphocytes with innate-like properties are potent producers of IL-17 and related pro-inflammatory cytokines (3–6). γδ T cells, NKT, and innate lymphoid cells have been shown to be major sources of IL-17 in host control of a variety of bacterial, viral, and fungal infections. However, dysregulation of these innate-like lymphocytes can also result in severe pathology in EAE and other models of autoimmunity. The role of IFN-γ in the pathogenesis of autoimmune diseases is more controversial. Like Th17 cells, transfer of myelin antigen-specific Th1 cells can induce EAE in naïve mice (7, 8). However, IFN-γ, the signature cytokine of Th1 and natural killer (NK) cells, has been shown to inhibit the function of pathogenic Th17 cells, as well as promoting development of encephalitogenic T cells during induction of EAE (8, 9). Immunotherapeutics that suppress the induction or function of Th17 cells have proved successful in treating psoriasis, but have had more variable success in MS patients (10). Based on recent studies on the role of innate-like lymphocytes in the pathogenesis of EAE, we propose that these cells may provide more selective and improved drug targets for the treatment of MS.

$CD4^+$ T Cells

Th1 cells were originally thought to be the main pathogenic cells in MS and EAE. This was in part attributed to the fact that IL-12p40$^{-/-}$ mice were resistant to EAE, and treatment of MS patients with IFN-γ exacerbated disease (11). However, mice deficient in IFN-γ or T-bet, which lack Th1 cells, were not protected from EAE (12, 13). The discovery of IL-23 partly resolved this paradox. IL-23 and IL-12 share a common p40 chain, which associates with a separate p19 chain to make IL-23 or with a p35 chain to make IL-12. Like IL-23p40$^{-/-}$ mice, IL-23p19$^{-/-}$ mice are resistant to EAE, whereas IL-12p35$^{-/-}$ mice are susceptible (14). IL-23 was then shown to be essential in driving the induction or the expansion of IL-17-secreting $CD4^+$ T cells, which were termed Th17 cells (15–17). IL-17-producing Th17 cells proved to have a key role in inflammation and autoimmunity when they were found capable of transferring EAE to naive mice (16).

In addition to IL-17A, Th17 cells produce an array of other inflammatory cytokines, including IL-17F, GM-CSF, IL-22, IL-21, IL-26 and TNF-α (16, 18–24). Since their discovery, Th17 cells have been implicated in the pathogenesis of most common autoimmune diseases, including psoriasis, rheumatoid arthritis (RA), and MS, and in animal models of these diseases. Despite the extensive studies on Th17 cells, the relative roles of Th1 and Th17 cells in the pathogenesis of MS and other autoimmune diseases are still unclear. Data from our laboratory and others show that both Th1- and Th17-polarized T cells are capable of transferring EAE (7, 8). Furthermore, $CD4^+$ T cells secreting both IL-17 and IFN-γ are detectable in the CNS of mice with EAE (25–27). Therefore, it is our opinion that both Th1 and Th17 cell subsets play important roles in autoimmune pathology, but that there is plasticity between these T cell types and that the pathogenic function of other immune cells, especially cells of the innate immune system should not be ignored.

γδ T Cells

γδ T cells represent around 2–5% of peripheral lymphocytes and are known to play an important role in innate and adaptive immunity at mucosal surfaces. γδ T cells have been described as polyfunctional; they produce an array of cytokines, including IL-17A, IL-17F, IFN-γ, IL-10, IL-22, IL-21, GM-CSF, and TNF-α (28–31). The IL-17-producing γδ T cells share many features with $CD4^+$ Th17 cells, including expression of RORγt, IL-1R1, IL-23R, and CCR6 (32). Although γδ T cells do express a unique T cell receptor (TCR), engagement of this TCR with MHC-antigen complexes is not a prerequisite for their activation. Unlike conventional αβ T cells, cytokine stimulation alone is sufficient for activation of IL-17-secreting γδ T cells, making these cells rapid and potent mediators of inflammation (28). γδ T cells have been shown to be pathogenic in a variety of autoimmune diseases, such as EAE, collagen-induced arthritis (CIA), and most recently in EAU (33–35). Before the discovery of Th17 cells and their signature cytokine IL-17, it was assumed that early IFN-γ derived from γδ T cells was the main pathogenic cytokine driving EAE; this was in part based on the established role of IFN-γ-secreting γδ T cells in enhancing $CD4^+$ and $CD8^+$ T cell responses in anti-tumor immunity (36). However, our studies, supported by recent results from other labs, suggest that the pathogenic function of γδ T cells is mediated by their production of IL-17 and related cytokines, including IL-21 and GM-CSF (28). γδ T cells can secrete IL-17 in response to IL-1, IL-18, and IL-23 without TCR engagement, promoting the induction of Th1 and Th17 cells and amplifying their encephalitogenic function during the development of EAE (28, 37, 38) (**Figure 1**). Studies from our group have demonstrated that dendritic cells (DCs) can enhance the ability of IL-1- and IL-23-activated γδ T cells to promote IL-17 production by Th17 cells (28). Furthermore, DCs express IL-17R and secrete IL-23 in response to IL-17, which was enhanced by LPS and blocked

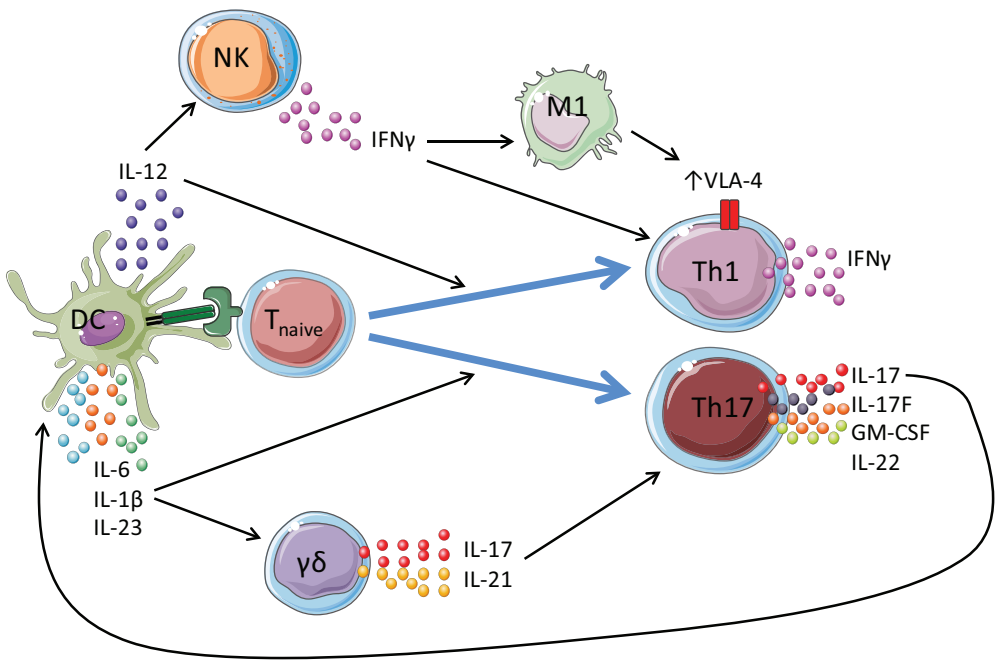

FIGURE 1 | Proposed roles for γδ T cells and NK cells in amplifying pathogenic $CD4^+$ T cell responses in EAE. Dendritic cells (DCs) are activated by TLR and NLR agonists. Antigenic peptide is presented by MHC class II molecules on DCs to the TCR on T cells. This, along with co-stimulatory signals, activates the T cell. Once activated, DCs secrete cytokines including IL-1β, IL-6, IL-23, and IL-12 that promote the polarization of naïve T cells into effector cells. IL-12 promotes the induction of Th1 cells, which are primarily IFN-γ producers. IL-1β, IL-6, and IL-23 promote the differentiation and expansion of Th17 cells, which secrete IL-17 (and IL-22, GM-CSF, IL-21) and mediate protection against extracellular pathogens, such as fungi, and are heavily implicated in the pathology of autoimmune diseases. γδ T cells secrete IL-17 and IL-21 following stimulation with IL-1β and IL-23 without TCR engagement, which act in an autocrine loop to promote further IL-17 production by Th17 cells in the development of EAE. NK cells provide an early source of IFN-γ to drive VLA-4 expression on Th1 and Th17 cells, allowing these cells to traffic from the peripheral lymphoid organs and into the CNS.

by anti-IL-17R. These findings suggest that γδ T-cell-derived IL-17 may act in a positive feedback loop involving DC activation leading to enhanced Th17 cell effector function during EAE. *In vitro* studies have also suggested a pathogenic role for γδ T cells in demyelinating diseases of the CNS, as γδ T cells are indirectly responsible for axonal demyelination through toxic destruction of oligodendrocytes, cells responsible for myelinating axons (39).

Importantly, data from our laboratory and others have shown that γδ T cells infiltrate the brain and spinal cord in large numbers during the course of EAE, where they produce IL-17 and related cytokines (28, 37, 38). Vγ4$^+$ T cells were identified as the main IL-17-producing γδ T cell in the brains of mice with EAE, but Vγ1 and Vγ6 T cells are also present (28). Vγ4$^+$ T cells are also key players in a variety of other autoimmune conditions, such as myocarditis, (40) psoriasis (41), and CIA (34). The pathogenic role of γδ T cells in EAE was demonstrated by a reduction in disease severity in TCRδ$^{-/-}$ mice (42). Furthermore, studies in the relapsing-remitting EAE model showed a significant reduction in clinical severity when mice were treated with a TCRδ depleting antibody immediately before disease onset or during the chronic phase of disease (33). In addition, experiments in the adoptive transfer model of EAE demonstrated that depletion of γδ T cells reduced clinical severity and delayed the onset of disease (43).

The pivotal role of γδ T cells in the pathogenicity of EAE is also reflected in MS, where clonal expansion of γδ T cells has been observed in the cerebrospinal fluid (CSF) of patients with recent disease onset (44). Furthermore, an increased frequency of γδ T cells have been detected in the peripheral blood of patients with MS (45) and an accumulation of γδ T cells has been described in acute brain lesions (46). Based on these findings, we propose that γδ T cells have a critical role in the active stages of both EAE and MS.

NK Cells

Natural killer cells are innate lymphocytes named for their cytolytic activity, which can control tumor growth and microbial infection. NK cells can produce the pro-inflammatory cytokines IFN-γ and TNF-α, as well as the immunosuppressive cytokine IL-10 and the growth factor GM-CSF in response to IL-12, IL-15, or IL-18 (47).

Human NK cells can be broadly separated into two types on the basis of their expression of CD16 and CD56. CD16$^+$CD56^{dim} cells express more intracellular perforin and are more efficient killers, whereas the CD16$^{dim/-}$CD56^{bright} subset produce greater amounts and a wider variety of cytokines, and are more regulatory in nature (48). The general consensus in the literature is that the CD56^{bright} NK cells play a protective role in MS. It has been reported that the ratio of CD56^{bright}:CD56^{dim} cells is higher in the CSF of MS patients relative to control subjects (49). Furthermore, the CD56^{bright} subtype is expanded in response to the MS disease modifying therapies IFN-β (50), daclizumab (51, 52) and natalizumab (53). There is also an established link between disease relapse and a decrease in the number of total circulating NK (CD16$^+$CD56$^+$) cells in peripheral blood of MS patients (54). Conversely, an increase in NK cell number and migratory capacity has been associated with remission (55). Therefore, it is possible that certain subsets of NK cells may have a role in controlling CNS inflammation in MS patients.

A potential mechanism underlying the protective effect of NK cells in MS was provided by the observation that these CD56^{bright} NK cells can kill activated, but not resting, autologous CD4$^+$ T cells by inducing apoptosis through degranulation (56). While less is known about the role of the CD56^{dim} subset in MS, the frequency of these cells in the circulation is enhanced in the progressive forms of the disease, (57) a phenomenon which also occurs with age (58). Therefore, it is possible that CD56^{dim} NK cells may contribute to neurodegeneration, however, further investigation is required to confirm this hypothesis.

Studies in the animal model EAE have generated more extensive data on the role of NK cells that has led to more controversy. The severity of EAE is enhanced in mice deficient in fractalkine receptor expression, which is required for NK cell recruitment to the inflamed CNS (59), suggesting that NK cells may play a role in limiting CNS inflammation. This is consistent with a more recent publication suggesting that a population of CNS-resident NK cells have a protective role in EAE through suppression of myelin-reactive Th17 cells (60). By contrast, IFN-γ from NK cells has also been shown to promote autoreactive Th1 responses and contribute to the pathogenesis of EAE (61).

Depletion of NK cells in EAE using either anti-NK1.1 or anti-asialo GM1, which induce apoptosis (62) or complement-dependent lysis (63), respectively, has generated conflicting reports of both exacerbation (60, 64–66) and amelioration (61, 67) of clinical disease. These discrepancies may reflect differences in the antibodies used, the depletion regimen, and a focus on disease peak. Data from our laboratory suggest that NK cells have a pathogenic role in disease induction; NK cells were found to infiltrate the CNS of mice with EAE before the onset of clinical symptoms, and depletion of these cells at this early timepoint led to a significant reduction in disease severity (8). The pathogenic role of NK cells was attributed to early IFN-γ production, as early depletion of NK cells did not affect the clinical course of EAE in IFN-γ$^{-/-}$ mice. IFN-γ from NK cells polarized macrophages to an M1 phenotype and thus conferred encephalitogenic potential on CD4$^+$ T cells by upregulating expression of the integrin VLA-4, which is required for CD4$^+$ T cell infiltration into the CNS (8) (**Figure 1**). We believe that NK cells play a critical pathogenic role in EAE by acting as an early source of innate IFN-γ in the initiation of disease. However, late in disease, IFN-γ production by Th1 cells, activated by NK cells, may have protective role through suppression of cytokine production by Th17 cells. This might explain the finding in MS patients of an association between reduced NK cells numbers and disease relapse (54) and increased NK cells and disease remission (55).

Conclusion

Understanding the Th17/IL-17 axis in both protective and dysregulated immunity has led to the development of many promising front line therapies for autoimmune diseases. However, we believe that research in this area has been too heavily focused on CD4$^+$ T cells and that further study on innate immunity

may provide vital insight into mechanisms of disease and improved therapies. Although much of the attention has been on Th17 cells, these are not the only source of the pro-inflammatory cytokine IL-17. Innate-like lymphocytes, such as γδ T cells and NK cells, provide an early source of IL-17 and IFN-γ, traffic to the CNS early during development of EAE, and provide an amplification loop for the activation of pathogenic $CD4^+$ T cells (**Figure 1**). IL-17 and IL-21 derived from γδ T cells enhances the pathogenicity of Th17 cells in EAE. Furthermore, IFN-γ derived from NK cells polarizes M1-type macrophages and enhances the encephalitogenic activity of $CD4^+$ T cells by upregulating VLA-4 expression. Treatment of MS patients with biological drugs designed to suppress the induction, migration, or function of $CD4^+$ T cells, such as natalizumab, come with an increased risk of infection, in particular progressive multifocal leukoencephalopathy (PML) (68). Given the important role of small populations of γδ T cells and NK cells in the pathogenesis of EAE, we propose that a better understanding of the activation and function of these innate-like lymphocytes and their secreted cytokines may lead to new and more selective therapeutic interventions for the treatment of MS.

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REVIEW
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The Emerging Roles of Gamma–Delta T Cells in Tissue Inflammation in Experimental Autoimmune Encephalomyelitis

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γδ (gamma–delta) T cells, a small population of unconventional T cells, have been found in central nervous system lesions of multiple sclerosis (MS) patients, but their function in disease activity is not clearly understood. Previous studies in experimental autoimmune encephalomyelitis (EAE) were inconsistent in identifying their specific roles in suppressing or promoting disease pathogenesis. Emerging advancements in the biology of γδ T cells especially in the context of their being the major initial producers of IL-17, suggested their crucial role in pathogenesis of EAE. In addition, γδ T cells express high levels of IL-23R and IL-1R, which further enhance their effector functions in the pathogenesis of EAE. Nonetheless, activated heterogeneous γδ T cells display functional dichotomy, which is crucial in determining the outcomes of tissue inflammation in EAE. In this review, we discussed recent advances in understanding the biology of γδ T cells in tissue inflammation as well as their roles in suppressing or promoting the development of EAE.

Keywords: gamma–delta T cells, Th17 cells, cytokines, inflammation, autoimmunity

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INTRODUCTION

γδ (gamma–delta) T cells comprise a small fraction (~1–5%) of the total blood lymphocytes of mice and humans and are more commonly localized in mucosal tissue and skin where they constitute a major population (up to 50%) of lymphocytes (1). The identification of an unusually rearranged γ chain of the T cell receptor (TCR) gene led to the discovery of γδ T cells (2, 3). After the identification of γδ T cells as a new subset of T cells, it became clear that these cell types, unlike their αβ (alpha-beta) T cell counterparts, possess features of both innate and adaptive immune cells (4, 5). Moreover, γδ T cells have also been recognized as non-conventional innate-like cells as they share several features of innate immune cells, such as surface expression of Toll-like receptors (TLRs) (6). In addition, γδ T cells acquire preactivated phenotypes of effector and memory T cells during their early development (6).

The antigen recognition, activation, and effector functions of γδ T cells are different than those of their αβ T cell counterparts. Unlike $\alpha\beta^+$ T cells, γδ T cells can be activated with or without their cognate TCR ligands and appear to induce an early burst of inflammatory cytokine that initiates effective and progressive αβ T cell responses in tissue inflammation during experimental autoimmune encephalomyelitis (EAE) (7–10). These unusual unique features of γδ T cells make them an early effector T cells during an immune response in inflamed tissue.

αβ$^+$ CD4$^+$ T cells are crucial for inducing tissue inflammation in EAE. It has been convincingly elucidated that IL-17-producing Th17 cells are the major driver in inducing pathogenesis of EAE. Ablation of Th17 cells or absence of IL-17 significantly reduces the severity of inflammation in EAE (11). Similarly, the absence of Th17 cell-associated genes, such as *Rorc*, a master transcription factor for Th17 cells development, and IL-23R also attenuate inflammation in EAE (12, 13). Interestingly, γδ T cells express higher level of IL-23R on their surface, which raised an interesting possibility that IL-23-responsive γδ T cells may contribute to the severity of tissue inflammation in EAE (8). Furthermore, GWAS studies suggested a genetic association of IL-23R with MS (14).

Although the *bona fide* antigens were identified for γδ T cells, still not much is known about their antigenic repertoire and restrictions (15). In addition to their antigens, γδ T cells can be activated by TLRs to induce various inflammatory cytokines, such as IFN-γ, IL-4, IL-17, IL-21, and IL-22 (6, 16).

Unlike αβ$^+$ T cells, antigen recognition by the TCR of γδ T cells does not require antigen processing and presentation by MHC molecules (17, 18). Moreover, deficiencies of MHC class II and β2 microglobulin do not affect the development of γδ T cells and their repertoire remain intact, which suggest that the generation of γδ T cells is apparently independent of both class I and II molecules (19, 20). Interestingly, non-classical MHC class Ib molecules T10 and T22 are described as the natural ligands for murine γδ T cells (21, 22). Similarly, human class I-like molecules MICA and MICB were also suggested as natural antigens for human γδ T cells (21, 23–25). Interestingly, alterations in the expression of these ligands are induced by infection or tissue inflammation or stress, which can provide early danger-signal to initiate the activation of γδ T cells even in the absence of αβ$^+$ T cells activation (15, 16).

The functions of γδ T cells in different pathophysiological conditions are driven by their tissue-specific distributions and tropism. At steady state, γδ T cells are predominantly localized in epithelial surfaces of liver, skin, and mucosal surfaces of digestive, respiratory, and reproductive organs (15, 16). Moreover, the distribution of γδ T cells to the above mentioned epithelial and mucosal surfaces is often driven by their specific expression of invariant or closely related γδ TCRs; for example, Vγ6Vδ1 TCR-expressing γδ T cells mostly accumulate in the lung, peritoneum, and reproductive organs, while Vγ5Vδ1-bearing γδ T cells predominantly reside in the epithelial surface of the skin (16). In addition to their tissue localization, cellular distribution, pathophysiological conditions, and inflammatory signals also determine the activation and phenotypic plasticity of γδ T cells.

Upon activation, γδ T cells can produce the effector cytokines of Th1, Th2, and Th17 cells, such as IFN-γ, IL-4, and IL-17, respectively, therefore contribute to specific effector function in Th1, Th2, and Th17 cell-associated tissue inflammation (26). Interestingly, IL-23 stimulation of γδ T cells rapidly induces IL-17 production (6, 13, 27) to initiate tissue inflammation and enhance CD4$^+$ αβ Th17 cells responses during EAE (7). It is apparent that γδ T cells play critical role in the induction and pathogenesis of EAE (15). Nonetheless, the regulatory role of γδ T cells is also suggested in EAE.

SUBSETS OF γδ T CELLS AND THEIR FUNCTIONS IN EAE

The functions of γδ T cells are not only critically required for elimination of intra- and extracellular pathogens and tissue surveillance in cancer but are also associated with multiple organ-specific autoimmunity, such as type 1 diabetes, arthritis, inflammatory bowel disease (IBD), and MS (16).

There are multiple subtypes of γδ T cells that are involved in the pathogenesis of EAE and can be identified based on the usage of their variable regions for both γ and δ genes (28, 29). Unlike the mucosal surfaces and the skin, which usually harbor higher frequency of γδ T cells, a smaller frequency of γδ T cells can be found within the central nervous system (CNS) in steady state of untreated naive mice (30, 31). Although the role of γδ T cells in the CNS at steady state is not precisely understood, it might be possible that their presence within the CNS could be required for carrying out immune surveillance function. Nonetheless, the frequency of γδ T cells profoundly increases within the CNS in EAE; and moreover, their distribution within the CNS can be classified based on their TCR usage during different phases of EAE (28). At the initial phase of EAE, CNS-infiltrating γδ T cells show a limited repertoire, including Vδ1, Vδ4, Vδ5, Vγ1–3, and Vγ6, while almost all the Vγ and Vδ transcripts can be found in the brain at the chronic or later phase of the disease (28). Although lymph nodes of EAE mice contained most of the Vγ transcripts during all phases of disease, a limited repertoire of γδ T cells was also observed within the CNS at the initial phase of the disease. Though Vγ6 (also known as DV7s6) expressing γδ T cells are predominantly located in mucosa, but they can also be found within the CNS at the initial phase of EAE. However, the precise antigen specificity of CNS-localized Vγ6 γδ T cells is not clearly understood in EAE. Since γδ T cells do not appear to recognize myelin basic protein (MBP) as antigen; therefore, Vγ6 T cells might be recruited to the CNS in EAE in response to the heat shock protein (HSP), which is expressed on stressed autologous cells (32). In addition, another possibility is that Vγ6 γδ T cells could recognize self-antigens that mimic bacterial peptide in the CNS during inflammation as this subset of γδ T cells is known to recognize microbial antigens (bacterial peptide) at mucosal surfaces (15, 33). Furthermore, γδ T cells are suggested to be functionally dichotomous on the basis of their TCR usage in EAE; Vγ1 subset preferentially regulates while Vγ4 subset further enhances tissue inflammation in EAE (34). Further analysis revealed that the Vγ1 subset is predominantly prevalent in spleen in all phases of EAE, and in fact, about 35–50% of total splenic γδ T cells are found to be Vγ1 in EAE (34). However, a small percentage of Vγ1 γδ T cells are also found in the CNS during EAE. Emerging literature suggested that Vγ1 γδ T cells act as regulatory cells and were shown to suppress tissue inflammation during the acute phase of EAE by enhancing the functions of Foxp3$^+$ regulatory T (Treg) cells. Moreover, it is proposed that Vγ1 subset of γδ T cells highly express CCL4, which can bind to CCR5 on Treg cells and promote their suppressive functions in EAE (34). Consistent with their regulatory role in EAE, CNS-sorted Vγ1 γδ T cells from EAE mice do not express high amounts of IL-17A, IL-17F, IL-23R,

and GM-CSF, which further reinforce their regulatory function in EAE (34). Thus, it is suggested that Vγ1 subset might shift the balance away from Th17 cells while promoting the proliferation and suppressive functions of Treg cells during EAE.

Yet, another subset of γδ T cells, Vγ4 predominates in the CNS during EAE. These cells typically responds to self-antigens by producing pro inflammatory cytokine, such as IL-17, which in turn can directly act on stromal cells and induce migration of lymphocytes across blood brain barrier in EAE (8, 27). Interestingly, the IL-17-producing Vγ4 γδ T cells also expressed other Th17 cell-associated molecules, such as Rorc, IL-22, IL-1R, and IL-1β (6, 34), which further suggested to contribute to inflammation and exacerbation of EAE (34). In addition to EAE, IL-17-producing Vγ4 γδ T cells are shown to promote collagen-induced arthritis (CIA), as antibody-mediated depletion of Vγ4 γδ T cells resulted in attenuated tissue inflammation in CIA (35). It is proposed that adjuvant rather antigen expands IL-17-producing Vγ4 γδ T cells in CIA.

The ability of γδ T cells to produce IL-17 innately in response to IL-23 in EAE could be attributed to Vγ4 subset of γδ T cells as they highly express IL-23R on their surface (6, 7, 27). Nonetheless, it is not clearly understood whether natural ligand or antigen of Vγ4 γδ T cells can induce strong IL-17 response in EAE. In addition to Vγ4 γδ T cells, Vγ6 γδ T cells, which primarily resides under the skin also express IL-23R on their surface, and therefore might be contributing to IL-17-mediated inflammation in the CNS of EAE mice (27).

Interestingly, in addition to IL-23R and Vγ4, the differential expression of CD27 can also identify γδT17 cells (IL-17-producing γδ T cells). $CD27^+$ γδ T cells produce IFN-γ while $CD27^-$ γδ T cells secrete IL-17 suggested that the surface expression CD27 can differentially mark IL-17- and IFN-γ-producing γδ T cells (36).

Furthermore, structural and functional heterogeneity of γδ T cells in EAE can be further contributed by different mice strain. Olive et al. have reported amplification of Vγ5 transcript in C57Bl/6 mice during EAE while this transcript was not detected in the CNS of SJL/J mice, suggesting that the infiltrating population of γδ T cells in CNS during disease can be varied on the basis of mouse strains (28).

Th17 CELLS DIFFERENTIATION AND IL-17-PRODUCING γδ T CELLS IN EAE

After the identification of Th17 cells as a separate lineage of helper T (Th) cells, it became clear that they, together, with Th1 cells, play a crucial role in EAE (37, 38). Before the identification of Th17 cells, IFN-γ-producing Th1 cells were thought to be the primary effector cell type involved in the disease induction of EAE, which has puzzled immunologist for a very long time as both IFN-γ- and IFN-γR-deficient animals had exacerbated tissue inflammation in EAE (39). In addition, the deficiencies of IL-12p35 (IL-12) and IL-12Rβ2 (IL-12 receptor), which are critically required for the development of Th1 cells, also enhanced the development of EAE (37). Taken together, it is clearly suggested that Th1 cells are not the primary effector T cell subsets involved in development of EAE. In fact, Th1 cell-associated molecules, such as IFN-γ, IL-12, and IL-12R, negatively regulate disease and tissue inflammation in EAE (11). Nonetheless, Th1 cells also critical for the development of EAE, as Th1 cells were found in the CNS in active EAE. In fact, a sizable population of IFN-γ and IL-17 double positive $CD4^+$ T cells was found within the CNS at the peak of EAE (40).

Seminal studies demonstrated that TGF-β1 and IL-6 are required for the differentiation of Th17 cells (41–43). IL-6 strongly induces IL-21 in Th17 cells, which creates feed forward loop to further amplify the generation of Th17 cells (44–46). The role of Th17 cells and IL-17 was further demonstrated by using IL-17-deficient mice, as *Il-17*$^{-/-}$ animals develop attenuated EAE with delayed onset. Moreover, the adoptive transfer of *Il-17*$^{-/-}$ $CD4^+$ T cells is inefficient in transferring EAE, suggesting that IL-17 is crucial for tissue inflammation and disease pathogenesis (47).

Similar to Th17 cells, IL-6 and TGF-β are also crucial for the generation of γδT17 cells (8, 48). *Tgfb*$^{-/-}$ and *Smad3*$^{-/-}$ mice harbor reduced precursor frequency of γδT17 cells in thymus (48). On the other hand, *Il6*$^{-/-}$ mice have shown reduced frequency of peripheral γδT17 cells (8). Taken together, similar to Th17 cells differentiation, TGF-β and IL-6 are crucial for the generation of γδT17 cells.

Importantly, the precise role of γδ T cells was demonstrated in EAE using *Tcrd*$^{-/-}$ mice (15, 49). Mice lacking TCR delta chain gene develop less severe EAE with reduced infiltration of $\alpha\beta^+$ T cells in their CNS (49). Similarly, depletion of γδ T cells by anti-GL3 antibody before the onset or at chronic phase of EAE reduces the severity and clinical signs of EAE (50). Moreover, antibody-mediated depletion of γδ T cells regulates the influx of proinflammatory cytokines, such as IL-1, IL-6, TNF-α, lymphotoxin, and IFN-γ, further suggesting an essential role of γδ T cells in contributing to the pathogenesis of EAE (50). Furthermore, it is demonstrated that the depletion of γδ T cells from MBP-reactive lymph node cells transferred attenuated EAE with reduced T cells proliferation and IL-12 secretion (51). Moreover, replenishing γδ T cells population not only enhanced the severity of EAE but also restored the IL-12 production and T cells proliferation (51).

In addition, a detailed systematic analysis of γδ T cells was performed to understand their distribution in different phases of EAE (52). Interestingly, an increased frequency of γδ T cells (up to 12% of total $CD3^+$ T cells) was found in the CNS during the acute phase while the percentage of γδ T cells decreased (from 12 to 5% of total $CD3^+$ T cells) during the recovery phase of EAE (52). Since the frequency of myelin-specific $Foxp3^+$ Treg cells increases during recovery phase of EAE, it is possible that the increased number of $Foxp3^+$ Tregs contributes in controlling the expansion of γδ T cells population during recovery phase of EAE (8, 53). Interestingly, the contraction of γδ T cells population was restricted only to the CNS, as their percentages in spleen remained low (~2% of total $CD3^+$ T cells) during all phases of EAE. This implies that γδ T cells selectively accumulate in the target tissue during tissue inflammation to enhance severity of inflammation in EAE (52).

Although $\alpha\beta^+$ $CD4^+$ T cells are suggested to be the primary source of IL-17 in infection and autoimmune inflammation, γδT cells can be a potent source of IL-17, and in some cases, even more dominant than Th17 cells (6, 33, 54). In fact, in the model of Fas-ligand-induced inflammation in which injecting

FasL-expressing tumor cells into peritoneum of mice induces enhanced production of IL-17 from non-conventional T cells (55). Interestingly, the majority of these IL-17-producing cells were γδ T cells as compared to αβ Th17 cells in this particular model (55). Similarly, γδ T cells isolated from *Mycobacterium*-infected lung and spleen produce massive amounts of IL-17 as compared to αβ Th17 cells (56). Furthermore, in other model of infection, such as *Escherichia coli*, *Bacillus subtilis*, and experimental sepsis, γδ T cells, rather than αβ$^+$ Th17 cells, are the primary source of IL-17 (33, 57). Hence, in certain conditions, γδ T cells appear to have an inherent ability to rapidly produce substantial amounts of IL-17 without being primed.

Although, initial studies identified that IL-17-producing γδ T cells are essential for clearing infections, the role of γδ T cells are also suggested for inducing autoimmune inflammation and propagation of autoimmune diseases, including EAE (15).

In addition to Th17 cells, Th1 cells were also implicated in the development of EAE (58). In fact, many studies suggested that myelin-specific Th1 cells adoptively transfer EAE (58). Interestingly, the initiation of EAE development by adoptively transferred myelin-specific Th1 cells resulted in recruitment of IL-17-producing host cells (IL-17hc) to the CNS (59). Further cellular characterization revealed that γδ T cells comprising almost 60% of the total IL-17hc (59). Moreover, in the absence of IL-17hc, myelin-specific Th1 cells transferred less severe EAE, suggesting the requirement of host production of IL-17, largely by γδ T cells, in the development of EAE (59).

PROINFLAMMATORY CYTOKINES THAT INDUCE IL-17 FROM γδ T CELLS IN INFLAMMATION IN CNS DURING EAE

Progression and development of tissue inflammation in EAE are primarily mediated by infiltrating mononuclear cells, which produce proinflammatory cytokines. Among other CNS-infiltrating cells, γδ T cells predominantly and rapidly produce proinflammatory cytokines to further enhance tissue inflammation in EAE. Like conventional αβ T cells, γδ T cells also expand in secondary lymphoid organs upon immunization with MOG/CFA. Once migrated to the CNS in β2 integrin-independent manner, these γδ T cells further expand and accumulate shortly before the peak of EAE and produce IFN-γ, TNF-α, and IL-17 to further enhance disease progression (60, 61).

Unlike αβ Th17 cells, which require primary (TCR), secondary (costimulation) and cytokine signals (TGF-β1 + IL-6) to produce IL-17, γδ T cells can produce IL-17 with cytokine signals (IL-23 and IL-1β) alone in the absence of primary and secondary signals (7). This peculiar feature of γδ T cells make them superior IL-17 producers by capturing the initial burst of proinflammatory cytokines produced by dendritic cells (DCs) and macrophages in response to TLR and NLR activation in EAE. The ability of γδ T cells to generate an initial burst of IL-17 in the absence of activation of αβ T cells is critical for initiating CNS inflammation, as *Tcrd*$^{-/-}$ mice develop less severe EAE with reduced production of IL-17 (7, 49). Moreover, αβ T cells from *Tcrd*$^{-/-}$ mice produce lower amounts of IL-17 as compared to αβ T cells from wild-type mice (8), which clearly suggested that the presence of γδ T cells is essentially required for optimal production of IL-17 by αβ T cells. Interestingly, *Il1r*$^{-/-}$ mice are substantially more resistant to EAE development (62); however, reconstituting IL-1R-sufficient γδ T cells into *Il1r*$^{-/-}$ mice prior to MOG immunization enhances progression of EAE, suggesting that IL-1β–IL-1R interaction on γδ T cells is essential for promoting tissue inflammation in EAE (7). Furthermore, stimulation of γδ T cells with IL-1β together with IL-23 synergistically enhanced IL-17 production in the absence of TCR stimulation (7). In addition to IL-17, other Th17 cell-associated cytokines, such as IL-17F, IL-21, and IL-22, were also produced by γδ T cells upon their activation with IL-1β and IL-23 (**Figure 1**). Consistently, culture supernatant of IL-1β– and IL-23-stimulated γδ T cells further enhanced IL-17 production from αβ$^+$ CD4$^+$ T cells (7, 8). Neutralization of IL-21 and IL-17 reduced IL-17 induction from αβ$^+$ CD4$^+$ T cells induced by culture supernatant of IL-1β- and IL-23-stimulated γδ T cells (7, 8). In fact, it is suggested that the combination of IL-1β- and IL-23-stimulated γδ T cells provides early burst of IL-21, which not only enhances production of IL-17 by the γδ T cells but it can also amplify the generation of Th17 cells (8, 11, 45) (**Figure 1**).

In addition to IL-21, another common γ chain family cytokine, IL-2 also play a role in generation of γδT17 cells. IL-2, which is known to suppress Th17 cells (63), promotes γδT17 cells generation, as *Il2*$^{-/-}$ and *Cd25*$^{-/-}$ mice selectively reduced the frequency of γδT17 cells (64). Interestingly, the new subset of IL-15-producing γδ T cells (γδT15) was recently identified in EAE (65). γδT15 cells suggested to enhance tissue inflammation in EAE by enhancing the functions of CD44^{hi} memory T and Th17 cells (65). However, whether these γδT15 cells express other inflammatory cytokines, such as IL-17 and GM-CSF, are not clear. In summary, various cytokines signals are required for the generation of γδT17 cells; and interestingly, some of these cytokines can directly activate γδ T cells without the requirement of TCR activation. Taken together, the initial burst of proinflammatory cytokines produce by γδ T cells is crucial for induction of EAE.

IL-18 PROMOTES IL-17 INDUCTION FROM γδ T CELLS IN EAE

IL-18, an IL-1 family cytokine, also known as IFN-γ-inducing factor. It has been shown that IL-18 further enhances the development of IL-12-induced Th1 cells. Moreover, Th1 cells sensitized with IL-18 enhance their disease promoting effector functions in EAE by activating IFN-γ-producing NK cells (66). The function of IL-18 in EAE was described using IL-18R1-deficient animals. *Il18r1*$^{-/-}$ mice were completely resistant to development of EAE, suggesting the role of IL-18R in inducing encephalitogenic T cells in disease (67). Moreover, the engagement of IL-18Rα on antigen-presenting cells is essential for generation of pathogenic Th17 cells during EAE (67). In fact, caspase-1-processed cytokines IL-1β and IL-18 predominantly promote innate production of IL-17 from γδ T cells in EAE (9). Immunization with CFA, which contains heat-killed cell wall of *Mycobacterium tuberculosis*, activates caspase-1 via NLRP3 inflammasome to induce active forms of IL-1β and IL-18 from DCs. Inhibition of

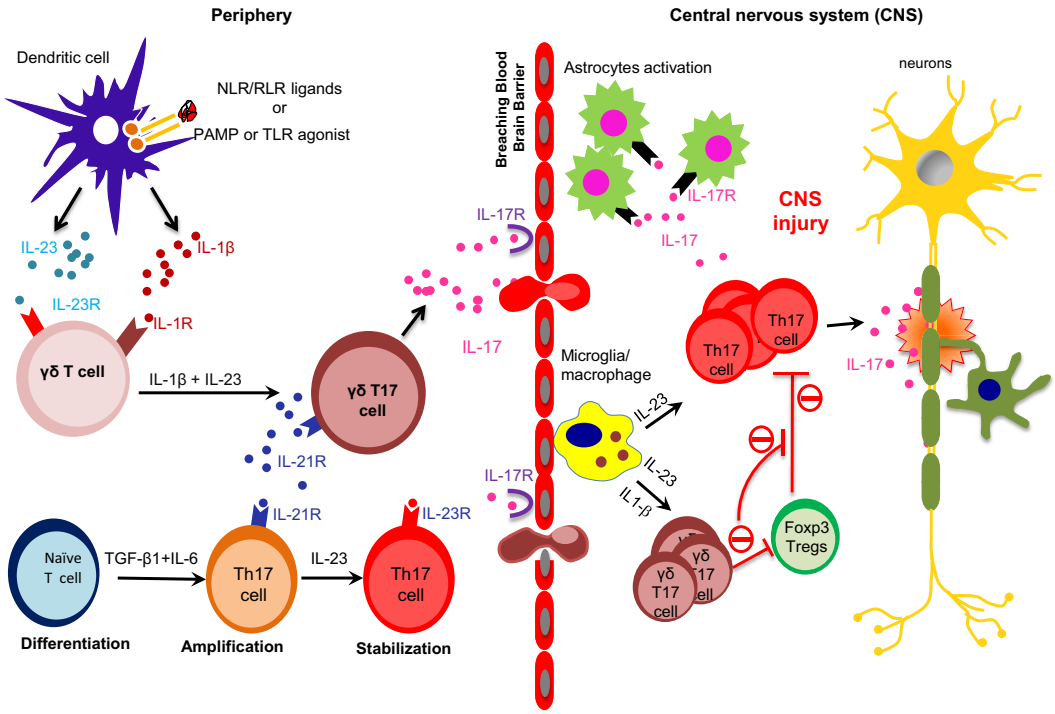

FIGURE 1 | Peripherally primed γδ T cells execute their effector functions in the CNS in EAE. TLRs and NLRs activated dendritic cells (DCs) and macrophages produce proinflammatory cytokines, such as IL-6, IL-23 and IL-1β. IL-23 and IL-1β are sensed by IL-23R-expressing γδ T cells, which in turn produce early burst of IL-17 during early phase of EAE. On the other hand, IL-6 together with TGF-β induce the differentiation of Th17 cells. γδT17 cells produce IL-21, which further amplify their own generation and also amplify the generation of Th17 cells. Differentiated γδT17 and αβ Th17 cells breach the blood brain barrier to execute their effector functions within the CNS during EAE. Activated microglia/macrophages produce IL-23 within the CNS to promote the generation of γδT17 and Th17 cells. Inflammatory γδT17 cells promote CNS injury in EAE by enhancing the effector functions of Th17 cells and restraining the suppressive functions of Tregs cells.

caspase-1 by its specific inhibitor suppresses EAE development and IL-17 production from γδ T cells (9). Similar to IL-23R, γδ T cells also express IL-18R constitutively on their surface even in the steady state (**Figure 2**). On the contrary, the expression of IL-18R on $CD4^+$ T cells is induced in inflammatory conditions during EAE, suggesting that γδ T cells, and not $CD4^+$ T cells, respond first to the IL-18 in order to induce IL-17 production. It has been shown that the combination of IL-18 together with IL-23 rapidly induced innate production of IL-17 from γδ T cells in the absence of TCR stimulation (**Figure 2**). This initial burst of IL-17 from γδ T cells may be required for initiation of EAE and the development of pathogenic Th17 cells. It is, however, unclear whether coexpression of IL-23R and IL-18R on γδ T cells make them more pathogenic in initiating EAE.

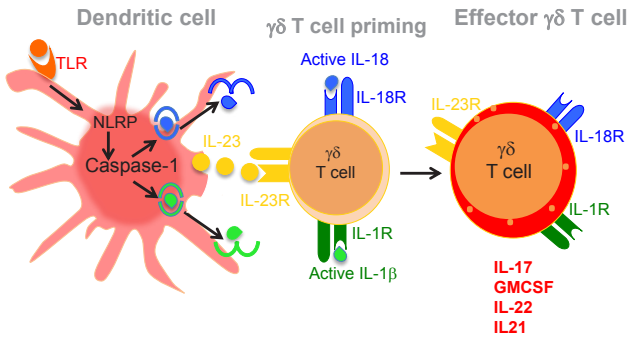

FIGURE 2 | Dendritic cell-derived IL-23, IL-1β, and IL-18 mediates induction of effector γδT17 cells. Ligation of TLRs on the surface of DCs induces caspase-1 activation in inflammasome-dependent manner. Activated caspase-1 cleaves pro IL-1β and IL-18 into their active forms as shown in the figure. Activated DCs also produce IL-23, which together with IL-1β or IL-18 promote the induction of proinflammatory cytokines, such as IL-17, GM-CSF, IL-21, and IL-22. These effector γδ T cells initiate disease induction and help $αβ^+$ $CD4^+$ T cells to induce EAE.

GM-CSF-PRODUCING γδ T IN TISSUE INFLAMMATION DURING EAE DEVELOPMENT

In addition to IL-17 and IFN-γ, GM-CSF is also essentially required for the development of EAE. GM-CSF-deficient mice are resistant to the development of EAE with reduced infiltration of effector T cells into the CNS (68). Rostami et al. reported that the neutralization of GM-CSF-attenuated tissue inflammation in EAE (69). Taken together, it is clearly suggested that GM-CSF is required for the induction of encephalitogenic T cells in EAE. In fact, both Th1 and Th17 cells were shown to produce GM-CSF, which can further enhance the encephalitogenicity of these effector T cells in

mediating the development of EAE. Moreover, it is proposed that GM-CSF is critical for the induction of pathogenic Th17 cells in EAE. Although both IL-12 and IL-23 can induce the production of GM-CSF by the effector T cells, it is clearly demonstrated that IL-23, but not IL-12, signaling is critically required for GM-SCF production in EAE (70–72). Similarly, exposure of IL-23 enhances the pathogenic functions of Th17 cells mediated by GM-CSF in EAE. In addition to CD4$^+$ T cells, macrophages, and NK cells, γδ T cells produce high amounts of GM-CSF, which contributes to neuroinflammation of CNS in EAE (68). In fact, γδ T cells are the major innate source of GM-CSF in the CNS during EAE development (10). Combination of IL-23 together with IL-1β promotes GM-CSF production from γδ T cells in the absence of TCR stimulation (10). Moreover, the production of GM-CSF induced by IL-23 and IL-1β was compromised in *Il1r*$^{-/-}$ γδ T cells. In fact, production of GM-CSF by CNS-infiltrating γδ T cells is abolished in *Il1r*$^{-/-}$ mice, suggesting that IL-1 signaling is crucial for generation of GM-SCF-producing γδ T cells in EAE (**Figure 2**) (10). Caspase-1, which is required for active IL-1β production, is also critical in inducing GM-CSF from γδ T cells, as *caspase1*$^{-/-}$ γδ T cells are defective in GM-CSF production. In fact, *caspase1*$^{-/-}$ and *Il1b*$^{-/-}$ mice share a similar EAE phenotype, suggesting a specific role of caspase-1 and downstream IL-1β in regulating the induction of GM-CSF during EAE (10). It is suggested that GM-CSF contribute to the development of EAE by enhancing the functions of CNS-resident myeloid cells, including microglial cells (70). Although IL-1 signaling is required for the generation of GM-CSF-producing γδ T cells within the CNS during EAE, it is not identified which subtype of γδ T cells predominantly produce GM-CSF during disease. Moreover, IL-1- and IL-23-mediated inductions of GM-CSF in γδ T cells are dependent on MyD88 signaling, as γδ T cells from MyD88-deficient mice severely reduced GM-CSF production (10). Since MyD88 is a major downstream signaling component of TLR signaling pathway, it might be possible that ligation of TLRs on γδ T cells can also induce IL-17 production (57). To precisely understand the role TLRs in generating γδT17 cells, Dong et al. used IL-17-RFP·KI mice to understand the cellular source of IL-17 in EAE in response to TLR4 ligation. Using a faithful IL-17 reporter system, Dong et al. clearly demonstrated that the expression of TLR4 is high on IL-17$^+$ as compared to IL-17-γδ T cells (31). In addition to IL-17 expression, IL-23-stimulation strongly enhanced the expression of TLR4 on γδ T cells. Moreover, the combination of IL-23 together with LPS further enhanced the secretion of IL-17 from γδ T cells (31). In addition to induced IL-17 production, TLR4 signaling also enhanced the survival of γδ T cells, which can further contribute in enhancing tissue inflammation in EAE. Taken together, TLRs especially TLR4 plays an essential role in inducing the development of IL-17-producing γδ T cells and their survival.

γδ T CELLS MAKE αβ$^+$ CD4$^+$ T CELLS REFRACTORY TO TREG SUPPRESSION IN EAE

Regulatory T cells are critical for maintaining immune homeostasis of the host as loss of these cells either by naturally occurring mutation or cellular ablation leads to overwhelming activation of effector T cell-mediated multiple organ failure of the host (73–75). The critical functions of Treg cells were described in various models of autoimmune diseases, including EAE (53, 76). Using MOG tetramer and Foxp3-GFP·KI mice, it has been demonstrated that myelin–antigen-specific Treg cells are primed and expanded during the priming phase of EAE (53). Similar to effector T cells, these myelin-specific Treg cells can effectively migrate to the CNS (53). Although their frequency within the CNS is lower during the peak of EAE, strikingly, the population of Treg cells outnumber the population of effector T cells within the CNS at recovery phase of EAE (53). These CNS-accumulated Treg cells produce both IL-10 and TGF-β, which help in resolving the inflammation at the recovery phase of EAE. Interestingly, both Tr1 and Treg cells were shown to produce IL-10 in the CNS during the recovery phase of EAE (53, 77). In spite of their presence in the CNS at the peak of EAE, Treg cells failed to suppress proliferation and effector functions of CNS-accumulated effector T cells (53). Interestingly, cytokine analysis of CNS-accumulated effector CD4$^+$ T cells revealed a strikingly higher production of proinflammatory cytokines, such as IL-6, TNF-α, and IL-21, which can be accounted for the failure of suppressive functions of Treg cells in EAE (53, 78). Interestingly, the higher frequency of γδ T cells together with αβ effector T cells found to be accumulated within the CNS (7, 8, 53, 76). In general, γδ T cells have high expression of IL-23R, in fact, all the γδ T cells present in the CNS at the peak of EAE exclusively expressed IL-23R (8, 13). Moreover, the frequency of IL-23R$^+$ γδ T cells contracts while the frequency of Tregs cells increases during the recovery phase of EAE (8, 53). This raised an interesting possibility that the presence of γδ T cells within the CNS might promote the functions of inflammatory αβ$^+$ T cells while hampering the suppressive functions of Treg cells in EAE (**Figure 1**). In fact, *Tcrd*$^{-/-}$ mice mount-attenuated effector αβ T cells response in EAE, supporting the fact that the presence of γδ T cells are essential for effective CD4$^+$ T cells effector functions in EAE (49). Interestingly, Korn et al. suggested a mechanism by which γδ T cells enhanced the effector functions of CD4$^+$ T cells during inflammation (8). Activation of γδ T cells with IL-23 produced soluble factors, which make αβ$^+$ T cells refractory to Treg cell-mediated suppression, as cellular supernatant of IL-23-activated γδ T cells inhibited the suppressive functions of Treg cell (7, 8). It has been demonstrated that Treg cells can lose their suppressive functions in the presence of inflammatory environment. In fact, IL-6 makes αβ effector T cells refractory to the suppressive activity of Tregs cells (8, 53, 78). In addition, IL-6 has also been shown to inhibit TGF-β-induced *de novo* conversion of conventional T cells into Treg cells (41, 42). Similarly, Kuchroo et al. has demonstrated that IL-21, in addition to IL-6, can also suppress TGF-β-mediated *de novo* conversion of conventional T cells into Treg cells. Interestingly, IL-23R-stimulated γδ T cells not only block the conversion of conventional T cells into Treg cells but also make αβ$^+$ effector T cells refractory to Treg cells suppression *in vivo* (8). This clearly indicates that the presence of γδ T cells at the site of tissue inflammation within the CNS indirectly promote the effector functions of αβ$^+$ T cells by restraining their *de novo* conversion into Treg cells and inhibiting

the suppressive functions of Treg cells in EAE (7, 8) (**Figure 1**). Similarly, the role of IL-23 in restraining the suppressive functions of Treg is well described in intestinal inflammation, as the frequency of inducible Foxp3$^+$ Treg (iTreg) cells increases in the absence of IL-23 (79). However, it is not clear whether appearance of increased frequency of iTreg cells in the absence of IL-23–IL-23R signaling in the intestinal inflammation is due to loss of IL-23R$^+$ γδ T cells functions, which are known to suppress the conversion of conventional T cells into Treg cells (8). Moreover, the importance of γδ T cells in mediating the inhibition of suppressive functions of Treg cells was further elucidated in *Tcrd*$^{-/-}$ mice, as these mice develop attenuated EAE with reduced production of IL-17 due to increased frequency of Treg cells (7, 49). Strikingly, anti-CD25 antibody-mediated depletion of Treg cells in *Tcrd*$^{-/-}$ mice enhanced the development of EAE with increased production of IL-17 (8). Altogether, it suggests that γδ T cells are crucial cellular component in promoting inflammation in EAE by restraining the regulatory functions of Treg cells and promoting the functions inflammatory αβ T cells (**Figure 2**).

TABLE 1 | Major γδ T cells subset in mouse.

γ/δ usage	Characteristic	Tissue location
Vγ1	Produce IL-4. Regulatory functions in EAE by promoting Treg cells functions (34)	Majorly found in circulation, lymphatics, spleen, lymph nodes
Vγ4	Produce IL-17 and express IL-23R. Promote EAE and CIA. Also promote virus-induced encephalitis (6–8, 27, 35, 83)	Lymphoid tissue and lung, also found in CNS in EAE
Vγ5	Regulation of skin inflammation by maintaining the epidermal homeostasis (84, 85)	Skin and epidermis
Vγ6	Produce IL-17, IL-22, IFN-γ, and express IL-23R (27)	Mucosal tissues, reproductive tract, tongue, lung and kidney. Also detected in CNS during EAE
Vγ7	Prevent colitis by protecting intestinal barrier functions (57, 86, 87)	IEL and intestine

γδ T CELLS: PATHOGENIC OR PROTECTIVE IN EAE?

While some models of EAE suggest that γδ T cells are pathogenic, others suggest that they modulate disease; thus, their precise role in pathogenesis is unclear. Both disease-promoting and disease-preventing functions of γδ T cells were documented in EAE. Deficiency of γδ T cells on B10·PL background develop a chronic EAE as compared to the development of monophasic acute EAE in the control mice (30). It has been further shown that γδ T cells regulate chronic inflammation by Fas–FasL-mediated killing of CNS-infiltrating inflammatory T cells (30). These studies clearly suggested the protective role of γδ T cells in EAE development.

Although recent literature on γδ T cells in context of IL-17 production implicated the pathogenic role of these cell types in EAE, a number of studies have ascribed the protective role of γδ T cells in EAE (30, 80, 81). A number of factors, such as using different mice strains in combination with either depleting antibodies or genetic manipulation of γδ T cells, might be contributing to these conflicting observations. Treatment of mice with UC7-13D5 anti-γδ antibody accelerates the onset of EAE (80). Similar results were obtained with the usage of UC7-13D5 antibody in other models of autoimmunity. It is partially identified that different subtypes of γδ T cells such as Vγ1 produce regulatory or Vγ4 and Vγ6 produce inflammatory cytokines (**Table 1**); therefore, it is possible that the treatment of UC7-13D5 antibody may alter this ratio and activate different subtypes of γδ T cell populations by cross-linking their TCR at different phases of EAE, which results in different outcome of disease. Nonetheless, it was not clearly understood whether anti-pan γδ T cells antibody depletes or activates γδ T cells by cross-linking their TCR in EAE (80). Using Tcrd-GFP knock-in mice, it has been clearly demonstrated that treatment with anti-pan γδ T cell antibodies activates, rather than depletes, γδ T cells and therefore exacerbating EAE (34). In addition, *Tcrd*$^{-/-}$ mice develop chronic inflammation in some mouse model of EAE (82). *Tcrd*$^{-/-}$ mice are devoid of δ TCR, which allow γδ T cells not to be activated by their TCR stimulation; however, the number of γδ T cells in these mice remains unchanged. This indicates that TCR-independent activation of γδ T cells can still occur in *Tcrd*$^{-/-}$ mice. We have discussed those different subsets of γδ T cells play opposite roles in EAE development. An interesting dichotomy has been established among Vγ1 and Vγ4 subsets of γδ T cells in EAE, which further provide a logical explanation for previously published contradictory results. Specific antibody-mediated activation of Vγ4 γδ T cells promote the development of EAE associated with enhanced production of IL-17 (34). On the other hand, specific antibody-mediated activation of Vγ1 γδ T cells suppressed EAE development (34). Interestingly, it has recently shown that γδ T cells can be activated with proinflammatory cytokines without the requirement of their TCR signals. To further identify the pathogenic or protective role of γδ T cells in EAE, a detailed study, including the involvement of various subtypes of γδ T cells, is required with more definitive tools. Nonetheless, accumulated literature in other autoimmunity has suggested that γδ T cells might play a pathogenic role in EAE. We have summarized the chief findings of γδ T cells in EAE in **Table 2**.

RELEVANCE OF γδ T CELLS IN MULTIPLE SCLEROSIS

Multiple sclerosis is demyelinating disease of CNS, which is caused by inflammatory T cells. In addition to αβ$^+$ CD4$^+$ T cells, γδ T cells were also clearly implicated in the disease pathogenesis in MS. It is shown that γδ T cells are accumulated in the MS plaques (90, 91). A restricted repertoire of γδ T cells was identified in MS lesions. CNS-restricted γδ T cells abundantly express variable gene segments Vδ1 and Vδ2. Furthermore, Vγ9$^+$ γδ T cells circulate abundantly in the blood of MS patients and can be

TABLE 2 | Chief findings of γδ T cells in EAE.

Gene deficiency/treatment	Consequence	Effect in EAE
Anti-γδ T cells (clone GL3) monoclonal antibody treatment in EAE	Reduction in disease pathology. Significant reduction in clinical sign in acute phase of EAE	Protection (52)
Anti-γδ T cells (clone UC7-13D5) monoclonal antibody treatment in EAE	Significant reduction in demyelination and reduction in limb paresis	Protection (88)
Active EAE development in delta (d) chain-deficient mice	Significant reduction in clinical score of EAE with enhanced frequency of $Foxp3^+$ Tregs	Protection (8, 49)
EAE induction by adoptively transferring MOG-specific Wt T cells into delta (d) chain-deficient mice	Significant reduction in clinical score of EAE with no cellular infiltration in CNS	Protection (49)
MBP-specific γδ T cells depleted (clone: GL3) lymph node cells were adoptively transferred to induce EAE	Significant reduction in clinical score in EAE with a significant reduction in IL-12 production	Protection (51)
Activation of Vγ4 subset with anti-Vγ4 TCR (UC3) antibody treatment in EAE	Worsen EAE with enhanced IL-17 response	Promote EAE (34)
Activation of Vγ1 subset by anti-Vγ1 TCR antibody (2.11) treatment in EAE	Significant reduction in clinical score of EAE with less proinflammatory cytokines production	Protection (34)
EAE in IL-23R-deficient mice and effect of IL-23–IL-23R axis on γδ T cells	IL-23R-deficient mice are resistant to EAE. γδ T cells constitutively express IL-23R. Almost all γδ T cells express IL-23R in CNS in EAE and produce IL-17	Protection (8, 13)
EAE in IL-18R-deficeint mice and effect of IL-18R on γδ T cells	IL-18R-deficient mice are protected from EAE. $IL\text{-}18R^{-/-}$ failed to produce IL-17	Protection (9, 67)
EAE in IL-1R-deficent mice and effect of IL-1R on γδ T cells	IL-1R-deficient mice are protected from EAE. $IL\text{-}1R1^{-/-}$ γδ T cells are defective in IL-17 and GM-CSF production in EAE	Protection (7, 10, 62)
EAE in caspase-1-deficient mice and effect of caspase-1 on γδ T cells	Significantly reduced clinical sign of EAE. Defective production of IL-17 and GM-CSF from caspase-1-deficient γδ T cells	Protection (9, 10, 89)

used as an indicator of disease activity (92). With the emerging literature on γδ T cells in EAE, it is indicated the involvement of γδ T cells in the pathogenesis of disease (see **Table 1**). Mouse data in EAE clearly indicated that IL-17-producing γδ T cells are crucial for disease induction and tissue inflammation in EAE (8, 10). Moreover, the role of IL-23, IL-1, IL-18, and caspase-1 is clearly indicated in enhancing IL-17- and GM-CSF-producing γδ T cells in EAE. Recent advancements in understanding the biology of Th17- and IL-17-producing γδ T cell and their implication in autoimmune diseases, including MS, could suggest new therapeutic targets for MS by targeting Th17- and IL-17-producing γδ T cells populations.

CONCLUSION

A number of studies have demonstrated a potential role of γδ T cells in the induction and maintenance of demyelinating CNS inflammation. γδ T cells are multifaceted cells, which are equipped with variety of functions to potentially influence all levels of inflammation by recognizing diverse array of antigens, rapid production of inflammatory mediators, and influencing the differentiation of their αβ counterparts. Equipped with functions of both innate and adaptive immune cells, γδ T cells can provide consequential functions in EAE development. Opposing roles of different subtypes of γδ T cells have been described in different mouse strains in EAE. Moreover, the identification of IL-17-producing inflammatory γδ T cells suggested their pathogenic role in EAE. In fact, many of the key questions in autoimmune inflammation, including EAE, were resolved by the discovery of IL-17-secreting Th17 cells. Moreover, clarification on the indispensible role of IL-23–IL-23R axis in Th17 cells also urged researchers to identify the role of IL-23–IL-23R signaling in γδ T cells as they have high expression of IL-23R receptor and therefore are responsive to IL-23 even in steady state – a characteristic which naive αβ T cells lack. This revisits the importance of IL-23 in the settings of EAE since it can influence the generation of two pathogenic subsets Th17 cells and γδT17 cells both of which contributes IL-17 to large extent. Synergistic action of IL-23, IL-1β, and IL-21 induces inflammatory IL-17-producing-γδ T cells, which not only enhance the generation and functions of $\alpha\beta^+$ Th17 cells but also obstructs the suppressive functions of Treg cells in EAE. Recently, significant progress has been made in understanding the pathogenic role of γδ T cells in tissue inflammation. Yet more substantial evidences are required on different subtypes of γδ T cells for defining their opposing roles in tissue inflammation and explaining the confounding findings on their pathogenic or protective role in EAE.

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REVIEW
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Mucosal-associated invariant T cells in multiple sclerosis: the jury is still out

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The immune system is strongly implicated in the pathophysiology of multiple sclerosis (MS), as demonstrated by the efficacy of therapies targeting various components of adaptive immunity. However, the disease still progresses despite these treatments in many patients, while others experience life-threatening adverse effects, urging for the discovery of new immune-targeting medications. Among the immune cell types participating to MS pathogenesis, decades of work have highlighted the prominent role of CD4 T cells. More recent data demonstrate the involvement of CD8 T cells as well. The existence of both pathogenic and protective CD8 T cells subsets has been suggested, adding an additional layer of complexity to the picture. Mucosal-associated invariant T (MAIT) cells are innate-like lymphocytes that make up to 25% of CD8 T cells in healthy subjects. They are specific for conserved microbial ligands and may constitute an important barrier against invasive bacterial and fungal infection. An increasing number of reports also suggest their possible involvement in chronic inflammatory diseases, including MS. MAIT cells could participate through their ability to produce IFNγ and/or IL-17, two major cytokines in the pathogenesis of several chronic inflammatory/autoimmune diseases. However, the mechanisms by which MAIT cells could be activated in these sterile conditions are not known. Furthermore, contradictory observations have been made, reporting either a protective or a pro-inflammatory behavior of MAIT cells in MS or its murine model, experimental autoimmune encephalomyelitis. In this review article, we will describe the current knowledge on MAIT cell biology in health and disease, and discuss the possible mechanisms behind their role in MS. The specific features of this new non-conventional T cell subset make it an interesting candidate as a biomarker or as the target of immune-mediated intervention.

Keywords: multiple sclerosis, adaptive immunity, innate-like T cells, MHC-related 1, IFN gamma

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Multiple sclerosis (MS) is a chronic immune-mediated disease of the central nervous system (CNS). MS is characterized by discrete white matter lesions in the brain and spinal cord. The cellular injury is primarily oligodendrocytes but MS also affects axons/neurons. This neural demise results in the progressive neurological disability affecting people with MS. The genetic factors providing susceptibility to the disease have been largely deciphered in recent years (1). Polymorphisms at more than 150 loci contribute to MS susceptibility. Importantly, the incriminated genes collectively point to a central role of the immune system in disease pathogenesis (2, 3). The most studied and also the strongest MS-susceptibility genes reside within the human leukocyte antigen (HLA) locus with a major

influence of the *DRB1*15:01-HLA-DRB5*01:01-DQB1*06:02* haplotype and significant impact of HLA class I alleles (4).

There is little doubt that multiple immune cell populations are implicated both at the initiation of the disease process and at the effector phase responsible for CNS tissue damage. However, the respective contribution of these various populations at the different phases of the disease remains only partly understood (5). Nevertheless, deciphering how the various CD4 and CD8 T cell subsets promote and regulate MS immunopathogenesis has benefited from progress in fundamental immunology and from experimental models (6–8). Much has been learned lately regarding the different functional subsets of CD4 T cells and regarding the pathogenic and regulatory influence of CD8 T cells. This has, in part, led to new therapeutic directions for the benefit of people with MS (9). However, newly identified innate-like T cell populations, such as innate lymphoid cells, invariant natural killer T (iNKT) cells, and mucosal-associated invariant T (MAIT) cells, have emerged as important actors in inflammatory diseases. They are positioned at the interface between the environment and the host and may, therefore, represent a key link for the amplification of an immune reaction against microbes. Understanding their exact contribution to pathogenesis will undoubtedly open innovative therapeutic possibilities.

Here, we review the current knowledge regarding the biology of MAIT cells and their possible involvement in MS. Future directions are suggested to better apprehend their precise role and their usefulness as therapeutic targets.

Mucosal-Associated Invariant T Cells: A New Innate-Like T Cell Subset

Mucosal-associated invariant T cells are a homogenous T cell subset displaying features of innate-like T cells, such as γδ or iNKT cells. Originally described in humans, they are phylogenetically conserved in distant mammal species, including mice (10–14). However, the frequency of MAIT cells in laboratory mouse strains is low, and there is evidence that they may be developmentally and/or functionally different from their human counterparts (15). These differences must be kept in mind when interpreting results obtained in mice. MAIT cells are mainly characterized by a highly restricted TCR repertoire, selected for by a monomorphic major histocompatibility complex (MHC) class I-like molecule known as MHC-related 1 (MR1) (10). Indeed, the vast majority of MAIT cells express an invariant TCRα chain (Vα7.2-Jα33 in humans and the homologous Vα19-Jα33 in mice) (13, 16). The second important feature of MAIT cells is their peripheral maturation/differentiation status; in one study, >90% of MAIT cells displayed an effector/memory phenotype in healthy adults (17). The ontogeny of MAIT cells in mice is dependent upon microbial colonization of the intestine soon after birth, suggesting that shared commensal bacterial antigens presented by MR1 drive the proliferation and maturation of memory MAIT cells (17). In humans, cord blood harbors a small population of naïve MAIT cells that apparently expand in early childhood, and differentiate into memory cells (17, 18), suggesting a similar mechanism of antigen-driven expansion after birth. Seminal studies performed by the Rossjohn and McCluskey laboratories led to the discovery of microbial antigens for MAIT cells (19, 20). These antigens are low molecular weight molecules derived from the intermediates of the riboflavin (vitamin B2) metabolism. The mammalian genome is devoid of the genes necessary to the synthesis of riboflavin; however, an important number of different bacterial and fungal species are riboflavin producers, and therefore, MAIT cell activators (21). It is, therefore, speculated that MAIT cells exit the thymus as naïve cells, and then encounter bacterial antigens (probably originating from the commensal flora), driving their early maturation in the periphery. However, a recent study challenged this hypothesis, showing evidences of MAIT cell proliferation and differentiation in the peripheral organs of second trimester human fetuses (22) This would suggest that MAIT cells can mature before bacterial colonization of the body with commensal microbes, and has profound consequences on our understanding of MAIT cells reactivity toward various cognate ligands and/or environmental cues; more studies are, therefore, needed to unravel these processes. Activation of MAIT cells leads to cytokine secretion, mostly interferon γ (IFNγ) and tumor necrosis factor α (TNFα), as well as induction of degranulation and cytotoxicity (23–25). Virtually all MAIT cells in humans express high levels of CD161, as well as the IL-23 receptor (IL-23R), the C-C chemokine receptor 6 (CCR6) and the transcription factor RAR-related orphan receptor gamma t (RORC2/RORγt), three markers associated with interleukin 17 (IL-17) producing subsets (**Figure 1**) (16, 18, 23). Indeed, IL-17-secreting MAIT cells can be found in some settings, mostly in pathological conditions (see below) (23, 26–31). The phenotype of MAIT cells in wild-type mice has been described recently (**Figure 1**). In peripheral tissues, such as the lung, they uniformly express a memory phenotype ($CD44^{hi}CD62L^{lo}$), the

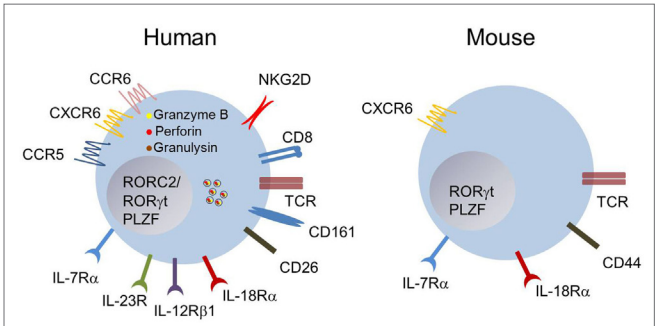

FIGURE 1 | Phenotype of human and mouse MAIT cells. Blood MAIT cells in humans are defined as TCRVα7.2^{+}CD161^{hi}-expressing T cells. Most MAIT cells are CD8^{+}, and express an effector/memory phenotype CD45RO^{+}CD62L^{lo}CCR7^{-} (not depicted). They express several chemokine- and interleukin-receptors at steady state. They are equipped with the cytotoxicity co-receptor NKG2D, and display intra-cytoplasmic granules containing granulysin, granzyme B, and perforin. The expression of CD161, IL-18Rα, or CD26 at high levels is usually sufficient to identify MAIT cells within the CD8^{+} subset in human blood. The phenotype of mouse MAIT cells is apparently more diverse, and dependent upon the tissues examined. Most of them are CD4^{-}CD8^{-} (double negative), display an effector/memory phenotype and the interleukin and chemokine receptors IL-7Rα, IL-18Rα, and CXCR6 (16, 17, 23, 24, 32).

cytokine receptors IL-7R and IL18R, and the C-X-C chemokine Receptor 6 (CXCR6) chemokine receptor, akin to humans (32). Other surface markers show a more diverse pattern of expression, suggesting that mouse MAIT cells may be more diverse than their human counterparts. In particular, most mouse MAIT cells lack both CD4 and CD8, but variable proportions of CD4 and CD8 cells are found in a tissue-specific fashion (32). In agreement with their anti-microbial reactivity, MAIT cells react against a wide array of bacteria *in vitro*, by producing IFNγ and lysing bacteria-infected cells (19, 24, 25, 33–36). *In vitro* analysis showed that their functional response is regulated by several cytokines, such as IL-7, IL-12, IL-18, and IL-23 (**Figure 2**) (26, 30, 37, 38). MAIT cells may be particularly involved in the immune response against *Mycobacterium tuberculosis*: patients with active tuberculosis show a depleted blood MAIT cell compartment resulting from their recruitment to the lung (33, 38–40). All evidence points out an important role for MAIT cells as a first line of defense against invasive bacterial infections, chiefly at mucosal surfaces (41).

An increasing number of studies have reported the likely involvement of MAIT cells in non-bacterial diseases. These include chronic viral infections like human immunodeficiency virus (HIV) (42–48), autoimmune diseases [such as inflammatory bowel diseases (IBD), systemic lupus erythematosus (SLE), MS, or psoriasis] (27, 31, 49–52), other inflammatory or hypersensitivity diseases, and even cancer (28, 29, 53–56). Although the significance of these observations is still unknown, they point out a possible role for MAIT cells in the pathogenesis of many inflammatory conditions.

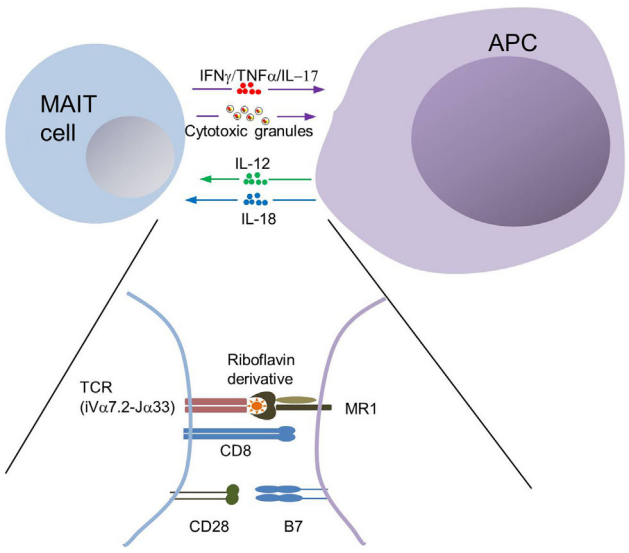

FIGURE 2 | MAIT cell interactions with MR1⁺ antigen-presenting cells. The semi-invariant TCR of MAIT cells allows recognition of a complex made of MR1 associated with a bacterial-derived riboflavin derivative. MR1-dependent interactions with MAIT cells has been formerly demonstrated with professional antigen-presenting cells (APCs), such as monocytes, macrophages, dendritic cells, and B cells as well as with epithelial cells. Professional APCs may influence MAIT cell response through secretion of IL-12 and IL-18, and by providing co-stimulation. MAIT cell effector functions primarily involve IFNγ secretion and degranulation of their cytotoxic content, thereby inducing killing of infected epithelial cells.

MAIT Cells in MS and EAE

The first study focusing on MAIT cells in the context of CNS inflammation was performed in humans. Because at the time there was no tool available to directly identify MAIT cells (such as TCR-targeting antibodies or tetramers), the transcripts encoding the MAIT cell-specific invariant TCRα chain *Vα7.2-Jα33* were searched for within the CNS (49). An accumulation of such mRNA species was found in autopsy lesions from MS patients in 50% of the analyzed cases, as well as in 73% of cerebrospinal fluid samples obtained from patients experiencing relapses. The authors concluded that MAIT cells are involved in the CNS inflammation. The same group further investigated the role of MAIT cells in the EAE mouse model. As MAIT cells are scarce in mice, they increased their number and frequency by generating a mouse transgenic (Tg) for the MAIT cell-specific TCRα chain (iVα19). Upon immunization with a myelin oligodendrocyte glycoprotein (MOG) peptide (57), iVα19 Tg mice showed dramatically reduced EAE incidence and severity, as compared with wild-type B6 or CD1d-deficient mice, which lack CD1d-restricted iNKT cells. A similar regulatory effect of MAIT cells was observed in regular B6 mice adoptively transferred with iVα19 Tg cells, as well as in MR1 knock-out (KO) mice compared to wild-type animals. iVα19 Tg cells induced IL-10 production by B cells in a MR1-independent manner *in vitro*, accompanied by a reduction in the production of inflammatory cytokines by MOG-specific T cells, probably accounting for the EAE-protecting effect of MAIT cells. Altogether, these data strongly suggested that MAIT cells display an immune-regulatory function in the context of EAE. The interpretation of these data was, however, hampered by the fact that iVα19 Tg mice were not crossed onto a Cα KO background, thereby allowing endogenous TCRα chains to be recombined and expressed. It is, therefore, difficult to evaluate how the alterations induced in the TCR repertoire of iVα19 Tg mice could have impacted the data obtained in the EAE model.

However, this striking paper triggered several studies evaluating MAIT cell numbers and functions in MS patients, which yielded contradictory results. Miyazaki et al. observed a dramatic reduction in the frequency of blood MAIT cells in patients with relapsing-remitting MS (RR-MS) (58). The frequency of blood MAIT cells inversely correlated with disease activity as it was lower in active disease as compared to stable patients. Interestingly, steroid treatment of active patients induced a rise in the frequency of MAIT cells. This may suggest that a reversible, altered distribution could be responsible for their depletion from blood in the acute phases of inflammation. It was suggested from *in vitro* experiments that MAIT cells suppress IFNγ production by other T cell subsets, akin to the data obtained with iVα19 Tg mice; however, this suppression was independent of B cells or IL-10 production. By contrast, Annibali et al. observed an increased frequency of $CD8^{+}CD161^{hi}$ T cells in the blood of MS patients (51). Several independent studies have clearly demonstrated that this T cell subset is almost exclusively composed of MAIT cells (16, 18). $CD8^{+}CD161^{hi}$ T cells from MS patients

displayed an inflammatory phenotype characterized by high IL-17 production, and could be found in post-mortem brain biopsies within perivascular cuffs and chronic active lesions. Thus, MAIT cells in these patients behaved oppositely from the previous reports, and suggested a pro-inflammatory pathogenic role in MS. These data were in part corroborated by an analysis of post-autologous hematopoietic stem cell transplantation in MS patients. This study showed that MAIT cells were depleted by a conditioning regimen including cyclophosphamide or alemtuzumab and did not recover for more than 2 years post-graft (59). Before immunosuppressive conditioning, the patients exhibited a high frequency of MAIT cells, together with a pro-inflammatory profile. Moreover, MAIT cells were identified within white matter inflammatory lesions from post-mortem samples of nine MS cases. In addition, a recent report described a significant but modest decrease of MAIT cells in the blood of RR-MS patients (60), and confirmed their presence in brain lesions. Stimulation of MAIT cells with IL-18 induced very late antigen 4 (VLA-4) up-regulation, providing a possible mechanism to explain their migratory behavior. In agreement with this, the decrease in blood MAIT cells was inversely correlated with IL-18 plasma levels (60). More recently, Held and collaborators made a strong effort to identify antigen-driven T cells expansion within brain lesions by combining laser microdissection and TCR pyrosequencing (61). They indeed observed massive expansion of T cell clones that persisted for several years. Surprisingly, TCRs closely related to, but distinct from, the MAIT cells' TCR were found, whereas the canonical TCR Vα7.2-Jα33 was present as a minor fraction (61). It is not clear at this point to what extent these TCRs allow any recognition of MR1, and how T cells expressing these specificities are indeed related to MAIT cells. Thus, opposite results are described with regard to MAIT cell frequency and functions in MS patients, and the reasons underlying these discrepancies are currently not clear. It should be pointed out at this point that the effect of immunosuppressive/immunomodulatory regimen on MAIT cells has never been studied, with the exception of the hematopoietic stem cell transplantation study mentioned above. The cohorts analyzed in these various articles included patients that were free of any current medication affecting the immune system, but no mention of previous treatments was made, with the exception of the Annibali's work, which analyzed mostly treatment-naïve patients. Therefore, differences in treatment history may hamper a valid comparison of these cohorts. Nevertheless, available data suggest that MAIT cells in MS gain the ability to traffic to the CNS, which in some cases may explain their depletion from blood, as seen in other pathological settings (see below).

MAIT Cells in Other Autoimmune/ Inflammatory Diseases

Several reports have described alterations in the frequency, phenotype, location and/or functions of MAIT cells in inflammatory diseases, mostly in human samples and sometimes in mouse models. Most studies found a decreased frequency of blood MAIT cells, in patients with SLE, celiac disease, or IBD (31, 52, 56). When studied, the function of MAIT cells was altered, with decreased IFNγ production in SLE and IBD, and increased IL-17 secretion in IBD. Increased MAIT cell frequency was found in the inflamed intestinal tissue of IBD patients as well as in skin lesions of patients with psoriasis (27, 31). MAIT cells appeared pathogenic in a mouse model of autoimmune arthritis (62). In this study, the authors showed that MR1 KO animals were less sensitive to both active (collagen-induced) and passive (antibody-induced) arthritis than wild-type animals. Moreover, they used adoptive transfer of MAIT cells from iVα19 Tg mice (with the limitations already described) to reveal the disease-enhancing role of MAIT cells in the passive arthritis model. Recently, two seminal studies documented the behavior of MAIT cells in obese patients. It appears that, similar to several autoimmune or infectious diseases, MAIT cell frequency gradually declines in the blood of obese patients, and increases in the adipose tissue where MAIT cells are prone to produce IL-17 (28, 29).

Putative Mechanisms of MAIT Cells Involvement in MS and Other Inflammatory Diseases

The diverse and clonal expression of the TCR by T cells implies that different clones react against different ligands. MAIT cells display very limited repertoire diversity and therefore should recognize a limited set of different ligands. However, akin to other innate T cell subsets (such as γδ T cells or CD1d-restricted iNKT cells), the antigens recognized by MAIT cells are highly conserved and potentially expressed by a large variety of bacteria and fungi (34). This property accounts for the activation of MAIT cells by various microorganisms, such as *Escherichia coli*, *Mycobacterium tuberculosis*, *Klebsiella pneumoniae*, *Shigella flexnerii*, *Salmonella typhimurium*, *Vibrio cholerae*, or *Francisella tularensis*, and certainly many others (20, 24, 33–35, 63–66).

However, our knowledge of the mechanisms by which MAIT cells are recruited and activated in non-infectious inflammatory diseases is minimal. The first general hypothesis postulates that MAIT cells are activated in a cognate manner, by recognition of their specific Ags. But what would be the origin of these antigens? In the case of intestinal inflammatory diseases, alterations in the permeability of the gut epithelial barrier and subsequent translocation of microbial products may promote MAIT cell activation. Further, recent findings suggest that this general mechanism is involved in the pathogenesis of non-intestinal inflammatory diseases, including MS (67, 68). However, it would be difficult to explain how a low level of microbial translocation would induce a strong MAIT cell activation, given the fact that their specific ligands appear to be extremely unstable (20), which could actually be a regulation mechanism. Alternatively, there may be endogenous ligands for MAIT cells, whose expression could be induced and/or increased in the context of inflammation. It must be pointed out that there is to date no demonstration that such endogenous ligands exist. However, this could be inferred from several studies analyzing MAIT cell ontogeny, which proved that

MAIT cells are positively selected on hematopoietic cells in the thymus in the presence of MR1 only (10, 69). Thus, although it is highly probable that such endogenous ligands for MAIT cells exist, their molecular characterization is still eagerly awaited.

The second hypothesis to explain such broad involvement of MAIT cells in inflammatory diseases involves a bystander mechanism, i.e., in the absence of their antigen recognition through their TCR. MAIT cells are effector/memory cells, and as such, are equipped with a panel of receptors involved in cell migration, or in the response to inflammatory mediators [Toll-like receptors (TLR), cytokine receptors, etc.]. Notably, MAIT cells can be activated to produce IFNγ in the mere presence of cytokines, specifically a combination of IL-12 and IL-18 (37). Jo et al. showed that the TLR7/8 agonist R848 stimulates monocytes to produce IL-12 and IL-18, which in turn activate IFNγ secretion by MAIT cells (70). Further, bacteria devoid of MR1-binding ligands, such as *Streptococcus pneumoniae*, also induce the production of these cytokines by antigen-presenting cell (APC) and subsequent MAIT cell activation. IL-12 is involved in many autoimmune diseases, including MS (and EAE); IL-18 is also a major cytokine whose role in driving autoimmune diseases but also hypersensitivity conditions is gaining strong interest (71, 72). Therefore, it could be proposed that the inflammatory milieu drives MAIT cell activation through cytokine responsiveness, leading to their recruitment in the inflamed lesions of the brain in MS. Indeed, IL-18 apparently up-regulates VLA-4 at the surface of MAIT cells, providing a molecular clue as to how the cytokine milieu might influence MAIT cells migratory behavior (60). Other cytokines, such as IL-1β, which shares with IL-18 an inflammasome-dependent processing, might also be involved.

Possible Roles of MAIT Cells in the Inflamed Brain During MS

Mucosal-associated invariant T cells can be found in the inflamed CNS lesions from MS patients; they are also observed in other inflamed tissues, such as the skin of psoriatic patients. This raises the obvious question of their functions within tissues and their relevance to the pathogenesis of these diseases in general. Very little information is available about MAIT cell functions in tissues, and we are currently led to speculate on this matter. It is of course possible that they are only innocent bystanders in the inflammatory lesions and do not play any important role. Blood MAIT cells are equipped with receptors involved in migration to the CNS, such as CCR5 (in the steady-state) and VLA-4 (after stimulation). Indeed, MAIT cells are usually identified in low frequency within target tissues of inflammation. On the other hand, it must be reminded that MAIT cells are effector/memory cells prone to produce inflammatory cytokines and to release cytotoxic granules. Several reports showed that circulating blood MAIT cells in diseases, such as IBD, type 2 diabetes (T2D), and MS, display increased cytokine-producing functions, in particular IL-17, as compared with their counterparts from healthy donors (29, 31, 51). Therefore, infiltrating MAIT cells in MS are functionally active, which may suggest their involvement in the disease. If so, the next question is: what role do MAIT cells play in the inflamed brain?

Upon activation, cytokine secretion by MAIT cells is mostly related to a Tc1/Tc17 pattern, i.e., IFNγ and/or IL-17 as well as GM-CSF and TNFα. All these cytokines are considered as major culprits in many autoimmune diseases, including MS. From there, it is tempting to speculate that MAIT cells are pro-inflammatory cells with deleterious effects in the disease process. Furthermore, given that MAIT cells are cytotoxic against bacteria-infected epithelial cells, it is possible that in MS they gain the ability to kill oligodendrocytes, axons/neurons, or even other CNS-resident cell types. Direct evidence supporting this hypothesis is, however, lacking. Although MR1 (the restriction molecule for MAIT cells) appears to be broadly expressed, its expression in CNS cells has never been investigated. This aspect needs to be addressed in MS as well as in other CNS inflammatory diseases. We think that the current knowledge regarding the functional properties of MAIT cells does not permit to predict whether these cells ultimately play a pro-inflammatory or regulatory role in MS. MS is probably a heterogeneous disease with regard to the precise mechanisms of pathogenesis. Although the issue is not totally resolved, there is increasing evidence that both inflammasome-dependent and inflammasome-independent mechanisms exist that would be differentially elicited in patients, and that this may underlie the differential response to therapy (73, 74). We postulate that this heterogeneity in the disease pathogenesis could partially explain the contradictory results found by different teams with regard to the pro-inflammatory or regulatory role of MAIT cells in MS and EAE. If this hypothesis holds true, we also anticipate that there could be a correlation between MAIT cells functions in MS (for instance, IFNγ versus IL-17 production) and response to disease-modifying therapies, suggesting a possible use as an immunological biomarker.

Concluding Remarks

The multifaceted nature of MAIT cells makes them promising candidates for therapeutic targeting and/or to use as biomarker of disease. A strong body of work strongly suggests that MAIT cells are involved in MS, fostering new studies aiming at deciphering their precise role in pathogenesis. Studies in human patients are obviously hampered by the limited access to tissue samples. One interesting avenue would be to analyze MAIT cells phenotype and functions in patients stratified according to their response to therapy. Limitations of the murine models available thus far have been already described. However, a report published while this review was in preparation described MAIT cells in wild-type mice, with the help of MR1 tetramers (32). The authors suggest that mouse MAIT cells more closely resemble their human counterparts than previously thought, although important differences remain, such as a much lower frequency and a more pronounced Tc17 skewing of cytokine secretion. There is no doubt that future analysis of MAIT cells in the EAE model will yield relevant data as to their role in this disease.

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