B CELLS IN INFLAMMATORY AND NEURODEGENERATIVE DISEASES OF THE CENTRAL NERVOUS SYSTEM

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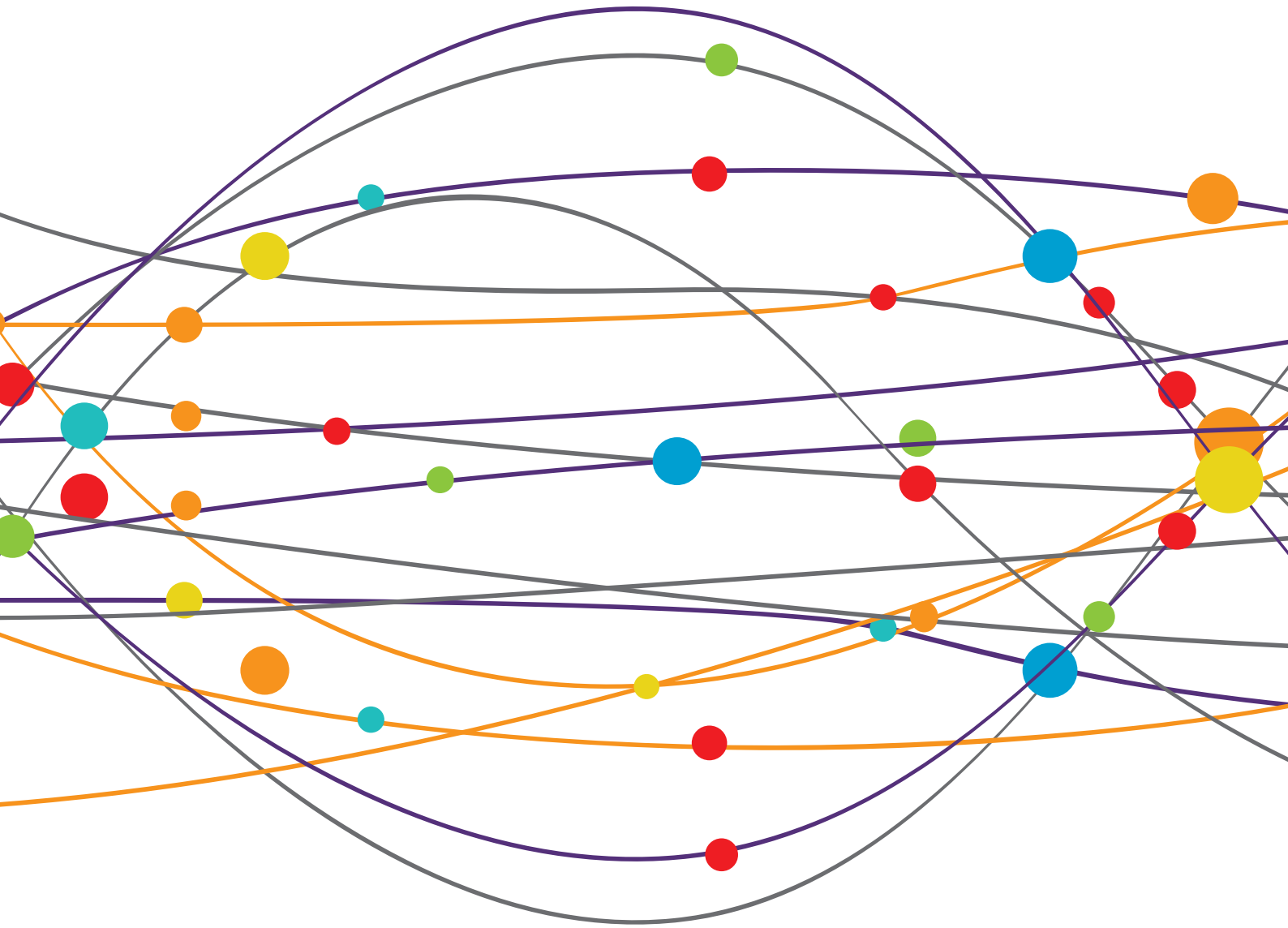

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B CELLS IN INFLAMMATORY AND NEURODEGENERATIVE DISEASES OF THE CENTRAL NERVOUS SYSTEM

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EDITORIAL
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Editorial: B Cells in Inflammatory and Neurodegenerative Diseases of the Central Nervous System

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Keywords: B cells, neurodegeneration, multiple sclerosis, antibodies, B cell depleting therapies

Editorial on the Research Topic

B Cells in Inflammatory and Neurodegenerative Diseases of the Central Nervous System

In recent years, the role of B cells in conditions affecting the central nervous system (CNS) has substantially expanded our perspectives on mechanisms of neuroinflammation and neurodegeneration. The success of B cell-depleting therapies (BCDT) in patients with diseases such as neuromyelitis optica and multiple sclerosis (MS) has underscored the role of B cells in both cellular and humoral-mediated CNS conditions. This Research Topic aimed to provide a comprehensive overview of the functions of B cells in the CNS during homeostasis and in the presence of inflammatory and/or neurodegenerative diseases. Ultimately, the contributions received for this Research Topic covered three main areas (1) antibodies, (2) B cells functions, and (3) therapies, mainly focusing on MS.

Two contributions are focused on the pathogenic role of antibodies. The presence of persistent oligoclonal bands (OCBs) and IgG deposition in demyelinating plaques are essential features of MS pathology. However, their role remains controversial. In their review, Yu et al. hypothesized that circulating IgG1 and IgG3 diffuse across a transiently damaged blood-brain barrier (BBB), contributing to the total intrathecal IgGs and increasing the risk of antibodies-mediated cytotoxicity to CNS cells. However, MS still does not meet the full definition of autoimmune disease, and more studies are needed to clarify the pathological role of antibodies in MS.

Understanding the immunopathogenic functions of antibodies is relevant in a wide range of CNS conditions other than MS. In their original research article, Hang et al. focus on the anti-leucine-rich glioma-inactivated 1 antibody (anti-LGI1) encephalitis, a common autoimmune encephalitis characterized by progressive cognitive impairment. By analyzing the clinical outcome of 21 patients, the authors conclude that considerable antibodies-mediated damage is seen in patients early in the disease, and early and long-term effective immunotherapy can obtain a better cognitive functional prognosis.

Five additional contributions in this Research Topic are focused on the role and function of B cells in CNS conditions. In their review, Chunder et al. discuss the involvement of B cells in two different neuroinflammatory scenarios by drawing parallels between MS and virus-induced neuroinflammation. Both conditions show similar signatures for B cell migration, retention, and regulation in the CNS. Thus, the authors conclude that the basics of B cell biology remain the same independently of the trigger of neuroinflammation, showing a balance between protective and pathogenic functions.

As the nature of the B cell response differs considerably between the stages of the disease,

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Holloman et al. explore the mechanisms by which B cells contribute to disease progression in primary-progressive MS (PPMS), specifically focusing on cytokine production, antigen presentation, and antibodies synthesis. Authors draw their conclusions based on the analysis of clinical trial data highlighting the existence of a subset of patients with PPMS. The latter have active inflammation contributing to their progressive disability and benefit from BCDTs such as Ocrelizumab. Altogether, the data indicate that BCDTs likely reduce disease progression in PPMS by reducing B cell-mediated inflammation.

Two more review articles focus on the function of specific B cell phenotypes in MS. In their review, Ran et al. unravel the role of regulatory B cells (Breg) and their diversification in demyelinating diseases. An increasing number of studies have confirmed that Breg improve MS. According to the inflammatory microenvironment and the interactions with surrounding cells, Breg can indeed be activated and differentiate into subgroups of cells with beneficial rather than pathogenic functions. Concurrently, DiSano et al. discuss the roles of memory B cells (Bmem), both within the periphery and inside the CNS compartment. Bmem are rising as a critical B cell phenotype in MS due to their antigen experience and rapid response to stimulation. Bmem display diverse effector functions, including antigen presentation to $CD4^+$ T cells and precursor to antibody-secreting cells (ASC). On this same line of thought, Negron et al. discuss the evidence supporting the interconnectedness of $CD4^+$ T cells, particularly follicular T helper (T_{FH}) cells and B cells. T cell-dependent B cell responses originate and take shape in germinal centers (GCs), specialized microenvironments that regulate B cell activation and subsequent differentiation into ASCs or Bmem, a process for which T_{FH} cells are indispensable. GCs represent a critical site of B cell tolerance, and their dysregulation has been implicated in the pathogenesis of several autoimmune diseases, including MS.

Finally, three contributions to this Research Topic focus on different aspects of MS treatment, two specifically discussing BCDTs. Roach and Cross critically review the results of past and ongoing clinical trials of anti-CD20 monoclonal antibodies (mAb) with lytic effects on B cells in MS: Rituximab, Ocrelizumab, and Ofatumumab. The authors review nicely and succinctly also safety profiles, the potential mechanism of action, and alternatives to interfere with B cell function, e.g., anti-CD19 mAb. Complementary to this contribution is the original work by Sempere et al., reporting their real-world experience in a retrospective analysis of 70 MS patients, both relapsing and progressive, treated with Ocrelizumab. Authors report clinical and MRI results, showing that 94% of relapsing patients achieved no evidence of disease activity (NEDA). Despite the overall low number of patients included, this study confirms the effectiveness of BCDTs in the treatment of MS.

The last article on this topic is shifting gear by proposing a new algorithm for progressive multifocal leukoencephalopathy (PML) risk stratification in patients treated with Natalizumab, an anti-α4 integrin mAb interfering with lymphocyte, both T and B cells, migration through the BBB. In this study by Toboso et al., 1,240 people with MS treated with Natalizumab were recruited in 36 European Hospitals to evaluate patients' clinical and demographic characteristics as predictors of PML occurrence. Thirty-five patients developed PML and based on the analysis of B cell-related parameters like anti-lipid specific IgM OCBs and anti-JC virus antibodies, besides disease activity and age, authors established a new algorithm as a PML risk stratification tool for individual patients.

In conclusion, this Research Topic highlights the involvement of B cells in neuroinflammatory diseases. It discusses the evidence supporting B cells' pathogenic immunomodulatory functions in neurological disorders, particularly B cell-directed therapies.

AUTHOR CONTRIBUTIONS

FG, RM, and LP wrote this editorial. All authors approved the submitted version of this editorial.

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REVIEW
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Regulatory B Cells and Its Role in Central Nervous System Inflammatory Demyelinating Diseases

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Regulatory B (Breg) cells represent a population of suppressor B cells that participate in immunomodulatory processes and inhibition of excessive inflammation. The regulatory function of Breg cells have been demonstrated in mice and human with inflammatory diseases, cancer, after transplantation, and particularly in autoinflammatory disorders. In order to suppress inflammation, Breg cells produce anti-inflammatory mediators, induce death ligand-mediated apoptosis, and regulate many kinds of immune cells such as suppressing the proliferation and differentiation of effector T cell and increasing the number of regulatory T cells. Central nervous system Inflammatory demyelinating diseases (CNS IDDs) are a heterogeneous group of disorders, which occur against the background of an acute or chronic inflammatory process. With the advent of monoclonal antibodies directed against B cells, breakthroughs have been made in the treatment of CNS IDDs. Therefore, the number and function of B cells in IDDs have attracted attention. Meanwhile, increasing number of studies have confirmed that Breg cells play a role in alleviating autoimmune diseases, and treatment with Breg cells has also been proposed as a new therapeutic direction. In this review, we focus on the understanding of the development and function of Breg cells and on the diversification of Breg cells in CNS IDDs.

Keywords: regulatory B cells, central nervous system, inflammatory demyelinating diseases, multiple sclerosis, neuromyelitis optica

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INTRODUCTION

The immune response feedback is an important mechanism that maintains the immune balance. Inflammatory diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and multiple sclerosis (MS) are hallmarks of immunologic imbalances. As a major component of the immune system, B cells play both positive and negative roles in innate and adaptive immunity, through effector molecules such as antibodies and cytokines as well as through antigen-presention. On the one hand, B cells can mediate several negative processes such as amplifying immune responses.

Mechanistically, they differentiate into plasmablasts that secrete effector antibodies (1), may modulate effector T cell response through antigen presentation (2) and production of inflammatory cytokines (3). In addition, there is also a subset of B cells that regulates immune response to pathogens and autoantigens. These regulatory B cells are core targets in autoimmune and infectious diseases as well as cancer. These cells have a huge therapeutic potential against the aforementioned diseases. In one study performed in 1974 on delayed-type hypersensitivity, it was found that when B cells were removed, adoptively transferred splenocytes induced more intense reactions and lost their ability to suppress the delayed-type hypersensitivity reactions. This suggests that B cells or their products mediates inhibition of excessive inflammatory response (4). In another study conducted in 1996, it was found that mice with experimental autoimmune encephalomyelitis (EAE) but lacking B cells displayed greater differences in disease onset, severity, and recovery compared with the wild type group (5). Other studies on colitis and arthritis have demonstrated that B cells have antibody-independent immunoregulatory function (6, 7). Elsewhere, researchers have suggested that B cells inhibits excessive inflammation. B cells associated with inhibitory functions are referred to as Breg cells. IL-10 has been found to play a crucial role in the recovery of EAE (8). Other studies have further demonstrated that IL-$10^{-/-}$ mice display a non-remitting course of EAE, similar to the B cell-deficient mice (9). Combined, these findings suggest that B cells regulatory functions are mediated by IL-10. B cell-derived IL-10 has indeed been shown to play a key role in controlling autoimmunity (10). Accordingly, expression of IL-10 has been widely used to define suppressive B cell populations in mice and humans (11). B cells also regulate inflammation by a variety of IL-10-independent mechanisms (12).

Central nervous system Inflammatory demyelinating diseases (CNS IDDs) is a term referring to several CNS disorders, characterized by damaged myelin sheath of neurons, thus impairing transmission of signal by affected nerves. CNS IDDs can be differentiated based on disease severity and temporal courses, imaging, laboratory test and pathological characteristics. CNS IDDs mainly include MS, neuromyelitis spectrum disorders (NMOSD), and myelin oligodendrocyte glycoprotein antibody-associated disease (MOG-Ab associated disease) (13). IDDs were considered to be primarily mediated by T lymphocytes. Given the success of therapeutic B cell depletion in MS (14) and NMOSD (15), there is growing concern on the role of B cells in the pathogenesis of IDDs. Studies on auto antibodies have improved our understanding of the role of B cells in the pathogenesis of immune-mediated diseases such as the appearance of oligoclonal IgG bands and deposition of IgG in the cerebrospinal fluid of MS, the presence of AQP4-IgG in NMOSD and antibodies against MOG in MOG-Ab associated disease. In addition, Breg cells also play a role in CNS IDDs. For instance, Breg cells deficiency is associated with severe symptoms of MS (16) and NMOSD (17), suggesting that Breg cells have the therapeutic potential to reduce immune-mediated inflammatory disorders. Subsequently, this review aimed at providing a summary of the current understanding on the development and function of Breg cells, and their role in the etiology of CNS IDDs.

DEVELOPMENT AND DIFFERENTIATION OF BREG CELLS

There are two distinct populations of B cells identified in mouse and human; the B1 and B2 subsets. Similar to other immune cells, B cells are derived from hematopoietic stem cells (HSCs), where they differentiate into progenitor B cells (Pro-B), precursor B cells (Pre-B) and immature B cells (**Figure 1**). Immature B cells undergo a "transitional" state, which is an early phase to the mature phenotype, after which they leave the bone marrow or fetal liver. B1 subset differentiates into mature B1a cells expressing CD5, and mature B1b cells. After stimulation with polysaccharides or lipids, mature B1 cells differentiate into antibody-secreting plasmablasts and short-lived plasma cells secreting antigen-specific antibodies. As for the B2 subset, they undergo three consecutive transitional B cells stages; transitional-1 (T1), transitional-2 (T2) and transitional-3 (T3). Transitional-B cells then migrates to the spleen and lymph node follicles, where they eventually differentiate into either follicular (FO) or marginal zone (MZ) B cells. The intermediate subset between T-2 B and MZ B cells are transitional-2 marginal-zone precursors (T2-MZP) B cells. Activated MZ B and FO B cells eventually differentiate into plasma cells, antibody producing B cells. Under special conditions, transitional B cells, MZ cells, T2-MZP cells, B1 cells, plasmablasts and plasma cells can all be activated to differentiate into Breg cells. Inflammatory microenvironment and intercellular interaction have been identified to activate this differentiation. Details of these processses will be discussed in the subsequent sections (**Figure 2**).

Inflammatory Microenvironment

The inflammatory microenvironment such as infiltration of cytokines and infection microenvironment can increase the expression as well as enhance the inhibitory property of Breg cells, implying that such factors play important roles in differentiation of Breg cells.

Numerous studies show that most inflammatory cytokines can indeed induce differentiation of Breg cells. As an immunosuppressive heterodimeric cytokine, IL-35 binds on its corresponding IL-35 receptor, activating signal transducer and activator of transcription (STAT) 1 and 3 to induce differentiation of resting B cells into IL-10 and IL-35-producing Breg cells. This suggests that IL-35 has the potential to induce autologous Breg cells as well as the treatment of autoimmune and inflammatory diseases (18, 19). More studies have demonstrated that IL-10-producing dendritic cells induced by IL-35 and phosphorylating STAT3 can induce immunosuppressive property of IL-10-producing B cells (20).

Similar to IL-35, IL-21 also induces production of IL-10 via phosphorylating STAT3. Accordingly, inhibition of phosphorylating STAT3 effectively blocks the production of IL-10 during the differentiation of the Breg cells. The effect of IL-21

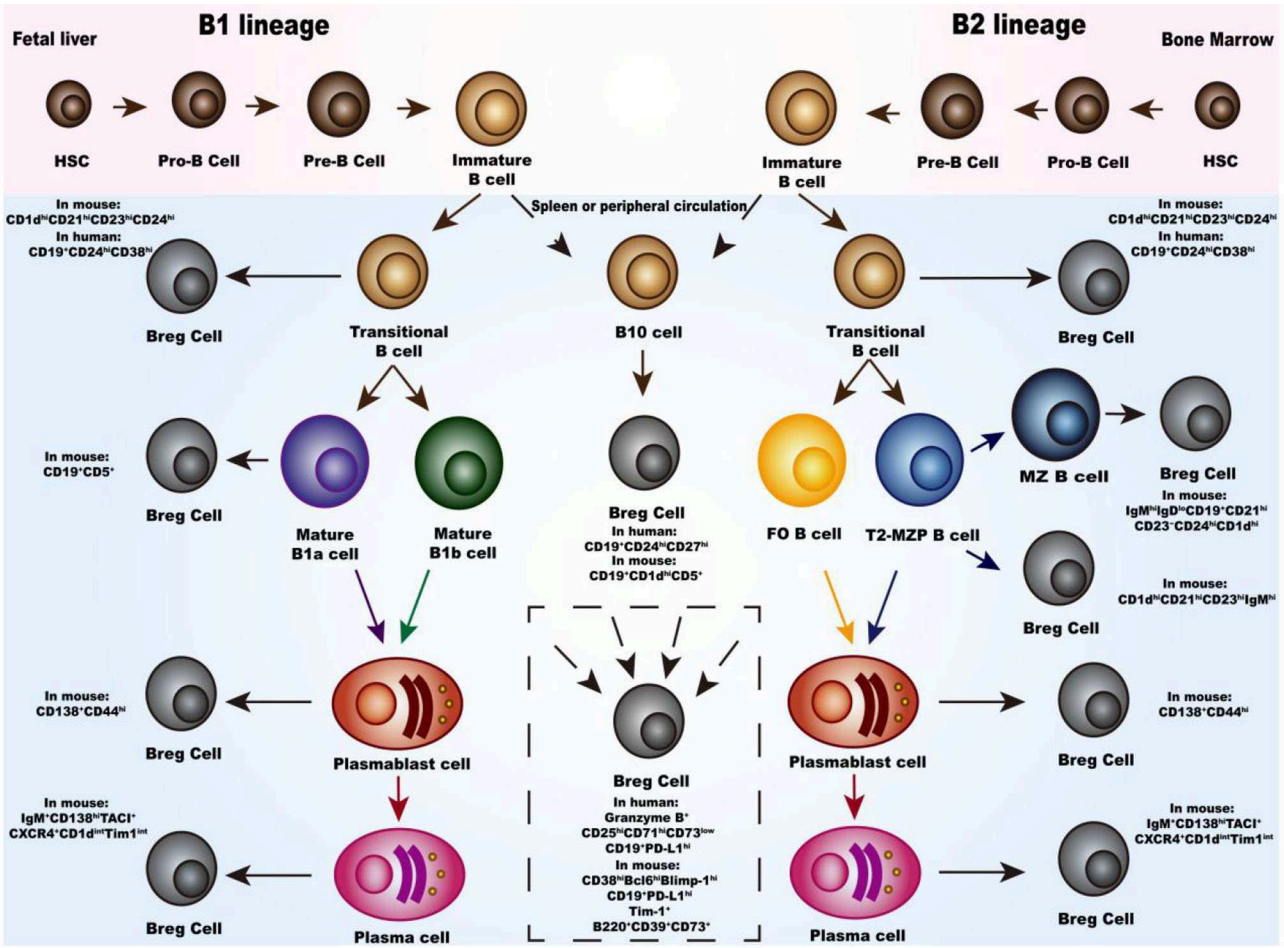

FIGURE 1 | Development and differentiation of Breg cells. In bone marrow or fetal liver, hematopoietic stem cells (HSC) differentiate through progenitor B cells (Pro-B), precursor B cells (pre-B), immature B cells and transitional B cells. For the B1 subset, migrating to the spleen or peripheral circulation, the transitional B cells differentiate into either mature B1a cells or mature B1b cells and eventually differentiate into plasmablast cells and plasma cells. Meanwhile, for the B2 subset, the transitional B cells differentiate either into follicular (FO) or marginal zone (MZ) B cells and eventually into plasmablast cells and plasma cells. Moreover, there is the transitional-2 marginal-zone precursors (T2-MZPs) B cells stage which is the precursor of MZ B cells. Studies have proved that the transitional B cells, MZ cells, T2-MZP cells, B1 cells, plasmablasts, and plasma cells can all differentiate into Breg cells with different phenotypes. Differentiation pathway of B10 cells in the middle of the photograph are listed separately because there are still not enough studies to classify them. In the dotted box, there are Breg cells with different phenotype and the source of them are still not clear.

on the differentiation of Breg cells strongly depends on additional signals including inhibitors of Toll-like receptors (TLR) and simulation of both B-cell receptor (BCR) stimulation and CD40 ligand. For instance, with the help of CD154 (CD40 ligand), IL-21 induces the differentiation of B cells into plasma cells or granzyme$^+$ B lymphocytes (an important type of Breg cells) (21). In addition, the maturation of Breg cells into effector cells that secrete functional IL-10 requires homologous interactions with T cells mediated by IL-21 and CD40 (22).

Other inflammatory cytokines critical for differentiation of Breg cells include IL-1β, IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF). In mice with arthritis, deficiency of B cell specific IL-6 or IL-1 receptors is shown to exacerbate the disease compared with the controls (23). GM-CSF and IL-15 are strong immunosuppressive molecules that can induce differentiation of naive CD19$^+$ B cells into Breg cells, a process that can reverse the neuropathology of EAE (24).

Surprisingly, immune response to infectious diseases does not always worsen autoimmune diseases. In some instances, response to infectious diseases drives the development of IL-10-producing Breg cells in both mice and humans. The helminth parasite *Schistosoma mansoni* contains TLR4 inhibitor, thus is able to induce secretion of IL-10 by B cells. This can then change the course of MS and reduce the severity of the disease (25). Similarly, *Mycobacterium tuberculosis* contains a TLR inhibitor, thus infection by this bacteria can aid in the recovery of EAE because it mediates the production of IL-10 by B cells. In a clinical trial, Bacillus Calmette-Guerin (BCG), a vaccine against tuberculosis disease, has been shown to alleviate clinically isolated syndrome (CIS) by reducing the number of lesions and

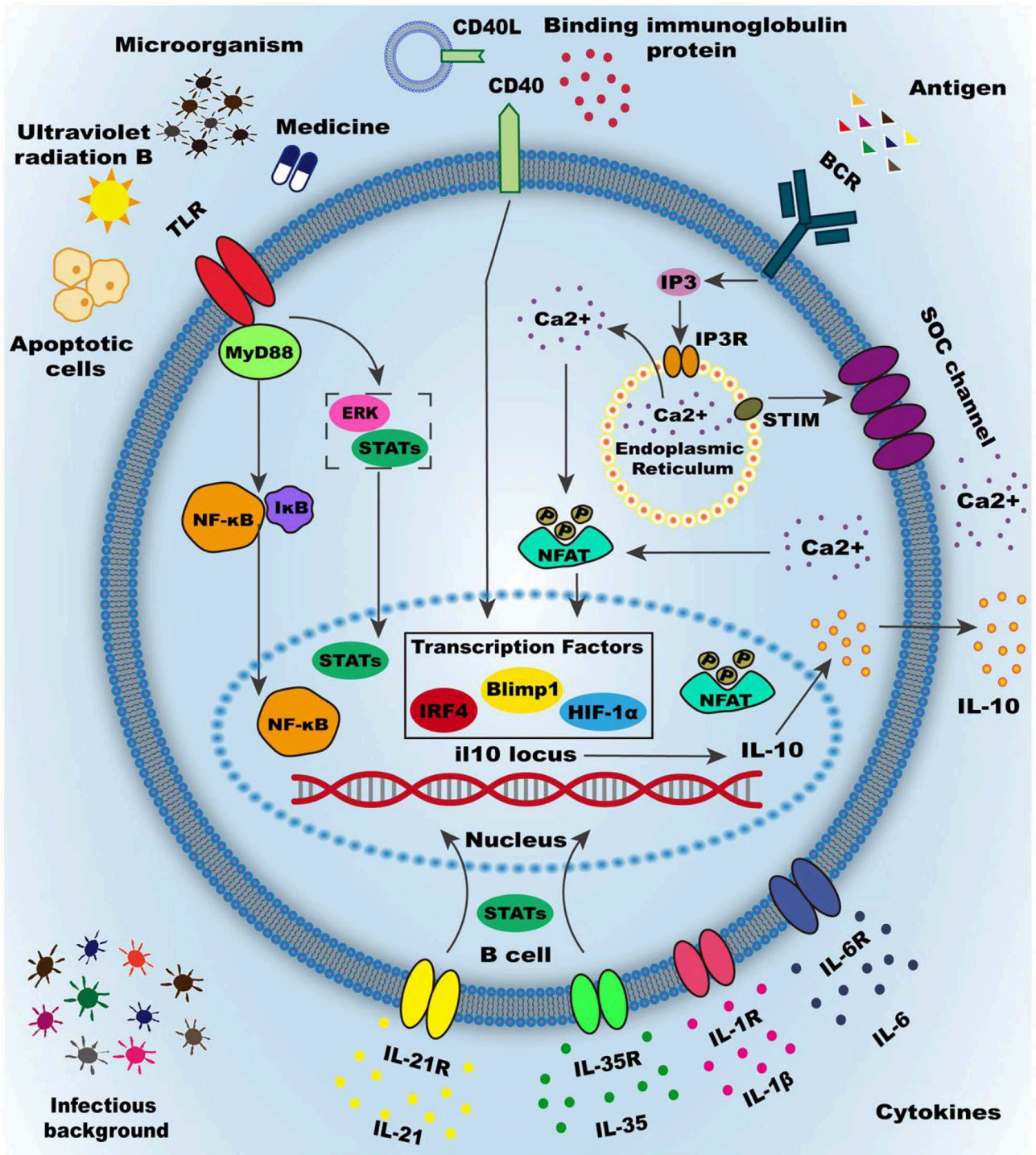

FIGURE 2 | Activation of Breg cells. Numerous mechanisms have been demonstrated to induce Breg cells, the most important of which are summarized as the inflammatory micro-environment and intercellular interactions. The inflammatory microenvironment is mainly produced by inflammatory factors, such as IL-35, IL-21, and other inflammatory cytokines. Cytokines often mediate activation of downstream signaling pathways by binding to cytokine receptors on the membrane. Microorganisms infection is another essential part of the inflammatory environment in the body, including invasive bacteria and commensal microbiota. Most microbial infections are activated by downstream molecular pathways mediated by membrane surface molecules, causing IL-10 transcription and translation and secretion. Intercellular interactions play a vital role in the induction of regulatory B cells, such as apoptotic cells or type3 innate lymphoid cells. Intercellular interactions mainly depend on the interaction of molecules on the surface of the membrane and the most classic surface molecules are Toll-like receptors (TLRs), CD40, B cell receptor (BCR). Many environmental and pharmaceutical factors can activate TLRs and cause downstream pathways activation, such as STATs and ERK orMyD88-NF-κB

(Continued)

FIGURE 2 | signaling pathway. CD40 can be activated by the CD40 ligand or binding immunoglobulin protein to mediate the activation of downstream signaling pathways STATs, and the downstream signaling pathways can be enhanced through synergy with other cell membrane surface molecules. BCR is thought to be involved in multiple functional processes of B cells, especially the induction of Breg cells. BCR combined with antigen can promote the release of calcium ions into the cell from the endoplasmic reticulum (ER) and also promote the activation of STIM on the endoplasmic reticulum, which open the calcium ion channels (SOC channel) on the cell membrane. A large amount of calcium influx increases the intracellular calcium ion concentration and promotes the phosphorylation of the nuclear factor of activated T cells (NFAT) in the downstream pathway, thereby increasing the transcription and translation secretion of IL-10. Other membrane surface molecules can promote Breg cell differentiation, such as Galectin-1and CD38. Research on regulatory B cell-specific transcription factors is still inconclusive, but the transcription factors such as Blimp1, IRF4, andHIF-1α have been shown to promote the transcription of IL-10.

improving long-term disease course (26). In MS, the severity of the disease significantly decreased after the reception with BCG vaccine (27). The underlying infection is not limited to invasive bacteria, but also includes the commensal microbiota in the intestines. These microorganisms have been shown to promote the differentiation of Breg cells in mesenteric lymph nodes and the spleen (23).

Intercellular Interaction

Intercellular interaction can also induce the differentiation of primary B cells into Breg cells, mainly through the activation of surface molecules on B cells (such as TLRs, CD40, BCR) and subsequent B cell downstream signaling pathway.

Gray M et al. found that apoptotic cells (ACs) affects the production of IL-10. This was demonstrated by injection of ACs into collagen-induced arthritis model, which induced the production of IL-10 by Breg cells, a process that alleviates inflammation (28). Gray M et al. also demonstrated the mechanism underlying secretion of IL-10 by B cells. Here, after recognizing the DNA containing complex on the surface of ACs, naturally occurring B cells (such as MZ B cells) bind and internalize the ACs surface chromatin complex, thereby activating TLR9 to regulate proliferation of B cells and secretion of IL-10 (29). Type 3 innate lymphoid cells (ILC3s) and innate B cells interact through IL-15 and B cell activating factors (BAFF), a process that promotes the development of ILC3s with CD40 ligand. CD40 positive ILC3s aid in the proliferation and differentiation of IL-10-secreting B cells. This mutually beneficial relationship between cells is important for maintaining immune tolerance, however, there are several deficiencies in this relationship in allergic asthmatic patients (30). By releasing IFN-α that interacts with CD40, dendritic cells can also drive the differentiation of immature B cells into IL-10-producing Breg cells. Conversely, Breg cells inhibits production of IFN-α by dendritic cells mediated by IL-10. In SLE, there are defects in this cross-talk, believed to be associated with abnormal activation of STAT1 and STAT3 (31).

TLRs are necessary for B cells to exert their inhibitory effects such as inhibition of inflammatory T cell responses and modulation of inflammation. TLRs-myeloid differentiation factor88 (MyD88) pathway is closely associated with the anti-inflammatory immune mechanism. In mouse and human, the activation of TLR2, TLR4, and TLR9 transduction signal can induce production of IL-10 in B cells. For instance, trametes versicolor is a medicinal fungus that can promote differentiation of B cells into $CD1d^+$ Breg cells in acute colitis, through the TLR2/4-mediated signaling pathway (32). Apart from chemical means, physical activation of B cells by factors such as ultraviolet radiation B has also been shown to induce differentiation of B cells into Breg cells. This process also suppresses the immune response through the TLR4-mediated signaling pathway (33). However, not all TLR stimulation can induce B cells to differentiate into Breg cells. For example, after activation through TLR7 and interferon-α, transitional B cells develop into pathogenic B cells, promoting the production of autoreactive antibodies (34). Studies on downstream mechanisms found that IFN-α can differentially regulate TLR7/8 and TLR9-activated STAT3 and ERK in B cells (35). More specifically, stimulation of B cells mediated by IFN-α and TLR7/8 inhibitors enhances phosphorylation of ERK1/2 and STAT3, which intern mediated production of IL-10 by B cells. Moreover, the activation of ERK and STAT3 is also important in TLR9- mediated IL-10 producing by B cells. However, IFN-α is not able to enhance the CpG-induced phosphorylation of ERK1/2 and STAT3 in B cells. MyD88 is a key downstream molecule in the inflammatory signaling pathway that also plays an important role in the regulation of cellular mediated immunity during infection (36). For instance, during *Helicobacter felis* infection, B cells activated by Helicobacter TLR-2 ligands can actuate IL-10-producing B cells in a MyD88 dependent manner (37). Endogenous TLR4 ligands are also found to be up-regulated, and activate B cells to produce IL-10 via TLR4-MyD88 signaling (38, 39). At transcription level, NF-κB plays an essential role in the inflammatory and immune response of cells, and the mis-regulation of NF-κB may cause autoimmune diseases, chronic inflammation and many types of cancer. Most importantly, with infectious diseases, activation of TLRs-MyD88-NF-κB can induce production of B cells specific IL-10 (40). On tumor research, one study found that activation of the TLRs-MyD88-NF-κB signaling pathway is necessary for Breg cells differentiation and the induced Breg cells with immunoregulatory functions can contribute to the suppression of anti-tumor immunity (41). This aside, IκBNS is a TLR-inducible nuclear IκB protein important in the TLRs-mediated IL-10 production in B cells. Mechanistically, IκBNS regulates inflammatory responses by inhibiting the induction of a subset of TLR-dependent genes through modulation of NF-κB activity (42). IκBNS-deficient B cells show reduced expression of Breg cells transcription factors including B lymphocyte induced maturation protein 1 (Blimp-1 protein) and interferon regulatory factor 4 (IRF4). They also fail to generate IL-10 producing $CD138^+$ plasmablasts (a subset of Breg cells), suggesting that IκBNS is selectively required for IL-10 production in B cells, responding to TLR signals (43).

CD40, a membrane-associated protein, is a member of the tumor necrosis factor (TNF) receptor superfamily. The activation of CD40 on B cells not only induces the maturation of B cells into antibody-producing B cells, but is also crucial for the activation of Breg cells. In transgenic mice, ectopic expression of the CD154 is associated with increased CD40 signaling, which can in turn induce activation of the STAT3 pathway and an increase in the proportion of Breg cells (44). In experimental lupus, stimulation with CD40 inhibitor can induce IL-10-producing T2-like B cells to suppress Th1 responses and induce suppressive capacity to $CD4^+$ T cells. This reduces the severity of the disease and delays IL-10 dependent progression of the disease (45). Furthermore, the synergistic effect of cell surface membrane molecules can activate B cells to perform their regulatory functions. For instance, co-stimulation of CD40 and TLRs has been shown to induce the highest proportion of IL-10 producing Breg cells, which plays a crucial role in recovery from MS relapse (46). Besides, an immunoglobulin protein (BIP), a member of the heat shock protein 70 family, can act in synergy with CD40 to induce the differentiation of Breg cells as well as suppress proliferation of T cells in a partially IL-10-dependent manner (47).

The BCR-STIM pathway is involved in most B cell function processes, such as activation, differentiation and antigen recognition, endocytosis, and presentation. BCR-stimulated B cells can maintain long-term tolerance and protect vulnerable mice from type 1 diabetes via an IL-10-dependent mechanism (48). At transcription level, many regulatory molecules are involved in IL-10 expression, including stromal interacting molecules (STIM) of endoplasmic reticulum (ER), nuclear factor of activated T cells (NFAT) family, IRF4 and a crucial cis-regulatory element CNS-9 that is located 9kb upstream of the transcription start site of *il10*. After BCR activation, calcium sensory proteins STIM-1 and STIM-2 induce store-operated Ca^{2+} (SOC) influx and proliferation to increase the intracellular calcium concentration. This can activate the signaling pathway of NFAT family and mediate secretion of IL-10 (49). Without STIM1 or STIM2 proteins, B cells fail to produce IL-10 due to the defect in the activation of NFAT after BCR stimulation. Besides, the CNS-9 region contains clusters of NFAT and IRF binding motifs, which enhances the expression of IL-10 mRNA through the synergy effect of NFAT1 and IRF4. Therefore, deficiency of Irf4 specific to B cells impairs secretion of IL-10 and abnormal differentiation of Breg cell in dLNs. This intern predispose one to EAE (50).

Some other surface molecules such as CD38, Galectin-1 (Gal-1). may also play a role in the expression and function of Breg cells. For instance, CD38 is a transmembrane protein expressed in B lymphocytes. It can induce proliferation, differentiation or apoptosis of Breg cells. Currently, studies have found that the expression and effect of CD38 are inconsistent in different diseases. Some studies have demonstrated that $CD38^{-/-}$ mice are more suitable for generating and expanding regulatory B10 cells than WT mice under appropriate stimulation (51). However, other studies have suggested that $CD1d^{hi}CD5^+$ Breg cells highly expresses CD38, and in the presence of a CD38 stimulator, the percentage of Breg cells and their IL-10 production function increases (52). Galectin-1 (Gal-1) is a class of protein necessary for B cell development in the bone marrow. It plays a role in inducing B cell regulatory functions. Compared with wild-type B cells, Gal-$1^{-/-}$ B cells have impaired IL-10 and Tim-1 expression, but with increased expression of TNF-α (53).

PHENOTYPES OF BREG CELLS

The activation process of immune cells is different in various disease states. Therefore, many surface markers used to identify Breg cells are either up- or down-regulated, which results in the non-uniform molecular expression of Breg cells. Studies in experimental animal models as well as in patients with autoimmune diseases have identified multiple subsets of Breg cells that exhibits diverse immune suppression mechanisms (see **Tables 1**, **2** for the function of various Breg cell subtypes in mice and humans). However, due to the intricate origins and activation pathways of Breg cells, there is an ever-increasing list of new phenotypic and functional markers associated with Breg cells.

Breg Cells in Mouse

Transitional-2 B Cells ($CD1d^{hi}CD21^{hi}CD23^{hi}CD24^{hi}$)

CD1d molecules are cell surface glycoproteins and the cytoplasmic tail of CD1d participates in signaling cascades associated with the transcription of IL-10 (82). In autoimmune diseases, the induced B-cell subpopulation is characterized by CD1d up-regulation, and the up-regulated CD1d can induce B-cell subpopulations to produce IL-10, promote antigen-specific regulatory T cell differentiation, and down-regulate inflammatory cascades associated with IL-1 upregulation and STAT3 activation (6). CD1d is expressed on a wide variety of cell types, of which three different B cell subsets express high levels of CD1d and have the potential to become Breg cells, including T2 B cells, MZ B cells and T2-MZP B cells. In the early stages of differentiation, B cells have already had the ability to differentiate into Breg cells. In SLE model, the adoptive transfer of T2 B cells can reverse autoimmunity and suppress the Th1 response (45). CD40 ligation halts the apoptosis of T2 B cells and prevents further differentiation into mature FO B cells (83), which can induce and expand the differentiation of $IL\text{-}10^+$ T2 B cells. In allograft rejection model, T2 B cells isolated from tolerant mice show higher survival rates and inhibit cytokine production of T cells, thereby prolonging graft survival, suggesting that T2 B cells have the potential to treat allograft rejection (54).

MZ B Cells ($IgM^{hi}IgD^{lo}CD19^+CD21^{hi}CD23^-CD24^{hi}CD1d^{hi}$)

MZ B cells with CD1d high expression play a role in the prevention of autoimmunity through the production of regulatory cytokines and natural antibodies. Besides the polyreactive BCRs, MZ B cells also express high levels of TLRs, such as TLR9 which recognizes hypomethylated CpG motifs in bacterial DNA or chromatin complexes expressed on the surface of apoptotic cells (29). MZ B cells can differentiate into IL-10-producing B cells and down-regulate the production of pro-inflammatory cytokines in response to stimulation of ligands or cytokines such as BAFF (84). Studies have demonstrated that through inflammatory stimuli, T-bet-expressing MZ B cells

TABLE 1 | The phenotype and function of mouse regulatory B cell subsets.

Name	Phenotype	Functions
Transitional2 B cells	$CD1d^{hi}CD21^{hi}CD23^{hi}CD24^{hi}$	1.Supress the Th1 responses (45)
		2.Inhibit T cell cytokine production to prolong graft survival (54)
MZ B cells	$IgM^{hi}IgD^{lo}CD19^{+}CD21^{hi}CD23^{-}CD24^{hi}CD1d^{hi}$	1.Suppress CD8 and CD4 effector functions (55)
T2-MZP B cells*	$CD1d^{hi}CD21^{hi}CD23^{hi}IgM^{hi}$	1.Inhibit inflammatory cytokines production, suppress Ag-specific T cell activation and reduce in cells exhibiting Th1-type functional responses (56)
		2.Regulate both $CD4^{+}CD25^{-}$ T cells and regulatory T cells (57)
		3.Promote tumor cell growth (58)
B10 cells	$CD19^{+}CD1d^{hi}CD5^{+}$	1.Suppress proliferation and differentiation of Th17 cells (59)
		2.Inhibit the production of Th1 cytokines, regulate the Th1/Th2 balance and maintain Treg cells (60)
		3.Inhibit microglial responses (61)
		4.Promote tumor cell proliferation (62)
B1a cells	$CD19^{+}CD5^{+}$	1.Inhibit macrophage proinflammatory responses and promote Treg cell responses (63)
		2.Regulate neutrophil infiltration, $CD4^{+}$ T cell activation and proinflammatory cytokine production (64)
		3.Inhibit proinflammatory cytokine secretion, mediate $CD4^{+}$ T cell apoptosis and prevent inflammation progressing (65)
		4.Negatively regulate anti-tumor immunity (66)
Plasmablast	$CD138^{+}CD44^{hi}$	1.Limit autoimmune inflammation and reduce disease severity (50)
Plasma B cells	$IgM^{+}CD138^{hi}TACI^{+}CXCR4^{+}CD1d^{int}Tim1^{int}$	1.Induce the Treg cell-mediated suppression (67)
		2.Activate macrophages to produce cytokines that reduce the anti-tumor immune response (68)
PD-$L1^{hi}$ B cells	$CD19^{+}$PD-$L1^{hi}$	1.Mediate the generation of regulatory T cells (69)
TIM-1^{+} B cells	TIM-1^{+}	1.Promote Th2 responses and regulate immune tolerance (70)
		2.Promote the generation of regulatory T cells (71)
$CD39^{+}CD73^{+}$B cells	$B220^{+}CD39^{+}CD73^{+}$	1.Suppress effector T cell functions (72)

**It is difficult to find a unified phenotype of T2 and T2-MZP B cells, so there are the most common and recognized cell phenotype of these two cells.*

secrete IL-10, suggesting that T-bet might contribute to the remission of autoimmune diseases by activating the regulatory potential of MZ B cells (85). In collagen-induced arthritis model, MZ B cells produce most of IL-10 in response to TLR stimulation or apoptotic cells, and the adoptive transfer of MZ B cells could protect mice from infection (28). Study of Leishmania donovani infection have found that MZ B cells can interact with parasites to secrete IL-10 in a MyD88-dependent manner, and MZ B cells are involved in the suppression of CD8 and CD4 effector functions (55).

T2-MZP B Cells ($CD1d^{hi}CD21^{hi}CD23^{hi}IgM^{hi}$)

Compared with MZ B cells, T2-MZP B cells also express highly CD1d but produce IL-10 in much larger quantities. It is difficult to find a unified phenotype of T2 and T2-MZP B cells, so the distinction between these two cells is worthy of further research and discussion. The immunomodulatory effects of T2-MZP B cells in a variety of immune-mediated pathologies including autoimmune diseases, allergy diseases and cancer. The regulatory function of T2-MZP B cells has been first demonstrated in experimental arthritis model, and the realization of its regulatory effect depends on IL-10 mediation, inhibition the production of inflammatory cytokines, suppression of Ag-specific T cell activation, and reduction of Th1-type functional responses (56). Moreover, the regulatory effect of T2-MZP B cells can ameliorate the cellular infiltrates and the inflammatory damage by increasing $Foxp3^{+}$ Treg cells and reducing the number of Th1 and Th17 cells (57). In Helicobacter felis infection model, T2-MZP B cells can induce the differentiation of T cells into a regulatory phenotype to ameliorate the inflammatory damage (37). In melanoma model, tumors initially signal via the lymphatic drainage to stimulate the preferential accumulation of T2-MZP Breg cells and this local response may be an early and critical step in generating an immunosuppressive environment to permit tumor growth and metastasis, suggesting T2-MZP B cells can promote tumor growth (58).

B10 ($CD19^{+}CD1d^{hi}CD5^{+}$)

With the continuous expansion of the research scope, the exposure of B cells to different inflammatory environments had limited the use of CD1d markers to identify Breg cells. The co-expression of CD1d and CD5 on B10 cells has been therefore used to characterize the spleen B cell population. In mice, although B10 cells only account for about 1–2% of spleen B cells and 7–8% of peritoneal B cells, they are the main source of IL-10 production. Similar to B1a cells,

TABLE 2 | The phenotype and function of human regulatory B cell subsets.

Name	Phenotype	Functions
Immature/Transitional B cells	$CD19^+CD24^{hi}CD38^{hi}$	1.Suppress effector T cell but enhance Treg cell functions (44, 73)
Memory B cells/B10 cells	$CD19^+CD24^{hi}CD27^+$	1.Induce Foxp3 expression on regulatory T cells (74)
		2.Have the value of evaluating the efficacy of biological drugs (75)
GrB^+ B cells	$IgM^+CD19^+CD38^+CD1d^+CD147^+$	1.Inversely related to disease activity and clinical characteristics (76)
		2.Negatively modulate Th1 and Th17 cells, induce T cell apoptosis and strongly suppress T cell proliferation (77)
		3.Suppress antitumor immune responses (78)
Br1 cells	$CD25^{hi}CD71^{hi}CD73^{low}$	1.Suppress antigen-specific $CD4^+$ T cell proliferation (79)
PD-$L1^{hi}$ B cells	PD-$L1^+$	1.Suppress pro-inflammatory cytokine production (80)
		2.Exhibit T cell suppressive capacity (69)
		3.Repress the proliferation and activation of $CD8^+$ T cells (81)

MZ B cells and T2-MZP B cells, B10 is able secrete a large amount of IL-10 and express similar surface markers such as CD19, CD1d, CD21, and CD24. However, each Breg cell type may have different stimulatory requirements for IL-10 production. *In vitro*, B10 cells stimulated via the TLR2 and TLR4 latter express cytoplasmic IL-10 at hour 5. Studies show that B10 cells have a regulatory function in suppressing immune responses such as IL-10-dependent regulation of T cell-dependent autoimmune responses (11). The adoptive transfer of B10 cells can suppress proliferation and differentiation of Th17 cells via the reduction of phosphorylating STAT3 and expression of retinoid-related orphan receptorγt (RORγt). This cascade of events delays the onset of inflammation and reduces clinical symptoms and inflammatory damage (59). In silicosis, a disease characterized by chronic lung inflammation and fibrosis, B10 can inhibit the production of Th1 cytokines, regulate the Th1/Th2 balance and maintain Treg cells (60). In viral infection, B10 cells can infiltrate the chronically infected brains and inhibit the microglial response (61). Generally, B10 cells are potent negative regulators of antigen-specific inflammation and T-cell-dependent autoimmune diseases. Therefore, the reinfusion of B10 cells to control disease progression may provide an effective treatment for both inflammatory and autoimmune conditions. B10 cells play a pro-tumorigenic role by promoting tumor cell proliferation. In pancreatic cancer, a recent research found that the bruton's tyrosine kinase signaling pathway can play a role in regulating differentiation of B10 cells, thereby controlling the cancer (62).

B1a Cells ($CD19^+CD5^+$)

B1a cells are another major source of IL-10, inhibiting the progression of both innate and adaptive immune responses, but at the cost of impeding pathogen clearance. The tissue-specific signals and unique pathogen-derived signals combine to determine whether the response of B1a cells is predominantly regulatory or proinflammatory. Gray M et al. found that in response to ACs, B1a cells can inhibit macrophage proinflammatory responses and promote Treg cell responses to self-antigens in an IL-10 dependent manner (63). In colitis model, IL-10 production by B1a significantly reduced disease severity by regulating neutrophil infiltration, $CD4^+$ T cell activation, and proinflammatory cytokine production during disease onset (64). In collagen-induced arthritis model, IL-10 produced by B1a cells inhibits proinflammatory cytokines secreted by activated macrophages and T cells in infectious lesions, and expressing Fas ligand (FasL) B1a cells can mediate $CD4^+$ T cell apoptosis and prevent inflammation progressing (65). In addition to secreting IL-10, in controlling immune homeostasis, B1a cells can also convert naive T cells into T cells with regulatory activity through cell-to-cell contact (86). In melanoma tumor immunity, B1a cells negatively regulate anti-tumor immunity by producing IL-10, suggesting they can be a target for immunotherapy of tumor (66).

Plasmablast ($CD138^+CD44^{hi}$) and Plasma B Cells ($IgM^+CD138^{hi}TACI^+CXCR4^+CD1d^{int}Tim1^{int}$)

Studies have shown that in later stages of B-cell development such as plasmablasts and plasma cells can also produce IL-10 and have the inhibitory capacity. In autoimmune diseases, plasmablasts in the dLNs serve as IL-10 producers to limit autoimmune inflammation, while the absence of $IL\text{-}10^+$ plasmablasts increases disease severity (50). In *Salmonella Typhimurium* infection, B cell-specific MyD88 signaling is essential for optimal development of IL-10-producing $CD19^+CD138^+$ B cells, especially in early stages of infection, and via MyD88 signaling, $CD19^+CD138^+$ B cells inhibit three key types of cells: neutrophils, natural killer cells, and inflammatory T cells (36). Similarly, plasma cells are also the major source of B-cell-derived IL-10 and IL-35 which can induce the Treg cell-mediated suppression (67). Plasma cells are found in the CNS of MS patients and the expression of IL-10 by plasma cells was necessary and sufficient to confer resistance toward inflammation, suggesting that plasma cells play an unexpected role in suppressing neuroinflammation (87). In hepatoma model, IgG-producing plasma cells activate macrophages to produce cytokines that reduce the anti-tumor immune response, while depletion of these plasma cells is able to prevent generation of activated macrophages, increase the anti-tumor T cell response, and reduce growth of tumor (68). As an essential regulator of plasma cell development, Prdm1 (encoding the Blimp-1 protein)

is strongly correlated with IL-10 production, and during the formation of plasma cells, the prolonged elevation of Blimp-1 expression can elicit IL-10 production (88). Simon Fillatreau et al. identified a natural plasma cell subset characterized by the expression of the inhibitory receptor LAG-3, CD200, PD-L1, as well as PD-L2. Via a TLR-driven mechanism, natural regulatory plasma cells upregulate IL-10 expression within hours and without proliferating, suggesting that this group of plasma cells may be a potential disease treatment (89).

Other Breg Subsets

Programmed death ligand-1 (PD-L1) is important in controlling immune function, and promoting the proliferation of antigen-specific T cells. Besides this, programmed cell death receptor-1 (PD-1) binds to PD-L1 thereby transmitting inhibitory signals that reduce T cell proliferation. In RA, PD-L1^{hi} B cells can suppress disease development by elevating the expression of PD-L1. Presence of PD-L1 on B cells is positively correlated with Treg cells but negatively correlated with effector T cells, implying that PD-L1 mediates the generation of Tregs, an important molecule on B cells (69). T cell immunoglobulin mucin domain-1 (Tim)-1 is a membrane surface glycoprotein mainly expressed on cells, and is associated with regulation of immune responses. Apart from an inclusive marker for IL-10^{+} Breg cells derived from T2-MZP B cells, B10 cells and CD138^{+} B cells (90), Tim-1 is also critical for the induction and maintenance of Breg cells. Co-stimulation of IL-21, anti-Tim1 and CD40L can induce IL-10 activity in B10 cells and inhibit the progression of experimental periodontitis (91). TIM-1^{+} B cells strongly express IL-4 and IL-10, and promote Th2 responses, which can directly regulate immune tolerance (70). Conversely, B cells with Tim-1 defects are unable to produce IL-10 in response to ACs or by specific ligation with anti-TIM-1, or are unable to increase production of proinflammatory cytokine such as IL-1 and IL-6. This effect promotes Th1 and Th17 responses. In addition, B cells with defective Tim-1 can inhibit the generation of regulatory T cells and enhance the severity of autoimmune diseases (71). Collectively, these studies suggest that TIM-1 is critical in both the maintenance and induction of Breg cells under varied physiological conditions. CD39 and CD73 on their part are two ectoenzymes that together catalyze the dephosphorylation of adenine nucleotides to adenosine. Adenosine is known to suppress effector T cell function by binding on several adenosine receptors. Circulating B220^{+}CD39^{+}CD73^{+} B cells can drive a shift from an ATP-driven pro-inflammatory environment to an anti-inflammatory milieu induced by adenosine (72). A recent research found that decreased CD73 expression and the adoptive transfer of CD73^{+} B cells can impair production of adenosine, which can reduce the severity of colitis. This implies that CD39^{+}CD73^{+} B cell adenosine can regulate autoimmune inflammation (92).

Breg Cells in Humans

In humans, Breg cells maintains immune homeostasis. Breg cells in both human and mice are predominantly identified based on their IL-10 producing property.

Immature/Transitional B Cells (CD19^{+}CD24^{hi}CD38^{hi})

Previous studies have demonstrated that transitional B cells can exert IL-10 mediated inhibition of the expression of IFN-γ and TNF-α in T cells (44). In healthy individuals, transitional B cells suppress proliferation of CD4^{+} T cell as well as release of pro-inflammatory cytokines, a function that partially mediated via the production of IL-10. However, in various autoimmune diseases such as primary Sjögren's syndrome (93) and diabetic nephropathy (94), there is an under production and defective functioning of transitional B cells, particularly during the active phase of the disease. In autoimmune diseases, defective transitional B cells have impaired IL-10 production upon activation via the CD40. Due to a defect in STAT3 phosphorylation (44), Breg cells are unable to suppress Th1 responses and fail to mediate differentiation of CD4^{+}T cells into functionally suppressive Treg cells. This suggests that Breg cells may fail to prevent the development of autoreactive responses and inflammation. The higher number of transitional B cells in patients receiving rituximab is associated with long-term remissions, suggesting that the re-aggregation of Breg cells may be associated with better disease outcome (95). Apart from autoimmunity, transitional B cells have been shown to play a key role in establishing transplant tolerance (96). Breg cells can inhibit effector T cell function during the immune response in transplantation (73). In infectious diseases such as with viruses, enhanced production of IL-10-producing transitional B cells positively correlates with the viral load. The Breg cells were also shown to suppress virus-specific CD8^{+} T cell responses but enhances function of regulatory T-cells via the production of IL-10 and possibly expression of PD-L1. This suggests that transitional B cells may contribute to immune dysfunction in virus infection, which can hinder the elimination of the infection (97).

Memory B Cells/B10 Cells (CD19^{+}CD24^{hi}CD27^{+})

Both memory B cells (also known as B10 cells in human) and transitional B cells are the major IL-10-producing B cells. They have similar functional characteristics such as suppressing proliferation of CD4^{+} T cells and inhibiting expression of pro-inflammatory cytokines. However, compared with transitional B cells, B10 cells have higher growth factor β (TGF-β) and shows stronger expression of granzyme B. B10 cells also express higher levels of surface integrins and CD39, suggesting the two Breg subsets have distinct functional characteristics (98). B10 cell subset additionally expresses high levels of TLR9, a receptor shown to be more sensitive to stimulation by CpG oligonucleotides. A proliferation-inducing ligand (APRIL) can stimulate signaling pathways activated by CpG (ERK1/2 and STAT3), which can induce an increase in the production of B10. This promotes production of IL-10 and induce expression of Foxp3 on regulatory T cells (74). Moreover, it is reported that miRNA-155 can regulate IL-10-Producing B10 cells in human by enhancing the expression of *il10* gene (99). Although B10 cells play a suppressive role, their function is altered differently in several autoimmune diseases such as Bullous pemphigoid (100). Moreover, B10 cells increases in RA patients treated with biopharmaceuticals, suggesting that B10 cells may represent

a predictive biomarker for response to the treatment (75). In transplant-related diseases, the number of IL-10-producing $CD24^{hi}CD27^{+}$ B cells decreases. The function of the same cells is also impaired in graft-versus-host disease (cGVHD) (101). Similarly, in liver transplantation, patients that suffered from acute allograft rejection had significantly decreased proportions of B10 cells, but they dramatically increased after anti-rejection therapies (102).

Granzyme B^{+} B Cells ($IgM^{+}CD19^{+}CD38^{+}CD1d^{+}CD147^{+}$)

Granzyme B(GrB) is a serine protease with several functions including antigen processing, matrix degradation, activation of inflammatory cytokines and immunoregulatory effects. GrB is dramatically elevated in chronic and inflammatory disorders. Secretion of GrB by B cells may play a significant role in early antiviral immune responses, regulation of autoimmune responses and in cancer immuno-surveillance. Studies show that co-stimulation of cytokines such as IL-21 and membrane surface molecules such as BCR, CD40 and TLRs induces B cells to differentiate into active forms that secrete cytotoxic serine protease (103). The immunoregulatory function of activated GrB^{+} B cells has been demonstrated in many human autoimmune diseases, suggesting that the impairment of this Breg cells subset is related to the pathogenesis of diseases (76). In RA, GrB-producing Breg cells are significantly decreased, and their proportion is negatively correlated with disease activity and clinical features. Moreover, the optimal level of these cells can be restored after effective therapy (104). Specifically, GrB^{+} B cells negatively modulate Th1 and Th17 cells, induce T cell apoptosis and strongly suppress T cell proliferation by downregulating the T cell receptor (TCR) zeta chain (77). Similar findings have been reported in tumor research. Other studies found that GrB^{+} B cells infiltrate tumor microenvironment and tumor-draining lymph nodes where they may participate in the suppression of antitumor immune responses (78). In transplant-related diseases such as in renal transplantation, the affected patients showed a diminished level of GrB^{+} B cells compared to healthy controls (105). In general, GrB^{+} B cells may participate in early cell-mediated immune responses during inflammatory and neoplastic processes. Therefore, a better understanding of the role of GrB-secreting B cells in the immune system may help develop and improve new immunotherapy methods for infectious, autoimmune and malignant diseases.

Other Breg Subsets

Type 1 regulatory B (Br1) cells are characterized by $CD25^{hi}CD71^{hi}CD73^{low}$. They maintain peripheral blood tolerance by producing IgG4 antibodies (106). After receiving allergen-specific immunotherpay, the proportion of BR1 cells increases, secreting high levels of IL-10 (107). In autoimmune diseases, the function of Br1 is impaired while the inhibition of Th2 response islimited, implying that Br1 plays an important role in tolerance induction (79). Many Breg subtypes such as $PD\text{-}L1^{hi}$ B cells are similar in both humans and mice. B cells can modulate T cell immune responses through the expression of regulatory molecules such as PD-L1. In autoimmune diseases, CpG induces expression of PD-L1 on human B cells, which suppresses pro-inflammatory cytokines produced from antigen-stimulated $CD4^{+}$ T cells (80). $PD\text{-}L1^{hi}$ B cells exhibiting T cell suppressive capacity are significantly decreased in untreated RA patients but normalize upon successful treatment (69). Besides, Tumor-infiltrating B cells that express high levels of PD-L1, IL-10 and TGF-β repress the proliferation and activation of $CD8^{+}$ T cells (81).

BREG CELL EFFECTOR FUNCTIONS

Breg cells identified in both mice and human have been shown to downregulate inflammation associated with numerous pathological processes and the ability of each Breg cell subtype to negatively regulate immune responses as previously described. Generally, the functional mechanism of Breg cells is split into two parts; the immunomodulatory function by mediators produced by B cells and the immune effects mediated by surface molecules on B cells (see **Figure 3**).

The Immunomodulatory Function of Mediator Produced by B Cells

As the hallmark of Breg cells, IL-10 cytokine is commonly used as a marker for identification of Breg cells. As an anti-inflammatory cytokine, IL-10 plays a pivotal role in controlling excessive inflammation and downregulating the immune response. Plasmacytoid dendritic cells interact with Breg cells, where they drive the differentiation of immature B cells into IL-10-producing Breg cells. Here, plasmacytoid dendritic cells releases IFN-α that interacts with CD40 on B cells. Conversely, Breg cells inhibits the production of IFN-α from plasmacytoid dendritic cells by secreting IL-10. This cross-interaction is however compromised in autoimmune diseases (31). There is a regulatory feedback loop between macrophages or microglia and Breg cells. In viral infection, B10 cells are able to inhibit microglia secreted cytokines, in addition to modulating microglial cell responses within the infected lesion (61). Moreover, B cells can dampen the activation and influence the migration of macrophages by secreting IL-10 (108). Mutually, M2 polarized microglia can enhance the proportion of Breg cells to protects against hyperactive autoimmunity (109). As for the T cells, IL-10 inhibits secretion of cytokines by Th1, thus suppressing these cells. On the other hand, IL-10 enhances polarization of Th2, thus generating and maintaining the regulatory T cell pool (57).

In addition to producing IL-10, Breg cells produce other immune-regulatory cytokines such as TGF-β and IL-35. TGF-β is a pleiotropic cytokine involved in both suppressive and inflammatory immune responses that has been shown to suppress differentiation of Th1 by inhibiting the expression of STAT4, but promotes the development of Treg cells (110). IL-35 is the newest member of the IL-12 family. It is a potent anti-inflammatory cytokine secreted by Treg cells (111). In autoimmune diseases, IL-35 can induce production of IL-10 and IL-35 by Breg cells, which inhibits pathogenic Th1/Th17 cells (112). This prevents progression of the diseases and increases the proportion of Treg cells (18). with regard to immunoregulatory

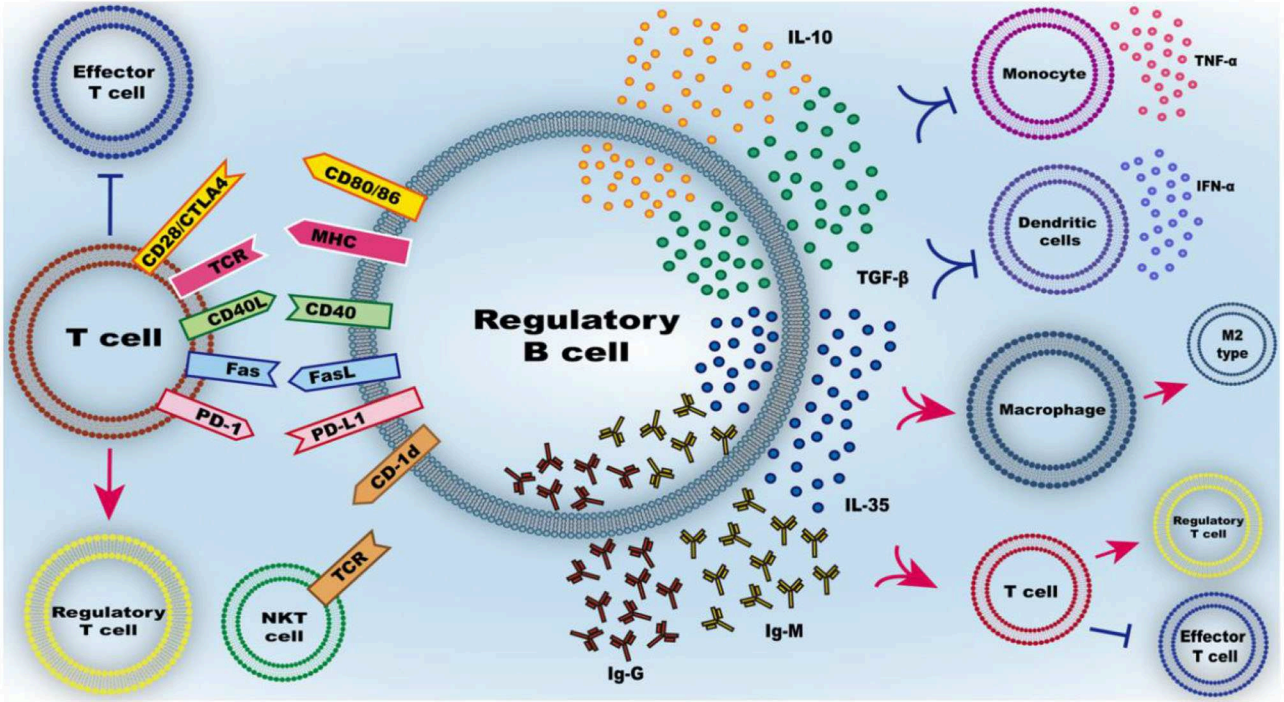

FIGURE 3 | Function of Breg cells. The mechanism of Breg cell inhibitory effect mainly includes the secretion of inhibitory mediators and the inhibitory effect of intercellular contact. IL-10, as the main secreted inhibitory cytokine of regulatory B cells, has a variety of effects such as inhibiting the release of pro-inflammatory cytokines from immune cells, inhibiting the inflammatory differentiation of macrophage/microglia and promoting the conversion of T cells to regulatory T cells rather than effector T cells. Similarly, TGF-β, IL-35, IgG, and IgM can also regulate the differentiation of T cells into regulatory T cells. Membrane-bound molecules at the interface between regulatory B cells and T cells include CD80/86, CD40, MHC, FasL, PD-L1, and CD-1d, can regulate the differentiation of T cells into regulatory T cells and activate natural killer T (NKT) cells with suppressive function.

property of IL-35, this cytokin, as well as its derivatives have been shown to have a therapeutic potential against autoimmune and infectious diseases (113). Besides this function, B cells are best known for their ability to produce immunoglobulins, essential in the induction of protective immunity against many pathogens. IgM promotes the removal of apoptotic cells, phagocytosis by macrophages and modulates activation of pro-inflammatory signals through the FcγR (114). Moreover, in allograft rejection, Peter I Lobo et al. found that high levels of IgM minimizes the rejection rate of renal and cardiac allografts transplants in recipient individuals. This tolerance is partially mediated by inhibition of NF-kB translocation into the nucleus by blocking TLR4. This inhibition in effect impairs differentiation of activated T cells (115). Besides IgM, IgG can suppress overwhelming immune response, important in maintaining tolerance (116). In particular, IgG4, described as "anti-inflammatory IgG" has the ability to shorten compliment processes and reduce proinflammatory responses in natural immune cells (117).

The Immune Effects Mediated by Surface Molecules of B Cells

Immunity by cell dependent mechanism can mediate the inhibitory function of Breg cells, aided by several cell surface molecules on Breg cells. These molecules on the surface of Breg cells can induce the inhibition of immune cell function, promote differentiation of regulatory T cell and apoptosis of target cells.

CD40-activated B cells produce Foxp3^{+} Treg cells more efficiently compared with other antigen presenting cells. The longer contact time enables IL-10^{+} B cells to up-regulate expression of Foxp3 on CD4^{+} T cells, thus converts effector T cells into Treg cells. Breg cells can inhibit the proliferation of effector T cells via the CD40/CD40L interaction, suppressing autoimmune inflammation (118). Moreover, patients with the bare lymphocyte syndrome who do not express major histocompatibility complex class II (MHC II) molecules have impaired programmed cell death of autoreactive mature naive B cells, suggesting that tolerance to peripheral B cell is dependent on MHC II- TCR interaction (119). CD80 and CD86 molecules in the B7 family are expressed on antigen presenting cells and are important in establishing immune synapses and activating adaptive immune responses. Blocking antibodies against CD80 or CD86 partially reduces the regulatory effect of transitional B cells with regard to cytokine production, which implies that production of cytokines by B cells depends on interaction between B and T cells during antigen presentation (120). PD-L1 is another member of the B7 family with the ability to inhibit activation of T cell by binding to PD-1. PD-L1^{hi} Breg cells negatively regulate differentiation of T cells. Adoptive transfer

of PD-L1^{hi} B cells can inhibit EAE, demonstrating that B cells can activate Treg cells through PD-L1, thus suppressing immune responses (121). In addition, Breg cells can trigger pathogenic Th1 cells to undergo apoptosis through Fas–FasL interaction and effectively downregulate pathogenic immunity of these cells (122). CD1d expressed on MZB cells are recognized by TCR on NKT cells, thus can exert their regulatory functions by activating NKT cells (123). However, in autoimmune diseases, studies have found that the expression of CD1d on B cells in individuals with SLE patients is defective. This effect impairs the presentation and recognition of glycolipids presented by CD1d via the TCRs on NKT. Consequently, this inhibits the proliferation and activation of iNKT cells. In addition, some studies have found that the proportion of iNKT cells and the expression of CD1d in B cells in individuals with SLE who positively responded to rituximab treatment were significantly increased compared with patients who did not respond to the drug.

BREGS AND CENTRAL NERVOUS SYSTEM INFLAMMATORY DEMYELINATING DISEASES

Breg Cells and Multiple Sclerosis

MS is an autoimmune disease which affects ∼2.5 million people worldwide. It is characterized by chronic inflammation of CNS and aberrant infiltration of inflammatory cells that eventually leads to demyelination and axonal damage. This disease is heterogeneous and complex, and thought to be caused by interactions of genetic and environmental factors (124). The disease was originally thought to be T cell-mediated, because activated T cells were abundantly present in MS lesions. In addition, EAE could also be induced by transfer of myelin-reactive T cells. However, accumulating evidence on abnormal increase of immunoglobulin levels in the cerebrospinal fluid of affected patients, Antibody-deposition in brain lesions and the successful alleviation of the disease by B cell-depleting therapies shifted the focus to B cells as key players in immune-pathogenesis of MS. Breg cells also play a key role in demyelinating diseases of the nervous system. For instance, the occurrence of a radiologically isolated syndrome (RIS) or a CIS usually precedes MS. Further to this, individuals with RIS or CIS are more prone to develop MS within 6 months if they are deficient of IL-10-producing B cells. This suggests that Breg cells have an inhibitory effect on the progression of MS (125, 126).

The B-cell-deficient mice failed to spontaneously recover from EAE. Interestingly, this was the first study to demonstrate that B cells modulate inflammation of the immune system (5). In addition, studies have shown that Breg cells are detected in CNS of EAE in a VLA-4 dependent manner, suggesting that Breg cells may contribute to regulation of CNS autoimmunity *in situ* (127). Recently, Simon Fillatreau et al. found that hypoxia-inducible factor-1α (HIF-1α) is a critical transcription factor for the production of IL-10 by B cells, and that HIF-1α-dependent glycolysis facilitates increase in the proportion of CD1d^{hi}CD5^{+} B cells. Mice with B cells lacking Hif1α have few IL-10-producing B cells, which exacerbates EAE (128).

When exploring the influence of external signals on the development and differentiation of immature B cells in the bone marrow, Simon Fillatreau et al. found that the bone marrow cells transiently stimulated by Toll-like receptor 9 can generate a new Breg cell subset CpG-proBs. CpG-proBs can slow down the development of EAE when transferred at the onset of clinical symptoms. Mechanistically, CpG-proBs can differentiate into mature Breg cells, trap T cells by releasing the CCR7 ligand and CCL19 and limit the immunopathogenesis of EAE through IL-10 production (129). Moreover, perforin-expressing regulatory B-cells (BRegs) are a new subset of Breg cells identified in patients with CIS and MS. BRegs exert their regulatory property on the disease by inhibiting proliferation of CD4^{+} T cells through the perforin/granzyme pathway (130). The proportion of BRegs in RRMS increased during relapse, suggesting that these new cells are associated with disease progression (131). Generally, the study on Breg cell subtypes and the mechanism with which they exert cell suppression have opened up interesting prospects on cell therapy during MS. Moreover, because some drugs used to treat MS patients were incidentally found to increase the production of IL-10 in human B cells, it provides promising prospect of regulating B cells for clinical treatment. As the first-line therapy against RRMS, IFN-β stimulates transformation of B lymphocytes into a subpopulation of regulatory transitional cells. Compared with untreated patients, the number of IL-10 producing transitional B lymphocytes in peripheral blood was significantly higher in IFN-β treated patients, demonstrating the role of IL-10-producing B cell populations in the disease therapy (132). Fingolimod is an immunosuppressant drug that modulates the sphingosine 1-phosphate receptor. It is the first oral, active disease-modifying drug approved for the treatment of MS. The proportion of IL-10 producing Breg cells is up-regulated while the migration capacity of the cells is enhanced after Fingolimod treatment (133). Alemtuzumab is another drug that inhibits monoclonal antibodies against CD52. It can restore the normal proportion of Breg cell subsets (CD19^{+}CD24^{hi}CD38^{hi} cells and CD19^{+}PD-L1^{hi} cells) in the peripheral blood of patients with relapsing MS patients. This suggests that CD19^{+}CD24^{hi}CD38^{hi} and CD19^{+}PD-L1^{hi} are promising candidate biomarkers for the efficacy of alemtuzumab therapy (134). Recently, siponimod, a selective sphingosine 1-phosphate receptor-1 and 5 modulator, was approved for active secondary progressive MS (SPMS). In one multi-centered randomized, double-blind, placebo-controlled clinical trial on SPMS patients, treatment with siponimod increased the number of transitional B cells and B1 cell subsets. In addition, the balance between Breg cells and memory B cells shifted in favor of Breg cells. Interestingly, it was a shift toward an anti-inflammatory and suppressive homeostatic immune state (135). However, in a phase 2 trial of atacicept (a recombinant fusion protein that suppresses B-cell function and proliferation), annualized relapse rates were higher in groups that received atacicept, compared to controls. One possible reason is that perhaps atacicept disrupt the B cell regulatory pathways but in turn stimulate T cell responses, a shift that sets in a proinflammatory environment and eventually relapses (136).

In conclusion, studies on the pathogenesis of MS have found that Breg cells regulates Th1/Th2 balance, induces apoptosis of effector T cells, neutralizes toxic substances, activates $CD4^+$ T cells or natural killer cells (NK), inhibit the activation of dendritic cells and clear apoptotic bodies among many other functions. Further research on the Breg cell subtypes and underlying mechanism of Breg cells in MS can elucidate on the complex immune response in MS. This could provide a more comprehensive and systematic insight into its pathogenesis of the disease, and provide a basis for further exploration of new immunotherapy targets.

Breg Cells and Neuromyelitis Optica Spectrum Disorders

Neuromyletis optica sectrum disorder (NMOSD) is a rare autoimmune disease of the CNS that primarily attacks the optic neuritis and longitudinally extended transverse myelitis (137). Epidemiologically, the disease has a globally distribution, and is more common among young and middle-aged women and results in high disability rate. A major advancement that helped to distinguish NMOSD from MS was the discovery that 75% of patients with NMOSD have detectable serum IgG auto-antibodies against the aquaporin-4 water channel (AQP4), an intergral water channel protein in astrocytes (138). After this discovery together with the pathogenic characteristics of the two diseases, it is believed that humoral mediated demyelination of astrocytes in the CNS is the major mechanism underlying the pathologenesis of the disease. The main features of the disease pathology can be reproduced using patient-derived monoclonal antibodies, thus reinforcing on the contribution of autoantibodies to the CNS injury associated with the disease (139). Moreover, studies on autoimmune diseases have found that impaired B cell tolerance potentially contributes to pathogenesis of the disease (140). In addition, accumulating evidence show that B cells play a vital role in NMOSD. This has been validated by B cell-targeted therapies such as rituximab, which have shown encouraging results for NMOSD (141).

The role of Breg cells in the pathogenesis of NMOSD has been extensively investigated. Some studies found that the proportion of Breg cells and expression of IL-10 are significantly lower in patients with NMOSD compared to those with MS, suggesting that the degree of impairment to B cell regulatory function can be considered as a distinctive marker between NMOSD and MS (142). $CD19^+CD24^{hi}CD38^{hi}$ Breg cells are less frequent in NMOSD patients positive for AQP4 antibodies than those without these antibodies. This phenomenon is also observed in $CD19^+CD5^+CD1d^{hi}$ Breg cells. In addition, NMOSD patients at acute relapse phase have lower IL-10 levels and significant impairment of $CD19^+CD24^{hi}CD38^{hi}$ Breg cells function (143). The advent of monoclonal antibodies has also provided a new direction in the treatment of NMOSD. For example, rituximab is an anti-CD20 chimeric monoclonal antibody shown to be well-tolerated, safe and efficient, with only minor risk of mild infusion reactions among NMOSD patients (141). After rituximab treatment, the functional balance between Breg cells and memory B cells inclines toward Breg cells as opposed to pro-inflammatory cytokines producing memory B cells (17). Tocilizumab on its part is a monoclonal antibody against IL-6 receptor shown to reduce relapse rate, neuropathic pain and fatigue in patients with NMOSD (144). Patients with autoimmune diseases treated with ocilizumab show an increased expression of TGF-β and CD25 molecule on the surface of B cells, reflective of activation of Breg cells (145). In summary, decrease in the proportion of Breg cells plays a role in the pathogenesis of neuroautoimmune diseases, and the number and function of Bregs can be restored after effective treatment.

Breg Cells and MOG-Ab Associated Demyelinating Disease

Myelin oligodendrocyte glycoprotein (MOG) is a glycoprotein located in the outer membrane of myelin, and is solely found within CNS, including in the brain, optic nerves and spinal cord. This implies that patients with encephalomyelitis attributed to MOG antibodies may develop bilateral optic nerve and lumbar spinal cord injury (146). Compared with NMOSD patients, patients with MOG antibody associated encephalomyelitis usually have a single course of the disease and show better recovery of neurological deficits after the attack. Based on clinical, immunological and histopathological evidence, encephalomyelitis associated with MOG antibodies has been regarded as a distinct disease entity different from MS and NMOSD (147).

In MOG antibody associated demyelinating disease, studies have demonstrated that the Breg cells such as $CD19^+CD24^{hi}CD38^{hi}$ and $CD19^+CD5^+CD1d^{hi}$ B cells are numerically low and functionally impaired (148). Moreover, research found that IL-6 secreted by the dendritic cells promotes the differentiation of naïve CD4 T cells into TFH cells, whereas IL-21 secreted by TFH cells induce differentiation of B cells into memory B cells and plasma cells. The latter process results in the production of antibodies and disproportion of the memory B cells/Breg cells ratio. Imbalance between the memory B cells and Breg cells promotes pro-inflammatory cytokine responses that ultimately contributes to active demyelination (148). As the first-line therapy against NMOSD, methotrexate has also been found to extend the remission period as well as reduce the recurrence rate of the disease. It also results in minor side effects. Elsewhere, methotrexate has been shown to stimulate specific immune tolerance to auto-antigens mainly by enhancing secretion of B lymphocytes that produce effector IL-10 and TGF-β (149). Studies on the role of Breg cells in the pathogenesis of MOG antibody associated demyelinating diseases remain scanty, which creates the need to explore more potential therapeutic uses of Breg cells.

DISCUSSION AND PERSPECTIVE

Regulatory B cells are important modulators of the immune response and further promotes immune tolerance. From the early immature stage to the late plasma cell stage, Breg subpopulations have been found to evolve from different stages of B cell development. Due to the different microenvironment,

Breg cells have different phenotypes, but they all display immunomodulatory functions. Activation of Breg cells via BCR, TLR, or CD40 and cytokines has been shown to activate and expand the function of these cells, but mechanisms that can stabilize and maintain Breg cells remain elusive, thus there is need for further research on the plasticity and functional stability of Breg cells. In addition, numerous studies suggest that the number and function of Breg cells are involved in the pathogenesis of many diseases. Interestingly, the number and function of Breg cells among diseases and in different states are not exactly the same, which also suggests that the role of Breg cells in many disease pathologies is complicated. Therefore, it is necessary to expand our understanding on the mechanisms underlying activation, proliferation and precise functional mechanism of Breg cells in healthy individuals, as well as individuals with various immune, inflammatory or tumor diseases. Nonetheless, the important role of Breg cells in the pathogenesis of CNS IDDs has been revealed, and with the growing research on the function and contribution of Breg cells in the pathogenesis of autoimmune diseases, the therapeutic potential of Breg cells is gradually gaining acceptance. Although the therapy encompassing depletion of B-cells in treating autoimmune diseases achieved some success, this approach may exhausts Breg cells involved in the suppression of inflammation. Consequently, it would be advantageous to selectively increase Breg cells depending on the condition of the disease. In his study, Simon Fillatreau et al. suggested that reprogrammed quiescent of B cells is a novel tool for suppressing undesirable immune responses. This presents a noble research prospect for Breg cells (150). Substantially, treatment with Breg cells has certain theoretical feasibility and prospective clinical application, but its ultimate goal in the practice remains elusive.

CONSENT FOR PUBLICATION

Written informed consent for publication was obtained from all participants.

AUTHOR CONTRIBUTIONS

ZQ-M and YH conceived and planned the review. ZR wrote the manuscript. ZQ-M and LY-B critically revised the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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Clinical Characteristics of Cognitive Impairment and 1-Year Outcome in Patients With Anti-LGI1 Antibody Encephalitis

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Introduction: Anti-leucine-rich glioma-inactivated 1 antibody (anti-LGI1) encephalitis is one of the most common autoimmune encephalitis. Anti-LGI1 encephalitis presented with subacute or acute onset of cognitive impairment, psychiatric disturbances, faciobrachial dystonic seizures (FBDSs), convulsions, and hyponatremia. The common sequela of anti-LGI1 encephalitis is cognitive disorder, but there are few studies on the recovery of cognitive function after immunotherapy. This study aimed to explore clinical characteristics of cognitive impairment and 1-year outcome in patients with anti-LGI1 encephalitis.

Methods: The clinical data and characteristics of cognitive impairment of 21 patients with anti-LGI1 encephalitis from 2016 to 2019 in Nanjing Brain Hospital were analyzed retrospectively. At the time of onset of hospitalization and 1 year after discharge, the cognitive functions in these patients were assessed using two cognitive screening scales—Mini-Mental State Examination (MMSE) and Montreal Cognitive Assessment-Basic (MoCA-B).

Results: Among the 21 patients, 13 were male and 8 were female, aged 51.10 ± 14.69 (age range 20–72) years. Nineteen patients, comprising 90.48%, had recent memory deterioration. Routine electroencephalography (EEG) results of 13 cases were abnormal. EEG results were epileptic or slow-wave activity involving the temporal lobes. Eleven cases of brain MRI were abnormal, and the focus involved the hippocampus and mediotemporal lobe. The decrease of short-term memory [recall scores: 0.57 ± 0.81 (MMSE), 0.76 ± 1.34 (MoCA-B)] is the most obvious at the time of admission. After intravenous (IV) injection of methylprednisolone and/or immunoglobulin, the clinical symptoms of the patients improved obviously. Total MMSE and MoCA-B scores of patients were significant increased after 1 year (21.19 ± 3.54 vs. 26.10 ± 3.02, $P < 0.001$; and 19.00 ± 4.38 vs. 25.19 ± 4.25, $P < 0.001$, respectively). Recall scores and orientation scores of MoCA-B were significantly improved after 1 year (0.76 ± 1.34 vs. 3.24 ± 1.48, $P < 0.001$; and 3.10 ± 1.26 vs. 5.00 ± 1.22, $P < 0.001$, respectively). However, 3/21 (14.29%) patients still have obvious short-term memory impairment (recall scores ≤ 1).

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Conclusion: Cognitive impairment is one of the most common manifestations of anti-LGI1 encephalitis, with the main prominent being acute or subacute short-term memory loss. Although most patients with anti-LGI1 encephalitis respond well to immunotherapy, a small number of patients still have cognitive disorders, mainly recent memory impairment, after 1 year.

Keywords: anti-LGI1 encephalitis, short-term memory impairment, cognitive outcomes, mini-mental state examination, montreal cognitive assessment-basic

INTRODUCTION

Autoimmune encephalitis (AE) is a rare and newly discovered inflammation disease (1–6) of the nervous system, which is related to specific autoantibodies (Abs). Among them, anti-LGI1 encephalitis (2) is a treatable etiology of AE. LGI1-Abs were found in 2010 (3), which may be the second most common cause of AE following anti-*N*-methyl-D-aspartate receptor (NMDAR) encephalitis and the most common cause of limbic encephalitis (LE) (4–6). The common manifestations of anti-LGI1 encephalitis are cognitive impairment or rapidly progressive dementia (7), psychiatric disturbances, convulsions (2, 8), faciobrachial dystonic seizures (FBDSs), and refractory hyponatremia (7). Anti-LGI1 encephalitis typically evolves and predominately affects middle-aged and elderly males over 50 years old (8, 9). Anti-LGI1 encephalitis has a good response to hormone and other immune system-based therapy (8, 9).

Cognitive impairment could be seen in most patients with anti-LGI1 encephalitis, and it is often (10), predominately, memory deterioration. It is reported (9–11) that about 25% of patients have complete recovery of cognitive function, whereas in others, mild disability may be a persistent sequela of the disease. There are more and more reports of patients with anti-LGI1 encephalitis (7–11); however, the characteristics of cognitive impairment in patients among the Chinese population with anti-LGI1 encephalitis have not been described.

The Mini-Mental State Examination (MMSE) is the gold standard of cognitive assessment for adults and the elderly. The MMSE has been proven to be effective and reliable in clinical and research settings, including adult, geriatric, hospital, and residential environments. The MMSE is the most extensively and widely validated tool (12) for cognitive assessment. The MoCA is a cognitive screening tool similar to MMSE, which pays more attention to and executive function of the frontal lobe. Montreal Cognitive Examination-Basic (MoCA-B) (13) is an improved version of MoCA, especially for the elderly subjects. MMSE and MoCA-B tests enable health-care providers to quickly assess patients' cognitive health and accurately make more informed medical decisions. MMSE and MoCA are two commonly used tools to measure cognitive impairment. A few studies have reported (9, 10) their application in AE cognitive assessment. In this study, the MoCA-B was used to compare the scores obtained by subjects to MMSE scores. The aim of this study is to characterize the clinical presentation and 1-year outcome, especially cognitive impairment in patients with anti-LGI1 encephalitis.

METHODS AND MATERIALS

Patients and Laboratory Tests, Electroencephalography, and Imaging Examination

This was an observational study conducted from January 2016 to December 2019 on hospital inpatients at the Affiliated Brain Hospital of Nanjing Medical University, China. We reviewed 21 patients who were diagnosed with anti-LGI1 encephalitis. All patients underwent a series of laboratory tests, including standard biochemistry, viral Abs (including herpes simplex virus 1 and 2, and herpes zoster virus), syphilis, HIV, thyroid function, rheumatic indicators, tumor biomarkers, and AE-related Abs [NMDAR, LGI1, GABABR, contactin-associated protein-like 2 (CASPR2), AMPA1R, and AMPA2R, and classical paratuberculosis Abs, such as Hu, Ri,Yo, Ma2, amphiphysin, CV2, ANNA-3, PCA-2, and Trand GAD], as well as other laboratory tests. Autoimmune encephalitis-related Abs of these patients also received a cerebrospinal fluid (CSF) test. The blood and CSF AE-related Abs were tested with commercial kits (Euroimmun, Germany) by indirect immunofluorescence testing (IIFT) as we previously described (6). All the 21 patients underwent chest CT, abdominal ultrasonography, brain magnetic resonance imaging (MRI), and routine electroencephalography (EEG) examinations. Clinical data from 21 patients who were diagnosed with anti-LGI1 encephalitis were collected and analyzed.

Clinical Evaluations

The MMSE and MoCA-B are routinely administered in our Department of Neurology. Both the MMSE and MoCA-B were conducted on the same day by a trained clinical psychologist. The MMSE and MoCA-B scores were used to assess the cognitive function of each patient at the time of early onset (the MMSE and MoCA-B collected within 1 week after admission) and at the time of follow-up 1 year later (the MMSE and MoCA-B collected 15 days before discharge and 1 year after discharge).

The MMSE scale consists of 30 questions, and the highest score is 30 points. Higher scores indicate better cognition. The MMSE tests five cognitive domains: time and place orientation (10 points), memory registration (3 points) and recall (3 points), attention and calculation (5 points), and language and praxis (9 points). MoCA-B measures nine cognitive domains including executive (1 point), abstraction (3 points), recall (5 points), verbal fluency (2 points), visuospatial (3 points), orientation (6 points), naming (4 points), calculation (3 points), and attention

(3 points). The MoCA-B score is between 0 and 30, the higher the score, the better the cognitive function. Subjects who scored 27 or more on MMSE and MoCA-B (14, 15) were considered cognitively normal; those with MMSE score of 21–26 and MoCA-B score of 18–26 suffer from mild cognitive impairment; those with MMSE score of 10–20 and MoCA-B score of 10–17 suffer from moderate cognitive impairment; and those with 9 points or less suffer from severe cognitive impairment. All subjects were assessed with the MMSE and the MoCA-B in addition to the required radiological and laboratory examinations.

Treatment

During hospitalization, all patients accepted first-line immune therapy [intravenous (IV) methylprednisolone], and 16 (76.19%) were treated with combination of IV methylprednisolone and immunoglobulins. The regimen was prednisolone at an initial dose of 60 mg daily, tapering (5 mg/half a month) within half a year until total withdrawal. Fifteen of 19 anti-LGI1 encephalitis patients were treated with chronic immunotherapy [mycophenolate mofetil (MMF)]. This study was approved by the Ethics Committee of the Affiliated Brain Hospital of Nanjing Medical University in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Statistical Analysis

Data of MMSE and MoCA-B scores were represented as mean ± SD and were examined for the homogeneity of variance. The paired samples *t*-test was used to compare the differences of MMSE and MoCA-B scores at symptom onset and after 1-year treatment. Correlations between serum and CSF anti-LGI1 Ab titers, and MMSE and MoCA-B scores were evaluated using a Pearson's correlation coefficient. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS version 16.0 software.

RESULTS

Demographic Data and Clinical Features

Among the 21 patients, 13 were male and 8 were female, aged 51.10 ± 14.69 (age range 20–72) years (**Table 1**). These patients had 11.76 ± 2.96 years of education. Interval from symptom onset of the disease to this admission was 44.67 ± 64.98 days and ranged from 5 for 270 days. Nineteen patients, comprising 90.48%, had recent memory deterioration; 15 (71.43%) patients had dysphrenia; 13 (61.90%) patients had hyponatremia; 15 (71.43%) patients had epileptic seizures; and 11 (52.38%) patients had FBDS. Routine EEG results of 13 cases were abnormal. EEG results were epileptic or slow-wave activity involving the temporal lobes. The brain MRI findings of 11 cases were abnormal, and the lesions involved the hippocampus and mediotemporal lobe. Two patients had tumor (one was thymoma and the other was an adrenal space-occupying lesion). LGI1 Ab was positive in the serum of 20 patients. LGI1 Ab was positive in CSF of 18 patients. Both serum and CSF LGI1 Abs of 17 patients were positive.

TABLE 1 | Demographic data and patient characteristics.

Demographic data and characteristic of the patients	
Age at onset, mean ± SD (range) (years)	51.10 ± 14.69 (20–72)
Male, *n* (%)	13, 61.90%
Education (years)	11.76 ± 2.96
Time from onset to diagnosis (range) (days)	44.67 ± 64.98 (5–270)
Memory decline, *n* (%)	19 (90.48%)
Seizure, *n* (%)	15 (71.43%)
Dysphrenia, *n* (%)	15 (71.43%)
Hyponatremia, *n* (%)	13 (61.90%)
FBDS, *n* (%)	11 (52.38%)
Tumor, *n* (%)	2 (9.5%)
Abnormal EEG, *n* (%)	13 (61.90%)
Abnormal brain MRI, *n* (%)	11 (52.38%)
Positive antibodies to LGI1 (Serum), *n* (%)	20 (95.24%)
Positive antibodies to LGI1 (CSF), *n* (%)	18 (85.71%)
Double positive to LGI1 (serum + CSF), *n* (%)	17 (80.95%)

n, number of patients; FBDS, faciobrachial dystonic seizures; CSF, cerebrospinal fluid; LGI1, leucine-rich glioma-inactivated 1; MRI, magnetic resonance imaging.

Brain MRI

The imaging manifestations of anti-LGI1 encephalitis patients mainly were high T_2 signal and fluid-attenuated inversion recovery (FLAIR) in the bilateral temporal lobe. Some patients had an abnormal signal in one or both sides of the hippocampus region. In addition, the basal ganglia region and temporal lobe are often involved. In this study, MRIs were abnormal in 11 (52.38%) of the 21 cases. The most common lesions involved the hippocampus and temporal lobe. Brain MRI in most patients with anti-LGI1 encephalitis shows a hyperintense signal in the unilateral or bilateral medial temporal lobes. Brain MRI (**Figure 1**) showed an abnormal signal in the left hippocampus region. Brain magnetic resonance spectroscopy (MRS) showed moderately decreased *N*-acetyl aspartic acid (NAA) and NAA/creatine (Cr) peak, and slightly elevated choline compound (Cho) and Cho/Cr peak. Brain MRI (**Figure 2**) showed the focus in the right temporal and insular lobes and the thalamus. On T_2WI and T_2FLAIR sequences, the right temporal and insular lobes and the right thalamus showed a slightly higher abnormal signal; and on the diffusion-weighted imaging (DWI) sequences, a slightly higher signal was seen. On T_2FLAIR sequence, there were abnormal hyperintensities in the right hippocampus and no obvious abnormal signal in the left hippocampus. Arterial spin labeling (ASL) showed significant hyperperfusion in the right temporal and insular lobes and the thalamus.

The Mini-Mental State Examination Scores

MMSE is the most commonly used cognitive function screening tool. **Figure 3A** and **Table 2** show the distribution of the MMSE scores for the 21 cases at the time of hospitalization and 1 year after discharge. Meanwhile, we counted the improvement of scores mean ± SD [95% confidence interval (CI)] (**Table 2**). Total MMSE scores of patients were significantly increased after 1-year

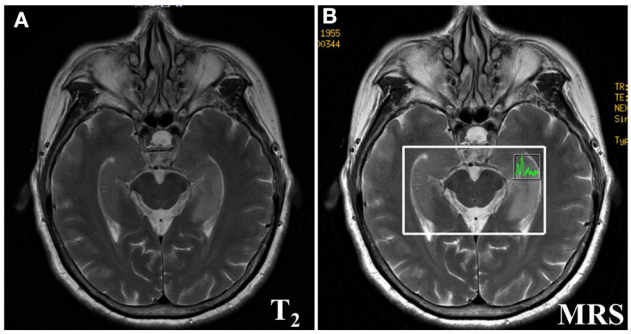

FIGURE 1 | Brain MRI **(A)** showed lesions in the left hippocampus. Brain Magnetic resonance spectrum (MRS) showed a bit increased slightly elevated Choline compound (Cho) and Cho/*Cr* peak, the moderately decreased the N-acetyl aspartic acid (NAA) and NAA/Creatine (*Cr)* peak **(B)** in the left hippocampus.

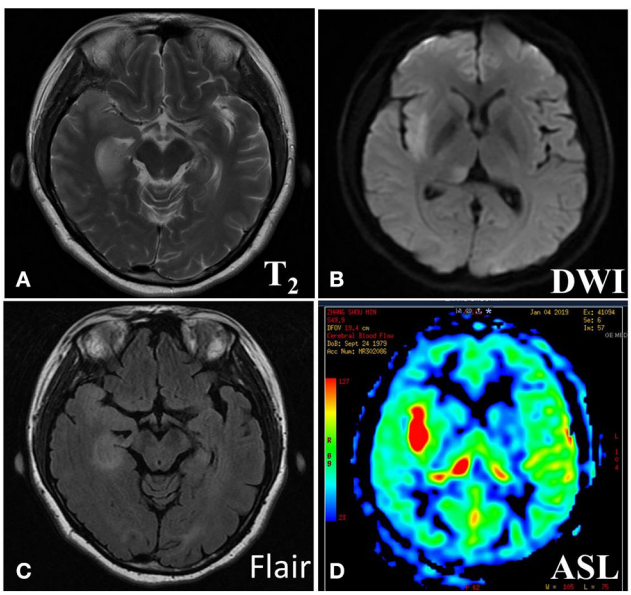

FIGURE 2 | Brain MRI **(A)** showed abnormal signal in right temporal and insular lobe, thalamus. On T2WI **(B)** and T2Flair **(C)** sequences, the right temporal and insular lobe, right thalamus showed slightly higher abnormal signal, the local cortex was slightly swollen, and on the DWI sequences, slightly higher signal was seen. On T2Flair sequence **(C)**, there was high abnormal signal in the right hippocampus and no obvious abnormal signal in the left hippocampus. Arterial spin labeling (ASL) sequence **(D)** showed significant hyperperfusion in the right temporal and insular lobe, thalamus.

follow-up (21.19 ± 3.54 vs. 26.10 ± 3.02, $P < 0.001$). After 1 year, the MMSE score of the patients improved by 4.90 ± 3.18 (3.46–6.35) compared with that of the patients at the time of onset, and the difference was statistically significant ($P < 0.001$). No moderate-to-severe cognitive impairment (MMSE ≤ 20) was determined at 1 year (**Figure 3A**).

Because the decrease of short-term memory (recall scores: 0.57 ± 0.81) is the most obvious at the time of admission (**Figure 3A**), we separately listed the distribution of patients with different recall scores of MMSE (**Figure 4A**). The MMSE recall items of 12 (57.14%) patients were scored 0 points at the time of onset, whereas only two patients were scored 0 points after 1 year. Only one patient had a normal recall of 3 points during the onset, and nine patients had 3 points in this item after 1 year. Orientation, registration, recall, and language scores of MMSE were significantly improved after 1-year follow-up (6.86 ± 1.82 vs. 9.14 ± 1.01, $P < 0.001$; 2.43 ± 0.51 vs. 2.71 ± 0.46, $P = 0.03$; 0.57 ± 0.81 vs. 2.14 ± 0.96, $P < 0.001$; and 7.00 ± 1.09 vs. 7.90 ± 1.09, $P = 0.01$, respectively). One year after immunotherapy, the patients' clinical symptoms improved obviously; however, 4/21 (19.05%) patients still have obvious short-term memory impairment (recall scores ≤ 1) (**Figure 4A**).

The MoCA-B Scores

Figure 3B and **Table 3** show the distribution of the MoCA-B scores for the 21 cases at the time of hospitalization and 1 year after discharge. Meanwhile, we counted the improvement of scores mean ± SD (95% CI) (**Table 3**). Total MoCA-B scores of patients were significant increased after 1-year follow-up (19.00 ± 4.38 vs. 25.19 ± 4.25, $P < 0.001$). After 1 year, the MoCA-B score of the patients improved by 6.19 ± 5.19 (3.83–8.55) compared with that of the patients at the time of onset, and the difference was statistically significant ($P < 0.001$). Moderate-to-severe cognitive impairment (MoCA-B ≤ 20) was determined in one of 21 patients at 1 year (**Figure 3B**).

Because the decrease of short-term memory (recall scores: 0.76 ± 1.34) is the most obvious at the time of admission, we separately list the distribution of patients with different recall scores of MMSE and MoCA-B (**Figure 4**). The MoCA-B recall items (**Figure 4B**) of 14 (66.67%) patients at the time of onset were scored 0 points, whereas those of only one patient after 1 year were scored 0 points. Only one patient had a normal recall of 5 points during the onset, and five patients had 5 points in this item after 1 year. Recall scores (0.76 ± 1.34) and orientation scores (3.10 ± 1.26) of MoCA-B decreased significantly at the symptom onset (**Table 3**). Recall scores and orientation scores of MoCA-B were significantly improved after 1-year follow-up (0.76 ± 1.34 vs. 3.24 ± 1.48, $P < 0.001$; and 3.10 ± 1.26 vs. 5.00 ± 1.22, $P < 0.001$, respectively). One year after immunotherapy, the patients' clinical symptoms improved obviously; however, 3/21 (14.29%) patients still have short-term memory impairment (recall scores ≤ 1) (**Figure 4**). There were three patients with poor cognitive function recovery in the current study. The three patients were diagnosed as anti-LGI1 encephalitis at 4, 5, and 9 months after onset and then immunotherapy. The time from onset to diagnosis was significantly delayed compared with that of most patients (average range: 44.67 ± 64.98 days) (**Table 1**). One patient was diagnosed with anti-LGI1 encephalitis 9 months after the onset of the disease, and brain MRI showed hippocampal atrophy. Because the effect of cognitive decline was not obvious, the immunotherapy was not continued after discharge.

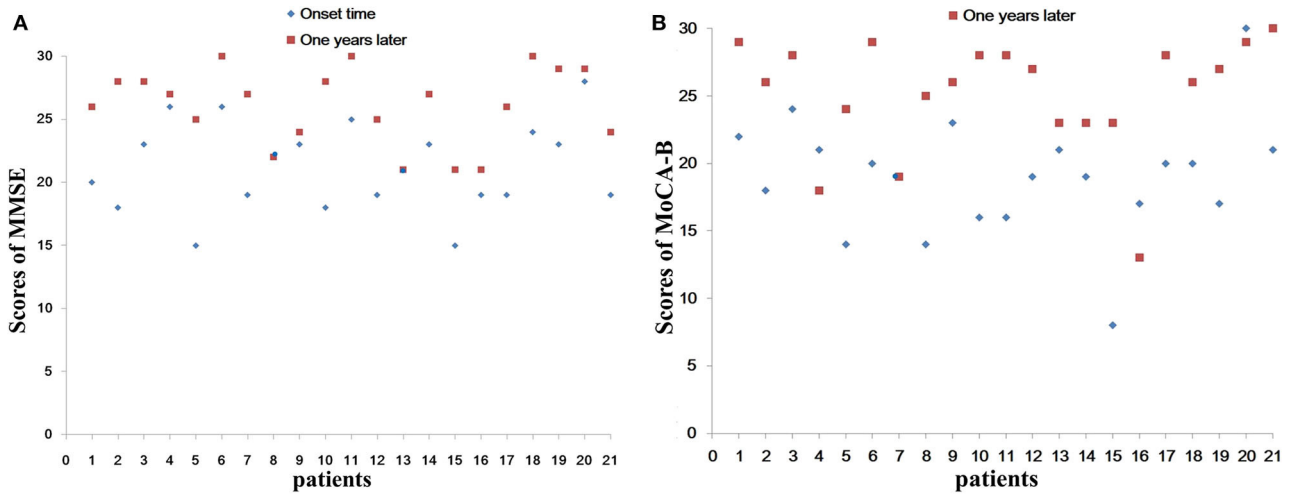

FIGURE 3 | Total scores of MMSE **(A)** and MoCA-B **(B)** of different patients. We separately list the different patient with total scores of MMSE and MoCA-B at the time of hospitalization and 1 year after discharge. **(A)** Show the distribution of the MMSE scores for the 21 cases. **(B)** Show the distribution of the MoCA-B scores for the 21 cases. No moderate to severe cognitive impairment (MMSE ≤ 20) was determined at 1 year **(A)**. MMSE, Mini-Mental State Examination; MoCA-B, Montreal Cognitive Assessment-Basic.

TABLE 2 | Domains of MMSE test in patients with anti-LGI1 antibody encephalitis.

MMSE	Onset time	1 year later	Improved score (95% CI)	*P*
Orientation (10 points)	6.86 ± 1.82	9.14 ± 1.01	2.57 ± 1.66 (1.46–3.11)	<0.001※
Registration (3 points)	2.43 ± 0.51	2.71 ± 0.46	0.29 ± 0.56 (0.03–0.54)	0.03※
Attention and calculation (5 points)	4.33 ± 0.58	4.57 ± 0.51	0.14 ± 0.57 (−0.12–0.40)	0.09
Recall (3 points)	0.57 ± 0.81	2.14 ± 0.96	1.57 ± 0.81 (1.08–2.06)	<0.001※
Language and praxis (9 points)	7.00 ± 1.09	7.90 ± 1.09	0.90 ± 1.04 (0.43–1.38)	0.01※
Total (30 points)	21.19 ± 3.54	26.10 ± 3.02	4.90 ± 3.18 (3.46–6.35)	<0.001※

MMSE, Mini-Mental State Examination; LGI1, leucine-rich glioma-inactivated 1.
※*Statistically significant value.*

Anti-LGI1 Antibody Titers and Cognitive Scores

No correlation was found between MMSE scores and serum anti-LGI1 Ab titers ($n = 20$) during the onset ($r = -0.139$, $P = 0.559$) or after 1 year ($r = 0.118$, $P = 0.621$). No correlation was found between MoCA-B scores and serum anti-LGI1 Ab titers ($n = 20$) during the onset ($r = 0.042$, $P = 0.860$) or after 1 year ($r = -0.099$, $P = 0.677$). There was no statistically significant correlation between MMSE scores and CSF anti-LGI1 Ab titers ($n = 18$) during the onset ($r = 0.095$, $P = 0.707$) or after 1 year ($r = 0.159$, $P = 0.529$). There was no statistically significant correlation between MoCA-B scores and CSF anti-LGI1 Ab titers ($n = 18$) during the onset ($r = -0.419$, $P = 0.083$) or after 1 year ($r = -0.066$, $P = 0.796$). There was no significant correlation between the serum and CSF Ab titer and the prognosis of cognitive impairment.

DISCUSSION

Autoimmune encephalitis accounts for 10–20% of cases of encephalitis (1), with anti-NMDAR encephalitis being the most common, accounting for about 80% of AE patients (6), followed by anti-LGI1 encephalitis and anti-γ-aminobutyric acid type B receptor ($GABA_BR$) Ab-related encephalitis. Anti-LGI1 Ab, anti-$GABA_BR$, and anti-AMPAR Ab-associated encephalitis mainly involve the limbic system and are called autoimmune LE (16). LE is an AE involving the limbic system, including the medial temporal lobe, amygdala, hippocampus, cingulate cortex, and insular lobe. LE is considered to be a disease associated with epilepsy, memory deterioration, and psychobehavioral disorders. LE is associated with Abs to the voltage-gated potassium channel complex (VGKC), and Abs mainly point to the VGKC-complex proteins, CASPR2, or LGI1 protein (17). The Abs involved are mostly LGI1, which is an anti-neuronal surface Ab, accounting for 30% (18) of LE-related Abs. LGI1 is mainly a non-malignant tumor (9–11) and thought to be responsive to immunotherapy.

The clinical manifestations of anti-LGI1 encephalitis are various. Cognitive impairment is the most common manifestation. Memory disorders, especially near memory disorders, are the most prominent (9–11, 19). Some researchers (19, 20) have confirmed that cognitive impairment was related to the course of disease before immunotherapy. Among the cases we studied, one case was diagnosed as anti-LGI1 encephalitis at the time of 9 months after the onset of the disease, and the effect

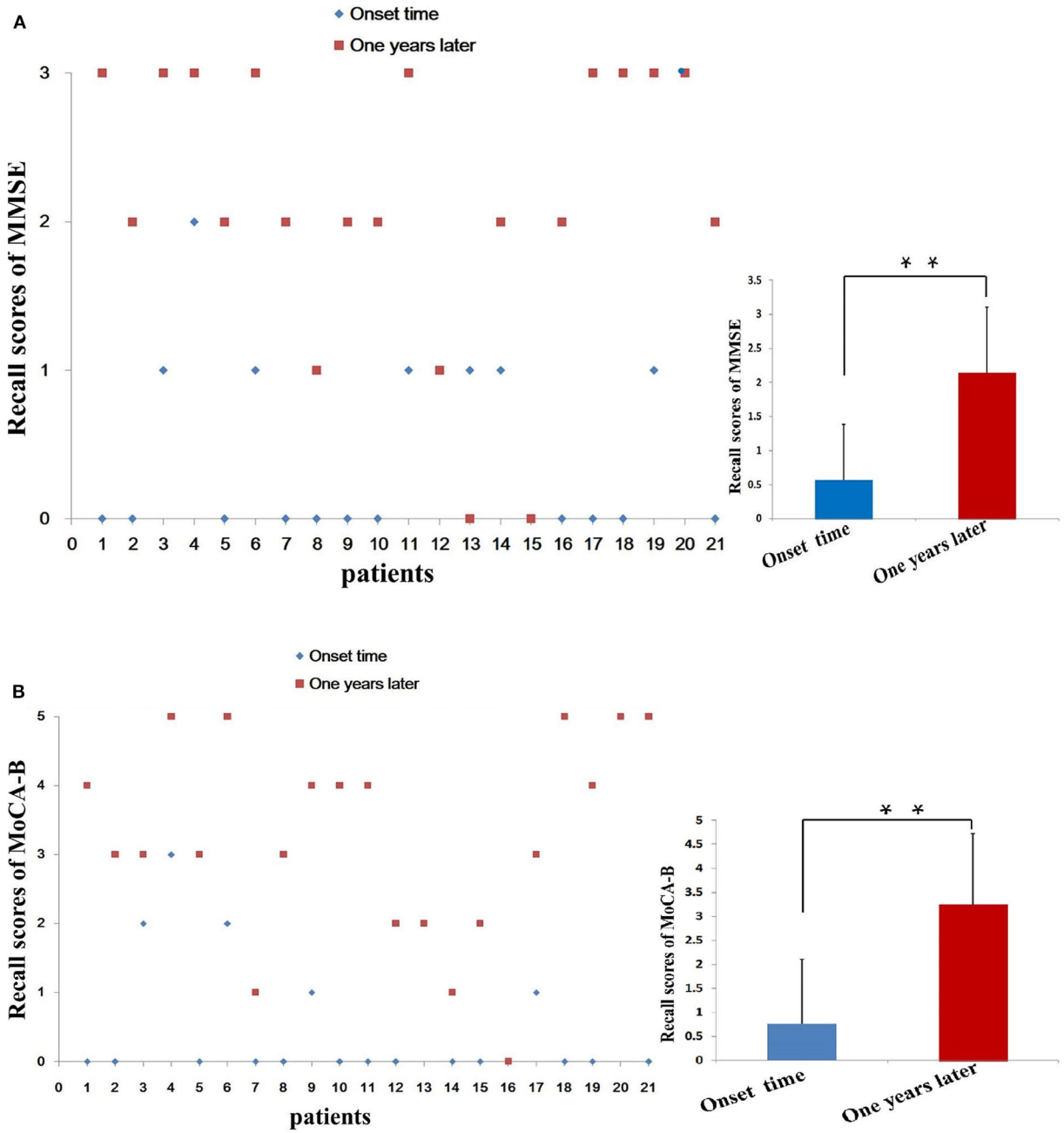

FIGURE 4 | Distribution of patients by short-term memory (recall) score. We separately list the distribution of patients with different recall scores of MMSE **(A)** and MoCA-B **(B)**. The MMSE recall items of 12 (57.14%) patients at the time of onset were scored 0 points, while only 2 patient after 1 year were scored 0 points. Only 1 patient had a normal recall of 3 points during the onset, and 9 patients got 3 points in this item after 1 year. MMSE, Mini-Mental State Examination; MoCA-B, Montreal Cognitive Assessment-Basic; $^{**}P < 0.01$.

of immunotherapy was poor. The MoCA and MMSE tests were done in all the 21 patients, and the results found that 19 patients, comprising 90.48%, suffered from memory deterioration. EEG and MRI were consistent with involvement of the limbic system. **Figures 1**, **2** show the abnormal MRI signals in the bilateral or unilateral hippocampus. This indicated that the abnormal signal changes in the hippocampus may be related to memory impairment of the patients.

In 2010, Irani et al. (3) first discovered that LGI1 Ab was involved in the pathogenesis of AE. Anti-LGI1 encephalitis is more common (8) in middle-aged and elderly males (over 50 years of age). In the current study, 13 were male and 8 were female, aged 51.10 ± 14.69 (age range 20–72) years. Most of them have acute or subacute cognitive disorder. Anti-LGI1 encephalitis is the main type of autoimmune LE, generally (7) associated with rapidly progressing cognitive impairment. The main symptoms

TABLE 3 | Domains of MoCA-B test in patients with anti-LGI1 antibody encephalitis.

MoCA-B	Onset time	1 year later	Improved score	*P*
Executive (1 point)	0.76 ± 0.44	0.90 ± 0.30	0.14 ± 0.48 (−0.07–0.36)	0.17
Abstraction (3 points)	2.43 ± 0.60	2.71 ± 0.46	0.29 ± 0.72 (−0.04–0.61)	0.08
Recall (5 points)	0.76 ± 1.34	3.24 ± 1.48	2.48 ± 1.57 (1.76–3.19)	<0.001※
Verbal fluency (2 points)	1.57 ± 0.60	1.86 ± 0.36	0.29 ± 0.72 (−0.04–0.61)	0.08
Visuospatial (3 points)	2.38 ± 0.59	2.62 ± 0.59	0.24 ± 0.62 (−0.05–0.52)	0.10
Orientation (6 points)	3.10 ± 1.26	5.00 ± 1.22	1.90 ± 1.48 (1.23–2.58)	<0.001※
Naming (4 points)	3.38 ± 0.80	3.62 ± 0.74	0.24 ± 0.94 (−0.19–0.67)	0.26
Calculation (3 points)	2.29 ± 0.85	2.57 ± 0.68	0.29 ± 1.06 (−0.19–0.77)	0.23
Attention (3 points)	2.33 ± 0.66	2.67 ± 0.58	0.33 ± 0.80 (−0.03–0.70)	0.07
Total (30 points)	19.00 ± 4.38	25.19 ± 4.25	6.19 ± 5.19 (3.83–8.55)	<0.001※

MoCA-B, Montreal Cognitive Assessment-Basic; LGI1, leucine-rich glioma-inactivated 1.
※*Statistically significant value.*

(8–11) were episodic memory impairment, temporal lobe seizures, asymmetric FBDS, and mental behavior abnormalities. As the most common type of VGKC-Ab encephalitis, cognitive disorders are common in anti-LGI1 encephalitis. In our study, 19 patients, comprising 90.48%, had recent memory deterioration.

Although there is no clear standardized treatment, immunotherapy, including first-line drugs (21)—IV methylprednisolone, plasma exchange, IV immunoglobulin, and other immune support—is strongly recommended. All 21 patients accepted first-line immune therapy (IV methylprednisolone), and 16 (76.19%) patients were treated with combination of IV methylprednisolone and immunoglobulin. The regimen was prednisolone at an initial dose of 60 mg daily after discharge, tapering (5 mg/half a month) within half a year until total withdrawal. Fifteen of 19 anti-LGI1 encephalitis patients were treated with chronic immunotherapy MMF 750 mg twice daily. After immunotherapy, the clinical symptoms of all 21 patients were improved in varying degrees. One year later, cognitive function had also been improved significantly (**Tables 2**, **3**). In the early stage, the patients are given the treatments of IV; in particular, immunoglobulin combined with hormone therapy is better than glucocorticoid alone (21, 22). At present, most patients with anti-LGI1 encephalitis have a relatively good prognosis after immunotherapy. FBDS can be quickly relieved, and most symptoms can be improved; however, cognitive status is slowly improved, and some patients (23) may have permanent memory impairment. The better understanding will be of great significance for early diagnosis, essentially immunotherapy, and even better prognosis. Some studies suggest (9–11, 23) that effective and long-term immunotherapy should be given to prevent long-term complications, including hippocampal atrophy (23) and sustained memory impairment (13). Second-line drugs can be added to the therapy of patients (23, 24) who did not respond well to the first-line drugs or had a recurrence, including rituximab, MMF, or cyclophosphamide. Once cognitive impairment is confirmed, patients should receive immunotherapy (9–11) and long-term maintenance therapy to relieve their symptoms (23, 24), improve prognosis, and avoid intractable epilepsy and hippocampal atrophy. Anti-LGI1 Ab encephalitis may recur or become chronic, as well as legacy cognitive sequelae.

In our current study, the decrease of short-term memory [recall scores: 0.57 ± 0.81 (MMSE), 0.76 ± 1.34 (MoCA-B)] is the most obvious at the time of admission. After the combined treatment of IV methylprednisolone and immunoglobulins, the patients' clinical symptoms improved obviously. Total MMSE and MoCA-B scores of patients at symptom onset were significant increased after 1 year (21.19 ± 3.54 vs. 26.10 ± 3.02, $P < 0.001$; and 19.00 ± 4.38 vs. 25.19 ± 4.25, $P < 0.001$, respectively). Recall scores of MMSE and MoCA-B were significantly improved after 1-year follow-up (0.57 ± 0.81 vs. 2.14 ± 0.96, $P < 0.001$; and 0.76 ± 1.34 vs. 3.24 ± 1.48, $P < 0.001$, respectively). However, 3/21 (14.29%) patients still have obvious short-term memory impairment (MoCA-B recall scores ≤ 1). The common sequela of anti-LGI1 encephalitis is cognitive impairment, especially recent memory impairment. Therefore, it is more necessary to add long-term immunotherapy including MMF to the first-line immunotherapy.

Limitations and Conclusions

Cognitive impairment is one of the most common manifestations of anti-LGI1 encephalitis, with the main prominent being acute or subacute short-term loss. The MMSE and MoCA-B scales can be used to evaluate cognitive function in patients with anti-LGI1 encephalitis. Although most patients of anti-LGI1 encephalitis had a good cognitive outcome, a small number of patients still have cognitive impairment, mainly short-term memory loss after 1 year. Early and long-term effective immunotherapy of anti-LGI1 encephalitis (10, 11, 20) can obtain better cognitive functional prognosis, so early diagnosis and early treatment of this disease are recommended.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Affiliated Brain Hospital of Nanjing Medical University. The

patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

HH, JL, and JS conceived and designed the study. HH and JL analyzed the data. HH and JL drafted the manuscript. HH, JZ, DC, JL, and JS critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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The Role of Antibodies in the Pathogenesis of Multiple Sclerosis

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The presence of persistent intrathecal oligoclonal immunoglobulin G (IgG) bands (OCBs) and lesional IgG deposition are seminal features of multiple sclerosis (MS) disease pathology. Despite extensive investigations, the role of antibodies, the products of mature CD19$^+$ B cells, in disease development is still controversial and under significant debate. Recent success of B cell depletion therapies has revealed that CD20$^+$ B cells contribute to MS pathogenesis via both antigen-presentation and T-cell-regulation. However, the limited efficacy of CD20$^+$ B cell depletion therapies for the treatment of progressive MS indicates that additional mechanisms are involved. In this review, we present findings suggesting a potential pathological role for increased intrathecal IgGs, the relation of circulating antibodies to intrathecal IgGs, and the selective elevation of IgG1 and IgG3 subclasses in MS. We propose a working hypothesis that circulating B cells and antibodies contribute significantly to intrathecal IgGs, thereby exerting primary and pathogenic effects in MS development. Increased levels of IgG1 and IgG3 antibodies induce potent antibody-mediated cytotoxicity to central nervous system (CNS) cells and/or reduce the threshold required for antigen-driven antibody clustering leading to optimal activation of immune responses. Direct proof of the pathogenic roles of antibodies in MS may provide opportunities for novel blood biomarker identification as well as strategies for the development of effective therapeutic interventions.

Keywords: multiple sclerosis, antibody, oligoclonal bands, immunoglobulin G, cytotoxicity, cerebrospinal fluid, serum, B cells

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INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease that affects the central nervous system (CNS), especially the brain, spinal cord, and optic nerves. About 1 million people in the US and 2.5 million worldwide live with a diagnosis of MS (1). After its first description in 1868 by Jean-Martin Charcot, MS has been classified into different types such as clinically isolated syndrome (CIS, a clinical syndrome highly suggestive of a first manifestation of MS), relapsing remitting (RR), secondary progressive (SP), and primary progressive (PP) (2). Extensive pathological studies have classified MS lesions as active, chronic active, inactive, and pre-active stages (3). Despite the heterogeneous features of MS lesions, a consensus has emerged that the pathogenic mechanisms of the disease are contributed by CNS inflammation and infiltration of peripheral immune cells, resulting in neuronal and glial cell injury and subsequent loss of myelin sheath around nerves, interruption of axonal communication, and neurologic deficits (4). However, the exact cause of MS is unknown, and currently there is no cure for the disease.

The presence of persistent CNS oligoclonal immunoglobulin G (IgG) bands (OCBs) and lesional IgG deposition are hallmarks of MS. OCBs consist of clonally restricted immunoglobulins detected by isoelectric focusing (IEF) and are a key feature of ongoing inflammatory events in CNS in a number of neuro-inflammatory conditions and viral infections (5). Although the pathological effects of OCBs have been implicated since their discovery (6), the role of antibodies in the pathogenesis of MS is controversial. In this review, we discuss pathological and immunological studies regarding the role of antibodies in MS. We propose a novel framework regarding the pathogenic mechanism of disease, which could be mediated by increased levels of serum IgG1 and IgG3 antibodies.

INCREASED INTRATHECAL SYNTHESIS OF OCB IS THE MOST CHARACTERISTIC FEATURE OF MS

Early biochemical studies of MS autopsy brain plaques with active lesions have demonstrated the presence of excessive amounts of IgG antibodies in both free/soluble and tissue-bound/particulate forms (7–9). The IgGs extracted from corresponding soluble and particulate samples displayed OCBs (9). Extensive pathological characterization of heterogeneous MS autopsy brain samples has demonstrated the co-localization of IgG antibodies, complement, and Fc gamma receptors (FcγR) in the active lesions, suggesting a role for these antibodies in the early stages of the disease (10, 11). Furthermore, complement activation is found in PPMS cortical gray matter lesions (12), indicating that antibodies may contribute to the worsening pathology that underlies the irreversible progression of MS. These lines of evidence suggest that the excessive presence of IgG antibodies in MS lesions may induce complement-mediated and immune-cell-mediated cytotoxicity, resulting in lesion formation.

The single most consistent laboratory abnormality in MS is the presence of OCBs in the cerebrospinal fluid (CSF) of up to 95% of patients (13, 14). Once present, the pattern of OCB is characteristic for each individual and does not change within patients over years, despite therapeutic interventions (5, 13, 15, 16). Besides OCBs, the intrathecal IgG can also be visualized with Reiber diagram, which uses CSF/serum quotient diagrams with hyperbolic discrimination lines for IgG (17). Plasma cells are found in the chronically inflamed MS CNS (18). Long-lived plasma cells were demonstrated in chronically inflamed CNS and chemokine CXCL12 is involved in plasma cell persistence (19). For detailed review regarding the complex nature of resident plasma cells and mechanism driving the persistence in CNS, please see the review by Pryce and Baker (5). Using a phage-displayed random peptide library approach, we demonstrated that CSF IgGs obtained longitudinally from MS patients recognized identical epitopes over time, supporting the notion of a temporal stability of CSF IgG specificity (20).

Accumulating evidence supports the pathological role of CSF immunoglobulins. CSF OCBs were found to be associated with increased levels of disease activity and disability, with the conversion from a CIS to early RRMS, with greater brain atrophy, and with increased levels of disease activity (21–28). Further, CSF of MS patients induced inflammatory demyelination and axonal damage in mice (29, 30). We demonstrated that a subset of myelin-specific recombinant antibodies constructed from clonal expanded plasma cells in MS CSF caused robust complement-dependent cytotoxicity in oligodendrocytes and induced rapid demyelination in mouse organotypic cerebellar slices (31, 32). These studies support the pathogenic effects of CSF IgGs in MS. Despite the significance of OCB in MS, no statistically significant differences of both number of OCBs and IgG index were found among subtypes of MS (CIS, RRMS, PPMS, and SPMS) (33). New technologies such as recombinant antibodies generated from clonally expanded single B cells/plasma cells and directly from IgG sequences of OCBs provided promises for determining the specificities of OCB, but have so far failed to reveal a common targets of MS (34–37) (https://www.jni-journal.com/article/S0165-5728(20)30298-8/pdf).

Except for myelin, convincing CNS target antigens for OCBs that are specific to MS are not known. Recently, it was shown that some OCBs targeted ubiquitous self-proteins and intracellular antigens (37–39), suggesting that CSF antibodies may develop as a passive response to CNS injury, rather than mediating primary pathogenic effects. Besides MS, CSF OCBs have been reported in a number of neuro-inflammatory conditions and viral infections (40). It has been argued that this intrathecal, poly-specific, and oligoclonal immune response possibly indicates that it is not a specific antigen that drives the development of OCBs in MS, but rather a non-specific activation of CSF-localized B cells (41).

THE SOURCES OF INCREASED INTRATHECAL IgG, A CONTROVERSY IN MS

Correlation of Serum Antibodies With Intrathecal IgGs

OCBs are thought to be produced by intrathecal parenchymal B lymphocytes, as the CSF Ig proteome and transcriptome of CSF-located B cells matched each other. In addition, intrathecal B cells show signs of somatic hyper-mutation and clonal expansion, pointing toward a germinal center-like reaction with antigen-driven affinity maturation within the CNS (42, 43). However, there is new evidence that terminally differentiated B cells in MS CSF are not solely derived from intrathecal maturation, but can emerge from the CNS compartment and interact with the peripheral immune system (44–46). Recent deep-immune repertoire studies revealed that MS CSF OCBs were not merely produced by CNS B cells, and some OCB specificities were related only to peripheral B cells, which indicate that disease-relevant B cells circulate between the CNS and peripheral compartments (47). We recently demonstrated that serum IgG in MS was significantly elevated and there was a strong correlation between CSF IgG and CSF albumin, and also between CSF IgG and serum IgG (48). Since CSF albumin is exclusively derived from the blood in MS, this correlation suggests that most of the CSF IgG is derived from the blood. It has to be noted that about 50% of MS sera did not show OCBs and patients with OCBs in sera

had patterns either partially similar or completely different from those seen in matching CSF (49). Because serum IgG is about 200 times more concentrated than CSF IgG (48), it is possible that serum OCBs are masked by massive amounts of polyclonal IgGs.

Another line of evidence supporting serum and intrathecal IgG exchange comes from the discrepancy between number of CNS B cells and the quantities of intrathecal IgG. A careful examination of MS plaques concluded that there were far too few cells in the plaques to contribute to IgG (50). As calculated by Tourtellotte's formula, the normal values of the CNS IgG synthesis rate were lower than 3.3 mg/day and the median value in MS patients was 29 mg/day (51). It would take 3.2 billion lymphocytes in MS to generate such large amounts of CNS IgG (30 mg in 500 ml CSF) (52). We and others have demonstrated that CSF leukocyte counts in most MS patients were <50 cells/μl (about 2.5 million cells in 500 ml CSF), of which 5% were B cells (48, 53, 54). Therefore, CSF lymphocytes could only account for <0.1% of the IgG in the MS CSF per day. The low number of lymphocytes in MS CSF and the high level of intrathecal IgG raise the question as to whether CNS B cells in MS can be responsible for the massive amounts of elevated intrathecal IgG. This apparent knowledge gap suggests that most of the intrathecal IgG in MS may in fact be derived from the blood.

MRI Detection of the Central Vein Sign in MS Lesions Supports a Peripheral Blood Contribution to Disease Activity

Early histopathological studies detected a unique character of MS lesions described as "centrifugally-spreading" that an MS plaque did not spread or grow at its edges. Plaques began as collars of demyelination around small veins and enlarged thereafter (50). This perivenous distribution of MS plaques was confirmed by ultra-high-field magnetic resonance imaging (MRI) (55–57). The MRI-detectable central vein sign inside white matter lesions can distinguish MS from other CNS inflammatory disorders and has been proposed as a biomarker for inflammatory demyelination (58). Further, the serum protein fibrinogen was found frequently and extensively to be present diffusely in both extracellular and intracellular spaces of MS motor cortex and in close proximity to blood vessels, and was related to the extent of neurodegeneration in progressive forms of MS (59). This line of evidence supports the notion of peripheral blood contribution of B cells and antibodies to intrathecal IgGs and their potential role in disease development.

We propose that MS intrathecal IgGs are derived from B cells in both the CNS and peripheral blood and may thus be contributed by serum antibodies (**Figure 1**). Inside the CNS compartments, clonally expanded antigen-experienced plasma cells produce antibodies that may target cell surface antigens and exert a pathogenic effect by activating complement-dependent or immune-cell dependent cytotoxicity. A subpopulation of these antibodies may direct against intracellular autoantigens released during tissue destruction. On the other hand, serum B cells and antibodies migrate across the blood barriers either by active transportation or by barrier breakdown. Some of these serum antibodies in MS are clearly pathogenic as reviewed below.

CIRCULATING ANTIBODIES CONTRIBUTE TO MS DISEASE PATHOGENESIS

Pathogenic Effect of Serum Antibodies

The benefit shown in therapeutic plasma exchange and immune-adsorption therapy in some MS patients (41, 60, 61) suggests that serum antibodies in MS are pathogenic. Patients who had lesions with prominent Ig deposition and complement activation profited most from plasma exchange (60, 62). However, direct proof of the pathogenic role of serum antibody in MS is complicated by the marked heterogeneity of the disease and the variability of experimental procedures.

Early *in vitro* demyelination studies have provided evidence supporting a pathogenic role of serum antibodies (50) and that there is strong correlation between disease activity and demyelinative activity of MS serum. Lumsden (50) investigated sera from 450 MS patients and controls over 7 years with 1,300 tests. He found that over 80% of MS sera with natural complement produced demyelination in live cultures of newborn rat cerebellum. Further, he found patients' immunoglobulins and complement were fixed anti-mortem to CNS components, indicating that circulating antibody in MS binds to myelin and causes demyelination. Lumsden's data indicate that serum antibodies in their natural state are pathogenic when they penetrate the CNS parenchyma. Using *ex vivo* assays, a number of laboratories have reported that some MS patients have serum factors that demyelinate myelinating explants (63–66). Later, complement-dependent demyelinating IgG response was detected with purified serum IgGs in ∼30% of 37 MS patients (67). However, the demyelinating effect of MS serum IgGs has been controversial, which is due in part to variable tissue culture and myelin imaging methods. Additionally, we postulate that the different results may reflect the sources of antibodies used. The antibody purification procedures may result in loss of the natural state of antibodies and may fail to efficiently recover specific IgG subclasses, resulting in a substantial reduction or a complete loss of demyelinating effect. Lumsden's work was carried out using unpurified native serum antibodies (50).

The potential pathogenic role of serum antibodies may also extend to enhancing inflammatory responses across the BBB in MS. For example, significantly higher levels of anti-endothelial cell antibodies and immune complexes were found in MS sera (68), and serum antibodies from MS patients were detected in micro-vessels in brain tissues and bound to endothelia cells (69, 70). Further, sera from RRMS and SPMS disrupt the BBB (71). In summary, over 50 years of extensive scientific investigations have provided accumulating evidence that serum antibodies in their natural state exert primary antibody-dependent cytotoxicity to glial cells, which leads to demyelinating effects that could contribute to MS disease pathogenesis.

Insights From B Cell Depletion Therapies

B cell depletion therapies using monoclonal antibodies against CD20; namely, Rituximab, Ocrelizumab, and Ofatumumab have shown profound success in controlling MS relapses (41). CD20 is a four-transmembrane protein expressed on the surface of B cells from the late pro-B cells through the memory cell

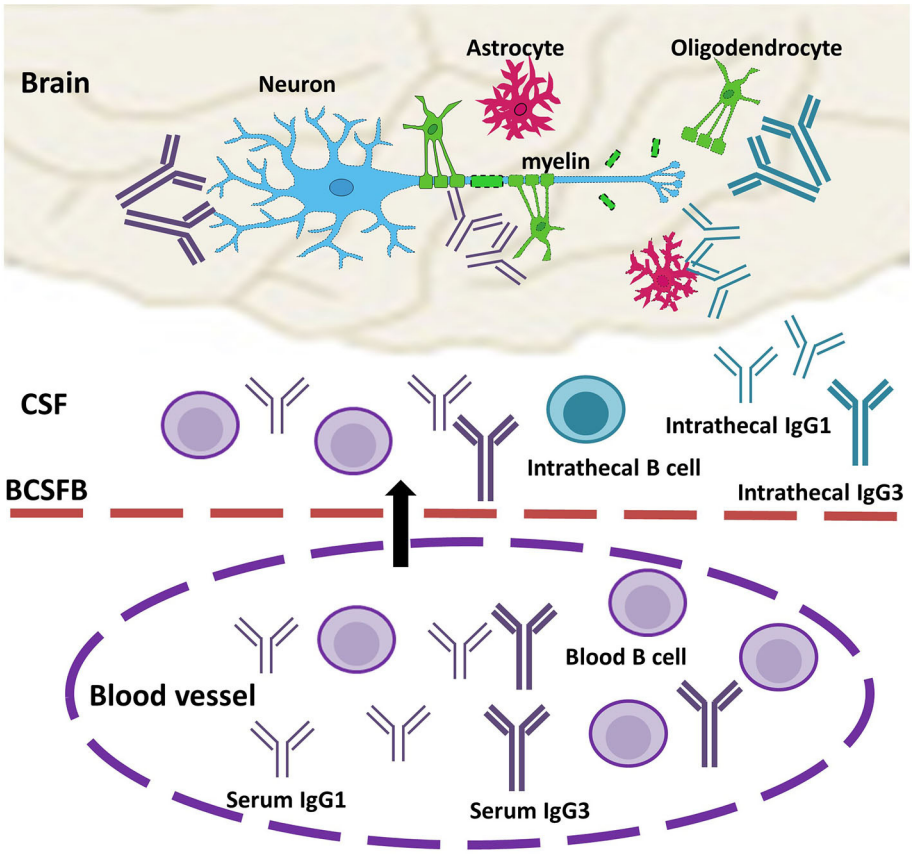

FIGURE 1 | Model of the role of serum antibodies in MS disease pathogenesis. Circulating serum antibodies (IgG1 and IgG3, purple) and antibody-producing B cells migrate across the impaired blood-barrier (arrow), and they are present in CSF OCBs and CNS lesion together with intrathecal IgGs (IgG1 and IgG3, turquoise). In the brain, IgGs recognize antigens on the cell surfaces of neurons or/and glial cells and form immune complexes with complement factors and/or immune cells. Elevated levels of IgG1 and IgG3 induce enhanced cytotoxicity or reduced threshold to trigger injury response to CNS cells, which, in turn, result in loss of myelin sheath outside of axons. BCSFB, blood-CSF barrier.

stages, but not on antibody-producing plasma cells. Thus, the efficacy of this B cell depletion therapy has been considered to be mediated by B cell function independent of antibody production, such as antigen-presentation for the activation of T cells and pro-inflammatory cytokine secretion (72). Indeed, serum antibody level and CSF OCB often persist despite CD20-antibody depleting B cells (5, 73). In some instances, certain serum antibodies were reduced in a proportion of patients after intravenous Rituximab treatment (74). CSF plasma cell depletion was observed following repeated intrathecal Rituximab injection (75, 76). These reductions are thought to result from secondary effects such as depletion of plasma cell precursors, depletion of survival factors, or possibly destruction of B cell niches rather than a direct influence on plasma cells. Interestingly, Laquinimod, a T cell targeting oral disease-modifying therapy, has been shown to modulate myelin antigen-specific B cell immune response and inhibit development of MOG-specific IgG antibodies (77). And treatment of Cladribine (another T cell targeted drug) in MS is associated with depletion of memory B cells (78).

However, reports of MS patients' failure to respond to anti-CD20 therapies, or even disease exacerbation thereafter, have also been published. Anti-CD20 therapies have limited efficacy in inhibiting disease progression (41). It is possible that these therapies do not effectively target the antibody-producing B cells, or do not significantly reduce the antibody levels in serum and CSF. Another issue concerns the increased risk of infection that is likely to accumulate with continuous B cell depletion with time. In MS, only a fraction of B cells and antibodies are pathogenic, while other subsets of B cells and antibodies exert essential regulatory functions to limit chronic inflammation. For in-depth reviews regarding B cell therapies and B cell biology between subtypes of MS, please see review papers by Gelfand et al. (79), Fraussen et al. (80), and Myhr et al. (81). It will be very important to develop innovative strategies selectively abrogating pathogenic B cells and antibodies. Thus, identifying the specific features of

pathogenic antibodies in MS is crucial for the development of successful therapeutic interventions.

ELEVATED LEVELS OF IgG1 AND IgG3 ANTIBODIES IN CSF AND SERUM, AN MS SPECIFIC FEATURE

Selective Elevation of IgG1 and IgG3 in MS

The glycoprotein IgG can be separated into four subclasses: IgG1 (60–70% in plasma), IgG2 (20–30%), IgG3 (5–8%), and IgG4 (1–3%) (82). A selective elevation of the IgG1 in MS CSF was observed (83, 84). The elevation of IgG1 and IgG3 indices in MS were found more frequently than the elevation of the general IgG index (21). Patients with a relapse were significantly more frequently seropositive for anti-MOG and anti-MBP IgG3 than those in remission (85). Further, the IgG3 allotype G3m was MS-specific and present in active brain plaques (86). Subsequent studies demonstrated that the susceptibility to MS was associated with an IgG3 restriction fragment length polymorphism (87), and a GWAS study showed that intrathecal IgG synthesis in MS was significantly associated with the intronic region of the IgG3 heavy chain gene SNPs (88). The significance of IgG3 in MS was recently highlighted by a findings that higher serum IgG3 levels may predict the development of MS from CIS (89) and IgG3 + B cells are associated with the development of MS (90). These data suggest that the presence of higher levels of IgG1 and IgG3 antibodies may play a significant role in MS disease activity.

Increased IgG1 and IgG3 Enhances Effector Functions

IgG3 has an extended hinge region with highest flexibility compared to other antibody subclasses. This subclass can probe less exposed antigens. This feature could contribute to the higher potential of IgG3, followed by IgG1, to antibody oligomerization and activation of effector functions, including enhanced antibody-mediated cellular cytotoxicity (ADCC); opsonophagocytosis; complement activation; and neutralization. Additionally, IgG3 has superior affinity to FcγR and the first component of complement cascade, C1q (78). Thus, increased IgG1 and IgG3 in MS serum and CSF may enhance immune-mediated cytotoxicity to CNS cells or may reduce the thresholds for antigen-driven antibody clustering for optimal activation of immune responses.

CONCLUSIONS

The role of antibodies in MS disease mechanisms has been disputed over several decades due to the lack of direct and reproducible proof of pathogenic effects. The limited efficacy of CD20-B cell therapies in progressive MS patients and in controlling disease progression implicates that CD20-negative antibody-producing B cells, as well as antibodies, play an important role in disease pathogenesis. The combined accumulating data that there is a strong correlation between serum and CSF IgG, insufficient B cells to produce large quantities of intrathecal IgG, MRI detection of the central vein sign in MS, and presence of pathogenic serum antibodies provide evidence for the hypothesis that circulating antibodies contribute to increased intrathecal IgG synthesis in MS. We further propose that (1) serum antibodies exert primary and pathogenic effects in MS development; (2) increased IgG1 and IgG3 result in enhanced cytotoxicity to CNS cells and produce antibody-mediated injury in MS pathogenesis (**Figure 1**). This novel hypothesis may help to resolve the current controversy regarding the roles of antibodies in MS and may draw attention to the possibly pathogenic role of IgG3. It may also provide novel opportunities for blood biomarker identification and the development of effective therapeutic interventions for MS.

AUTHOR CONTRIBUTIONS

XY and YL wrote and edited the manuscript. PK and MG critically reviewed and edited the manuscript. All authors contributed to the manuscript.

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REVIEW
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B Cells in Multiple Sclerosis and Virus-Induced Neuroinflammation

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Neuroinflammation can be defined as an inflammatory response within the central nervous system (CNS) mediated by a complex crosstalk between CNS-resident and infiltrating immune cells from the periphery. Triggers for neuroinflammation not only include pathogens, trauma and toxic metabolites, but also autoimmune diseases such as neuromyelitis optica spectrum disorders and multiple sclerosis (MS) where the inflammatory response is recognized as a disease-escalating factor. B cells are not considered as the first responders of neuroinflammation, yet they have recently gained focus as a key component involved in the disease pathogenesis of several neuroinflammatory disorders like MS. Traditionally, the prime focus of the role of B cells in any disease, including neuroinflammatory diseases, was their ability to produce antibodies. While that may indeed be an important contribution of B cells in mediating disease pathogenesis, several lines of recent evidence indicate that B cells are multifunctional players during an inflammatory response, including their ability to present antigens and produce an array of cytokines. Moreover, interaction between B cells and other cellular components of the immune system or nervous system can either promote or dampen neuroinflammation depending on the disease. Given that the interest in B cells in neuroinflammation is relatively new, the precise roles that they play in the pathophysiology and progression of different neuroinflammatory disorders have not yet been well-elucidated. Furthermore, the possibility that they might change their function during the course of neuroinflammation adds another level of complexity and the puzzle remains incomplete. Indeed, advancing our knowledge on the role of B cells in neuroinflammation would also allow us to tackle these disorders better. Here, we review the available literature to explore the relationship between autoimmune and infectious neuroinflammation with a focus on the involvement of B cells in MS and viral infections of the CNS.

Keywords: B cells, multiple sclerosis, neuroinflammation, central nervous system, viral infection, EBV

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INTRODUCTION

Historically, the primary focus of B cells as enhancers of autoimmunity was their exclusive ability to differentiate into plasma cells and produce autoantibodies. Over the last few decades our understanding that B cells are merely responsible for the production of autoantibodies has been challenged and antibody independent effector functions of B cells are now greatly appreciated (1–8). Based on preclinical and clinical data, mounting evidences suggest that B cells effectively collaborate with T cells to initiate and fine-tune T cell-dependent responses

in the development of several autoimmune diseases (9–11). B cells are also known to act as negative sensors of autoimmunity that regulate immunological functions by suppressing T cell proliferation, secreting anti-inflammatory cytokines (12, 13) and controlling monocyte activity (14–18). Consequently, B cells have now emerged to take center stage as cells with effector as well as immunoregulatory potential.

Indeed, a large volume of literature emphasizes on the heterogenous roles of B cells in autoimmunity and peripheral inflammation, yet our understanding of the extent of B cell involvement in autoimmune neuroinflammation remains incomplete.

Neuroinflammation can be defined as a coordinated and complex interaction between CNS-resident cells and the peripheral immune system and is characterized by a host of cellular and molecular changes within the CNS (19, 20). Neuroinflammation is a prominent feature in the etiology of a number of neurological disorders and diseases including multiple sclerosis (MS), and viral encephalitis (21) where the inflammatory response is generally recognized as a disease-escalating factor (22, 23). A common denominator for neuroinflammatory disorders is the impairment of the integrity of the endothelial, epithelial, and glial brain barriers that together compartmentalize the CNS from the periphery (24–26).

Cells of the innate immune system are typically the focal point for any discussion of neuroinflammation (19, 27), while B cells are not considered as the first responders of an inflammatory insult within the CNS. However, recent evidence suggests that B cells, which are largely absent in the CNS parenchyma or sparsely present in the cerebrospinal fluid (CSF) of healthy individuals (27), rapidly accumulate in the CSF (28, 29) during neuroinflammation and their numbers increase by several folds in the CNS parenchyma or the perivascular spaces (30).

Taken together, it is only recently that the importance of B cells as multifunctional players in neuroinflammatory disorders is being acknowledged with several outstanding questions requiring elucidation (31–33).

In this article, we discuss the literature available on how B cells are involved in two different instances of neuroinflammation by highlighting their beneficial and detrimental roles in ameliorating or aggravating disease pathophysiology, respectively. On the one hand, we focus on MS, which is a classical example of autoimmune neuroinflammation and on the other hand we extend our discussion by drawing parallels between MS and virus-induced neuroinflammation with respect to the involvement of B cells.

GENERAL INTRODUCTION TO B CELL BIOLOGY

As B cells are in the focus of this review, this chapter will briefly summarize the principles of B cell biology as well as provide an overview of different B cell subsets and their main functions. B cells belong to the population of lymphocytes and they are part of the adaptive immune system. They express clonally diverse antigen recognition molecules known as immunoglobulins (Igs). Membrane-bound Ig on the surface of B cells acts as a receptor, the so-called B cell receptor (BCR), that recognizes specific antigenic epitopes.

Very briefly, the development and differentiation of a B cell begins in the bone marrow from a pro-B cell to an immature naïve B cell (34, 35). At this stage of development, B cells undergo various checkpoints including clonal deletion and receptor editing, which prevents the development of auto-reactive cells (36–38). B cells that successfully complete these checkpoints leave the bone marrow as transitional B cells (39). However, the checkpoints can be imperfect and B cells capable of self-directed autoimmune responses are common and exist as a part of the healthy immune repertoire (40, 41). An immature naïve B cell migrates into a secondary lymphoid organ where it then develops into a mature naïve B cell, expressing a BCR with single antigenic specificity (42). A mature naïve B cell can generally be divided into three further subsets: B-1 B cells, marginal zone (MZ) B cells and follicular B cells, with the B-1 B cells being further subdivided into B-1a ($CD4^+$ helper T cell-dependent) and B-1b ($CD4^+$ helper T cell-independent) B cells (43). B-1a cells provide protection against bacterial infections while B-1b cells function independently of T helper cells and provide adaptive immune response to polysaccharides, for instance lipopolysaccharide, and other T cell-independent antigens (44). When mature naïve B cells encounter their cognate antigen in the secondary lymphoid tissue, they become activated. While the primary signal for B cell activation is the binding of antigen to its antigen-specific receptor expressed by the B cell, secondary signals are also required. A B cell response to the antigen is successful only by the synergy between the engagement of their BCR and co-receptors like Toll-like receptors (TLRs) and CD40, which control class switching and affinity maturation in these activated B cells (45, 46). Following activation, some of these B cells—in conjunction with $CD4^+$ T cell help—take part in germinal center (GC) reactions within the lymphoid follicles.

Lymphoid follicles in secondary lymphoid tissue act as a site of antigen-induced B cell proliferation and they have a complex microenvironment, which consists of immune cells, adhesion molecules and antigen-antibody complexes. GCs are specialized areas within these lymphoid follicles where B cells undergo somatic hypermutation leading to affinity maturation to eventually develop into memory B cells or antibody secreting plasma cells (47, 48). The adaptive immune system can evoke an enhanced response to a previously experienced pathogen. This response depends on memory lymphocyte populations of which memory B cells are a part. The improved responsiveness of memory B cells is attributed to class switching and high affinity BCR on their surface which they develop within the GC. However, it is important to note that memory B cells are a heterogenous population and can be further differentiated into T cell-dependent/GC-dependent memory B cells or GC-independent memory B cells (49).

AUTOIMMUNE NEUROINFLAMMATION: A FOCUS ON MULTIPLE SCLEROSIS (MS)

An Overview of the Disease

MS is a neuroinflammatory demyelinating disorder of the CNS in genetically predisposed individuals (50). MS is considered to be a heterogenous disease with different clinical courses depending on the subtype (51). While ∼85–90% of MS patients present with a relapsing-remitting form of MS (RRMS), most of these patients develop secondary progressive disability (SPMS) in the course of the disease. The rarer form of MS is primary progressive MS (PPMS) which has an insidious disease onset and is characterized by a steady increase in neurological disability (52). The pathogenic role of inflammation in all the subtypes of MS remains undisputed (53–55), and the inflammatory reaction in MS is said to be a cumulative effect of a number of factors including cells of the innate and adaptive immune system, their mediators and effector molecules like cytokines and antibodies (56–58).

Evidence of B Cells in MS

Despite historically being dubbed as a "T-cell mediated disease," emerging evidence suggests that B cells contribute to MS pathogenesis in more than one way (59–62). The multifaceted roles of B cells as "shapers" in MS disease progression include antibody production, pro- and anti-inflammatory cytokine secretion and antigen presentation (32, 63, 64).

One of the earliest indications that B cells contribute to disease pathogenesis comes from the identification of persistent oligoclonal bands (OCBs) in the CSF of > 90% of all patients diagnosed with clinically definite MS (65, 66). In general, the presence of OCBs suggests abnormal intrathecal production of clonally expanded IgGs which is an indication of the pathogenic role of B cells in neuroinflammatory and infectious diseases of the CNS (67). In MS, a direct link between CSF-infiltrating B cells as the source of Igs associated with these OCBs has been established (68). Two studies have demonstrated that a significantly increased accumulation of B cells in the CSF of MS patients strongly correlates with intrathecal synthesis of IgG (69, 70). Furthermore, these B cells have been characterized to be of the $IgM^{-}IgD^{-}$ class-switched memory and plasmablast phenotypes. In line with the findings above, other studies have separately identified that clonally expanded B cells in the CSF of MS patients show evidence of somatic hypermutation and affinity maturation (71, 72). Indeed, the presence of B cells is not just restricted to the CSF but overlapping B cell populations are common between the periphery and the different CNS compartments (58, 73, 74) providing proof that clonally related B cells participate in bidirectional exchange across the brain barriers in the case of MS. In another study Ig gene repertoire sequencing of CSF and peripheral blood B cells in treatment-naïve MS patients has also revealed a clonal relationship between the B cell populations in the two compartments (75).

The involvement of both B cells and autoantibodies in MS also comes from neuropathological analysis of lesions from patients. For instance, one of the most frequent patterns in MS lesions is characterized by antibody deposition and complement activation (76). Although the presence of complement supports a pathogenic role of the antibodies in correlation with areas of demyelination (76, 77), the antigenic targets for these autoantibodies remain unclear. Moving from the detection of antibodies to B cells in autopsied CNS tissue from MS patients, immunohistochemical stainings have indicated the accumulation of B cells and plasma cells in perivascular spaces of the brain which are associated with active demyelination (78). A more recent study has revealed a prominent presence of $CD20^{+}$ B cells in the lesions of patients with acute MS (79), indicating that B cells may be important in the overall inflammatory process and also in the early stages of the MS.

Furthermore, in the secondary progressive stages of MS, lymphoid-like B cell follicles have been detected in the inflamed meninges of up to 40% of patients (80–82). These ectopic follicles containing a complex network of B- and T cells, plasma cells as well as follicular dendritic cells (83) are preferentially localized within the subarachnoid space, attached to the pial membrane and their presence is often associated with a more aggressive disease progression (60, 81). A connection between the presence of these meningeal B cell follicular aggregates and the sustenance of B cell maturation locally within the CNS leading to a compartmentalized humoral immune response has been made (61). In addition to these ectopic B cell follicles in the leptomeninges of SPMS patients, meningeal $CD20^{+}$ B cell infiltrates have also been reported in patients with PPMS which correlate with a higher degree of cortical demyelination (84). However, as yet there is little knowledge on the (immuno)phenotype of these B cells, their molecular characteristics or the precise role they play within these follicular structures or aggregates. Of importance are the difficulties faced in studying these aggregates because of limited availability of appropriate B cell follicle containing tissue, poor quality of tissue and technical difficulties of detecting these follicles due to the easy detachment of the meninges during autopsy (85).

A number of antigen experienced B cell clones have also been detected within the CNS parenchyma in MS patients with a chronic progressive or secondary progressive disease course (86). Furthermore, a more recent study demonstrated the presence of B cell follicles in the spinal meninges of SPMS patients that were associated with demyelination and axonal loss (87). These findings suggest that B cells are probably not just localized in the extraparenchymal tissue of the brain but also populate different areas of the CNS tissue, including the spinal cord.

Despite there being little doubt regarding the presence of B cells in the different compartments of MS patients, the precise site(s) or trigger(s) of B cell activation remain fairly speculative (31, 63). One hypothesis for their activation could be that B cells encounter their cognate antigen in the peripheral deep cervical lymph nodes—which is the site of CSF-mediated drainage of brain antigens—where they differentiate into memory B cells or plasmablasts before migrating into the CNS (31). In the inflamed CNS, these plasmablasts or memory B cells may further differentiate into plasma cells. This differentiation may be even in the absence of specific antigens but rather in an antigen non-specific manner by a polyclonal stimulus (88). For example, human herpesvirus 6 (HHV-6), which is an infectious

agent implicated in the pathogenesis of MS, may be involved in polyspecific B cell activation (89, 90). One of the obvious manifestations of these antibody secreting plasma cells within the CNS is in the form of OCBs as seen in the CSF of MS patients. It may also be plausible that naïve B cells enter the CNS and are activated within the CNS (for example, by taking part in GC reactions within meningeal B cell follicles) and complete the circle of eventually differentiating into plasma cells.

To summarize, the studies mentioned above indicate that the number of B cells and their location possibly depends on the disease course and duration, with a substantial amount of variation between individual cases. It supports more careful screening of autopsied CNS tissue from MS patients with a chronic disease course with the purpose of characterizing the B cells beyond their CD20 marker. The literature strongly suggests that B cells are involved in MS and are present in all the different compartments within the CNS and in the periphery. Yet, in what ways these B cells establish themselves in the inflamed brain, where and how they are activated has not yet been clearly elucidated with only a limited number of studies addressing these questions (91–93).

Role of B Cells in MS

The importance of different antibody-independent functions of B cells in the pathogenesis of MS is highlighted by the success story of treatment with monoclonal anti-CD20 antibodies. It has been shown that depletion of circulating B cells by the chimeric anti-CD20 monoclonal antibody rituximab effectively led to rapid reduction in gadolinium (Gd)-enhancing lesions and MRI lesion load as well as relapse activity in RRMS patients (94, 95). This anti-CD20 monoclonal antibody has also shown high efficacy in the removal of $CD20^+$ B cells from the peripheral and CSF compartments (96, 97). However, the reduction of B cells in the CSF was comparatively much lower than in the periphery (30, 98, 99). Ocrelizumab, a humanized anti-CD20 antibody, has demonstrated high efficacy in reducing relapse rates in RRMS patients in different clinical trials and is also associated with lower rates of clinical and MRI progression in patients with progressive MS (100, 101).

Since plasma cells do not express CD20, they are not directly depleted by anti-CD20 therapy (96). Therefore, the decrease in disease activity following treatment of MS patients with anti-CD20 antibodies is possibly linked to one or more antibody-independent functions of B cells such as antigen presentation (to T cells) or cytokine production (32).

B cells can function as effective antigen presenting cells (APCs) when they recognize the same antigen as T cells (102), which is important for the activation of effector T cells (103). As a part of this B- and T- cell cognate interactions, the combination of co-stimulatory signals plays a key role in defining the T cell response of which the interaction between CD80/CD86 and CD28 is among the best characterized (32). One such antigen presenting potential of B cells in the context of MS comes from reports indicating that during MS disease exacerbations, the number of $CD80^+$ B cells abnormally increases (59, 63, 104). Exactly what set of triggers is responsible for this upregulation of CD80 in B cells of MS patients is, however, less known. One of the possibilities is that interferon (IFN)-β induces CD80 expression (104, 105), which is a cytokine produced by innate immune cells like macrophages and non-immune cells like fibroblasts and epithelial cells. It has been shown that IFN-β therapy noticeably reduces the number of circulating CD80 B cells (59, 104) in MS patients. Secondly, ligands for TLR 1/2, 4, 7/8 are also known to induce a strong activation of B cells and upregulation of CD40 and CD80 (106). In the context of MS, TLRs indeed play a major role in the initiation of disease as well as in the triggering of relapses (107, 108). Furthermore, a variety of cytokines like IL-4 and IL-2 that are relevant in the context of MS, are also known to induce CD80 expression on B cells (109).

The potential of memory B cells from RRMS patients to activate T cells has also been demonstrated by Jelcic et al. and Harp et al. (110, 111), where T helper cells promoted B cell proliferation and differentiation, thus establishing a bidirectional B- and T cell interaction that plays a key role in MS pathogenesis (110).

Furthermore, circulating B cells of untreated MS patients exhibit an abnormal balance of pro- to anti-inflammatory cytokine responses (112–114). These abnormalities in the effector-cytokine production by MS B cells in turn also affects the myeloid as well as the T cell compartments. Of note, *in vitro* studies using B cells from MS patients demonstrate the ability of granulocyte-macrophage colony-stimulating factor (GM-CSF) expressing B cells to efficiently enhance myeloid cell pro-inflammatory responses in a GM-CSF dependent manner (115). Another example comes from anti-CD20 depletion studies where changes in the number of pro-inflammatory B cells correlated with a persistent decrease of T cell lineage pro-inflammatory responses (116). These studies have demonstrated that B cells from MS patients in comparison to healthy controls cannot only produce a myriad of pro-inflammatory cytokines (114, 115), but these cytokines also have the ability to modify responses of other immune cell populations (115, 117).

As mentioned earlier, cortical demyelination in a subgroup of MS patients is associated with ectopic B cell follicles in the meninges which implies that B cells may be involved in cortical injury by secreting cytotoxic factors (63). *In vitro* studies using B cells from RRMS patients substantiate that they are capable of killing oligodendrocytes and neurons in an antibody-independent manner involving apoptosis (118, 119), while the identity of the cytotoxic products remains to be clarified.

However, it may also be necessary to note that the beneficial effects of anti-CD20 therapy in MS patients cannot solely be attributed to the depletion of B cells but rather $CD20^+$ T cells may also be targeted (120). Although CD20 is a hallmark cell surface marker of B cells, a proportion of $CD3^+$ T cells also expresses this marker (121) which are found in an increased number in the peripheral blood and CSF of MS patients (122). While it has been proposed that T cells present in the blood may acquire CD20 from B cells by a process called trogocytosis and are therefore $CD3^+CD20^+$, Schuh et al. have elaborately demonstrated that indeed a subset of T cells transcribes CD20 but no other molecules typically found on B cells (120). CD20 expressing T cells have been reported to be a highly activated pro-inflammatory cytokine-producing cell population

with pathogenic potential (120, 121). Furthermore, several studies have elaborately demonstrated that this population of $CD20^+$ T cells can be effectively depleted by rituximab and ocrelizumab in patients with RRMS (122–124) suggesting that depletion of this cell population might be an important consideration in the overall clinical effectiveness of anti-CD20 directed therapies (125).

Animal Model(s) of MS: Experimental Autoimmune Encephalomyelitis (EAE)

There are of course limitations of studying the pathomechanisms of disease development in human subjects. Scientists have therefore turned to using EAE, which is one of the best characterized and most frequently used animal models for studying neuroinflammation in the human disease MS. A wide range of EAE models have been induced in a number of different species (including rats, mice, and primates) with varying degrees of efficacy to study different aspects of MS pathogenesis (126–129). Yet, most of these models are biased towards a $CD4^+$ T cell-restricted immune response and no single experimental model covers all the immunological and pathological features of the human disease (130, 131). In particular, some aspects of MS, especially the progressive stage of MS, have so far been poorly covered in commonly used experimental rodent models.

As discussed above, there is a growing appreciation of the involvement of B cells in the later stage of MS where aggregates of B cells have been found in the leptomeninges of SPMS patients (81, 83). These B cell aggregates feature a complex follicle-like structure and are most likely instrumental in strong meningeal inflammation. Modeling this B cell aspect of the human disease in the conventional EAE models has yielded varying results between the different strains of rodents and with regard to the immunizing antigen(s) (85, 132).

One of the more robust mouse models that is both B cell- and antibody-dependent on the C57BL/6 background is the MP4-induced EAE (133). MP4 is a fusion protein that consists of the human isoform of myelin basic protein (MBP) and the three hydrophilic domains of proteolipid protein (PLP). Using this model several studies have successfully demonstrated both antibody-dependent and -independent roles of B cells in EAE (which mirrors aspects of the human disease as well). This includes induction of demyelination through complement activation (76, 134) and a pathogenic role for antibodies (133, 135). Of interest, B cell infiltrates are also present in the spinal cord, brain and cerebellum of MP4-immunized mice (136). In particular, aggregation of B cells that acquired features of lymphoid tissue in the chronic disease stage was detected in the cerebellar parenchyma. A detailed characterization of these B cell aggregates in MP4-induced EAE revealed that the lymphoid structures in MP4-induced EAE were segregated into a B cell and T cell zone, which is similar to secondary lymphoid tissues where B cells reside in the follicles and T cells in the parafollicular zone. Furthermore, in MP4-induced EAE, high endothelial venules (HEVs) expressing the addressins CCL19 and CCL21 were also detected in addition to the chemoattractant CXCL13 (83, 137). Heavily proliferating B cells were also found indicating recent and clonal activation (137, 138). Collectively, these findings from the MP4-induced EAE model support a strong role for B cells in MS that is not only restricted to their antibody secreting ability. While the limited availability of human tissue in conjunction with the fact that autopsied brain tissue of MS patients only provides a "snapshot," this B cell-dependent EAE model can be exploited to answer a number of disease relevant questions. For example, time course experiments on the development of B cell follicles and studies to investigate whether B cells play a different role depending on the disease stage at which they are found can be demonstrated using this EAE model.

Role of B Cells in MS: Lessons From Rodent Models of EAE

Studies done in other EAE models have also revealed some important aspects of B cell involvement in disease progression and pathogenesis with some of the examples mentioned below.

As mentioned earlier, B cells can function as effective APCs especially when they recognize the same antigen as T cells (139). This antigen presenting capacity of B cells has been highlighted in different B cell-dependent EAE-based studies. In EAE induced by recombinant myelin oligodendrocyte glycoprotein (rMOG) protein, activated B cells have been shown to serve as APCs that promote the differentiation and proliferation of Th1 and Th17 cells. Accordingly, anti-CD20-mediated depletion of B cells inhibited B cell-dependent activation of pathogenic T cells contributing to the overall reduction of CNS inflammation (140). Furthermore, using an adoptive transfer model of EAE, it has been demonstrated that the development of autoimmune attacks within the CNS is facilitated by induction of MHC class II on B cells followed by pathogenic cognate interactions between B- and T cells (141). Similarly, B cell-specific MHC class II knock-out (KO) mice have been found to be resistant to rMOG-induced disease indicating that B cells provide critical cellular functions independent of their humoral involvements (142).

A more favorable role of B cells in EAE has been elaborated in mice which did not express the anti-inflammatory cytokine IL-35. These mice also lost their ability to recover from T cell-mediated EAE confirming the importance of IL-10/IL-35 secreting B cells in ameliorating disease progression (143). Regulatory roles for B cells during EAE immunopathogenesis have also been discussed by other groups (144). A recent study highlights that non-selective depletion of B cells using anti-CD20 therapy concurrently abolishes preexisting regulatory B cells which are important for limiting chronic disease progression (145). Efforts to expand our understanding of this regulatory population of B cells in improving EAE severity and reducing neuroinflammation is a current topic of interest.

Taken together, there is plenty of evidence from studies done in animal models and from MS itself which repeatedly points towards a definite role of B cells in aggravating disease pathogenesis in more than one way. In addition, there is also indication of an anti-inflammatory cytokine secreting "beneficial" population of B cells in both MS patients (repopulating IL-10 secreting B cells following CD20 depletion therapy) (32) and its EAE animal model (143). Nevertheless,

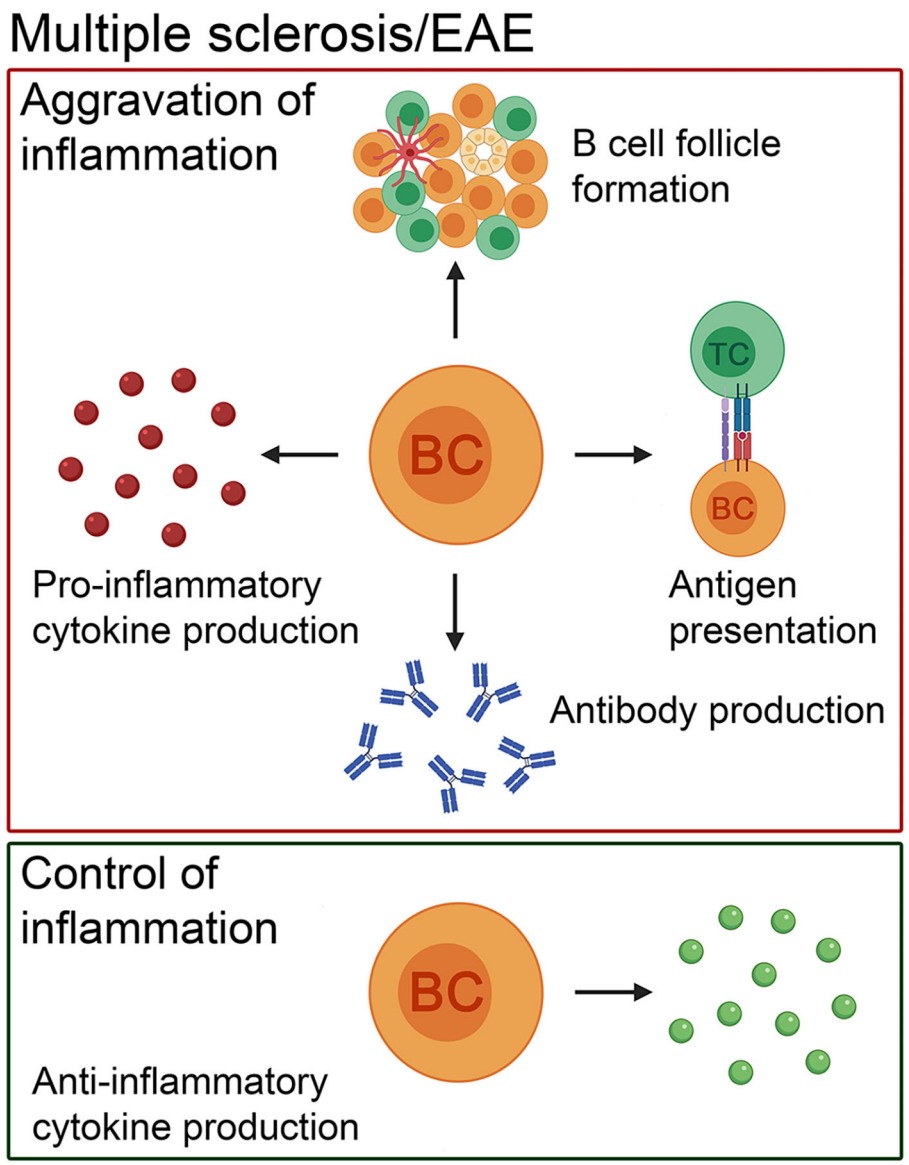

FIGURE 1 | The pathogenic and beneficial effects of B cells in MS patients and in its animal model, EAE. In addition to antibody production, B cells present antigens to T cells, form ectopic lymphoid structures consisting of B- and T-cell compartments, follicular dendritic cells and high endothelial venules and produce pro-inflammatory cytokines to exacerbate the disease course. Besides the negative role of this cell population, there is evidence that B cells positively influence the disease course by secreting anti-inflammatory cytokines. BC, B cell; TC, T cell.

several unanswered questions remain including, whether the pathogenic B cell subset(s) in MS patients can be selectively depleted, based on a more detailed characterization of this cell population. Longitudinal studies to monitor changes in the pro- vs. anti-inflammatory B cell subsets in the different compartments of MS patients (or in relevant EAE models) would also provide new insights into how B cells promote or reduce neuroinflammation, respectively. Finally, it would also be interesting to explore whether these new findings can be translated into therapeutic potentials and treatment options for patients (**Figure 1**).

Infectious Neuroinflammation: A Focus on Viral Diseases

As evident by the studies discussed above, the involvement of B cells as multifunctional players in MS as an example of autoimmune neuroinflammation is clear. However, as mentioned earlier, neuroinflammation can result from several other insults to the CNS which is not just restricted to being autoimmune (146). Infectious diseases of the CNS also result in neuroinflammation as an inherent host-defense mechanism to restore the normal function of the brain against the infecting pathogen (147). The importance of B cells, in general, in

providing several lines of defense against a variety of pathogens and the ability of antibodies as their effector molecules in eliminating viral particles is very well established and has been discussed elsewhere (148–151). Therefore, neuroinflammation can be considered a common denominator between these two infectious and autoimmune triggers where B cells play a significant role. It is also intuitive that the "gaps" in our understanding of the contribution of B cells in autoimmune neuroinflammation, as discussed earlier, can be compensated by drawing parallels between the findings in infectious and autoimmune neuroinflammation with a focus on the involvement of B cells in both cases.

In that context, we discuss a few examples of viral infections of the CNS where B cells have been indicated to play a role in either clearing of the pathogen or progression of the infection. Furthermore, we have included examples of viral infections that are particularly relevant for MS. However, details of B cell activation in the different types of viral infections related to the CNS is beyond the scope of this review.

Viral Infections of the CNS

Viral infections of the CNS are the most prevalent cause of encephalitis, meningitis as well as meningoencephalitis and the number of cases surpasses all bacterial, fungal, protozoal infections combined (152, 153). Following viral infections of the CNS, inflammation can occur in different anatomical regions including the meninges, brain parenchyma, the spinal cord or simultaneously in multiple regions. Examples of viral infections affecting the CNS include herpes simplex virus, adenoviruses, arboviruses, flaviviruses, and enteroviruses (154). The complexity of these viral infections is influenced by a number of different factors including the tropism of the viruses, their routes of CNS entry as well as the overall "health" of the immune system (152).

John Cunningham virus (JCV) is an important example to learn about the interplay between opportunistic viral replication and the adaptive immune system. JCV infection of the CNS is associated with improper functioning of the adaptive immune system with relation to both the B- and T cell compartments (155). The occurrence of progressive multifocal leucoencephalopathy (PML), an oftentimes deadly demyelinating disease caused by JC virus replication in the brain, has been linked to immunomodulatory treatments in patients with autoimmune diseases (156), and is also observed in immunocompromised individuals or those with hematological malignancies. B cells appear to play a complex role in mediating disease pathogenesis of PML because on the one hand, they represent a potential reservoir for JCV and on the other hand they likely play a role in the control of the infection (157). Evidence from clinical studies and those done in animal models suggests that B cells not only influence the T cell response through cytokine secretion but are also able to mount an effective humoral response against the virus which together allows the control of infection (157). Although being a very rare event, the occurrence of PML has been linked to anti-CD20 depletion therapies, indicating a potential importance of B cells in controlling JCV infection (155, 158). In general, it has been suggested that profound perturbation of B cell homeostasis by anti-CD20 therapies (as in the case of rituximab) could contribute to the development of PML (155). For instance, following anti-CD20 depletion the reconstituted B cell pool is mostly considered to be IL-10$^+$ (112, 115), with IL-10 being an anti-inflammatory cytokine that suppresses both T cell- and innate cell-mediated inflammatory responses. Whether this change in the overall B cell cytokine profile together with the phenotype of the newly appearing B cells aggravates the pathogenesis of PML is a current topic of investigation (155). Nevertheless it is important to stress that anti-CD20 therapy associated PML in MS patients is an extremely rare complication compared to treatment with, for instance, natalizumab (156).

Animal Models of Virus-Induced Neuroinflammation

Owing to the limited availability of patient material, the involvement of B cells in most viral infections of the CNS comes from the relevant animal models (159, 160). Through these various models it becomes well-established that B cells can play both detrimental as well as beneficial roles during CNS infection with encephalitic RNA viruses, such as Sindbis virus (SINV), Semliki forest virus (SFV), West Nile virus (WNV), neurotropic coronavirus, and murine cytomegalovirus (MCMV).

For instance, infection of mice with SFV suggests that brain infiltrating B cells contribute to myelin injury in SFV encephalomyelitis in both an antibody-dependent and -independent manner (161).

Extending the role of B cells beyond their capacity to modulate T cell functions, the study by Mutnal et al. is of note, where the authors demonstrate a distinctive subset of CD5$^+$ B regulatory cells to infiltrate brains of mice chronically infected with MCMV. This population of regulatory B cells was found to control macrophage-dependent pro-inflammatory responses while absence of this cell population resulted in exacerbated T cell-mediated neuroinflammation post viral infection (162). In another mouse model using attenuated rabies virus it has been shown that the production of rabies-specific antibody by CNS tissue infiltrating B cells is essential for the complete elimination of the virus (163). Furthermore, studies done in mice infected with WNV have shown that B cells are critical in providing defense against early spread of infection in these mice as well as limiting infection in the CNS (164).

While the data mentioned above highlight some of the dual functions that B cells play in virus-induced neuroinflammation, numerous studies using viral models have focused on specific chemotactic signatures that allow B cell migration into the CNS.

Infection of the CNS with the neurotropic strain of mouse hepatitis virus (JHMV) in a murine model results in an acute CNS inflammatory response containing B cells (165). Antibody secreting cells were directed toward the CNS in a virus-induced chemotactic manner where CXCL9 and CXCL10 were identified as two such chemokines induced by JHMV (165). On the other hand, CXCR3 has also been identified as a chemokine receptor recruiting plasmablasts to the CNS in the same murine viral model (166). In response to another viral strain, SINV, a similar trend was noticed where CXCL13 and CCL19 were induced

in the brains of mice infected with the virus (167). Similarly, during MCMV infection of the brain, $CD19^+$ B cells isolated from the brain expressed chemokine receptors CXCR3, CXCR5, CCR5, and CCR7 (168). Overall, results from the different animal models of viral infections suggest CNS infiltrating B cells during viral infection migrate into the CNS in a CXCR3-, CXCR5-, and CCR7-dependent manner whose ligands are also upregulated within the CNS (169).

While most of the above-mentioned studies highlight CXCL13 (the ligand for CXCR5) as an essential B cell chemotactic factor, interestingly, during coronavirus encephalomyelitis infection in mice, naïve and early activated IgD^+ B cells were able to migrate into the CNS independent of CXCL13-driven signals (170). This of course suggests a more complex chemokine kinetics over the course of an infection representing several possible "windows of trafficking" for B cells into the CNS. Accordingly, the subset and phenotype of B cells which migrate into the CNS may change depending on the time point.

Not only do viral models of inflammation give clues on migration patterns of B cells into the CNS but studies done in different viral models also present evidence that the CNS provides the necessary signals, including the expression of B-cell activating factor (BAFF), for sustained B cell viability and maintenance of a repertoire of virus-specific antibody secreting cells within the CNS (165, 168, 171). Additionally, an increased expression of BAFF mRNA in the CNS also coincides with long-term maintenance of virus-specific antibody secreting B cells in the brain (167). Sustained local antibody secretion by already infiltrated B cells in the brain seems to be an effective strategy in case of chronic viral infections of the CNS since the passage of antibodies from the periphery through intact brain barriers is insufficient (168). Another example of the CNS fostering B cell survival and differentiation comes from the Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD) model. TMEV-IDD induced by injecting a virus into susceptible mice strains captures several aspects of chronic inflammation as seen in the progressive stages of MS (172). Using this model, during chronic infection, the predominant B cell phenotypes accumulating in the CNS were characterized to include isotype-switched B cells, memory B cells and antibody secreting cells. Mature and isotype-switched B cells were detected in the meninges and perivascular space and B cell relevant chemokines and tropic factors were elevated in the CNS in the absence of ectopic B cell follicles. Therefore, results from these studies revealed that the CNS has the ability to promote accumulation of isotype-switched B cells as well as intrathecal antibody synthesis independent of ectopic B cell follicle-like structures during chronic inflammation (173, 174) (**Figure 2**).

Viral Infections Related to MS

On one hand, the involvement of B cells in the context of viral infections resulting in neuroinflammation can be emphasized by the examples mentioned above. On the other hand, viruses as infectious agents in the etiology of MS have been suspected for several decades (175). Here, we discuss the interplay between viral infections and MS with a special focus on Epstein-Barr virus (EBV).

Epstein-Barr Virus (EBV)

Among infectious factors, EBV has the strongest epidemiological and serological connection to MS (176–178) and a relationship between EBV infection with the MS brain has long been explored. While some studies suggest that EBV may be responsible for breaking immune tolerance to CNS myelin antigens through molecular mimicry (179), others focus on the ability of the virus to infect and promote immortalization of antibody secreting B cell clones (180). It has also been suggested that the virus can act as a possible antigenic stimulus of lasting immune response within the CNS with a link to the presence of persisting OCBs (181).

EBV is a ubiquitous B-lymphotropic virus with the ability to infect, activate and latently persist in B cells for the lifetime of the infected individual (182). Furthermore, EBV is known to drive an infected B cell out of its resting state to become activated into a B cell blast and eventually become a memory B cell that can circulate in the blood (182). Suggestions have been made that when EBV-infected B cells from the periphery migrate into the CNS, they play a crucial role in propagating CNS-compartmentalized neuroinflammation (183, 184). Given that the general opinion for development of MS pathology is thought to involve interactions between T- and B cells, whether EBV-infected B cells can also activate T cells in the periphery is an attractive hypothesis (183).

Elaborate histopathological evidence demonstrating a direct link between EBV and B cells comes from the work of Aloisi and others (185) where they repeatedly identified the presence of EBV-infected B cells "exclusively" in the brain of MS patients (180, 181, 186) and not in corresponding control patients. In particular, areas with heavy B cell infiltrates have been identified as major sites of viral persistence (186).

Interestingly, a link between EBV infection and induction of human endogenous retoviral proteins on B cells has also been made (187). For example, the activation of the human endogenous retrovirus (HERV) has been suggested to be made in the presence of EBV infection where high quantities of HERV-W proteins are said to be expressed on the surface of B cells in patients with active MS (188).

Nevertheless, it is important to note that other studies have failed to establish any relationship between EBV infection, B cells and MS (189, 190) leaving this question to what extent (if at all) EBV might be involved in MS open-ended (**Figure 3**).

Cytomegalovirus (CMV)

CMV is a latent virus that is known to cause chronic activation of the immune system (191) with the seroprevalence of CMV in the general population being between 45 and 100% (192). An association between CMV infection and MS risk has been made in the past but with inconsistent results (193, 194).

Nevertheless, most studies have found that CMV seropositivity is negatively associated with MS (195, 196) with reports suggesting that CMV infection modulates the immune response to a regulatory type (196, 197). More recently it has been demonstrated that CMV infection regulates the distribution of B cell subsets in MS patients to a reduced pro-inflammatory phenotype (198)—a finding similar to what has

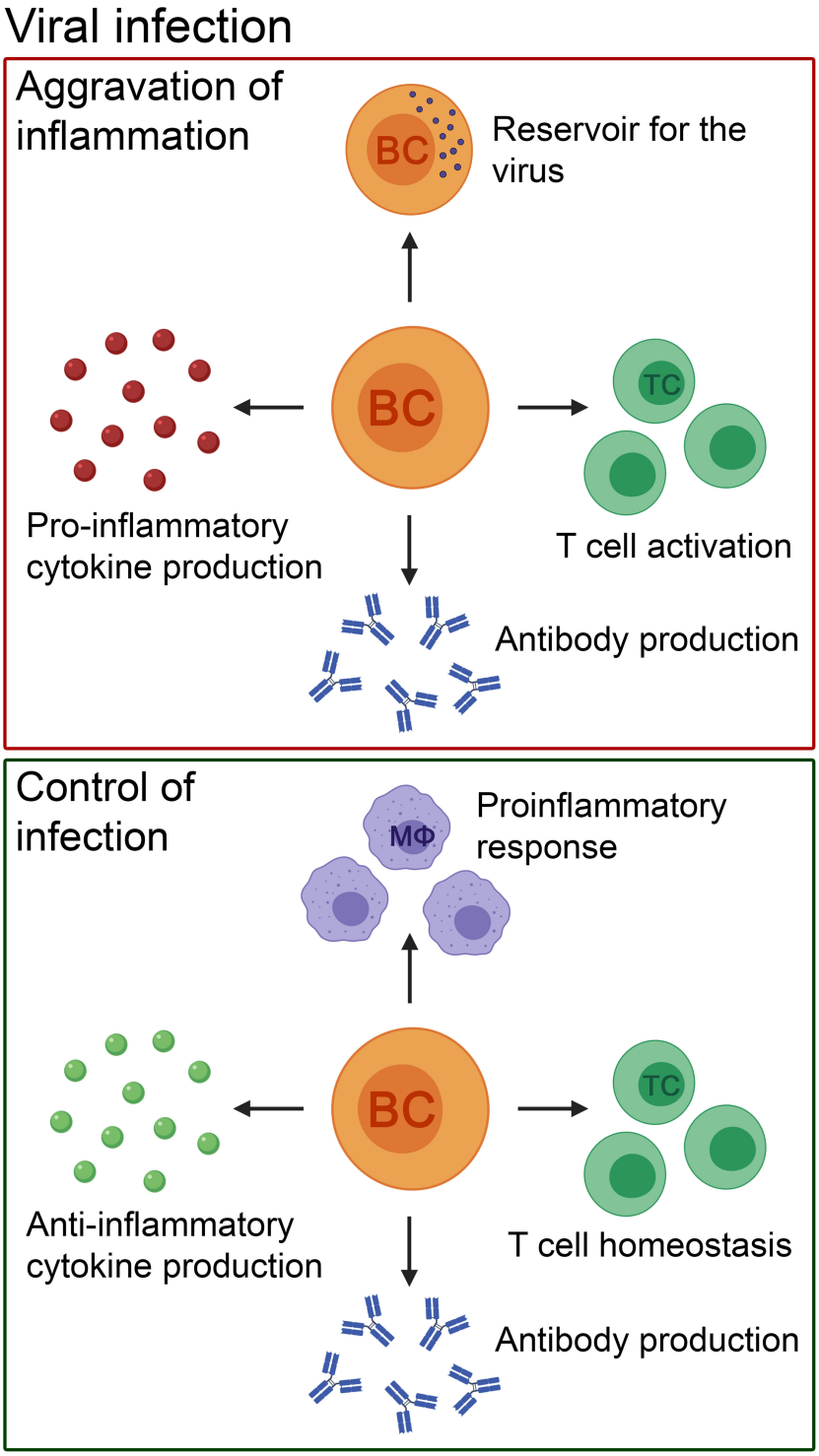

FIGURE 2 | The pathogenic and beneficial effects of B cells in patients with viral infections of the CNS and their corresponding animal models. B cells can cause aggravation of inflammation by pro-inflammatory cytokine and antibody production. In addition, they can act as a reservoir for the virus, and activate T cells. Nevertheless, the role of B cells in viral infections is heterogenous. B cells control the infection by producing anti-inflammatory cytokines and antibodies to eliminate the virus. Furthermore, they have a positive effect on other immune cells by promoting T cell homeostasis and controlling innate immune cell-mediated pro-inflammatory responses (e.g., by macrophages). BC, B cell; TC, T cell; MΦ, macrophage.

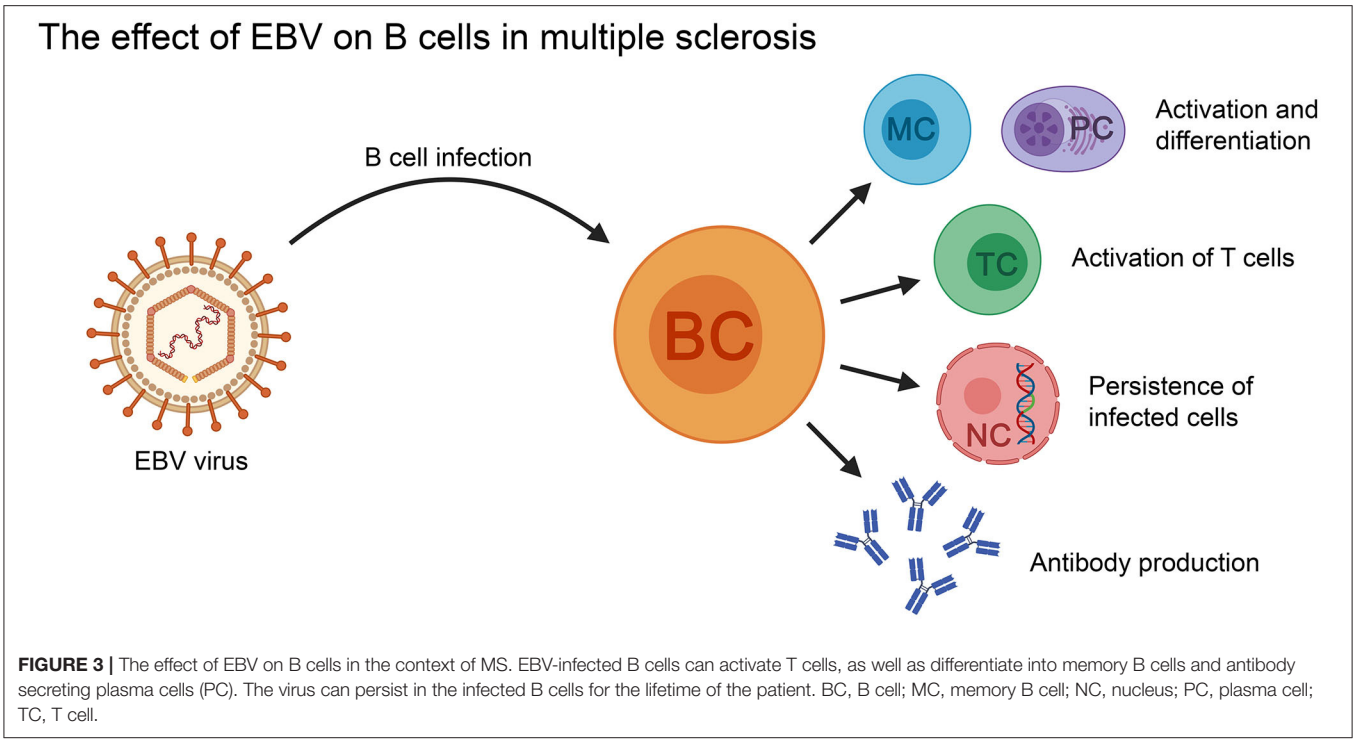

FIGURE 3 | The effect of EBV on B cells in the context of MS. EBV-infected B cells can activate T cells, as well as differentiate into memory B cells and antibody secreting plasma cells (PC). The virus can persist in the infected B cells for the lifetime of the patient. BC, B cell; MC, memory B cell; NC, nucleus; PC, plasma cell; TC, T cell.

been previously described in the case of chronic CMV infection (199). Another hypothesis of how CMV may result in milder MS symptoms could be that in patients that are CMV/EBV double seropositive, there is a balance in the immune response between these two viruses (196). However, in CMV seronegative patients, EBV could drive the immune system towards a more aggressive MS disease phenotype.

Other mechanisms by which CMV might influence MS pathogenesis can be *via* molecular mimicry or bystander activation (196, 200).

In summary, the interaction between different viruses and the immune system—in particular B cells—in MS patients seems complex with contradictory findings. Further longitudinal studies with larger patient cohorts and rigorous methodologies are required to unravel the relationship between viral infections and disease initiation as well as progression in MS.

MS vs. Virus-Induced Neuroinflammation

It is reasonable to say that the basics of B cell biology remain the same independent of the trigger of neuroinflammation. Therefore, transferring the findings from one field of research to another may not only allow us to tackle the "unknown" better but also to look at the disorder from another perspective. As evident from the different studies mentioned above, several parallels can be drawn between virus-induced and autoimmune neuroinflammation. Here we discuss a few such examples.

Studies done in murine models of neurotropic viral infections indicate that B cells enter the CNS during acute viral infection with early infiltrating B cells expressing CXCR3 and CXCR5 (among others) and upregulation of the corresponding ligands in the CNS (169). A similar situation is observed in MS where CXCL12 and CXCL13 are elevated in actively demyelinating MS lesions (91, 201), fostering B cell entry into the CNS. Additional evidence suggests the chemokines CXCL10, CCL2, and CCL3 also to be involved in attracting B cells into the CNS in MS (202). Therefore, B cells in general appear to have a specific and common chemotactic signature that allows them to migrate into the CNS under neuroinflammatory conditions whether the source is infectious or autoimmune.

Moreover, if one was to apply the findings in the viral model (as mentioned above) by the group of Phares et al. (170) to MS, it is indeed plausible that there is a variation in the chemotactic factors in the CSF/serum over the course of the disease. This plausible time-dependent change in chemokines in MS patients may also affect the phenotype of B cells migrating into the CNS. However, given the difficulties of following this "range" of migration pattern of B cells into the CSF/CNS compartment in MS patients, the question remains open.

While these are a few instances of the chemotactic "signature" behind the migration of B cells into the CNS, there are also similarities between viral models and MS with respect to how the B cells may be able to establish themselves within the inflamed brain. For example, in line with findings from viral models (167), strong astrocytic expression of BAFF in MS lesions (87) supports B cell survival making them active participants of "trapped inflammation" (in the case of SPMS) (203). Enough circumstantial evidence suggests that through the expression of the necessary B cell survival factors, the MS brain creates an environment that is conducive for the retention of B cells within the CNS (87, 91, 202).

To what extent B cells can "establish" themselves within the CNS and the role(s) they play from within this compartment

have been discussed using both viral models and EAE. Using the TMEV-IDD model DiSano et al. suggest that aggregates of B cells, independent of ectopic lymphoid-like follicle structures, are sufficient to drive B cell differentiation and also contribute to intrathecal antibody synthesis (173). This is an important finding because while there may not be an obvious presence of ectopic B cell follicles in all cases of progressive MS (189, 204), prominent $CD20^{+}$ B cells infiltrates or clusters are detected in a higher percentage of MS patients (79, 189). Although the significance of these B cell clusters has not been well-discussed in the field of MS research, it might be interesting to see if these B cells also

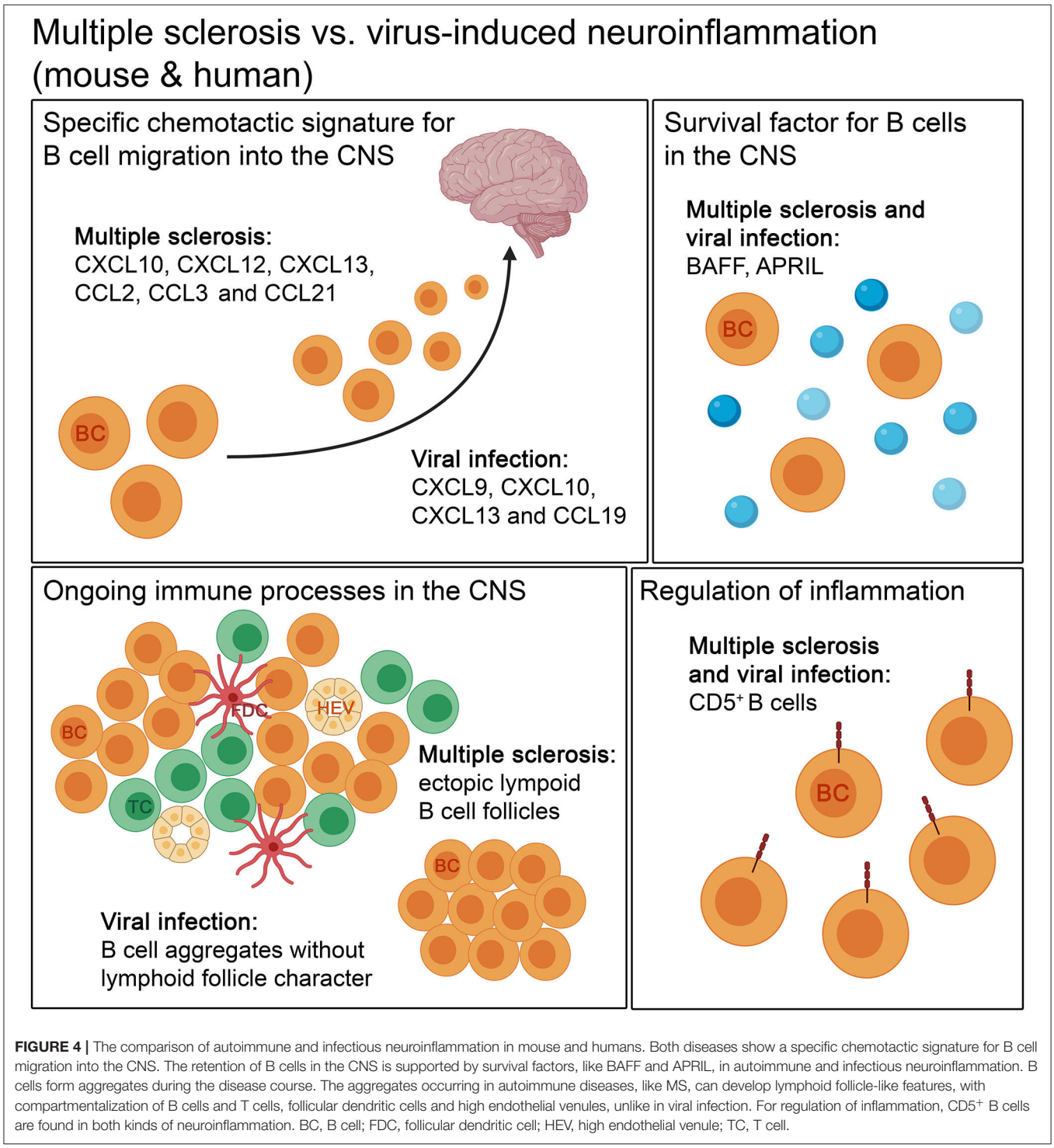

FIGURE 4 | The comparison of autoimmune and infectious neuroinflammation in mouse and humans. Both diseases show a specific chemotactic signature for B cell migration into the CNS. The retention of B cells in the CNS is supported by survival factors, like BAFF and APRIL, in autoimmune and infectious neuroinflammation. B cells form aggregates during the disease course. The aggregates occurring in autoimmune diseases, like MS, can develop lymphoid follicle-like features, with compartmentalization of B cells and T cells, follicular dendritic cells and high endothelial venules, unlike in viral infection. For regulation of inflammation, $CD5^{+}$ B cells are found in both kinds of neuroinflammation. BC, B cell; FDC, follicular dendritic cell; HEV, high endothelial venule; TC, T cell.

participate in similar functions as observed in the TMEV-IDD model.

The significance of a specific regulatory subset of $CD5^+$ B cells in the CNS following chronic viral infection has been demonstrated in animal models (162). The role of $CD5^+$ B cells with a potent regulatory capacity (205, 206) has been reviewed previously (207, 208). Interestingly, clinical data from MS patients suggest that regulatory B cells with increased expression of CD5 predominantly repopulate following anti-CD20 treatment, which—when activated—secrete more anti-inflammatory IL-10 (209). Among other functions, the immunosuppressive cytokine IL-10 is also associated with T cell exhaustion allowing control of aggressive disease progression and preventing excessive tissue injury (210). Indeed, in MS, where demyelination and aggressive disease progression are associated with the presence of T cells (211), exploiting this immunoregulatory subset of IL-10 secreting $CD5^+$ B cells to dampen neuroinflammation remains a current research focus (**Figure 4**).

CONCLUDING REMARKS

The contribution of B cells to CNS neuroinflammatory diseases is unambiguous, as demonstrated by the examples mentioned above. One can say that the B cell response in neuroinflammation is complex and comprises a combination of both beneficial and detrimental phenotypes. Furthermore, the nature of the B cell response differs considerably between the different stages of the disease.

Following limitations of human studies, it is often necessary to extend our understanding of the involvement and importance of a particular cell type by performing experiments in appropriate animal models. Most of the animal disease models used to identify mechanisms that underlie neuroinflammation are induced artificially while mimicking all aspects of a human disease in a single animal model is not feasible. Researchers working on a particular disease often tend to use models which are within their specific area of research, while neglecting the relevance of the findings from other disease models. As an example in this review we have compared studies done in animal models of virus-induced neuroinflammation to findings in MS, although similar comparisons between other instances of neuroinflammation can also be established. It can be assumed that application of the findings from infectious neuroinflammation (with respect to the involvement of B cells) to the field of autoimmune neuroinflammation may facilitate the development of novel therapies to tackle neuroinflammatory disorders like MS and vice versa.

AUTHOR CONTRIBUTIONS

RC and VS outlined the subject of the review, searched for and interpreted the literature, prepared the figures, wrote the manuscript, and gave final approval of the version for publication. SK edited and revised the manuscript for important intellectual content and gave final approval of the version for publication. All figures were created with BioRender.com. All authors contributed to the article and approved the submitted version.

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REVIEW
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Ectopic Lymphoid Follicles in Multiple Sclerosis: Centers for Disease Control?

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While the contribution of autoreactive $CD4^+$ T cells to the pathogenesis of Multiple Sclerosis (MS) is widely accepted, the advent of B cell-depleting monoclonal antibody (mAb) therapies has shed new light on the complex cellular mechanisms underlying MS pathogenesis. Evidence supports the involvement of B cells in both antibody-dependent and -independent capacities. T cell-dependent B cell responses originate and take shape in germinal centers (GCs), specialized microenvironments that regulate B cell activation and subsequent differentiation into antibody-secreting cells (ASCs) or memory B cells, a process for which $CD4^+$ T cells, namely follicular T helper (T_{FH}) cells, are indispensable. ASCs carry out their effector function primarily via secreted Ig but also through the secretion of both pro- and anti-inflammatory cytokines. Memory B cells, in addition to being capable of rapidly differentiating into ASCs, can function as potent antigen-presenting cells (APCs) to cognate memory $CD4^+$ T cells. Aberrant B cell responses are prevented, at least in part, by follicular regulatory T (T_{FR}) cells, which are key suppressors of GC-derived autoreactive B cell responses through the expression of inhibitory receptors and cytokines, such as CTLA4 and IL-10, respectively. Therefore, GCs represent a critical site of peripheral B cell tolerance, and their dysregulation has been implicated in the pathogenesis of several autoimmune diseases. In MS patients, the presence of GC-like leptomeningeal ectopic lymphoid follicles (eLFs) has prompted their investigation as potential sources of pathogenic B and T cell responses. This hypothesis is supported by elevated levels of CXCL13 and circulating T_{FH} cells in the cerebrospinal fluid (CSF) of MS patients, both of which are required to initiate and maintain GC reactions. Additionally, eLFs in post-mortem MS patient samples are notably devoid of T_{FR} cells. The ability of GCs to generate and perpetuate, but also regulate autoreactive B and T cell responses driving MS pathology makes them an attractive target for therapeutic intervention. In this review, we will summarize the evidence from both humans and animal models supporting B cells as drivers of MS, the role of GC-like eLFs in the pathogenesis of MS, and mechanisms controlling GC-derived autoreactive B cell responses in MS.

Keywords: germinal center, GCR, ectopic lymphoid follicles, ELF, follicular T helper cells, TFH, B cell, Th17 (T helper 17 cell)

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INTRODUCTION

Multiple sclerosis (MS) is a neuroinflammatory autoimmune disease affecting nearly 2.3 million people globally (1). MS most commonly presents as episodes of neurological dysfunction followed by periods of clinical recovery known as remission. The accumulating damage resulting from the persistent repetition of relapse and remission is thought to eventually lead to a continuous phase of increased neurological dysfunction and disability without remission, known as secondary-progressive MS (SPMS). About 10% of patients immediately enter this phase after clinical onset in form of primary-progressive MS (PPMS) (2).

Evidence from human samples as well as from the animal model of MS, experimental autoimmune encephalomyelitis (EAE), has established that multiple cell types contribute to disease pathogenesis, with $CD4^+$ T cells as the primary drivers of autoimmune pathology. However, the remarkable clinical success of Rituximab (RTX), a B cell-depleting monoclonal antibody (mAb) targeting CD20, challenged this long-held assumption, demonstrating that the role of B cells in MS may have been underappreciated (3). This proposition is further supported by studies showing that B cells are a major target of previously established disease-modifying therapies (DMTs), and specifically, that positive therapeutic responses are strongly associated with the elimination of pathogenic B cell subsets. The advent and efficacy of B cell-depleting therapies (BCDTs) has necessitated the reevaluation of the mechanisms underlying the pathogenesis and progression of MS.

Despite the considerable success of B cell-targeting therapeutics, clinical outcomes remain varied, similar to previously established DMTs (4). More importantly, the progressive forms of MS are refractory to nearly all currently approved DMTs. Most likely, the inability to halt disease progression is in large part a consequence of our incomplete understanding of the mechanisms responsible for progressive MS.

Along these lines, highly organized structures resembling secondary lymphoid organs (SLOs), known as ectopic lymphoid follicles (eLFs), were initially described in the 1980s and subsequently reported as a common feature of several chronic inflammatory autoimmune diseases (5–8). It is thought that these structures facilitate the perpetuation of autoreactive B cell responses. Interestingly, meningeal eLFs are found in a substantial proportion of SPMS patients, and aggregates of B and T cells were also observed in PPMS and RRMS patients, however, these notably lack features of more developed follicles such as follicular dendritic cells (FDCs), distinct T and B cell zones, and high endothelial venules (HEVs) (9–12).

In this review, we will summarize the progress made in understanding mechanisms of MS immunopathology, with particular emphasis on the role of eLFs as drivers of disease progression, cell types potentially involved in eLF development in MS. Furthermore, we will discuss treatments either currently available or in development that specifically target molecular or cellular mediators of eLF formation or function. Lastly, we will discuss key questions that remain unanswered.

THE GERMINAL CENTER REACTION

SLOs, such as the spleen and draining lymph nodes (DLNs), are specialized structures within which T cell- and B cell-dependent immune responses initiate and develop/mature. This is due to their ability to support germinal center (GC) reactions. GC reactions primarily serve to refine the B cell component of the adaptive immune response through selection and expansion of high-affinity B cell clones and subsequent differentiation into either ASCs, such as plasmablasts (PBs), and plasma cells (PCs), or into memory B cells (13–17). ASCs are effectors that function in both primary and subsequent immune responses. PBs are typically short-lived and serve to neutralize an acute threat by infectious pathogens, while PCs are long-lived, and reside in sites that are specially equipped to support their persistence (18). Memory B cells are rapidly activated upon secondary antigen encounter (19).

GCs are compartmentalized into a dark zone, within which B cell clones proliferate and undergo affinity maturation, and a light zone, where B cells undergo selection, differentiation, or are directed to return to the dark zone to undergo further rounds of affinity maturation and proliferation (16).

$CD4^+$ T cells, specifically follicular T helper (T_{FH}) cells, are principal orchestrators of this process and direct B cell fate decisions through the provision of surface-bound and soluble stimulatory and inhibitory signals (20–22). Additionally, several of these signals, such as interleukin-21 (IL-21) and CD40L, influence class-switch recombination (CSR), thus directing the nature of the B cell effector response. FDCs are a second specialized cell type that display antigen bound in form of immune complexes or with complement and therefore provide B cell receptor (BCR)-mediated survival signals to high affinity B cell clones.

Following resolution of the primary response, circulating and resident memory T and B cells are on stand-by for secondary antigen encounters, upon which they can undergo rapid differentiation and restoration of effector function. Importantly, these encounters can also result in the development of new GCs.

MS PATHOGENESIS

$CD4^+$ T Cells in MS

MS has long been thought to be primarily mediated by autoreactive $CD4^+$ T cells directed against central nervous system (CNS) antigens, such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), or aquaporin-4 (AQP4) (23, 24). The pathogenic role of T cells is undisputed and is based mostly on the following observations: (1) The EAE model of MS can be induced by adoptively transferring myelin-reactive T cells into a healthy recipient animal; (2) the association of MS with human leukocyte antigen (HLA) DRB1*15:01; (3) the exacerbation of MS following treatment with an altered peptide ligand of myelin basic protein (MBP) that activated MBP-reactive T cells and led to disease exacerbations; (4) the *de novo* onset and the re-activation of MS during immune checkpoint inhibitors for cancer therapy; (5) the beneficial effects of T cell depleting pharmacotherapies, such as alemtuzumab, or therapies

that sequester T cells out of the CNS, such as natalizumab; (6) the clonal expansion of $CD4^+$ T cells infiltrating the CNS (25–35).

The importance of $CD4^+$ T cells has been substantiated by studies from both humans and the animal model of MS, EAE. Indeed, $CD4^+$ T cells are enriched in lesions of MS patients and EAE studies further revealed two pathogenic T helper subsets important for disease: interferon gamma (IFN-γ)-producing type 1 T helper (T_H1) cells and IL-17 producing type 17 T helper (T_H17) cells (36). In line with this assertion, both IFN-γ and IL-17 are detected in the lesions of MS patients (37). IFN-γ also positively correlates with increased disease activity and increased disability (38). Moreover, T_H1 cells were found localized in CNS lesions in MS patients and are also increased in the CSF of RRMS patients during relapse compared with remission (39).

Taken together, experimental evidence from human MS patients and experimental animal studies have led to a proposed mechanism in which an unknown trigger results in the aberrant activation of autoreactive $CD4^+$ T cells in the immune periphery, after which these encephalitogenic $CD4^+$ T cells enter the CNS from the choroid plexus (CP), are reactivated by local APCs in the CNS, and initiate a proinflammatory cascade that results in increased permeability of the blood-brain barrier (BBB), subsequent recruitment of proinflammatory immune cells, and subpial cortical damage (40).

A Trail of Breadcrumbs: Initial Evidence of Antibody-Mediated B Cell Involvement

A potential role for B cells in the pathogenesis of MS was initially suggested by the discovery of IgM and IgG antibodies in the CSF of around 40% and 95% of MS patients, respectively (24, 41). Intrathecal IgM and IgG, which are collectively referred to as oligoclonal bands (OCBs), are considered a diagnostic hallmark of MS due to their association with disease activity and persistence throughout the entire course of disease. A study comparing the CSF immunoglobulin (Ig) proteome and the Ig transcriptome of B cells within the CNS showed a strong overlap, demonstrating that ASCs generated from clonally expanded B cells within the CSF are the major source of intrathecal OCBs (42–44). Consequently, B cells were thought to contribute to MS primarily via the production of autoreactive antibodies targeting CNS antigens. In support of this, IgM antibodies targeting myelin lipids have been identified in MS patients and the presence of these antibodies is associated with a more aggressive disease course (45). Moreover, there was evidence of substantial IgG and complement deposition, as well as the presence of macrophages containing myelin-bound antibodies in patients exhibiting the most common demyelination pattern, pattern II, which is present in 60% of MS patients (46, 47).

Surprisingly, in stark contrast to classically antibody-mediated autoimmune diseases such as myasthenia gravis or Goodpasture's syndrome, identification of a disease-specific antigenic target remains elusive, and accumulating evidence supports reactivity toward a variety of self-antigens, from ubiquitously expressed intracellular proteins to neurofilament proteins (24, 48–50). However, antibodies targeting viruses have also been observed, such as the MRZ pattern, which consists of antibodies targeting the measles, rubella, and zoster viruses (51). Moreover, evidence suggests that these reactivities may be unique for different patients (52). The contribution of autoantibodies was further challenged by the finding that plasmapheresis was primarily beneficial in patients exhibiting pattern II demyelination (53).

BCDTs: Ushering in a New Age

A phase 2 clinical trial testing the efficacy of the B cell-depleting mAb RTX as a treatment for RRMS showed that RTX was able to suppress inflammatory disease activity and reduce relapse rates (54). The striking results vindicated the previously overlooked pathogenic relevance of B cells and in doing so challenged our understanding of the mechanisms involved in MS pathogenesis and ushered in a new wave of therapeutics specifically targeting B cells. Following RTX, three subsequent anti-CD20 mAbs, each slightly varying in structure and specificity, have been developed in an effort to optimize safety and therapeutic efficacy: ocrelizumab (OCR), ofatumumab (OFT), and ublituximab (UTX). Both OCR and OFT have been approved and UTX is currently undergoing phase 3 trials (ClinicalTrials.gov number, NCT03277248) (55).

However, the benefit of BCDTs went beyond their obvious clinical efficacy. By studying the compositional changes in the CSF and periphery associated with successful clinical outcomes to BCDTs, our understanding of the dynamic involvement of B cells in MS has greatly advanced. One of the most impactful observations contributing to this advancement was that the positive clinical responses elicited by BCDTs took place without alterations in intrathecal OCBs. While this could have been anticipated due to the lack of CD20 expression on mature PCs, it indicated that B cells primarily exert their pathogenic function not by autoantibody secretion, but rather by antibody-independent mechanisms such as antigen presentation and pro-inflammatory cytokine secretion (56).

Memory B cells can function as potent APCs and therefore may contribute to the reactivation of CNS-reactive $CD4^+$ T cells due to their superior ability to capture and present antigens present at very low concentrations compared with dendritic cells (24, 57–63). In strong support of this, memory B cells are not only increased in MS patients but also display elevated surface levels of MHCII and the costimulatory molecules CD80 and CD86 (64–66). Furthermore, these cells secrete proinflammatory cytokines such as IL-6, GM-CSF, and TNFα upon restimulation (67–71). Although B cells and even some subsets of ASCs such as IgA^+ PCs are capable of secreting anti-inflammatory cytokines such as IL-10, TGF-β, and IL-35, MS patients are abnormally deficient in these regulatory-type B cell subsets, which further amplifies the effects of the aforementioned proinflammatory cytokines (68, 72–77). Subsequent studies investigating the cell populations predominantly affected by BCDTs as well as previously existing DMTs also point to memory B cells as a major pathogenic B cell subset in MS (56).

Importantly, the discovery of bidirectional exchange of B cell clones between the CNS compartment and the periphery gave strong credence to the possibility that in MS patients, memory B cells contribute to MS pathology by acting as the APCs that reactivate encephalitogenic $CD4^+$ T cells and subsequently

produce proinflammatory cytokines that further contribute to inflammation and damage within the CNS (43, 78).

The remarkable success of BCDTs in treating MS is blunted however by heterogeneous clinical outcomes, and more-so by the inability of these treatments to halt advancement of disease progression (4). Even treatment with OCR and OFT, which have been approved for the treatment of PPMS and active SPMS, only slow rather than halt progression. However, the inability of these antibodies to cross the BBB may provide a possible clue to their failure in arresting disease progression (56).

OCBs: Pathognomonic Yet Poorly Understood

Among the changes in our conceptual understanding of MS pathogenesis, it is now acknowledged that MS involves both peripheral as well as compartmentalized inflammatory processes in the CNS. While our understanding of the mechanisms leading to and sustaining compartmentalized inflammation remains largely incomplete this process is thought to be driven by tissue resident populations (12, 79–81).

OCBs are thought to be produced by ASCs derived from the local antigen-driven reactivation of memory B cells within the CNS, indicated by mutations highly concentrated within the CDR3 regions (82). This finding has been corroborated by other studies (83, 84). This evidence of a CSF-restricted humoral response demonstrates that B cells participate in and potentially contribute to compartmentalized inflammation seen during later disease stages (85).

Importantly, the discovery of B cell-rich follicles in the meninges of up to 40% of SPMS patients pointed to the possibility that these structures might be involved in the reactivation of encephalitogenic $CD4^+$ T cells (12). Although initially not considered as a pathognomonic feature of MS, these aggregates correlate strongly with cortical pathology and disease severity in PPMS and SPMS patients. Moreover, the continual antigen-driven expansion of B cells in MS patients strongly implicated eLFs as a prospective driver of MS progression and warrants their investigation. Therefore, given their resemblance to eLFs seen in other chronic inflammatory conditions, these structures might offer a potential explanation as to the source of continual OCB production seen in MS.

ECTOPIC LYMPHOID FOLLICLES IN MULTIPLE SCLEROSIS: CENTERS FOR DISEASE CONTROL

The development of structures analogous to SLOs has been reported in peripheral tissues at sites of chronic inflammation, serving as a reservoir for autoreactive B and T cell reactivation. These structures are known by a variety of monikers, such as tertiary lymphoid organs (TLOs), ectopic lymphoid structures (ELS), and tertiary lymphoid tissues (TLTs), but will be referred to as eLFs in this review (86, 87).

eLFs support the continuous antigen-driven expansion of B cells in sites of chronic inflammation and are therefore a common feature of several B cell-mediated autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and Sjögren's syndrome (24). eLFs have been demonstrated in the meninges of approximately 40% of SPMS patients (10, 80, 88). Moreover, recent evidence suggested that these aggregates are not restricted to late disease stages but rather are also present in early stages of MS (12, 89). Indeed, meningeal inflammation strongly correlates with subpial cortical injury in nearly all disease stages.

While eLFs share structural and functional similarities with SLOs, the mechanisms underlying their initiation and establishment as well as the cellular players involved and required are quite different. Moreover, due to the specialized nature of the CNS, meningeal eLFs warrant special considerations that set them apart from eLFs in other disease settings. Molecular and cellular traffic to and from the CNS is stringently regulated by the blood-CSF and BBB, barriers that inadvertently provide a significant level of protection for eLFs established within this restricted tissue (90).

Here, we will detail (i) the similarities and differences regarding the establishment and maintenance of SLOs and eLFs, (ii) the unique nature of the CNS as a site of chronic inflammation and eLF formation, and (iii) current evidence supporting the potential role for eLFs in driving MS disease progression.

SLO vs. eLF Establishment

SLOs are ideally suited entities for facilitating immune surveillance and the adaptive immune responses, namely the GC reaction. As a result of their importance in mediating such a nuanced and vital process, the development and location of these tissues is genetically preprogrammed. Broadly, SLO formation involves three main phases: the establishment of chemotactic gradients to facilitate B cell and T cell homing and clustering, stimulation of tissue remodeling and angiogenesis, and the formation of a stromal reticular network.

Lymphoid organogenesis is catalyzed by the interaction of lymphoid tissue-inducer (LTi) cells with lymphoid tissue-organizer (LTo) cells via the binding of lymphotoxin (LT)$\alpha_1\beta_2$ to the LTβ receptor (89). This stimulates LTo cells to produce the chemokines CCL19, CCL21, CXCL13, and CXCL12, as well as growth factors such as VEGF-C and FGF2. The resulting chemotactic gradient facilitates immune cell homing and compartmentalization, while the growth factors stimulate the development of lymphatic vessels and HEVs, allowing B and T cell ingress. Importantly, the chemokines secreted by LTo cells also continue to recruit LTi cells, forming a positive feedback loop important for the maintenance of this process. LTo cells also begin to express intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 in order to aid in immune cell retention upon entry. Finally, LTo cells will differentiate into FDCs, fibroblastic reticular cells (FRCs), and marginal reticular cells, which comprise the stromal reticular network (89, 91, 92).

The formation of eLFs follows the same basic developmental steps as the formation of SLOs, however the first key distinction is that eLF formation is triggered in response to inflammation and thus can occur in a variety of non-lymphoid tissues and the resulting structures are not encapsulated (90). In this

context, immune cells have the capacity to function in a manner analogous to LTi cells. For example, in the context of pulmonary inflammation, the development of inducible bronchus-associated lymphoid tissue (iBALT) was dependent on T_H17-derived IL-17 (90). B cells have also demonstrated this LTi-like ability in a model of colitis, but in a $LT\alpha_1\beta_2$-dependent manner (93). In a similar fashion, the role of LTo cells is taken on by stromal organizer cells such as fibroblasts and endothelial cells that are activated by the inflammatory milieu. In addition to providing the aforementioned homeostatic chemokines, these activated stromal cells can also produce survival factors such as B cell-activating factor (BAFF) and cytokines capable of influencing the T cell response, such as IL-6 which promotes T_H17 responses (93).

Partially due to their formation being initiated by inflammation, eLFs can form and dissipate quickly. As a direct consequence of this transient nature, eLFs can display significant organizational and cellular heterogeneity ranging from small and disorganized aggregates of B and T cells to highly organized structures containing compartmentalized T and B cell zones, HEVs, FDCs, and a developed stromal reticular network (10, 11, 80, 90, 91). It is important to note, however, that once the reticular network has formed and an eLF has reached an advanced state of maturation, eLFs become fairly stable and are less likely to dissipate (94). The dependency of eLFs on the inflammatory context is apparent in conditions such as RA, where inflammation in articular joints is chronic and promotes self-sustaining eLFs. Additionally, in diseases such as RA and myasthenia gravis, the presence of disease-specific autoantigens enables the long-term persistence of eLFs. Thus, the extent of organization of an eLF is a consequence of the extent and persistence of inflammation (87, 94, 95). Furthermore, in MS mature meningeal eLFs are exclusively found in SPMS patients as compared with PPMS and RRMS patients (10, 11, 80).

Importantly, smaller and less developed eLFs are still able to support typical GC-related B cell processes such as affinity maturation, proliferation, and differentiation (90). This might be a result of the inflammatory microenvironment, as well as of the tendency of eLFs to be comprised primarily of memory B and T cell populations, which differ from their naïve counterparts in regard to signaling requirements. Moreover, GC-related processes in eLFs can occur independently of T_{FH} cells, and are instead facilitated by a T_{FH}-like population known as peripheral T helper cells, which lack the canonical T_{FH} cell markers CXCR5 and BCL6 (96).

Nevertheless, it must be noted that inflammation is not the sole prerequisite for eLF formation. Rather, the permissiveness of a tissue to the influx and aggregation of lymphocytes is an equally important consideration during this process (97, 98). This quality is particularly apparent in the context of MS, as the CNS is unique in its structural and circulatory properties, both of which can dramatically change in the context of inflammation.

Immune Cell Access to the CNS: Keys to the Kingdom

The CNS is a vital system and the regulation of cellular and molecular influx and efflux is accordingly more complex than in most other tissues, a characteristic reflected in the structures within and the barriers surrounding it, e.g., the BBB. The CNS parenchyma is enveloped by the meninges, a structure consisting of the dura mater, the arachnoid mater, and the pia mater. The dura contains fenestrated blood vessels as well as lymphatic vessels, both of which facilitate trafficking of lymphocytes between the CNS and the deep cervical lymph nodes (dCLN). The two innermost layers, the arachnoid mater and the pia mater, are collectively known as the leptomeninges and are separated by the subarachnoid space, a cavity filled with CSF (99).

Produced by the CP, CSF plays an important role in remote immune surveillance of the CNS due to its role in the glymphatic system in which the interstitial fluid, which contains molecules drained from the parenchyma, is taken up by the CSF and flows via the lymphatic vessels into the dCLN. This is thought to be important for tolerance, as it facilitates the presentation of parenchymal self-antigens in the absence of inflammation (100). Additionally, it also provides a medium by which lymphocytes circulate within and surveil the subarachnoid space.

Lymphocyte entry to the CNS is regulated by two specific barriers: the BBB and the blood-cerebrospinal fluid barrier (BCSFB). The BBB, which separates the leptomeningeal and deep parenchymal capillaries from the perivascular subarachnoid and Virchow-Robin (VR) spaces, is made up of endothelial cells connected by tight junctions. In the parenchymal capillaries, cell infiltration of the parenchyma is further restricted collectively by the pia mater, the glia limitans, which is a thin barrier comprised of astrocytic endfeet, and the parenchymal basal lamina.

The BCSFB regulates entry to the CSF-filled ventricles from the capillaries embedded within the CP stroma. In contrast to the BBB, this barrier is comprised of the fenestrated endothelium of the choroidal capillaries, and the ependymal cells, which are connected by tight junctions.

The BBB and BCSFB restricts lymphocyte access through the dynamic expression of specific adhesion molecules such as VCAM-1 and ICAM-1. In steady-state conditions, these two barriers allow minimal lymphocyte entry. In response to inflammatory signals, these barriers can become more permeable, increasing infiltration by lymphocytes (89). Furthermore, the leakiness of these barriers increases efflux of molecules such as chemokines and cytokines, resulting in further recruitment of potentially proinflammatory immune cells (101). Collectively, these barriers stringently regulate entry and egress of cells as well as macromolecules such as antibodies.

Evidence of eLFs in MS

In 1979, Prineas (9) observed what they described as "reticular-like cells embedded within lymphoid-like structures and lymphatic capillaries within old plaques" in the CNS of MS patients. Subsequently it was shown that lymphocytic aggregates, found in the meninges of SPMS patients, appeared proximal to subpial lesions and correlated with disease severity and progression (10, 80, 88). Following the seminal findings by Prineas, Magliozzi, and Serafini, Lucchinetti et al. showed that perivascular T and B cell infiltrates could also be detected in acute and RRMS patients proximal to cortical plaques; however

limited tissue availability prevented probing for other cell types characteristic of eLFs, such as FDCs (12, 89).

Interestingly, eLFs found in MS patients resemble those described in other chronic inflammatory autoimmune diseases such as RA, SLE, and Sjögren's syndrome. Moreover, the CSF of MS patients during disease relapses contains elevated levels of LTα and CXCL13, both of which are critical for lymphoid organogenesis, and the latter of which also correlates with the levels of intrathecal Ig and the frequency of B cells and PBs in the CSF. Furthermore, these follicles have also been reported to contain CXCL13, FRCs, and FDC-like $CD35^+$ cells as well as HEVs (10, 90, 102).

The presence of B cell clusters surrounded by T cells makes meningeal eLFs ideal environments to facilitate GC reactions. Indeed, high-throughput Ig repertoire analyses of B cell clones from paired CNS and SLOs showed that antigen-driven affinity maturation can occur within the CNS (103). This view is further supported by the expression of activation-induced cytidine deaminase (AID), a required transcription factor for affinity maturation in the GC, in B cells from the CSF (104). Proliferating $Ki67^+$ centroblasts have also been observed in the CSF but not the peripheral blood of MS patients, further indicating a compartmentalized GC reaction (105). Additionally, Ig repertoire analyses show a higher degree of somatic hypermutation, specifically in the CDR3 region, in CSF-derived IgM and IgG compared with those from peripheral blood, indicating antigen-driven affinity maturation within the CNS of MS patients (78, 82). The cytokine milieu in eLFs specifically supports these processes and the survival of B cells. The high concentration of BAFF, a potent B cell survival factor, is particularly notable, due to its ability to rescue self-reactive B cells from deletion (106). In RA, the abundance of survival factors such as BAFF has been attributed to the resistance of eLFs to BCDTs such as RTX (107). It is important to note that meningeal inflammation observed in early stage MS has been associated with pronounced subpial cortical pathology and is associated with more aggressive disease course (80). In light of these findings, and further supported by the correlation between meningeal inflammation and cortical pathology throughout all stages of MS, it is plausible that meningeal eLFs could serve as a supportive niche for the reactivation and persistence of autoreactive $CD4^+$ T cells and memory B cells, thereby representing an insidious mechanism driving disease progression (24).

Catch Me If You Can: Hurdles in Studying eLFs in MS

Despite the evidence detailed above, the ability to concretely demonstrate the relationship between eLFs and progression is mired by three critical limitations.

In MS, most observations are derived from analyzing post-mortem tissue samples, which are understandably limited in their availability. Importantly, these samples are typically obtained at later stages of disease when inflammation is possibly less pronounced (89). Therefore, while heterogeneous observations between patients regarding cellular composition and structural organization can partly be explained by the transient nature of eLFs, it is more likely a consequence of differences in disease stage and varying degrees of residual CNS inflammation between patients (91).

Conceivably, EAE studies may provide a viable alternative model to study eLFs. Indeed, EAE models have provided critical insights into the immunological mechanisms involved in MS and all therapeutics (such as natalizumab) have been developed as a direct result of EAE studies (108). An additional advantage is that the disease manifestations, including the involvement of specific cell types, can be adjusted based on the immunogen as well as the strain of mice. But despite their proven merit, current EAE models remain incomplete models of MS (90).

In regard to studying eLFs in the CNS, only few EAE models are able to form eLFs similar to those observed in humans (90). Even so, these models exhibit substantial variability, both between models and within the same model. The kinetics of eLF formation and maturation is a major factor in this, since the relatively short disease courses used may not provide enough time for eLF maturation.

Another limitation involves inter-species differences. One of the cell types strongly associated with eLF formation and subsequent GC-like responses is the T_{FH} subset. In humans, T_{FH} cells are substantial producers of CXCL13, a cytokine that facilitates eLF formation and maintenance as well as recruitment of CXCR5-expressing B cells. Murine T_{FH} cells, however, do not produce CXCL13 and instead parenchymal and stromal cells are the primary producers of this cytokine (109, 110). While interspecies differences like these are not uncommon by any means, a recent study might impart physiological relevance to this discrepancy: using a model of EAE in which MOG-specific T_H17 cells are adoptively transferred to naïve mice, a model known to yield a high frequency of eLFs correlating to disease severity, Quinn et al. (111) showed that T_{FH} cells induced eLF formation in a manner that required the CXCL13-mediated homing of circulating memory T_{FH} cells (90). In line with these findings, the use of a blocking antibody to target CXCL13 could theoretically prevent eLF formation in humans. However, this species-specific functional difference calls the translational nature of the proposed axis into question.

Despite these limitations, clinical evidence still provides a strong argument for the involvement of eLFs in driving progression. Furthermore, the findings derived from EAE studies still absolutely merit consideration and could still provide critical insight into this potential link.

IF THE SHOE FITS: EVIDENCE SUPPORTING A ROLE FOR eLFs IN MS

The persistent interrogation of the composition of treated and untreated patient blood, serum, and CSF continues to reveal new biomarkers and implicate new B and T cell subsets and functions contributing to disease severity. These insights have consequently necessitated an evolving, flexible view of the mechanisms underlying MS pathogenesis and progression. Along these lines, a plethora of cytokines and cell types upregulated in MS patients strongly implicate eLFs as drivers of disease progression.

T_H17 Cells: Jack of All Trades

The functions and phenotypes of $CD4^+$ T helper subsets have canonically been viewed simplistically, with each subset associated with a handful of signature cytokines, chemokine receptors, and typically a single transcription factor. However, $CD4^+$ T cells are now known to display a remarkable degree of plasticity and versatility, qualities exemplified by T_H17 cells.

T_H17 cells are thought to be the primary T helper subset driving MS pathogenesis. Initially described in EAE models, this hypothesis is also supported in humans as T_H17 are elevated in the CSF of MS patients, specifically during relapse (91). Several T_H17-associated cytokines are associated with MS pathology. One study showed an increase in IL-22, a cytokine which coincidentally shares with IL-17 the ability to promote BBB breakdown, in the serum of patients experiencing relapse (112). Moreover, IL-6 and IL-23, both of which are required for T_H17 maturation and maintenance, are also overrepresented in the CSF of MS patients (113, 114).

As detailed above, infiltration of the CNS is tightly regulated and varies depending on both the point of entry as well as on the inflammatory context. CCR6, a chemokine receptor that is required to cross the blood-CSF barrier in the choroid plexus, is highly expressed by T_H17 cells (115, 116). Additionally, CCL20, the ligand of CCR6, was recently found to be upregulated in the CSF of MS patients (117). Taken together, the strong association of numerous cytokines and chemokines specifically related to the T_H17 subset makes the CNS of MS patients an auspicious locale for the function and persistence encephalitogenic T_H17 cells.

In addition to the more overt pathogenic contributions of this subset, several recent findings have suggested that T_H17 cells might play a more inconspicuous role, namely in orchestrating GC-like responses and inducing the formation of meningeal eLFs in MS.

T_H17 cells are known to secrete large amounts of IL-21, a cytokine typically secreted by T_{FH} cells (118, 119). T_{FH} cells, which are known to be upregulated in MS patients, are a specialized subset required for directing B cell responses within the GC reaction, such as proliferation, CSR, and differentiation into memory B cells and ASCs. Since IL-21 is primarily associated with T_{FH} cells, it would stand to reason that T_H17 cells may have the capacity to function in a T_{FH}-like capacity. Indeed, a study by Mitsdoerffer et al. (120) which showed that, upon adoptive transfer to T cell-deficient mice, T_H17 cells were able to initiate GCs, promote isotype switching, and induce a pronounced antibody response, confirmed this theory. Further establishing the B-helper capacity of T_H17 cells, a recent study showed that IL-17, when combined with BAFF, a cytokine also upregulated in MS patients, promoted B cell survival, proliferation, and differentiation into PCs, providing a second method by which T_H17 cells can promote GC-like B cell responses within the CNS (120).

The ability to induce eLF formation has been shown in other contexts such as iBALT formation and occurred in an IL-17-dependent manner (97). Likewise, this capacity was also demonstrated in an EAE study that showed that adoptive transfer of MOG-reactive T_H17 cells induced the formation of eLFs within the CNS through stimulating the production of CXCL13 by stromal cells (121). In a model of spontaneous arthritis, T_H17-derived IL-17 was also shown to be critical for the development of autoreactive GCs (122). A separate study also showed the ability of IL-17 to induce meningeal fibroblast remodeling *in vivo* and *in vitro* (123). In mucosal tissues, IL-22 was also shown to induce eLF formation by stimulating the production of homeostatic chemokines by stromal cells (124, 125). This introduces the possibility that the elevated levels of CXCL13 in the CSF of MS patients may be due to T_H17-derived IL-17 and IL-22.

B Cells: Bad Memories

Several chemokines and cytokines that are upregulated in the CSF of MS patients are known to be important for facilitating B cell migration, activation, differentiation, and survival. The inflamed CNS of MS patients therefore seems to provide a microenvironment that is particularly conducive for facilitating B cell-mediated responses.

Memory B cells, specifically a subset known as IgD^-CD27^- "double-negative" (DN) memory B cells, are abnormally overrepresented in the peripheral blood and CSF of MS patients. This subset of memory B cells has also been associated with SLE and RA and is associated with disease severity (83). The pathogenic relevance of memory B cells in MS has been substantially corroborated by studies exploring the immunological aspects of previously established non-B-cell-targeting DMTs. Indeed, depletion of memory B cells was associated with the efficacy of IFN-β, glatiramer acetate (GA), and dimethyl fumarate (DMF) (126). Furthermore, the clinical success of natalizumab has also been associated with its ability to reduce the frequency of memory B cells in the CNS.

As previously stated, memory B cells from MS patients secrete abnormally high quantities of IL-6, TNFα, and GM-CSF (67). This particular milieu promotes inflammation by increasing the permeability of the CNS vasculature, stimulating the production of IL-6 and IL-12 by myeloid cells, and the maintenance of pathogenic T_H17 effector responses. Of note, B cell-derived IL-6 has also been shown in a model of SLE to be required for the formation of spontaneous eLFs (127). A recent study also demonstrated that B cells from the CSF of RRMS and progressive MS patients secrete large amounts of VEGF and LTα, respectively. Importantly, both of these growth factors promote the development of eLFs by stimulating lymphangiogenesis (128). Additionally, the enhanced production of neurotoxic factors by B cells from MS patients could offer further explanation for the strong association between meningeal inflammation and subpial cortical damage.

DN memory B cells have enhanced APC functions, indicated by elevated levels of MHCII along with the costimulatory molecules CD80, CD86, and CD40 (129). This was demonstrated by a study which found that B cells from MS patients were able to activate T cells in the presence of neuroantigens, unlike B cells from healthy controls. Memory B cells within the CNS of MS patients therefore have an enhanced capacity to serve as potent APCs to encephalitogenic memory $CD4^+$ T cells and strongly contribute to the proinflammatory milieu within the CNS. The reactivation of encephalitogenic $CD4^+$ T cells will result in the reciprocal reactivation of the presenting memory B cell. It is

important to note that while reactivation of memory B cells is thought primarily to result in ASC generation, these cells are also fully able to undergo further affinity maturation in secondary GC-like reactions.

In addition to their proinflammatory functions within the CNS, memory B cells from MS patients also seem to have enhanced brain-infiltrating potential. In line with the observations from natalizumab treatment, B cells from MS patients express high levels of very late antigen-4 (VLA-4) (130). These cells also express ICAM-1 and activated leukocyte cell adhesion molecule (ALCAM), both of which facilitate migration across the BBB and BCSFB (131, 132). Interestingly, the absence of B cells within the parenchyma is supported by the enhanced expression of molecules that preferentially facilitate migration through meningeal vasculature.

In summary, memory B cells in MS patients display several phenotypic and functional traits that support not only an enhanced ability to migrate to, stimulate, and perpetuate inflammatory responses within the meningeal spaces, but also to promote the formation of eLFs.

T_{FH} Cells: Hurting More Than Helping?

T_{FH} cells are broadly identified as $CXCR5^+PD\text{-}1^+BCL6^+ICOS^+$ and their effector function is primarily associated with the secretion of IL-21 (91). This subset is most strongly linked to GC B cell responses and has only recently been included as a disease-relevant subset in MS and other disease conditions (133, 134). T_{FH} cells have been associated with RA, SLE, and Sjögren's syndrome in a manner dependent on their ability to support GC B cell responses (135). T_{FH} and B cells provide critical signals to each other including signals important for development, effector function, and survival. Important T_{FH}-derived signals include IL-21, which stimulates CSR, CD40L, which delivers costimulatory signals via CD40, and inducible T cell costimulator (ICOS). Secretion of BAFF, a potent B cell survival factor, by mouse T_{FH} cells was reported; however, BAFF secretion has so far not been reported by human T_{FH} cells (136). Additionally, a link between T_{FH} cells and AID expression in GC B cells has been suggested.

GWAS studies have shown that polymorphisms in IL-21, CXCR5, and PD-1 are genetic risk factors for MS. Moreover, about 20% of the $CD4^+$ T cells in the CSF of MS patients express CXCR5, and active lesions have been shown to contain $IL\text{-}21^+$ as well as $CD40L^+$ $CD4^+$ T cells (102, 137). Additionally, IL-21, BAFF, and CXCL13 are all abnormally elevated in MS patients (89, 138). However, these observations only supported the involvement of T_{FH} cells indirectly, due to the fact that many of these markers are also linked to other cell populations that are known to play a role. For example, IL-21 is known to be secreted by T_H17 cells, a significant and well-known driver of MS (139).

The link between T_{FH} cells and MS was solidified in a 2013 study by Christensen et al. (140) reporting an increase in T_{FH} cells in RRMS and SPMS as well as a correlation with progression. Importantly, the same study showed that the T_{FH} cells were $ICOS^+$ and correlated with the frequency of PBs. A subsequent study also reported the elevation of T_{FH} cells in the blood of MS patients as well as a positive correlation with disease severity (133). In further support of the importance of T_{FH} cells in MS, several studies showed that circulating T_{FH} cells are among the cells that are most prominently affected by DMTs, including fingolimod, and abatacept (141, 142). These studies indicate that a decrease in circulating T_{FH} cells is a prominent feature accompanying positive clinical responses.

In light of this cumulative body of evidence, it is plausible that T_{FH} cells in MS patients play a substantial role in eLF formation. It is particularly intriguing that T_{FH} cells share many features with T_H17 cells, including the secretion of IL-21 and the ability to support GC responses. In fact, similar to studies showing the ability of T_H17 cells to become T_{FH} -like T_H17 cells, T_{FH} cells can become T_H17-like T_{FH} cells, which express the transcription factor RORγt, the chemokine receptor CCR6, and secrete IL-17 as well as IL-21 (143). Notably, T_H17-like T_{FH} cells display a formidable ability to induce antibody production (143). In MS patients, DMF treatment was shown to decrease the frequency of T_H17-like T_{FH} cells and increase that of T_H2-like T_{FH} cells, giving credence to the relevance of this unique subset (144). This reinforces evidence that T_H17-like T_{FH} cells were increased in PPMS patients (140).

Importantly, the potential humoral dysregulation resulting from an overrepresentation of T_{FH} cells may be further amplified in MS patients due to a decrease in T_{FR} cells, the regulatory counterpart of T_{FH} cells, reported in a recent study (145). Similar to what has been observed regarding T_{regs}, T_{FR} cells in MS patients also exhibit reduced suppressive capacity, indicated by abnormal IgG production in the blood, and CSF (146). Notably, a recent study reported that eLFs found in SPMS patients are devoid of T_{FR} cells, despite detection in the CSF. The lack of T_{FR} was also shown in the less-defined eLF aggregates from PPMS patients (147).

Taken together, these observations strongly support a potential contribution of T_{FH} cells to the progression of MS, namely via stimulating eLF formation, orchestrating GC-like responses in the CNS, and providing signals that support multiple inflammatory populations in the CNS. Of particular interest is the ability to adopt T_H17-like effector functions, for this consequently amplifies the encephalitogenic potential of T_{FH} cells. The complex and multifaceted involvement of T_{FH} cells may represent the next paradigm shift in the journey toward understanding the pathogenesis and progression of MS.

THE (UN)USUAL SUSPECTS: UNIQUE INFLAMMATORY CELL SUBSETS AND THEIR POTENTIAL CONTRIBUTION TO eLF FORMATION

Studies attempting to elucidate the mechanisms underlying MS have led to a single unanimous conclusion: it's complicated. MS pathogenesis is a complex process involving a vast array of cell types, all of which vary in their phenotype and function, and thus their pathogenic contribution, depending on the stage of disease. However, the continuous advances in multiparametric analyses have enabled us to more closely interrogate the characteristics of specific immune cell subsets associated with MS. This ability has revealed two subsets of particular relevance to progression,

potentially via their contribution to eLF formation: T_H1-like T_H17 cells and T-bet$^+$ memory B cells. In this section, we will describe the distinguishing features of these subsets and the evidence supporting their involvement in MS pathogenesis and progression, with a particular focus on their role in eLF formation.

$T_H17.1$ Cells: Potently Detrimental

Mechanisms of T cell plasticity are still enigmatic, and attempts to define novel T helper subsets, though regularly proposed, often lack sufficient evidence to merit their inclusion in the established lineup. However, numerous studies have not only confirmed the existence of T_H1-like T_H17 cells, but also established their functional relevance in both pathogenesis and progression of MS.

T helper subsets are conventionally characterized by the expression of a signature transcription factor, cytokine, and chemokine receptor. As mentioned above, T_H17 and T_H1 cells are considered the two primary encephalitogenic T helper subsets in MS and are identified as RORγt$^+$IL-17$^+$CCR6$^+$ and T-bet$^+$IFN-γ^+CXCR3$^+$, respectively.

T_H1-like T_H17 cells are a recently described subset of T_H17 cells that, as the name suggests, express both T_H1- and T_H17-associated signature molecules and are identified as T-bet$^+$RORγt$^+$IFN-γ^+IL-17$^+$CXCR3$^+$CCR6$^+$. T_H1-like T_H17 cells have been identified in MS lesions and are selectively expanded in RRMS patients with more severe disease (148).

Recently, a variant of this subset, distinguished primarily by the additional expression of GM-CSF, was identified. Termed $T_H17.1$ cells, this subset is associated specifically with early disease activity and correlated with the transition from clinically isolated syndrome (CIS) to clinically definite MS (CDMS). This observation is in line with the known role of GM-CSF as a critical proinflammatory mediator early in disease.

It is important to note that, although all three cytokines are expressed, $T_H17.1$ cells express a relatively lower amount of IL-17. Therefore, the authors posit that IFN-γ and GM-CSF are considered the major proinflammatory cytokines responsible for association with the transition from CIS to CDMS. Interestingly, $T_H17.1$ cells isolated from the CSF of relapsing patients express IL-17 to a degree similar to IFN-γ, while GM-CSF expression is decreased, suggesting that IL-17 is more important for progression than for onset. This shift in cytokine secretion may be attributable to the T_H17-promoting milieu of the inflamed CNS increasing the production of IL-17, the regulation of which is antagonistic to that of GM-CSF in humans (149). Alternatively, this could be a result of IL-21 signaling, which induces the downregulation of T-bet and GM-CSF (149, 150).

In addition to the secretion of proinflammatory cytokines known to be elevated in MS patients, the encephalitogenicity of $T_H17.1$ cells is also attributed to the enhanced brain-homing potential imparted by the simultaneous expression of CXCR3, CCR6, and VLA-4, all of which are important for trafficking to the inflamed CNS. In support of this, CXCL10, CCL20, and VCAM-1, the corresponding ligands, are highly upregulated in the CSF of MS patients during inflammation. Interestingly, $T_H17.1$ cells have also been shown to acquire CCR2 expression as disease progresses. The expression of this additional receptor further amplifies this subset's ability to infiltrate the CNS. In further support of their clinical relevance, a recent study investigating the effects of DMF on immune cell populations found that $T_H17.1$ cells are indeed downregulated as a result of treatment (144). Additionally, $T_H17.1$ cells were shown to be markedly accumulated in the blood of patients who clinically responded to natalizumab treatment, further implicating their pathogenicity (151).

ABC's and OCB's: T-Bet$^+$ DN Memory B Cells and Their Potential Role in eLFs

The evidence that OCBs target ubiquitous intracellular self-antigens in a patient-specific manner would suggest that these likely originate in response to dead cell debris (49). Therefore, since OCBs are considered to be derived from the reactivation of DN memory B cells, this begs the question: where did these autoreactive memory B cells come from? Autoreactive B cells are present in the periphery of healthy individuals, but their aberrant activation and subsequent differentiation into memory B cells or autoantibody secreting PBs and PCs is prevented through several peripheral tolerance mechanisms within the GC. Therefore, the existence of memory B cells with such reactivity indicates a breach of these GC-related peripheral tolerance mechanisms (147).

As detailed above, DN memory B cells are considered a major pathogenic cell type in MS. However, similar to other immune cell types, more in-depth characterization of memory B cells has revealed several functionally distinct subsets, including recently described "atypical" memory B cell subsets. Atypical memory cells expressing CD11c and T-bet are associated with autoimmune diseases including SLE (152). Although initially described as age-associated B cells (ABCs), these cells are present in both healthy donors as well as aged mice and humans (153, 154). Functionally, these T-bet$^+$ memory B cells are excellent APCs (155).

T-bet$^+$ memory B cells are thought to be generated in a manner similar to extrafollicular responses (156). The expression of T-bet in B cells is induced by IFN-γ stimulation. The inflamed CNS of MS patients provides a microenvironment that supports the differentiation and persistence of these cells, due to the abundance of IFN-γ. Additionally, the reactivity of OCBs toward antigens derived from dead cell-debris provides evidence that the inflamed CNS also contains molecules that can stimulate Toll-like receptor 9 (TLR9), the second signal required for T-bet$^+$ memory B cell development. Indeed, in all categories of MS, T-bet$^+$ memory B cells, are elevated. Importantly, T-bet$^+$ memory B cells display the same proinflammatory attributes that have been described for memory B cells in MS. In addition, T-bet$^+$ memory B cells also express high amounts of CD20 and is therefore a major target of BCDTs. T-bet expression is also strongly associated with IgG1 and IgG3 class-switching, which are isotypes that are associated with MS (157).

The expression of CXCR3 in addition to CXCR5 enhances the brain-homing potential of these cells, enabling migration toward CXCL10 and CXCL13, both of which are elevated in the CSF of MS patients. Additionally, *in vitro* studies have demonstrated an

enhanced ability of T-bet$^+$ memory B cells to migrate through human brain endothelial layers. Similar to what was found regarding $T_H17.1$ cells, T-bet$^+$ memory B cells also accumulated in the blood of natalizumab-treated patients.

Although the enhanced antigen-presenting capabilities and proinflammatory characteristics of memory B cells from MS patients have been well-established, these new findings provide further evidence supporting the likelihood that T-bet$^+$ memory B cells reactivate encephalitogenic CD4$^+$ T cells in the brain. Importantly, a recent study showed that DN memory B cells from MS patients express ICOSL at levels only slightly lower that of mature naïve B cells (158). This enables direct interaction with T_{FH} cells within the CNS, which, in concert with the milieu of the inflamed CNS may promote meningeal eLF formation and propagate GC-like responses therein.

FUTURE PERSPECTIVES: POTENTIAL NOVEL APPROACHES TO TARGETING eLFs IN MS

Despite the vast progress made regarding our understanding of MS, the ability to halt disease progression remains an elusive and enigmatic target. Several currently approved DMTs target B cells and may affect development of eLFs in MS; however, effects on eLFs may be limited by their access to the CNS. Nevertheless, the collaboration between T_{FH} cells and memory B cells, which underlies eLF formation, offers attractive therapeutic targets, especially in light of evidence implicating eLFs in driving MS progression. In this section, we will describe potential novel approaches to prevent formation of eLFs in MS by targeting B cells and T_{FH} cells.

BTK Inhibitors

The rationale for pursuing therapeutics that selectively target B cells is clear, given the demonstrated efficacy of BCDTs. However, the inability of these mAbs to efficiently cross the BBB and BCSFB poses a significant problem in the treatment of the progressive forms of MS, as these barriers do not exhibit the same degree of permeability as seen in earlier disease stages. As a result, much interest has centered on pursuing compounds that can penetrate the intact BBB and BCSFB in the CNS of progressive MS patients, of which Bruton's tyrosine kinase (BTK) inhibitors have led the charge.

BTK is a tyrosine kinase that is essential for conveying signals necessary for B cell maturation, activation and survival, and BTK inhibitors showed efficacy in treating RRMS. Several different inhibitors are currently being investigated, including evobrutinib and PRN2246.

Evobrutinib has successfully completed phase 2 trials and showed positive results in treatment of RRMS (ClinicalTrials.gov number, NCT02975349) (159). Nevertheless, its degree of CNS penetration has not been assessed yet. However, PRN2246, another BTK inhibitor, can effectively penetrate the CNS and achieve therapeutic levels (160).

A third BTK inhibitor, fenebrutinib (GDC-0853), is very selective and potent as compared with previous inhibitors, and phase 3 clinical trials evaluating its efficacy in RRMS (FENhance 1 and FENhance 2) and PPMS (FENtrepid; ClinicalTrials.gov number, NCT04544449) are currently underway (161).

Targeting T_{FH} Cells via CD28 and ICOS

The relevance of T_{FH} cells to MS has been established in both animal models as well as in human studies. As detailed above, their potential involvement in the progressive phase of MS via contributing to eLF formation makes them an attractive therapeutic target, specifically by exploiting the importance of the costimulatory receptors CD28 and ICOS for T_{FH} development and maintenance as well as for interacting with B cells (162, 163).

Abatacept is a fusion protein composed of the Fc region of human IgG1 and the extracellular domain of CTLA4, which binds CD80 and CD86, the ligands of CD28 as well as CTLA4. Abatacept has been efficacious in the treatment of RA, psoriasis vulgaris, and type 1 diabetes and is thought to act by abrogating autoimmune T cell responses through blocking costimulation through CD28. CD28 signaling is also thought to play a major role in T_{FH} cell development (164). Similar to MS, an increase in circulating T_{FH} cells has been associated with type 1 diabetes (165–167). A recent study showed that abatacept was able to decrease T_{FH} cells in a mouse model of type 1 diabetes, even after the disease is established (134). Furthermore, abatacept reduces circulating T_{FH} cells in RA and in Sjögren's syndrome (168, 169). While ACCLAIM, a phase 2 clinical trial studying the efficacy of abatacept in patients with RRMS, showed no clinical benefit, a subsequent study of samples obtained from patients participating in that trial showed that T_{FH} cells and T_{reg} cells were selectively decreased, the latter of which may be a significant disadvantage of this treatment (142, 170). Abatacept was followed by the development of belatacept, which has a higher affinity for both CD80 and CD86, and MEDI5256, which binds CD80 with greater affinity than CD86, although these have not been studied in the context of MS (171, 172). Interestingly, in a nonhuman primate model of transplantation, crosstalk between T_{FH} cells and B cells was more potently affected by treatment with a CD28 antagonist, FR104, compared to belatacept, suggesting that targeting CD28 directly might be more beneficial (162, 163, 172–175).

The increase in ICOS$^+$ T_{FH} cells in MS is mirrored in several autoimmune diseases, such as SLE, Sjögren's syndrome, and type 1 diabetes (176–179). ICOS is a critical signal for T_{FH} cell development, functions such as IL-21 secretion, and is highly expressed on T_{FH} cells as well as on T_H17 cells, albeit to a lesser extent (180, 181). Importantly, and in contrast to CD28 and CTLA4, ICOS expression is thought to be restricted to T_{FH} cells and antigen-experienced CD4$^+$ memory T cells and is upregulated during reactivation (172, 182). In MS, the increase in IL-21- and ICOS-expressing CD4$^+$ T cells would suggest a potential benefit in targeting ICOS-ICOSL interactions.

Prezalumab, a human mAb that binds ICOSL and blocks its interaction with ICOS, has been tested in SLE and arthritis; however results from a phase 2 clinical trial in patients with Sjögren's syndrome showed no clinical improvement and its development for the treatment of rheumatic diseases has been discontinued (162, 172). In light of this result and considering the restricted expression of ICOS to T_{FH} cells and CD4$^+$

memory T cells, targeting ICOS might prove more effective than targeting ICOSL.

MEDI-570 is a mAb that binds ICOS, blocking its interaction with ICOSL. Additionally, MEDI-570 is afucosylated, a modification in the Fc region that enhances antibody-dependent cellular cytotoxicity by NK cells and macrophages (172, 183). In the context of autoimmunity, this mAb has only been evaluated in SLE; however the phase 1 study was terminated due to commercial considerations (ClinicalTrials.gov number, NCT01127321) (172). Nonetheless, the selective elimination of T_{FH} cells and $CD4^+$ memory T cells, two $CD4^+$ T cell populations strongly associated with disease activity, bolsters the rationale for further exploring this class of therapeutics.

Given the importance of both the CD28 and ICOS signaling pathways in T_{FH} cells, the recent development of a first-in-class dual inhibitor targeting CD28 and ICOS named ALPN-101 is particularly noteworthy, as it may offer the ability to compound the benefits observed using CD28 - and ICOS-targeting mAbs individually. While this compound has only completed phase 1 safety trials, preliminary evidence using an adoptive-transfer EAE model has yielded promising results as it was able to significantly ameliorate disease severity (ClinicalTrials.gov number, NCT03748836). An important consideration is that despite its molecular weight (80.8 kDa) being much smaller than that of traditional mAbs (∼150 kDa), the BBB will likely still impede CNS access and limit its effectiveness in MS.

Targeting eLF-Associated Molecules: IL-17, IL-22, IL-23, IL-21, and CXCL13

As described above, the induction of eLFs is coordinated by cytokines associated with T_{FH} cells and T_H17 cells, all of which are overexpressed in MS. Both IL-17 and IL-22, which are produced by T_H17 cells, facilitate BBB disruption and potentially induce the production of CXCL13 by meningeal stromal cells (111, 112, 125, 184). In EAE, these cytokines have also been shown to promote expansion of the reticular network (89, 121). A proof-of-concept study of secukinumab, a mAb targeting IL-17A, showed a reduction in annualized relapse rates in patients with RRMS (185). However, follow-up studies have not been reported. Currently, secukinumab and ixekizumab, a second anti-IL17A mAb, are approved for the treatment of psoriasis (186). A mAb targeting IL-22, fezakinumab, is available and has undergone clinical trials for psoriasis (ClinicalTrials.gov number, NCT00563524), RA (ClinicalTrials.gov number, NCT00883896), and atopic dermatitis (ClinicalTrials.gov number, NCT01941537); unlike secukinumab, this antibody has not been investigated in MS (187).

Meningeal stromal cells also secrete IL-23, required for T_H17 maintenance, in inflammatory conditions (113, 114, 121, 188). IL-23 promotes the release of IL-22 by synovial fibroblasts in a model of arthritis (125). IL-23, which structurally shares the p40 subunit with IL-12, has been explored as a target for RRMS treatment in a phase 2 trial with ustekinumab, which targets p40 and thus exhibits dual specificity for IL-23 and IL-12 (189). Notably, guselkumab, a first-in-class mAb specific for the IL-23-exclusive p19 subunit and has been approved for treatment of psoriasis (190).

IL-21 is expressed by and induces the expansion of both T_{FH} cells and T_H17 cells (191). IL-21 also promotes the generation of T-bet^+ DN memory B cells (130, 156). An anti-IL-21 mAb, known as NNC01140006 or BOS161721, is currently being investigated in SLE in a phase 2 trial, but has not been explored in MS (ClinicalTrials.gov number, NCT03371251) (187, 192).

Lastly, CXCL13 is overexpressed in MS patients, strongly correlates with disease activity as well as the frequency of B cells and PBs in the CSF, and can be expressed by T_{FH} cells in humans. Reduction in CXCL13 levels in the CSF can be accomplished by several DMTs, including natalizumab and fingolimod (193, 194). Quinn et al. (91, 111) showed that blocking CXCL13 protected against disease development in the T_H17-mediated adoptive transfer EAE model by reducing the influx of T_{FH} cells into the CNS, which resulted in a reduction of B cell-mediated inflammation in the CNS. Additionally, a neutralizing mAb directed against CXCL13, MAb 5261, inhibited CXCL13 function *in vitro* (195). However, it has not been explored in MS.

Given the association of these cytokines with MS and their ability to support the continuous recruitment and differentiation of inflammatory effector subsets, targeting these cytokines is an approach that warrants investigation. Importantly, efficient crossing of the BBB or BCSFB still remains a formidable hinderance to the efficacy of these drugs. Although therapeutic mAbs delivered intravenously can be detected in the CSF, the concentration is vastly smaller than that in the serum (196). RTX, for example, only reaches concentrations in the CSF $<$ 0.1% of that in the serum (197). While intrathecal administration has been investigated, an abundance of efflux transporters such as the neonatal Fc receptor (FcRn) present on the BBB endothelium results in the rapid clearance of therapeutic monoclonal antibodies from the CSF into the blood, preventing the meaningful retention of these therapeutics within the CNS (198–201). Indeed, human and animal studies show that intrathecal RTX is rapidly cleared from the CSF and accompanied by a concomitant increase in serum concentration (197, 200, 202, 203). Thus, CNS penetration remains a paramount issue to address in the advancement of DMTs.

CONCLUDING REMARKS

The insights afforded by the more in-depth characterization of disease-relevant immune and non-immune cell populations bring us closer to an understanding of the mechanisms driving MS relapses and progression. Specifically, the evidence supporting the interconnectedness of T_H17, T_{FH}, and B cells and the remarkable plasticity of each lineage could offer a possible inroad for unraveling the puzzle of the factors that induce and promote MS.

Ample evidence suggests that memory B cells in MS patients are ideally equipped for the reactivation of encephalitogenic $CD4^+$ T cells, a process which can occur in the CNS or in the dCLNs. In the CNS, the inflammatory microenvironment that

results from the reactivation of $CD4^+$ T cells can stimulate the CXCL13-mediated recruitment of T_{FH} cells to the CNS, which is a particularly important link in the context of progression, due to the strong association of this subset with eLFs seen in other chronic inflammatory autoimmune diseases.

Importantly, CXCL13 will also result in the recruitment of naïve B cells into the CNS. As the inflammation in the CNS persists, it is possible that these infiltrating naïve B cells could encounter dead cell debris containing myelin-derived proteins and nucleic acids, the latter as potent ligands for TLR9 and TLR7. The combination of these signals along with those received from IFN-γ and T_{FH} cell-derived IL-21, will result in the T cell-independent generation of proinflammatory T-bet^+ DN memory B cells (130). The generation of these autoreactive clones has major implications for subsequent relapses, as these cells are now not only more adept in their capacity to infiltrate the CNS but they are also potent APCs that can potentially precipitate a secondary break in $CD4^+$ T cell tolerance. This can lead to the development of GC-like reactions and the expansion of further autoreactive B and T cell clones (204). These autoreactive responses are well-supported in the MS CNS due to the presence of proinflammatory cytokines and the abundance of BAFF, which is known to be elevated in MS (130). Furthermore, in the absence of TLR signaling, these cells will preferentially differentiate into PBs upon stimulation with IFN-γ and IL-21, thus representing a source of OCBs that may be unrelated to CNS autoantigens (204). This would be in line with a recent study that suggested that novel OCBs in RRMS patients result from the clonal expansion of memory and PB/PC populations in the CSF (84).

As stated previously, these cells express ICOSL at levels slightly lower than naïve B cells. The expression of ICOSL is noteworthy in light of a recent study which found that naïve B cells are able to reactivate effector memory $CD4^+$ T cells from SLE and RA patients in an ICOSL-dependent manner even in the absence of T cell receptor triggering (205). Even more significant is that ICOSL preferentially stimulates effector T cells to produce IFN-γ, IL-17, and IL-22, all of which are highly expressed in the inflamed CNS of MS patients. While it must be noted that the effector memory cells expressed CD69, indicating they were recently activated, this finding nonetheless has important implications in the context of MS and could suggest that the reactivation of encephalitogenic $CD4^+$ T cells can be carried out not only by non-cognate T-bet^+ DN memory B cells, but also by naïve B cells, which are present in the inflamed and steady-state CNS. The result would be a population of autoreactive B and T cell clones that would expand with each relapse (206).

While this concept is speculative, the identification of these subsets and the evidence supporting their association with MS, specifically regarding reactivations that initiate relapses, provides a new lens through which we could view the inflammatory events that lead up to progression. Considering the paucity of inflammation during the progressive phase of MS, these potential mechanisms may be superseded by other mechanisms during those stages. Nonetheless, these findings help shed light on which cell populations may have an important impact on promoting relapses and would thus represent promising therapeutic targets.

AUTHOR CONTRIBUTIONS

AN, OS, and TF wrote and revised the manuscript. All authors reviewed and approved the manuscript.

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New Algorithms Improving PML Risk Stratification in MS Patients Treated With Natalizumab

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Overview: We assessed the role of age and disease activity as new factors contributing to establish the risk of progressive multifocal leucoencephalopathy in multiple sclerosis

patients treated with natalizumab in 36 University Hospitals in Europe. We performed the study in 1,307 multiple sclerosis patients (70.8% anti-John Cunninghan virus positive antibodies) treated with natalizumab for a median time of 3.28 years. Epidemiological, clinical, and laboratory variables were collected. Lipid-specific IgM oligoclonal band status was available in 277 patients. Factors associated with progressive multifocal leucoencephalopathy onset were explored by uni- and multivariate logistic regression.

Results: Thirty-five patients developed progressive multifocal leucoencephalopathy. The multivariate analysis identified anti-John Cunninghan virus antibody indices and relapse rate as the best predictors for the onset of this serious opportunistic infection in the whole cohort. They allowed to stratify progressive multifocal leucoencephalopathy risk before natalizumab initiation in individual patients [area under the curve (AUC) = 0.85]. The risk ranged from <1/3,300 in patients with anti-John Cunninghan virus antibody indices <0.9 and relapse rate >0.5, to 1/50 in the opposite case. In patients with lipid-specific IgM oligoclonal bands assessment, age at natalizumab onset, anti-John Cunninghan virus antibody indices, and lipid-specific IgM oligoclonal band status predicted progressive multifocal leucoencephalopathy risk (AUC = 0.92). The absence of lipid-specific IgM oligoclonal bands was the best individual predictor (OR = 40.94). The individual risk ranged from <1/10,000 in patients younger than 45 years at natalizumab initiation, who showed anti John Cunningham virus antibody indices <0.9 and lipid-specific IgM oligoclonal bands to 1/33 in the opposite case.

Conclusions: In a perspective of personalized medicine, disease activity, anti-lipid specific IgM oligoclonal bands, anti Jonh Cunninghan virus antibody levels, and age can help tailor natalizumab therapy in multiple sclerosis patients, as predictors of progressive multifocal leucoencephalopathy.

Keywords: multiple sclerosis, demyelinating diseases, biomarkers, natalizumab, progressive multifocal leucoencephalopathy, disease modifying treatments

INTRODUCTION

The use of natalizumab, a highly effective therapy approved for the treatment of active relapsing-remitting multiple sclerosis (1), is limited by the risk of progressive multifocal leucoencephalopathy (PML), a serious opportunistic infection of the central nervous system caused by John Cunninghan virus (JCV), appearing in about 1/250 treated patients (2, 3).

The factors most frequently used to stratify PML risk in multiple sclerosis patients treated with natalizumab are the presence of anti-JCV antibodies or high anti-JCV indexes in serum; prior immunosuppressive therapies; and duration of natalizumab treatment (3–7). These factors have proven to be effective in reducing the risk of PML in the clinical setting (8, 9). However, these strategies present some limitations. They depend on treatment duration and anti-JCV antibody levels, or negative anti-JCV status may change a long time, and this modifies patient prognosis (10, 11). Therefore, the search for new factors to stratify PML risk is of great clinical relevance. A highly inflammatory disease, revealed by the presence of lipid-specific oligoclonal IgM bands (LS-OCMB) in cerebrospinal fluid (CSF), associates with a lower PML risk in natalizumab treated patients (10). However, it remains unknown if clinical data indicating high inflammatory course prior natalizumab onset can also predict PML risk.

It was also demonstrated that mean age is higher in multiple sclerosis patients suffering PML during natalizumab treatment (10, 12, 13). However, the role of age as PML risk factor has not been fully explored.

We studied in a multicenter cohort of multiple sclerosis patients treated with natalizumab whether patients' clinical and demographic characteristics can be useful in predicting PML onset. Moreover, we further investigated the utility of LS-OCMB for the stratification of PML risk in combination with other clinical and laboratory variables.

MATERIALS AND METHODS

This was a multicenter cross-sectional study including 1,307 patients treated with natalizumab (natalizumab treatment duration: 3.73 ± 2.13 years, mean ± *SD*) in 36 European hospitals. The study was approved by the ethical committee of Ramon y Cajal University Hospital. All patients signed and informed consent before entering.

Patients were followed every 3–6 months in the neurology clinics at every participating center, with additional visits in

case of relapses. Demographic, clinical, and laboratory data prospectively collected at every center were anonymized and sent to the coordinator center. All patients signed an informed consent obtained according to the Declaration of Helsinki before entry.

Inclusion Criteria

We established the following inclusion criteria:

Patients had to be treated with natalizumab for at least a year to avoid the effect of a short time of treatment as confounder factor.

Clinical data had to be obtained prospectively since disease onset to avoid the lack of accuracy of retrospective data acquisition.

Data Collection

We established a minimum sample size of 1,000 patients to analyze all the variables projected. A form was sent to the participating centers comprising the following variables: sex, age at first relapse, age at natalizumab initiation, time between multiple sclerosis onset and natalizumab initiation, duration of natalizumab treatment, Expanded Disability Status Scale (EDSS) at natalizumab initiation, Multiple Sclerosis Severity Scale (MSSS) (14) at natalizumab initiation, relapse rate measured from multiple sclerosis onset to natalizumab initiation, previous treatments, serum anti-JCV antibody status (positive or negative), anti-JCV antibody index (which is proportional to serum anti-JCV antibody levels) (5), IgG oligoclonal bands (OCGB), and PML onset. LS-OCMB were available in a sub-cohort of 277 patients recruited at 29 different hospitals. LS-OCMB were determined by isoelectric focusing and immunoblotting, as previously described (15).

After receiving the first set of results, the database was debugged three times to complete data collection and correct inconsistent results. Finally, 69 patients were excluded, because of incomplete data or treatment duration shorter than 1 year. All the analyses were performed in the remaining 1,240 multiple sclerosis patients. Missing data were found in the following variables: Anti-John Cunninghan (JC) antibodies were only available in 1,174 patients (97.5%). Thirty-four of them developed PML, and 1,140 did not. Of note, in two PML cases anti-JC antibodies were negative 4 and 6 months before PML onset, when the last control test was performed. In both cases, the anti-JC test became positive at PML diagnosis. Anti-JC antibody levels were only available in 1,016 patients (82%). Twenty-seven developed PML, and 989 did not; relapse rate before natalizumab initiation was only obtained in 1,224 cases (98.7%). Thirty-five developed PML, and 1,189 did not. Finally, data on OCGB were only available in 756 patients (61%). Thirty-two developed PML, and 726 did not. Data collection comprised from 31 March 2017 to 15 June 2018.

Statistical Analysis

Results were analyzed with STATA v.14 (StataCorp.2014. Statistical Software: Release 14. College Station, TX, USA). $p < 0.05$ were considered as significant.

Normality of the different variables in PML and not PML groups was assessed with Kolmogorov–Smirnov test. No variable passed normality test in PML group. Thus, Mann–Whitney *U*-test (two tailed) was applied for non-parametric tests and Fisher exact test (two sided) was used for comparisons of categorical variables between groups. Univariate tests based on logistic regression were used to explore variables associated to PML risk and to calculate odds ratios (OR) and confidence intervals (CI). Significant results obtained in the univariate analyses were explored by multivariate tests, and minimal models were established by eliminating variables loosing statistical significance.

To assess PML risk in individual patients, a nomogram was generated from the minimal model logistic regression results. In this analysis, the program assigns a score to every factor increasing PML risk. It also creates two parallel scales with total scores and the correspondent probability of PML. To explore individual risk in a patient, the total score is calculated and the corresponding risk read in the probability scale. To avoid overestimating PML risk, probabilities were corrected by a factor obtained by dividing previously described PML frequency in natalizumab treated patients (3) and the one obtained in our cohorts.

Data Availability

The study protocol, statistical analysis plan, and data not provided in the article because of space limitations will be shared upon request by any qualified investigator for purposes of replicating procedures and results during 3 years after publication.

RESULTS

We included in the study 1,240 multiple sclerosis patients treated with natalizumab at 36 different hospitals. Thirty-five developed PML during natalizumab treatment, and 1,205 did not suffer this opportunistic infection. Clinical and demographic data of the patients classified according to PML onset are shown in **Table 1A**. The highest differences were found in age at natalizumab initiation ($p = 0.004$), relapse rate before natalizumab ($p < 0.0001$), anti-JCV antibody positivity ($p = 0.004$), and anti-JCV index levels ($p < 0.0001$). PML patients were older at treatment initiation, showed a lower relapse rate, a higher proportion tested positive for anti-JCV antibodies before PML, and had increased anti-JCV antibody indices. We also found that PML group showed an increased proportion of males ($p = 0.04$) and had longer disease duration at natalizumab initiation ($p = 0.02$). No variation in other clinical or demographic variables was associated with PML, including prior immunosuppression or duration of natalizumab treatment. We further explored if the values of this variable could change depending on anti-JC antibody values. The median time of treatment was 3.28 years in the whole cohort, the range going from 1.00 to 13.40 years, and the interquartile range (IQR) from 2.06 to 4.82 years. These values did not change substantially in patients with anti-JC antibody levels higher (median = 3.32, range: 1.00–11.46, IQR:

TABLE 1 | Demographic and clinical data.

	(A) Total group (N = 1,240)			(B) LS-OCMB group (N = 277)		
	Pml (n = 35)	**NoT PML (n = 1,205)**	***P***	**PML (N = 24)**	**NOT PML (N = 253)**	***p***
Sex (M/F)	16/19	358/847	0.04	10/14	78/175	0.28
Age at 1st relapse (y)	30.1 ± 9.5 (23–36)	28.2 ± 8.7 (22–33)	0.33	31.1 ± 9.6	28.1 ± 8.41	0.14
Disease duration at NTZ onset (y)	11.2 ± 7.4 (4.7–17.9)	8.3 ± 6.3 (3.4–11.9)	0.02	12.7 ± 7.8	6.7 ± 5.7	0.0002
Age at NTZ onset (y)	41.3 ± 8.9 (33.2–49.2)	36.5 ± 9.4 (29.9–42.7)	0.004	43.8 ± 8.7	34.8 ± 8.9	<0.0001
Duration of NTZ treatment (y)	3.4 ± 1.5 (1.1–7.7)	3.8 ± 2.1 (1.0–13.4)	0.77	3.3 ± 1.6	3.47 ± 2.0	0.82
EDSS at NTZ onset	3.3 ± 1.4 (2–4)	3.2 ± 1.6 (2–4)	0.68	3.6 ± 1.4	3.1 ± 1.6	0.07
MSSS at NTZ onset	4.3 ± 2.5 (2.2–6.8)	4.8 ± 2.4 (2.8–6.6)	0.24	4.4 ± 2.6	5.1 ± 2.4	0.23
Relapse rate before NTZ onset	0.8 ± 0.95 (0.25–0.93)	1.4 ± 1.4 (0.53–1.56)	<0.0001	0.6 ± 0.5	1.6 ± 1.7	<0.0001
Prior IS (yes/no)	7/28	139/1066	0.13	5/19	34/219	0.32
Anti-JCV Abs (pos/neg)*	32/2	844/331	0.004	21/2	162/87	0.010
Anti-JCV Ab levels*	2.2 ± 1.2 (1.23–3.18)	0.9 ± 1.1 (0.09–1.45)	<0.0001	1.9 ± 1.3	1.0 ± 1.1	0.0047
OCGB (pos/neg)	30/2	651/73	0.48	22/2	234/19	0.88
LS-OCMB (pos/neg)				1/23	162/91	<0.0001

For continuous variables values are expressed as mean ± standard deviation (interquartile range).
**The last measure before study completion; 1st, first; Anti-JCV Ab, anti-John Cunningham virus antibodies; EDSS, expanded disability status scale; F, female; IS, immunosuppression; LS-OCMB, lipid-specific oligoclonal IgM bands; M, male; MSSS, multiple sclerosis severity score; neg, negative; NOT PML, not progressive multifocal leukoencephalopathy; NTZ, Natalizumab; OCGB, oligoclonal IgG bands; PML, progressive multifocal leukoencephalopathy; pos, positive; y, years.*

2.01–5.03 years) or lower (median = 3.26, range: 1.00–13.40, IQR: 2.08–4.3 years) than 0.9.

To better define associations of the different variables with PML onset, we first performed univariate analyses (**Table 2A**). Cutoff values were established using receiver operating characteristic (ROC) curves in case of age, time until natalizumab initiation, and relapse rate before treatment or pre-established cutoffs for anti-JCV antibody levels and EDSS and MSSS scores. The strongest association was found with high anti-JCV index values, being the clearest one obtained for anti-JCV indices higher than 0.9 (OR = 18.29, $p < 0.001$). Additionally, having anti-JCV index levels >1.5 (OR = 8.58, $p < 0.001$) and the presence of anti-JCV antibodies (OR = 6.27, $p = 0.012$) also associated with PML onset. Age at natalizumab initiation ≥45 years also increased PML risk (OR = 3.20, $p = 0.001$). A disease duration higher than 10 years at natalizumab initiation (OR = 2.37, $p = 0.012$) and an MSSS score lower than 3 (OR = 2.25, $p = 0.019$) was also associated with PML onset. Finally, male sex associated modestly with an increased PML risk (OR = 1.99, $p = 0.046$). On the other hand, having an annualized relapse rate higher than 0.5 before treatment initiation clearly diminished PML risk (OR = 4.47, $p < 0.001$).

Based on univariate analyses, we performed three different multivariate analyses according to anti-JCV antibody classification. First, we included all the significant factors and anti-JCV antibodies classified according to the positive or negative results (**Table 3**). In the minimal model, anti-JCV antibody positivity (OR = 6.04, $p = 0.014$), annualized relapse rate before natalizumab <0.5 (OR = 4.25, $p < 0.001$), and age at natalizumab initiation ≥45 years (OR = 2.33, $p = 0.022$) significantly impacted on PML appearance. In this model area under the ROC curve was 0.78.

The second multivariate analysis included anti-JCV antibodies classified using the level of 0.9 as cutoff value. In the minimal model, only anti-JCV antibody levels ≥0.9 (OR = 18.72, $p < 0.001$) and annualized relapse rate before natalizumab initiation <0.5 (OR = 4.66, $p < 0.001$) had an effect on PML risk. Although only these two factors were significant in this model, the area under ROC curve was higher (0.85).

Finally, we performed a multivariate analysis using 1.5 as cutoff value for anti-JC antibody levels. Anti-JCV antibody levels ≥1.5 (OR = 7.85, $p < 0.001$), annualized relapse rate before natalizumab initiation <0.5 (OR = 3.73, $p = 0.001$), and age at natalizumab initiation ≥45 years (OR = 2.31, $p = 0.048$) significantly increased PML risk in this model. The area under the ROC curve was 0.84.

We made a nomogram analysis of the second multivariate analysis (cutoff: anti-JCV antibody levels of 0.9) to explore the contribution of each variable to PML risk. Data are shown in **Figure 1A**. We adjusted the risk using a correction factor obtained calculating the ratio between the numbers of PML cases per 1,000 patients reported after commercialization (4.16‰) and that of our cohort (28‰). Patients with anti-JCV antibody levels lower than 0.9 and annualized relapse rate higher than 0.5 prior

TABLE 2 | Univariate analysis to explore the ability of different clinical and demographic variables for predicting PML onset during natalizumab treatment.

	(A) Total patient group (n = 1,240)			(B) Patients with a LS-OCMB study (n = 277)		
	OR	**95% CI**	***P***	**OR**	**95% CI**	***p***
Male sex	1.99	1.06–4.17	0.046	1.60	0.68–3.77	0.28
Age at NTZ onset ≥45 years	3.20	1.60–6.39	0.001	7.36	3.06–17.72	<0.001
Disease duration at NTZ onset ≥10 years	2.37	1.21–4.66	0.012	4.60	1.94–10.90	0.001
NTZ treatment for >2 years	1.02	0.46–2.27	0.96	1.69	0.61–4.70	0.31
NTZ treatment for >3 years	1.25	0.63–2.48	0.53	1.29	0.56–2.99	0.55
NTZ treatment for >4 years	0.99	0.43–1.98	0.97	0.91	0.36–2.27	0.84
NTZ treatment for >5 years	0.43	0.15–1.23	0.12	0.41	0.09–1.80	0.24
Positive anti–JCV Abs	6.27	1.50–26.33	0.012	5.64	1.29–24.61	0.021
Anti-JCV Ab levels ≥0.9	18.29	5.46–61.19	<0.001	9.08	2.54–32.45	0.001
Anti-JC Ab levels ≥1.5	8.58	3.59–20.54	<0.001	4.78	1.71–13.33	0.003
EDSS at NTZ onset <3	0.75	0.38–1.51	0.42	0.43	0.17–1.07	0.07
EDSS at NTZ onset <6	1.15	0.35–3.82	0.82	1.31	0.29–5.90	0.72
MSSS at NTZ onset <3	2.25	1.14–4.43	0.019	2.25	0.95–5.32	0.06
MSSS at NTZ onset <6	0.95	0.47–1.94	0.90	0.86	0.35–2.09	0.74
Relapse rate before NTZ onset <0.5	4.47	2.26–8.86	<0.001	6.77	2.80–16.35	<0.001
Prior immunosuppression	1.92	0.82–4.47	0.13	1.70	0.59–4.84	0.32
LS-OCMB Negative				40.94	5.44–308.20	<0.001

Anti-JCV Abs, anti-John Cunningham virus antibodies; CI, confidence interval; EDSS, expanded disability status scale; LS-OCMB, lipid-specific oligoclonal IgM bands; MSSS, multiple sclerosis severity score; NTZ, natalizumab; OR, odd ratio; PML, progressive multifocal leukoencephalopathy.

TABLE 3 | Factors predicting PML onset in the total group of patients.

	OR	**95% CI**	***P***
Minimal model with anti-JCV antibodies classified as positive/negative			
Anti-JCV antibodies (positive)	6.04	1.43–25.53	0.014
Relapse rate before natalizumab	4.25	2.08–8.69	<0.001
onset <0.5	2.33	1.13–4.80	0.022
Age at natalizumab onset ≥45 years			
Area under ROC curve: 0.78			
Minimal model with anti-JCV antibodies classified using a level of 0.9 as cut off value			
Anti-JCV antibody levels ≥0.9	18.7	5.56–63.02	<0.001
Relapse rate before natalizumab	4.66	2.10–10.35	<0.001
onset <0.5			
Area under ROC curve: 0.85			
Minimal model with anti-JCV antibodies classified using a level of 1.5 as cut off value			
Anti-JCV antibodies levels ≥1.5	7.85	3.25–19.00	<0.001
Relapse rate before natalizumab	3.73	1.67–8.34	0.001
onset <0.5	2.31	1.01–5.28	0.048
Age at natalizumab onset ≥45 years			
Area under ROC curve: 0.84			

Multivariate analyses.
Anti-JC antibodies, anti-John Cunningham virus antibodies; CI, confident interval; OR, odd ratio; PML, progressive multifocal leukoencephalopathy; ROC, Receiver operating characteristic.

natalizumab initiation showed a PML risk lower than 0.3‰. If the annualized relapse rate was lower than 0.5, the PML risk increased to 1.5‰, and if, in addition, anti-JCV antibody levels were higher than 0.9, the risk was augmented to 2%. These values are independent of the sex, disease duration, time on natalizumab treatment, or previous treatment with anti-suppressive drugs.

Role of Lipid Specific Oligoclonal IgM Bands in Risk Stratification

Two hundred seventy-seven patients (22.3% of the whole cohort) were examined for LS-OCMB. Twenty-four of them (8.7%) developed PML. Clinical and demographic data of these patients are shown in **Table 1B**. One hundred and sixty-two of the 253

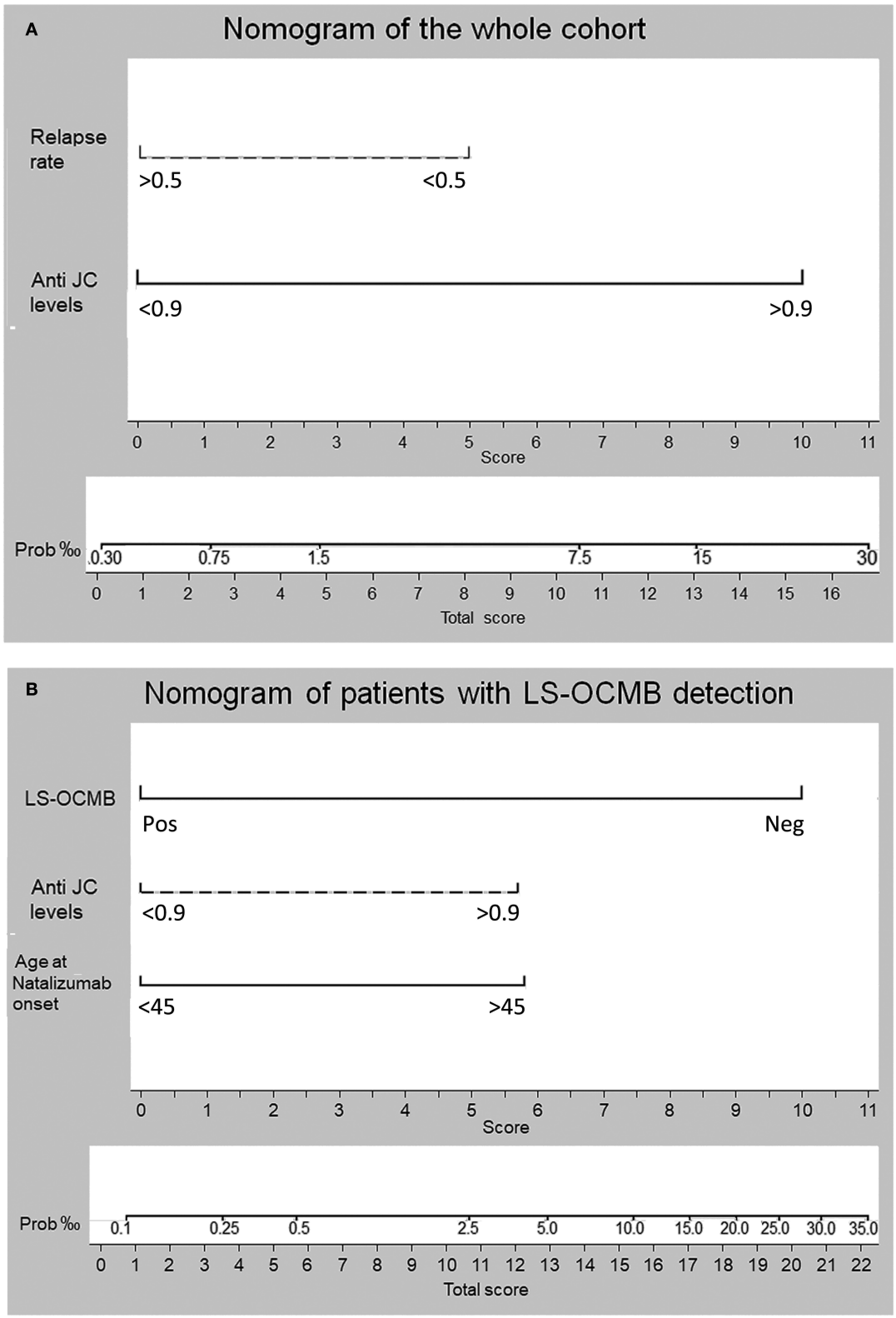

FIGURE 1 | Nomogram for predicting progressive multifocal leukoencephalopathy (PML) onset in individual MS patients. The multivariate logistic regression analysis assigns a score to every variable included in the minimal model. The sum of the scores obtained by a patient is interpolated in the total score point-probability line at

(Continued)

FIGURE 1 | the bottom of each nomogram and gives the individual PML risk. **(A)** PML risk in the total cohort. Having a relapse rate lower than 0.5 gives a score of 5 and showing anti-John Cunningham virus antibody levels (anti-JC levels) higher than 0.9 provides a score of 10. Individual patient scores range from 0 to 15 and their PML risk from <1/3,300 to 1/50, respectively. **(B)** PML risk in the patients with lipid-specific oligoclonal IgM band (LS-OCMB) detection. Being negative (Neg) for LS-OCMB gives a score of 10. Showing anti-JC levels higher than 0.9 provides a score of 5.75. Being older than 45 years gives a score of 5.75. Individual patient scores range from 0 to 21.5 and their PML risk from <1/10,000 to 1/30, respectively.

TABLE 4 | Factors predicting PML onset in the group of patients with LS-OCMB detection.

	OR	95% CI	*P*
Minimal model with anti-JCV antibodies classified as positive/negative			
LS-OCMB negative	30.44	3.94–234.91	<0.001
Age at natalizumab initiation ≥45 years	4.80	1.76–13.14	0.002
Relapse rate before natalizumab initiation <0.5	3.21	1.19–8.66	0.022
Area under ROC curve: 0.90			
Minimal model with anti-JCV antibodies classified using a level of 0.9 as cut off value			
LS-OCMB negative	26.83	3.33–216.29	0.002
Age at natalizumab initiation ≥45 years	6.74	2.00–22.73	0.002
Anti-JCV antibodies levels ≥0.9	6.52	1.64–25.85	0.008
Area under ROC curve: 0.92			
Minimal model with anti-JCV antibodies classified using a level of 1.5 as cut off value			
LS-OCMB negative	31.18	3.81–255.16	0.001
Age at natalizumab initiation ≥45 years	8.85	2.64–29.60	<0.001
Anti-JCV antibodies levels ≥1.5	4.38	1.33–14.43	0.015
Area under ROC curve: 0.92			

Multivariate analyses.

Anti-JCV antibodies, anti-John Cunningham virus antibodies; CI, confident interval; LS-OCMB, lipid-specific oligoclonal IgM bands; OR, odd ratio; PML, progressive multifocal leukoencephalopathy; ROC, Receiver operating characteristic.

patients not developing PML (64.0%) were LS-OCMB positive. By contrast, only one of the 24 PML patients (4.2%) displayed these antibodies ($p < 0.0001$). Similarly to the whole cohort, patients suffering PML were older ($p < 0.0001$), had a longer disease duration ($p = 0.0002$) at natalizumab initiation, and had a lower relapse rate before natalizumab ($p < 0.0001$). A higher percentage of these patients were anti-JCV positive ($p = 0.010$), and they also displayed higher anti-JCV antibody levels ($p = 0.0047$).

We followed the same approach described for the entire cohort. First, we performed univariate analyses (**Table 2B**). The conditions associated with PML risk were the following: absence of LS-OCMB (OR = 40.94; $p < 0.001$); levels of anti-JCV index ≥0.9 (OR = 9.08, $p = 0.001$) or ≥1.5 (OR = 4.78, $p = 0.003$); or positive anti-JCV antibodies (OR = 5.64, $p = 0.021$); age at natalizumab initiation ≥45 years (OR = 7.36, $p < 0.001$); annualized relapse rate before natalizumab ≤0.5 (OR = 6.77, $p < 0.001$), and disease duration at natalizumab initiation ≥10 years (OR = 4.60, $p = 0.001$).

Again, we made three different multivariate models according to anti-JCV antibody classification (**Table 4**). First, we included all the significant variables and anti-JCV antibodies classified according to having positive or negative results. In the minimal model, only absence of LS-OCMB (OR = 30.44, $p < 0.001$), age at natalizumab initiation ≥45 years (OR = 4.80, $p = 0.002$), and relapse rate before natalizumab initiation <0.5 (OR = 3.21, $p = 0.022$) had an effect on PML risk. The area under the ROC curve was 0.90.

In the second multivariate analysis we used an anti-JCV index of 0.9 as cutoff value. In the minimal model the variables that significantly impacted PML development were absence of LS-OCMB (OR = 26.83, $p = 0.002$), age at natalizumab initiation ≥45 years (OR = 6.74, $p = 0.002$), and anti-JCV antibody index ≥0.9 (OR = 6.52, $p = 0.008$). In this model, the area under the ROC curve was 0.92.

Finally, we performed a multivariate analysis using 1.5 as cutoff value for anti-JCV antibody levels. Again, absence of LS-OCMB (OR = 31.18, $p = 0.001$), age at natalizumab initiation ≥45 years (OR = 8.85, $p < 0.001$), and anti-JCV antibody levels ≥1.5 (OR = 4.38, $p = 0.015$) significantly increased PML risk. The area under the ROC curve was 0.92.

Finally, we repeated a nomogram analysis of the second multivariate analysis (cutoff: anti-JCV antibody levels of 0.9). Data are shown in **Figure 1B**. We adjusted the risk using a correction factor obtained calculating the ratio between the numbers of PML cases per 1,000 patients reported after commercialization (4.16‰) and that of our cohort (87‰). Patients with LS-OCMB, anti-JCV antibody levels lower than 0.9, and age at natalizumab initiation younger than 45 years showed a PML risk lower than 0.1‰. When anti-JCV antibody levels were

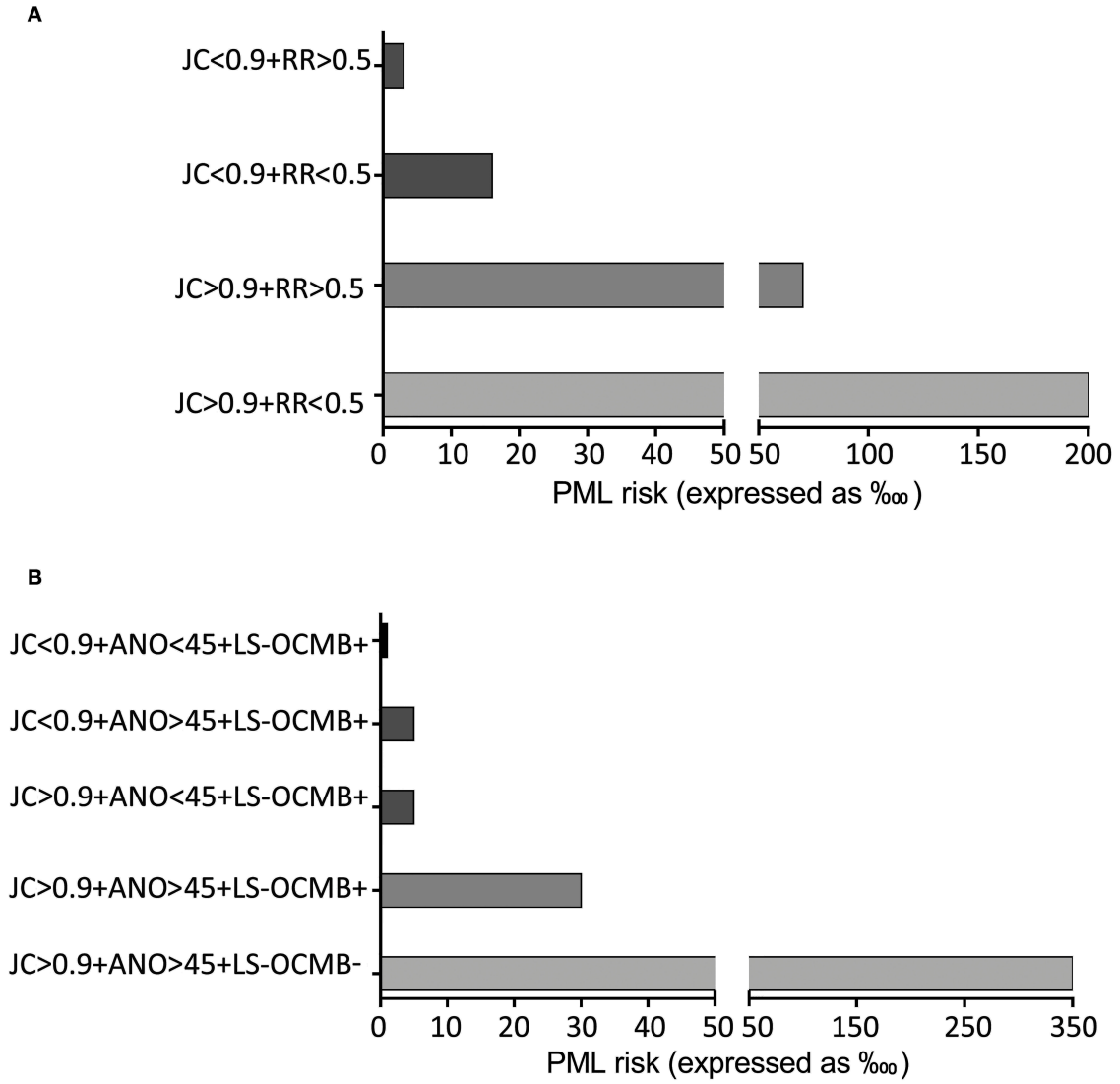

FIGURE 2 | Illustration of predicting progressive multifocal leukoencephalopathy (PML) risk depending on the results of the nomograms. **(A)** In the whole cohort PML risk associates with the anti-John Cunningham virus antibody levels (JC) and the relapse rate (RR). **(B)** In patients with lipid-specific oligoclonal IgM band (LS-OCMB) detection, PML risk associated with the LS-OCMB, and JC status, and the age at natalizumab onset (ANO).

higher than 0.9 or patients were older than 45 at natalizumab onset, the risk was augmented to 0.5‰. If both conditions were present, it rose to 3.5‰. If LS-OCMB were negative too, the risk increased to 3%. Again, these values were independent of sex, disease duration, prior immunosuppression, or the duration of natalizumab treatment.

A graphic representation of PML risk in the two cohorts depending of the results of the nomograms is shown in **Figure 2**.

Finally, we studied if OCMB could add some advantage to previous risk factors in the 47 patients (29 female/19 male) with anti-JCV antibody levels >1.5, who were treated with natalizumab for more than 2 years (2.32, 2.01–4.32 years; median, interquartile range). Results are shown in **Table 5**. Only one of 23 patients showing OCMB developed a PML. By contrast, 10 out of 24 OCMB negative patients suffered this opportunistic infection (Pearson chi square = 9.12, $p = 0.003$).

TABLE 5 | Value of OCMB for predicting PML onset in patients with anti JC antibody levels >1.5 and treated with natalizumab for more than 2 years.

	PML+	PML-
LS-OCMB+ (*n*, %)	1, 4.35%	22, 95.65%
LS-OCMB- (*n*, %)	10, 41.67%	14, 58.33%
Total (*n*, %)	11, 23.40%	36, 76.60%
Pearson $chi^2 = 9.12$ $p = 0.003$		

LS-OCMB, lipid-specific oligoclonal IgM bands; PML, progressive multifocal leukoencephalopathy.

DISCUSSION

The appearance of highly effective immunotherapies has changed disease course of patients with aggressive multiple sclerosis (1, 16–18). However, efficacy associates with higher risk of deleterious side effects (19–22). Finding biomarkers that allow the best balance between efficacy and safety for individual patients has become a challenge of the most clinical relevance in multiple sclerosis research.

In case of natalizumab, the most important side effect is the appearance of PML, an opportunistic infection of the brain appearing in about one of every 250 treated patients (3, 23). It may cause patient death or considerable increase of disability. This has limited the use of this drug. Safety concerns, in both patient and neurologist sides, often make it difficult to administer this treatment for long. This is unfortunate, since the clinical efficacy of this drug in the long term was demonstrated (24).

Additional factors reflecting patient inflammatory status can contribute to further stratify PML risk. Decreased CD4+ T cell expression of L-Selectin (CD62L), a molecule implicated in leukocyte adhesion to the endothelium, during natalizumab treatment was found to associate with an increase of PML risk (25). Although validation studies gave no uniform results (26, 27), probably due to the difficulty of measuring this biomarker in cryopreserved cells, these data may reflect that a decrease in cells migrating to the central nervous system may increase PML risk. Another factor indicating that patient inflammatory status may contribute to stratifying PML risk is one of the actual risk factors, prior immunosuppression. Previous treatments inducing a strong immunosuppression increase PML risk (4, 6).

Age, another factor associated with inhibition of the adaptive immune response in multiple sclerosis and with reduced lymphocyte migration into the central nervous system (CNS), also relates to a higher PML risk in multiple sclerosis patients treated with different biological drugs (10–12, 28). By contrast, a highly inflammatory disease course revealed by the presence of LS-OCMB greatly diminishes PML risk (10). We studied here if clinical data reflecting disease activity may contribute to stratify PML risk in a cohort of 1,240 patients treated with natalizumab in 36 European hospitals. We also studied the value of these variables in combination with LS-OCMB in a sub-cohort of 277 patients in which these antibodies were analyzed.

We did not find any significant association between prior immunosuppression and PML risk in our cohort, although the proportion of patients showing prior immunosuppression was higher in the group of PML patients (20%) than in those not developing this opportunistic infection (11%). The lower number of immunosuppressed patients in both PML and not PML cases compared with previous studies (5) may account for the lack of significance of this variable in our cohort. However, anti-JCV antibodies and mostly anti-JCV indices higher than 0.9 continued to increase the probability of PML in these patients. In addition, clinical data associated with disease activity also contribute to identify patients at higher risk. Thus, an MSSS score lower than 3 or relapse rates lower than 0.5 since disease onset is associated with increased probability of PML. Age older than 45 years at natalizumab onset also identified patients at higher PML risk. When including all factors giving significant results in the total cohort, in a multivariate logistic analysis to identify variables that were statistically independent, the best predictive model to assess PML risk included anti-JCV levels higher than 0.9 and annualized relapse rate below 0.5. We assessed individual PML risk by a nomogram analysis. When anti-JCV levels where below 0.9 and relapse rate over 0.5, PML risk was below 1 in every 3,300 treated patients. If the results were the opposite, it rose to 1/50.

By contrast, natalizumab treatment duration did not associate with PML risk in our study. The divergence of these results with those previously published can be partly due to the absence of patients treated for less than a year, who have extremely low PML risk, in our cohort. The relatively low number of patients included in this study (1,306) compared to other cohorts with more than 5,000 patients (5) may also contribute to the loss of significance of treatment duration for PML risk stratification. In addition, the particular characteristics of our cohort which includes mainly active (median relapse rate 0.88 with a low interquartile range of 0.51) and relatively young patients (median age at natalizumab onset = 36.5 years, with a high interquartile range of 42.8 years) also can contribute to the loss of significance in this variable. If these data are confirmed in larger cohorts, they could indicate that treatment duration impact on PML risk could be modulated by younger age and high disease activity.

The presence of LS-OCMB further contributed to stratify PML risk. When we performed a multivariate logistic analysis in the sub-cohort of patients in which these antibodies were assessed, the best predictive model to assess PML risk changed. It included LS-OCMB as best individual predictor and anti-JCV levels higher than 0.9 and age older than 45 years as factors that equally contributed to PML risk. Nomogram analysis showed that patients with CSF restricted LS-OCMB, anti-JCV antibodies below 0.9, and age younger than 45 years at natalizumab onset had a PML risk below 1 in every 10,000 treated patients. If anti-JCV antibody levels were higher than 0.9 or age at natalizumab onset over 45 years, PML risk was only 1/2,000 in LS-OCMB positive patients. When these two factors coincided in a patient, the risk rose to 1/300 despite LS-OCMB positivity and even increased to 1/33 in LS-OCMB negative patients. These data are clinically relevant since they show that patients with a more inflammatory disease, who get more clinical benefit of this highly active drug, are at lower PML risk during natalizumab treatment.

In conclusion, these data allow to introduce a new algorithm in which PML risk can be established for individual patients attending to clinical and laboratory data measured prior to natalizumab treatment initiation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Hospital Ramon y Cajal, Madrid, Spain. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LV, IT, RA-L, RA, HH, FD, SS, JA-C, GI, DPa, PO, BC, EA-M, DF, MG, OF, PU, JG-D, FR, AL, AU, AP-S, AS, YB, DG, ES, CE, XM, LR, FPau, IG, YA, EÁ, CR, AC, CC, PE, AB-B, LR-T, EQ, JM-R, AO, CL, LC, LL, JF, GB, PM, MH, JP, DPé, MO, FPad, JG-M, LN, AM, LF, and MC: sample collection, collection of clinical data, and critical review of the manuscript. All authors contributed to the article and approved the submitted version.

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Ocrelizumab in Multiple Sclerosis: A Real-World Study From Spain

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Objectives: The aim of this study was to describe the tolerability, safety, and effectiveness of ocrelizumab for primary progressive multiple sclerosis (PPMS) and relapsing multiple sclerosis (RMS) in a clinical practice setting.

Methods: In this retrospective observational study, we analyzed clinical and MRI data in all patients with PPMS and RMS who had received at least one infusion of ocrelizumab in two health areas in south-eastern Spain. Patients involved in any ocrelizumab trial and those patients with a follow-up shorter than 6 months were excluded.

Results: The cohort included 70 patients (42 women) who had received ocrelizumab; 30% had PPMS and 70%, RMS. At baseline, patients' mean age was 47.1 years in the PPMS group and 39.2 years in the RMS group, while the median EDSS was 3.0 and 2.5, respectively. Median follow-up was 13.6 months. The median number of treatment cycles was three. Most patients remained free from clinical and MRI activity after ocrelizumab initiation. Baseline MRI showed T1 Gd-enhancing lesions in 57% of the patients; by the first MRI control at 4–6 months, all patients except one were free of T1 Gd-enhancing lesions (69/70, 98.6% $P < 0.001$). The proportion of patients with NEDA was 94% in the group of RMS patients who were followed for at least 1 year. Ocrelizumab was generally well-tolerated; the most common adverse events were infusion-related reactions and infections, none of which were serious.

Conclusions: Our real-world study supports the tolerability, safety, and effectiveness of ocrelizumab in clinical practice.

Keywords: multiple sclerosis, drug therapy, ocrelizumab, safety, tolerability, real-world, effectiveness, MRI

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INTRODUCTION

The humanized anti-CD20 B cell-depleting antibody ocrelizumab is approved in Europe for treating adults who have relapsing forms of multiple sclerosis (RMS) with active disease or early primary progressive multiple sclerosis (PPMS) with imaging features characteristic of inflammatory activity (1). The pool of PPMS patients who are candidates for this drug differs from the population studied in the pivotal phase 3 randomized clinical trials (RCT) with respect to the requirement of evidence of inflammatory activity from magnetic resonance imaging (MRI) (T1 Gd-enhancing lesions and/or new or enlarging T2 lesions), which was not present in the RCT inclusion criteria (2).

In the 96-weeks OPERA I and II trials in patients with RMS, ocrelizumab significantly reduced annualized relapse rates vs. interferon β-1a by 46% and the number of gadolinium-enhancing lesions by 94% (3). Likewise, in the ORATORIO trial in patients with PPMS, ocrelizumab significantly reduced the risk of confirmed disability progression relative to placebo (2). Ocrelizumab was generally well-tolerated in these studies, with mild to moderate infusion-related reactions and infections being the most common adverse events (4).

Although RCTs are essential to establish the efficacy of a new drug, they have limited validity because their results may not be widely generalizable, since the enrollment of patients with different comorbidities or previous treatments may be limited by the inclusion criteria. Real-world studies can thus provide useful information on the treatment tolerability, effectiveness and safety (5). Real-world data on ocrelizumab is limited as only a few studies have been published in Europe (6–8). The aim of this study was to describe the tolerability, safety and effectiveness of ocrelizumab for PPMS and RMS in clinical practice in a different geographical setting.

METHODS

Patients and Study Design

This retrospective, observational study was performed in two health areas in the province of Alicante: Marina Baixa and Alicante, both situated in south-eastern Spain with a combined population of about 500,000. Patients with multiple sclerosis were attended at Marina Baixa General Hospital and Alicante General Hospital. The patients of both centers were evaluated jointly, under the same protocol. The healthcare system in Spain is universal and free at the point of service.

The main inclusion criteria was a history of initiation of ocrelizumab. Patients involved in any ocrelizumab trial and those patients with a follow-up shorter than 6 months were excluded. We retrospectively analyzed data in all patients with PPMS and RMS who had received at least one infusion of ocrelizumab. Multiple sclerosis was diagnosed according to the McDonald criteria (9). Clinical relapse, disease progression, and adverse events during ocrelizumab treatment were assessed by reviewing medical reports until September 18, 2020.

The standard patient follow-up included visits at 3, 6, and 12 months and every 6 months thereafter. During follow-up visits, clinicians considered new relapses and assessed patients using the Expanded Disability Status Scale (EDSS). Trained examiners with Neurostatus certification (APS, LBR) performed all EDSS assessments.

Patients underwent brain MRI scans before ocrelizumab initiation (baseline); at 4–6 months (before the second cycle of ocrelizumab), at 12 months, and at 24 months. Spinal cord and brain MRI scans, using 1.5 T and 3 T scanners, were done on an individual basis. At least contiguous, 3-mm axial sections, T2-weighted, FLAIR and gadolinium-enhanced T1-weighted scans through the whole brain were acquired in all patients according to published guidelines (10). MRI scans were read by experienced radiologists.

Baseline data collected from medical records were as follows: (a) demographic variables, (b) type of multiple sclerosis, (c) disease-modifying therapy before starting on ocrelizumab, (d) EDSS score, (e) number of relapses in the previous year, (f) time since diagnosis, (g) number of gadolinium-enhancing lesions on MRI, and (g) reason for starting ocrelizumab. Variables and outcomes assessed during follow-up were: (a) duration of follow-up, (b) number of relapses, (c) EDSS at the last visit, (d) number of ocrelizumab cycles, (e) adverse events, (f) number of gadolinium-enhancing lesions on the first MRI after ocrelizumab initiation (4–6 months), (g) number of new T2-lesions and T1 gadolinium-enhancing lesions in the annual MRI, and (h) discontinuation of ocrelizumab.

Clinical and MRI Outcomes

A relapse was defined as new or recurrent symptoms and objective typical findings of multiple sclerosis with a duration of at least 24 h, in the absence of fever or infection (9). Disability progression was defined as a sustained (≥3 months) increase in the EDSS score, of: 1.5 points if the baseline EDSS score was 0; 1 point if the baseline score was 1–5.5; and 0.5 points if the baseline EDSS score was 6.0 or more. Disability improvement was defined as a sustained (≥3 months) decrease in the EDSS score, of: 0.5 points if the baseline EDSS score was 6.5 or more, or one point if the baseline score was 6.0 or less (11).

Clinical activity was defined as relapse and/or disability progression, and MRI activity was defined as the presence of T1 gadolinium-enhancing lesions at any time point or new T2 lesions on the annual MRI (compared to the MRI performed at 4–6 months). Highly active disease was defined as one or more relapse in the previous year and one or more T1 gadolinium-enhancing lesion on the baseline MRI.

No evidence of disease activity (NEDA) outcome was assessed in RMS patients who were followed for at least 1 year. NEDA status was defined as the combined absence of clinical (relapses and disability progression) and MRI activity (12).

Treatment Protocol

Ocrelizumab was administered according to the schedule recommended in its summary of product characteristics (1). Before ocrelizumab administration, all patients were evaluated by their attending neurologist about symptoms suggestive of COVID-19 after Covid pandemic. The initial 600 mg cycle was administered as two separate intravenous infusions of 300 mg, at a 2-weeks interval. Subsequent cycles were administered as a single 600 mg intravenous infusion every 6 months. The premedication for all cases consisted of 100 mg intravenous methylprednisolone, 10 mg of cetirizine or 5 mg of dexchlorpheniramine, and 1,000 mg of paracetamol. Patients were monitored at hospital during the infusion and for 1 h after its completion. Infusion-related reactions included all symptoms and events occurring during or within 24 h of the infusion (in hospital or at home) and were graded as mild, moderate, severe, or life-threatening according to Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 (13).

Statistical Analysis

Quantitative variables are described using the mean ± standard deviation (SD) or median and range and they were compared with Student test or Mann–Whitney *U* depending on the normality of the distribution. Qualitative variables are presented as absolute and relative frequencies and were compared with chi-squared test. We compared the number of patients with T1 gadolinium-enhancing lesions on MRI at baseline and follow-up using McNemar's test. All calculations were performed with a statistical significance of 5% and for every relevant parameter, we calculated the confidence interval (CI) of 95%. The statistical package used was the IBM SPSS Statistics version 25.

RESULTS

Cohort Characteristics

A total of 70 patients (42 female and 28 male), who had received at least the first cycle of ocrelizumab and with a follow-up longer than 6 months were included. There were no significant differences in baseline demographics and clinical characteristics (age, sex, EDSS, disease duration) for the two centers. Their clinical characteristics are summarized in **Table 1**. Twenty-one patients (30%) with a mean age 47.1 years had PPMS, and 49 patients (70%) with a mean age of 39.2 years, RMS. Relevant comorbidities according to the treating neurologist were present in 24% of patients (**Table 2**).

The main reason for switching to ocrelizumab for RMS was treatment failure due to clinical relapse, MRI activity or both (36/39, 92%). One patient on fingolimod was switched due to hepatic toxicity, and another one (also on fingolimod) because of persistent vomiting after bariatric surgery. The patient on rituximab was switched due to serum sickness.

In the RMS group at baseline, median EDSS at was 2.5, the annualized relapse rate in the previous year was 1.3 ± 0.65, 63% (31/49) of patients had gadolinium-enhancing lesions on MRI, and 61% (30/49) had highly active disease. In the PPMS group at baseline, median EDSS was 3.0 and 43% (9/21) of the patients had gadolinium-enhancing lesions on MRI.

Ninety percent of PPMS patients were treatment-naive, compared to 20% of RMS patients. Before starting ocrelizumab, patients' most recent treatments included beta-interferon ($n = 12$), dimethyl fumarate ($n = 11$), fingolimod ($n = 10$), teriflunomide ($n = 2$), cladribine ($n = 2$), glatiramer acetate ($n = 2$), rituximab ($n = 1$), and alemtuzumab ($n = 1$). No patient switched from natalizumab to ocrelizumab. There was no washout period after beta-interferon and glatiramer acetate, but for patients on fingolimod, it was 1 month; on teriflunomide, 2 weeks, after undergoing the accelerated elimination procedure with cholestyramine; and on dimethyl fumarate, 1 week, except for one patient with lymphopenia that required a longer washout interval. The washout period for the patient on rituximab was 6 months, and the patient on alemtuzumab began ocrelizumab 16 months after the second cycle of alemtuzumab. No patient experienced a relapse during the washout period.

Clinical Course After Treatment Initiation With Ocrelizumab

The clinical course was assessed in all the patients who began treatment with ocrelizumab, with a mean follow-up of 13.6 months (range 6–32). Follow-up was longer in the patients with PPMS compared to those with RMS (17 vs. 12 months, $p < 0.05$). No patient was lost to follow-up. The median number of treatment cycles was 3 (range 2–6).

The clinical and MRI outcomes after ocrelizumab initiation are outlined in **Table 3**. Among the 21 patients with PPMS, one patient (5%) experienced disability progression and discontinued treatment. In the 49 patients with RMS, only one had a relapse, none experienced disability progression, and nine showed disability improvement (18%, 95% CI 10–31%). The annualized relapse rate fell from 1.3 ± 0.65 before ocrelizumab initiation to 0.02 ± 014 after ($P < 0.001$). There was no evidence of clinical activity (relapses and/or disability progression) in 98% of RMS patients.

Baseline MRI showed T1 Gd-enhancing lesions in 57% of the patients (RMS: 63%, PPMS: 43%). All patients except one were free of T1 Gd-enhancing lesions at the first control MRI

TABLE 1 | Baseline characteristics in our cohort of 70 patients with multiple sclerosis treated with ocrelizumab.

Patients	RMS ($n = 49$)	PPMS ($n = 21$)
Age at ocrelizumab start	39.2 ± 10.9	47.1 ± 10.5
Sex (Female)	69%	38%
Time since diagnosis (years)	7.7 ± 6.7	2.8 ± 4.1
Baseline EDSS; median (IQR)	2.5 (2–3)	3.0 (3–4.8)
ARR previous year	1.3 ± 0.65	–
Treatment naive *n/N* (%)	10/49 (20%)	19/21 (90%)
Patients with at least one Gd-enhancing lesions, *n/N* (%)	31/49 (63%)	9/21 (43%)

ARR, anualized relapse rate; EDSS, Expanded Disability Status Scale; IQR, interquartile range.

TABLE 2 | Comorbidities in patients treated with ocrelizumab ($n = 70$).

Comorbidity	*N* patients
Bipolar disease	1
Cerebral palsy	1
Chronic migraine	1
Diabetes mellitus	2
Heart disease	2
Hepatitis B inactive carrier	1
Hodgkin's lymphoma in remission	1
Hypertension	2
Morbid obesity	2
Pituitary adenoma	1
Psoriasis	1
Thrombocythemia	1
Uveitis	1

TABLE 3 | Clinical and MRI outcomes ($n = 70$).

Outcome	
Relapses in RMS patients	
ARR 12 months prior to study inclusion	1.3
ARR after ocrelizumab initiation	0.02
Disability progression (EDSS)	1/70 (1.4%)
MRI	
Patients with Gadolinium-enhancing lesions at:	
Baseline	40/70 (57%)
4–6 months	1/70 (1.4%)
12 months	0/46 (0%)
New or enlarging T2-hyperintense lesions at 12 months	1/46 (2.2%)

ARR, annualized relapse rate; RMS, relapsing multiple sclerosis.

TABLE 4 | Adverse events in 70 patients treated with ocrelizumab.

Adverse event	*n* (%)
Any adverse event	37 (53%)
Infusion-related reactions*	30 (43%)
Mild	14 (20%)
Moderate	16 (23%)
Severe	0
Infections	9 (13%)
Urinary tract infections	5
Pneumonia	1
Cellulitis	1
Gastroenteritis	1
Dental phlegmon	1
Others	2 (3%)
Alopecia areata	1
Biliary colic	1

**Infusion-related reactions included pruritus, sore throat, rash, flushing, urticaria, erythema, headache, irritability and myalgias.*

performed at 4–6 months (69/70, 98.6% $P < 0.001$). At the MRI at 12 months, all patients were free of T1 Gd-enhancing lesions (0/46, $P < 0.001$), and only one patient showed new T2 lesions compared to the previous MRI (2.2%).

The proportion of patients with NEDA was 94% (31/33) in the group of RMS patients who were followed for at least 1 year.

Tolerance and Safety

Just over half (37/70, 53%) of the patients reported adverse events, none of which were serious (**Table 4**). The risk of adverse events was higher in the group of patients with previous DMT (59%) than in the group of patients who were treatment-naïve (45%) but the difference was not statistically significant ($P = 0.257$). The most frequent adverse events were infusion-related reactions: 43% (95% CI 32–55%) reported at least one; all of these were mild to moderate and were treated by reducing the infusion rate and administering symptomatic therapy if needed. The rate of this complication decreased from 40% (28/70) in the first cycle to 16% (11/70) thereafter. Aspirin 300 mg was included in the premedication protocol in some patients to prevent flushing.

Nine patients had infections: five had urinary tract infections and one each pneumonia, gastroenteritis, cellulitis, and dental phlegmon. No patient developed symptoms suggestive of COVID-19. No patient required hospitalization, and no malignancies were detected. The switch from rituximab to ocrelizumab due to rituximab-induced serum sickness was well-tolerated and the patient did not develop serum sickness after the first cycle (two infusions) of ocrelizumab.

Two patients (2.9%) discontinued ocrelizumab; one due to pregnancy and the other one because of lack of efficacy, but none did so because of an adverse event or tolerability.

DISCUSSION

Ocrelizumab has recently been approved in Europe for the treatment of patients with multiple sclerosis, but European data on its real-world use are limited (6–8). Our results support the safety and effectiveness of ocrelizumab in a clinical practice setting.

The results of clinical trials of ocrelizumab may not be generalizable to clinical practice if patients' baseline characteristics are significantly different from those of trial participants. With regard to age, disease duration and the percentage of treatment-naïve patients, our cohort of PPMS patients was similar to that in the ORATORIO phase 3 trial of ocrelizumab. The number of patients with gadolinium-enhancing lesions on the baseline MRI was slightly higher (43.5 vs. 27.5%). Only one of the 21 patients with PPMS in our cohort experienced confirmed disability progression (mean follow-up of 17 months). A recent real-world data study confirmed that ocrelizumab can stabilize disability progression in patients with PPMS and three out of 17 patients even showed clinically relevant improvement in disability status (8). In the ORATORIO trial, pre-specified non-powered subgroup analyses indicated that patients who were younger or had T1 Gd-enhancing lesions at baseline had a greater treatment benefit than older patients or those without T1 Gd-enhancing lesions, which may explain the low rate of disability progression in our cohort (14).

Our results confirm the rapid suppression of new focal brain MRI lesion activity with ocrelizumab. In our cohort, 98.6% of patients were free of T1 Gd-enhancing lesions at the first control MRI performed at 4–6 months. The analysis of phase 2 MRI data of the ocrelizumab 600 mg dose revealed near-complete suppression of T1 Gd-enhancing lesions by week 12 (15). MRI data were lacking in the already published ocrelizumab real-world studies (6–8).

The overall annualized relapse rate of patients with RMS in the study by Ellwardt et al. was 0.17 (95% CI 0.10–0.24), which was very similar to that of the OPERA 1 phase 3 clinical trial (0.16, 95% CI 0.12–0.20). In our cohort, the proportion of patients with NEDA was 94% in the group of RMS patients who were followed for at least 1 year. The greater treatment benefit observed in our study may be due to the higher number of patients with highly active disease (61%). Subgroup analyses comparing ocrelizumab and other disease-modifying therapies (natalizumab, alemtuzumab, fingolimod, cladribine,

teriflunomide, and dimethyl fumarate) have found higher efficacy in patients with more active disease (16–22).

About three quarters of the RMS patients included in the OPERA trial were treatment-naive, and the most common previous therapies were interferon and glatiramer acetate (3). In contrast, in our cohort and in other observational studies most RMS patients had been previously treated with other disease-modifying therapies (6, 7). Nonetheless, prior treatment *per se* did not impact the magnitude of the beneficial effect of ocrelizumab although previous therapies in the pivotal trial and in the observational cohorts were rather different (16). Ocrelizumab in the observational cohorts showed efficacy not only after switching from first-line injectable treatments but also after switching from highly effective therapies such as alemtuzumab, natalizumab, fingolimod, and cladribine, although the participant numbers were small.

As observed in the phase 3 trials and in the real-world studies, mild to moderate infusion-related reactions and mild infections were the most common adverse events. The percentage of infusion-related reactions in our study (43%) was similar to that of the pivotal clinical trials (ORATORIO: 39.9%, OPERA 1: 30.7%, OPERA 2: 37.6%) and higher than in other observational studies (6, 7). The premedication protocol in the three observational cohorts included intravenous methylprednisolone, antipyretics, and antihistamines, but the dose of methylprednisolone was different: we used 100 mg as indicated in the summary of product characteristics while 250 mg was used in the other two studies. Whether the reason for the observed difference in the rate of infusion-related reactions resides in the different doses of methylprednisolone or underreporting from patients warrants further study.

The most common infections observed in the clinical trials of ocrelizumab were upper respiratory tract and urinary tract infections. Minor infections were reported in 8 and 5% of patients in other observational cohorts (6, 7). In our cohort, this proportion was higher (13%) which may be explained by the longer follow-up with ocrelizumab in our series. Besides, it is likely that in our case there was underreporting of upper respiratory tract infections since most patients do not consult their physicians for symptoms of nasopharyngitis. While most reported infections to date have been minor, there have been a few isolated case reports of severe viral infections, such as a fulminant hepatitis associated with echovirus 25 and HSV-2 encephalitis, in patients on ocrelizumab (23, 24). We did not observe any serious infections, while the rate was 1.3% in the OPERA trial and 6.2% in the ORATORIO trial (2, 3).

We observed a very low treatment discontinuation rate with ocrelizumab, consistent with findings from the phase 3 trials and other observational studies. Only two patients (2.9%) discontinued ocrelizumab; one due to pregnancy and the other one because of lack of efficacy, but none did so due to safety issues. The rate of treatment discontinuation due to adverse events was 3.2% in the 96-weeks OPERA 1 trial and 4.1% in the $\geq$120-weeks ORATORIO trial (2, 3). The annual discontinuation rate (3%) was lower for rituximab, another anti-CD20 B cell–depleting antibody, compared to other DMTs in patients with newly diagnosed RMS in a real-world study from Sweden (25).

The effectiveness of ocrelizumab in this study is similar to that of rituximab in a similar general hospital setting although there are some differences concerning secondary infectious adverse events and discontinuation rate (26). The discontinuation rate in this study was 2.8% and no patient required hospitalization due to infectious adverse events while the discontinuation rate was 14.4% in the rituximab observational study from Sweden and four patients (4.8%) required hospitalization due to infectious adverse events. However, the follow-up was longer and the patients slightly older in the Swedish cohort which may explain the observed differences.

The main limitations of this study are the small sample, its retrospective design, a short time of follow-up and the absence of a control group. On the other hand, the study provides MRI and NEDA data that are not available from other real-world studies and a longer time of follow-up. Besides, the study was conducted in a general hospital setting with universal healthcare access, eliminating the bias of a tertiary referral center or unequal access to healthcare or DMTs.

In conclusion, our data confirm the short-term effectiveness, tolerability, and safety of ocrelizumab in real-world clinical practice. Further studies are needed to assess patient outcomes with longer follow-up periods.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Ethics Committee of the Hospital General Universitario de Alicante (reference number: PI-2019-116). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS and LB-R: study design, analysis, drafting and revision of manuscript. IB-S: study design and drafting. AB-SJ: revision of manuscript. LC-A and LV: MRI analyses and revision of manuscript. MA: contributed patients and revised the manuscript. AP-B: performed the statistics and revised the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Anti-CD20 B Cell Treatment for Relapsing Multiple Sclerosis

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Several clinical trials have demonstrated the efficacy of lytic therapies targeting B cells in the treatment of relapsing multiple sclerosis (MS). More modest efficacy has been noted in the primary progressive subtype of MS. Clinical success has increased interest in the role of B cells in the pathogenesis of MS and in ways to potentially improve upon current B cell therapies. In this mini review, we will critically review previous and ongoing clinical trials of anti-CD20 monoclonal antibodies in MS, including rituximab, ocrelizumab, ofatumumab, and ublituximab. Side effects and adverse event profiles will be discussed. Studies examining the proposed mechanisms of action of B cell depleting therapies will also be reviewed.

Keywords: multiple sclerosis, anti-CD20 agent, rituximab, ofatumumab, ocrelizumab, ublituximab

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INTRODUCTION

Four lytic monoclonal antibodies that target the CD20 molecule on B cells have now undergone clinical trials in relapsing multiple sclerosis (RMS). These successful trials showed B cell depletion to be an effective treatment for RMS, focusing scientific attention on the role of B cells in MS. Here we summarize trials using anti-CD20 therapies in RMS and discuss proposed mechanisms of action. Although rituximab (RTX) and ocrelizumab (OCR) have been studied in and OCR is approved for primary progressive MS (PPMS) (1, 2), due to word limitations PPMS studies will not be discussed.

CLINICAL TRIALS OF B-CELL THERAPIES IN RELAPSING MULTIPLE SCLEROSIS

Rituximab

Rituximab is a chimeric mouse-human monoclonal lytic antibody directed at CD20 (3, 4). Two early phase clinical trials of B-cell depletion using RTX as a therapy in relapsing-remitting MS (RRMS) (5, 6) are summarized in **Table 1A**. An early phase 2 trial in 30 RMS patients with contrast-enhanced lesions (CELs) on brain MRI used RTX at oncology dosing (375 mg/m^2 weekly, 4 doses) as add-on to injectable disease modifying therapies (DMTs) (5). It showed 88% reduction of number of CELs on brain MRIs after RTX treatment ($p < 0.0001$).

The HERMES trial was the first double-blind, placebo-controlled trial of RTX in RMS, and demonstrated a significant reduction in CELs ($p < 0.001$) along with an almost 50% reduction in ARR at 48 weeks ($p = 0.04$) vs. placebo (6). CELs remained near zero at 48 weeks, despite no further treatment. These and concurrent observational studies (10) led to increased interest in targeting B cell therapies for the treatment of MS.

As a mouse-human chimeric mAb, development of anti-drug neutralizing mAbs is of concern. RTX is approved for chronic use in several diseases, such as rheumatoid arthritis and Wegener's

granulomatosis (but not for MS in the United States). Depending on the disease, the duration of exposure to RTX and the assay methodology, anti-RTX Abs (not all of which were shown to be neutralizing) have been reported in 11 to >50% of people taking RTX chronically (11).

Ocrelizumab

Ocrelizumab (OCR) is a fully humanized lytic mAb targeting CD20 (14). As a fully humanized mAb, it evokes less anti-drug antibody formation. A phase 2 randomized, double-blind trial compared 600 mg OCR and 2,000 mg OCR delivered intravenously on days 1 and 15 with placebo and with IFNβ-1a 30 micrograms IM weekly 1:1:1:1 in 218 RRMS subjects (**Table 1A**) (14). The study showed 89% reduction in the total number of CELs at 24 weeks in the OCR 600 mg group ($p < 0.0001$) and 96% reduction in the 2,000 mg group ($p < 0.0001$) vs. placebo. ARR at 24 weeks was 0.13 in the 600 mg dose OCR group, significantly <0.64 for placebo and 0.36 for IFNβ-1a. ARR was also reduced in the 2,000 mg dose OCR group compared to placebo but did not reach statistical significance compared to IFNβ-1a.

TABLE 1A | A summary of the phase 2 clinic trials of anti-CD20 therapies in RRMS.

Anti-CD20 mAb	Primary endpoint	Intervention/ control groups	Patient number (% Female)	Mean age ± Standard deviation	ARR [relative reduction] (*p*-value)	Disability progression (*p*-value)	Mean new CELs (*p*-value)	Mean new T2 lesions (*p*-value)	References
RTX (HERMES Trial)	Number of CELs at weeks 12, 16, 20, and 24	RTX 1,000 mg IV	69 (52%)	39.6 ± 8.7	0.20 [50%] ($p = 0.04$)	NR	0.2 ($p < 0.001$)	NS	(6)
		Placebo	35 (29%)	45.5 ± 8.5	0.40	NR	4.5	NS	
RTX	Number of CELs on 3 pre-treatment MRIs vs. 3 post-treatment MRIs	RTX 375 mg/m^2 weekly × 4 doses as add on to IFNβ or glatiramer acetate.	30 (73.3%)	43.5 (20–50)	0.23	NR	88% reduction post-treatment vs. pre-treatment	NR	(5)
OCR	Number of CELs at weeks 12, 16, 20, 24	OCR 600 mg	55 (64%)	35.6 ± 8.5	0.13 [79%] ($p = 0.0005$)	NR	0.8 ($p < 0.0001$)	0.0 ($p < 0.0001$)	(7)
		OCR 2,000 mg	55 (69%)	38.5 ± 8.7	0.17 [73%], ($p = 0.0014$)	NR	0.8 ($p < 0.0001$)	0.0 ($p < 0.0001$)	
		IFNβ-1a 30 mcg/week IM	54 (59%)	38.1 ± 9.3	0.36 [43%] ($p = 0.07$)	NR	7.2	1.8	
		Placebo infusions days 1 and 15, received OCR at 24 weeks	54 (67%)	38.0 ± 8.8	0.64	NR	6.6	1.4	
OFA	Safety	OFA 100, 300, or 700 mg × 2 doses followed by placebo	26 (61.5%)	36.3 ± 7.9	NS	NS	8–24 weeks 0.04 ($p < 0.001$) 24–48 weeks 0.12	8–24 weeks 0.12 ($p < 0.001$) 24–48 weeks 0.12	(8)
		Placebo followed by OFA 100, 300, or 700 mg ×2 doses	12 (50%)	36.0 ± 9.1	NS	NS	8–24 weeks 9.69 24–48 weeks 0.09	8–24 weeks 10.67 24–48 weeks 0.09	
UTX	B cell depletion	Ublituximab 150 mg IV followed by 400 or 600 mg at weeks 2 and 24	36 (reported in subgroups only)	Reported in subgroups only	0.07	12 weeks 7.0% 24 weeks 17.0%	0.00	24–48 weeks 0.2	(9)
		Placebo	12 (NR)	NR	NR	NR	NR	NR	

ARR, annualized relapse rate; CEL, contrast enhancing lesions; mAb, monoclonal Antibody; NS, not significant; NR, not reported; OCR, Ocrelizumab; OFA, Ofatumumab; RRMS, Relapsing Remitting MS; UTX, Ublituximab.

TABLE 1B | A summary of the phase 3 clinical trials of anti-CD20 therapies in RRMS.

Anti-CD20 mAb	Primary endpoint	Intervention/ Control groups	Patient number (% Female)	Mean age ± Standard deviation	ARR [relative reduction] (*p*-value)	Disability progression (*p*-value)	Mean new CELs (*p*-value)	Mean new T2 lesions (*p*-value)	References
OCR (OPERA I)	ARR	OCR 600 mg every 6 months	410 (65.9%)	37.1 ± 9.3	0.156 [46%] ($p < 0.0001$)	12 weeks pooled for OPERA I and II: 9.1% [60%] ($p < 0.001$) 24 weeks pooled 6.9% [60%] ($p = 0.003$)	0.016 ($p < 0.0001$)	0.323 ($p < 0.0001$)	(12)
		IFNβ-1a	411 (66.2%)	36.9 ± 9.3	0.292	12 weeks pooled 13.6% 24 weeks pooled 10.5%	0.286	1.413	
OCR (OPERA II)	ARR	OCR 600 mg every 6 months	417 (65%)	37.2 ± 9.1	0.155 [47%] ($p < 0.0001$)	Pooled data above	0.291 ($p < 0.0001$)	0.325 ($p < 0.0001$)	
		IFNβ-1a	418 (67%)	37.4 ± 9.0	0.290	Pooled data above	0.416	1.904	
OFA (ASCLEPIOS I)	ARR	OFA 20 mg every 4 weeks after 20-mg loading doses on days 1, 7, and 14	465 (64.4%)	38.9 ± 8.8	0.11 ($p < 0.001$)	3 months pooled for ASCLEPIOS I and II: 9.3% [66%] ($p = 0.002$) 6 months pooled 7.5% [68%] ($p = 0.012$)	0.012 ($p < 0.001$)	0.72/year ($p < 0.001$)	(13)
		Teriflunomide 14 mg daily	462 (68.6%)	37.8 ± 9.0	0.22	3 months pooled 13.7% 6 months pooled 10.6%	0.452	4.0/year	
OFA (ASCLEPIOS II)	ARR	OFA 20 mg every 4 weeks after 20-mg loading doses on days 1, 7, and 14	481 (66.3%)	38.0 ± 9.3	0.10 ($p < 0.001$)	Pooled data above	0.032 ($p < 0.001$)	0.64/year ($p < 0.001$)	
		Teriflunomide 14 mg daily	474 (67.3%)	38.2 ± 9.5	0.25	Pooled data above	0.514	4.15/year	

ARR, annualized relapse rate; CEL, contrast enhancing lesions; mAb, monoclonal Antibody; NS, not significant; NR, not reported; OCR, Ocrelizumab; OFA, Ofatumumab; RRMS, Relapsing Remitting MS; UTX, Ublituximab.

Two identically designed phase 3 trials (OPERA I and II) compared OCR with IFNβ-1a in RRMS (12). These multicenter, double-blind, double-dummy, parallel-group trials enrolled 1,656 RRMS patients (OPERA I 821; OPERA II 835) who were randomized 1:1 to receive OCR 600 mg every 24 weeks or IFNβ-1a 44 micrograms subcutaneously injected three times per week over 96 weeks. Patients were between 18 and 55 years of age, with EDSS score ≤5.5, and had at least two documented clinical attacks within 2 years, or 1 within 1 year prior to screening. The primary endpoint, ARR, was reduced relative to IFNβ-1a by 46 and 47%, CELs were reduced by 94 and 95%, the number of new and/or enlarging T2 lesions was reduced by 77 and 83%, and rate of brain volume loss was reduce by 22.8 and 14.9% in OPERA I and OPERA II, respectively. In pre-specified pooled analyses, the percentage of subjects with 3 months confirmed disability worsening (CDW) and 6-months CDW was 40% lower in the OCR groups compared with IFNβ-1a (**Table 1B**).

Ofatumumab

Ofatumumab (OFA) is fully human mAb targeting CD20-expressing cells (15). An early small phase 2, placebo-controlled, double-blind trial demonstrated no safety concerns in 38 RRMS subjects OFA or placebo 2 weeks apart (**Table 1A**) (8). This study was followed by a larger, phase 2, multicenter, double-blind study of OFA (17) that enrolled 232 RRMS subjects randomized to receive subcutaneous OFA 3, 30, or 60 mg every 12 weeks, OFA 60 mg every 4 weeks, or placebo every 12 weeks or every 4 weeks for 24 weeks. After the 12-weeks placebo-controlled period, the placebo group received a single 3 mg OFA dose while the remaining subjects continued their original dose of OFA. At 12 weeks, mean cumulative new CELs was reduced 65% for all OFA groups compared with placebo ($p < 0.001$). *Post-hoc* analysis excluding weeks 1–4 estimated a ≥90% reduction in CELs at 12 weeks for all groups that received ≥30 mg OFA.

Two identically designed multicenter, double-blind, double-dummy, parallel-group phase 3 trials compared 20 mg subcutaneous OFA every 4 weeks to 14 mg oral teriflunomide daily, randomized 1:1 (13). The trials enrolled 927 and 955 subjects, respectively. Subjects enrolled were mostly RRMS, but a small percentage (5.9% in ASCLEPIOS I and 5.6% in ASCLEPIOS II) had active secondary progressive MS (SPMS). Inclusion required at least 2 documented clinical attacks within 2 years or 1 within 1 year of screening, or a CEL on MRI in the year before randomization. EDSS scores at baseline were $\leq$5.5. Both studies met their primary endpoint with similar and significant relative reductions in ARR in the OFA arms. ARR for the OFA arms were reduced by over 50% relative to the teriflunomide arms (0.11 vs. 0.22 and 0.10 vs. 0.25 in ASCLEPIOS I and II, respectively). Significant (>30%) reductions in CDW relative to teriflunomide were seen in pooled 3 and 6-months CDW analyses (10.9 vs. 15.0% and 8.1 vs. 12.0%, respectively). Six-months sustained disability improvement favored the OFA arm in both trials but did not reach statistical significance. Significant reductions in mean CELs per scan (0.01 vs. 0.45, and 0.03 vs. 0.51 in ASCLEPIOS I and II, respectively) and new or enlarging T2 lesions per year (0.72 vs. 4.00, and 0.64 vs. 4.15, respectively) were seen in the OFA vs. teriflunomide groups. Serum neurofilament light chain concentrations were reduced in the OFA arm relative to teriflunomide arm by 7% at month 3, 27% at month 12, and 23% at month 24 in ASCLEPIOS I and by 11% at month 3, 26% at month 12, and 24% at month 24 in ASCLEPIOS II (**Table 1B**).

Ublituximab

Ublituximab (UTX) is a chimeric mAb targeting CD20 that has been glycoengineered to remove sugar molecules, resulting in enhanced lytic potency (18) (**Table 2**). In a phase 2, placebo-controlled trial of UTX, 48 RRMS subjects were randomized 3:1 to receive UTX IV or placebo on day 1, day 15, and week 24 (9). No CELs were seen at weeks 24 and 48, and a 10.6% reduction in T2 lesion volume was seen in the UTX group vs. placebo. Further studies of this drug are ongoing. As a chimeric mAb, the potential development of anti-UTX Abs will need to be monitored.

SAFETY CONSIDERATIONS

Infusion Reactions

Infusion reactions were the most common adverse events in the OCR and RTX phase 3 trials. These were mostly mild to moderate in severity and decreased in rate and severity with subsequent dosing. There were no fatal or life-threatening reactions in the phase 3 trials. Longer-term safety event reporting data suggest that infusion related reactions occur at similar rates in MS patients treated with RTX and OCR at 4.82 and 4.76%, respectively (19). Injection reactions with subcutaneous OFA occurred at 16.1 and 24.1% in the ASCLEPIOS I and II trials, respectively and were largely confined to the first dose.

Infections Including Progressive Multifocal Leukoencephalopathy (PML) and COVID-19

The most common minor infections seen in the phase 3 trials of RTX, OCR, and OFA were upper respiratory infections, nasopharyngitis, and urinary tract infections (UTIs). These occurred at similar rates in the anti-CD20 groups of these trials. Recent real-world safety reporting data showed nearly a 2-fold higher rate of minor infections in OCR compared to RTX with significantly higher rates of UTIs, nasopharyngitis, and oral herpes (19).

As of end of January 2020, nine cases of definite PML according to AAN criteria have been reported in MS patients receiving OCR (20). Seven of these cases were in anti-JCV antibody positive patients who had previously received natalizumab; one case occurred in a patient previously treated with fingolimod. One reported case of PML occurred in a patient treated with OCR that had not received prior DMTs, but this was confounded by older patient age (78 years) and low absolute lymphocyte counts prior to OCR treatment.

A potential risk of anti-CD20 therapies in people with MS infected with SARS-CoV-2 has been reported. A retrospective study of 784 MS patients with SARS-CoV-2 infection conducted by the Italian MS and COVID-19 registry found increased risk of severe COVID-19 in people treated with OCR or RTX with an odds ratio of 2.59 ($p = 0.002$) (21). A multi-center retrospective French study with only 347 total patients did not find an association of severe COVID-19 with anti-CD20 therapies (22). The North American COViMS Registry has reported 858 MS patients with SARS-CoV-2 infection (23). Multivariable logistic regression analysis demonstrated an OR of 2.31 ($p < 0.002$) for those on anti-CD20 to have higher chance of death, ICU or hospitalization compared with those on other DMTs. None of these early reports have true denominators. Research in this area is ongoing; COViMS Registry and several other worldwide efforts continue to accrue data.

Malignancy

Fifteen malignancies were observed over the 96-weeks study periods in patients randomized to OCR compared to 4 in the IFNβ-1a or placebo groups in the phase 3 trials in RRMS and PPMS. The latest OCR package insert (dated May 2020) states that "an increased risk of malignancy, including breast cancer, may exist with OCREVUS." We recommend that all patients taking anti-CD20 mAbs closely adhere to standard cancer screening guidelines, including periodic skin checks for skin cancers.

MECHANISM OF ACTION OF ANTI-CD20 MONOCLONAL ANTIBODIES IN MULTIPLE SCLEROSIS

The most consistent laboratory abnormality found in MS patients is increased intrathecal production of antibodies (Abs), which is most sensitively detected as cerebrospinal fluid (CSF)-restricted oligoclonal bands (OCB). CSF-restricted OCBs are present in

TABLE 2 | A summary of anti-CD20 antibody type, target, and mechanisms of lysing B lymphocytes.

mAb	Antibody type and target	Mechanism of action	References
RTX	Mouse/human chimeric IgG1 mAb that targets CD20	Lyses B cells by direct signaling of apoptosis, complement activation, and ADCC	(3, 4)
OCR	Humanized IgG1 mAb that targets CD20	Lyses B cells by ADCC and complement mediated lysis	(14)
OFA	Fully human IgG1 mAb that targets CD20	Lyses B cells by CDC and ADCC	(15)
UTX	Mouse/human chimeric IgG1 mAb glycoengineered for high affinity for FcγRIIIa	Enhanced lyses of B cells via ADCC compared to RTX Similar effects on apoptosis and CDC compared to RTX	(16)

Ab, Antibody; ADCC, antibody dependent cellular cytotoxicity; CDC, complement dependent cytotoxicity; mAb, monoclonal antibody; OCR, Ocrelizumab; OFA, Ofatumumab; RTX, Rituximab; UTX, Ublituximab.

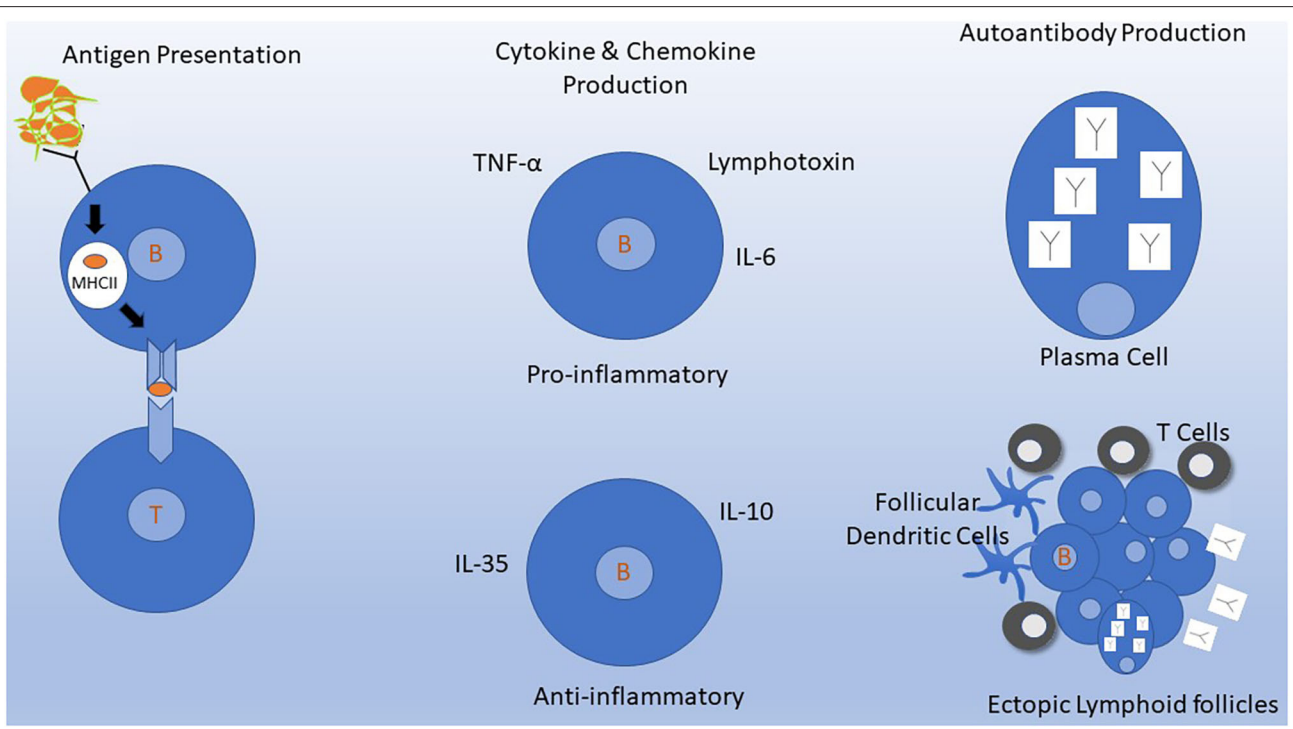

FIGURE 1 | Mechanisms of B cells in MS pathogenesis. Depletion of B cells by targeting CD20 molecules on the B cell surface inhibits several of these mechanisms either directly or indirectly. (Left) Antigen Presentation. B cells efficiently process and present antigens to T cells. B cells constitutively express MHC molecules and T cell costimulatory molecules that allow for interaction and activation of autoreactive T cells. This process occurs in lymph nodes and may also occur in meningeal ectopic lymphoid follicles in the meninges (bottom right). (Middle) Naive and memory B cells can produce cytokines and chemokines that have various downstream effects on the immune system. B cell signaling via B cell receptor engagement and CD40 leads to production of several pro-inflammatory cytokines and down regulation of IL-10. (Upper Right) Intrathecal antibody production detected by CSF oligoclonal banding is the most consistent laboratory abnormality identified in MS. Antibodies are produced primarily by plasma cells which do not express CD20 and thus are not depleted by anti-CD20 monoclonal antibodies in the short term. (Lower Right) Ectopic lymphoid follicles containing B cells, T cells, follicular dendritic cells, and plasma cells develop at sites of chronic inflammation. In MS, these can develop in the meninges, where their presence has been associated with a worse clinical course.

more than 90% of persons with definite MS (24). Elevated levels of CSF IgG and IgM, and number of OCBs have been correlated with worse MS prognosis (25, 26). This indirectly implicates B lymphocytes, as B cells produce Abs. However, plasma cells (which differentiate from B cells but do not express CD20) are the long-lived cells that produce most Abs.

Some insights into the mechanisms of action of B cell depletion with anti-CD20 mAb derived from the Phase 2 study we performed using oncologic doses of RTX (375 mg/m^2 weekly × 4 weeks). In this MRI-blinded open-label study (5), 26 of the 30 subjects underwent CSF and blood collection before and 6 months after RTX treatment. B cells declined in the CSF after RTX treatment in 20 of the 26 subjects ($p < 0.0001$ by Wilcoxon matched pairs test). In the remaining six subjects, B cells were undetectable in CSF prior to or after RTX. CSF Abs as measured by IgG index, IgG concentration, and oligoclonal band number

did not decline 6 months post-RTX (27). Because the major producers of Ab are plasma cells that do not express CD20, this was not surprising. Given the rapidity of the beneficial effects of anti-CD20 mAbs in RRMS, these studies suggested that reduced Ab levels are unlikely to be critical for the mechanism of action of anti-CD20 mAb therapies.

However, B cells have other functions aside from their role in Ab production (**Figure 1**). B cells comprise several subtypes, such as naïve and memory B cells, and including B cells that produce proinflammatory (e.g., IL-6), or anti-inflammatory (e.g., IL-10) cytokines (28). Memory B cells are strongly implicated in the underlying pathophysiology of MS (29, 30). For this reason, studies to test the possibility of tailoring anti-CD20 treatments to target continued absence of circulating memory B cells are being pursued (31). Bar-Or and colleagues showed that B cell signaling via the combination of B cell receptor engagement and CD40 leads to production of several pro-inflammatory cytokines (e.g., lymphotoxin and tumor necrosis alpha), while reducing B cell production of IL-10 (32). Two chemokines, CXCL13 and CCL19, were significantly decreased ($P = 0.002$, $P = 0.03$, respectively) in post-RTX CSF (27). Lysis of B cells using anti-CD20 eliminates their production of cytokines and chemokines and may contribute to the mechanism of action of anti-CD20 treatments.

T cells were also reduced in the CSF of 81% of subjects 6 months after RTX treatment (27, 33, 34). The mean reduction of CSF B cells was 95% and of CSF T cells was 55%. T cells were reduced in the CSF to a larger degree than the 12% reduction observed in blood, suggesting reduced T cell trafficking into the CNS. Activated T cells express CXCR5, the receptor for the chemoattractant factor CXCL13. CXCL13 is increased in CSF during several CNS inflammatory conditions, including MS (35) and CXCL13 was reduced 6 months post-RTX (27). Reduced trafficking of T cells into the CSF appears to be an indirect consequence of B cell elimination, especially since B cells themselves do not produce CXCL13 (36). A better understanding of the manifold effects of anti-CD20 mAb therapy in MS is expected from a multicenter longitudinal study that is underway (NCT02688985).

B cells are constitutively able to process and present antigen to T cells, and they are extraordinarily efficient at this when presenting their own cognate antigen to T cells recognizing the same antigen (37, 38). B cells that target myelin recognize it via surface B cell receptors, which enables efficient antigen capture of a self-antigen that is at low concentration. As B cells constitutively express MHC-I and MHC-II and the T cell costimulatory molecules CD86 and CD80, they are ready to process and present antigens to pathogenic autoreactive T cells (39). A process by which B cells that capture low concentration myelin antigens and then serve as antigen-presenting cells (APCs) to activate myelin-reactive T cells is postulated to be a trigger of MS activity. The interaction of T with B cells further cross-activates the B cells. Several groups have reported evidence that this process can occur in deep cervical lymph nodes (40, 41). In established MS, the process may occur in meningeal ectopic lymphoid-like structures (42).

Yet another mechanism through which anti-CD20 mAbs may act is the elimination of CD20-expressing T cells. A small proportion of circulating T cells express surface CD20; these cells are eliminated by anti-CD20 treatments. $CD20^+$ T cells comprise only 3–5% of circulating T cells of healthy persons (43), but comprise a slightly higher proportion (up to 10%) in MS patients (44, 45). $CD4^+$ and $CD8^+$ $CD20^+$ T cells produce pro-inflammatory cytokines, such as interferon gamma, TNF-alpha and GM-CSF, which could contribute to MS pathogenesis (45). In MS, CSF T cells are enriched for those expressing $CD20^+$, but are still <50% of CSF T cells (45) suggesting that the CSF T cell reduction observed after RTX treatment cannot be fully explained by their lysis by anti-CD20 mAb.

An early report using the lytic anti-CD19 mAb, inebilizumab, in MS showed benefit on MRI activity to a similar strong degree as seen with anti-CD20 therapies (46). This study may provide insights, as the CD19 molecule is expressed on B cells but not on T cells. Results using inebilizumab hinted that lysis of $CD20^+$ T cells is not responsible for all beneficial effects of anti-CD20 mAb treatments.

OTHER EMERGING B CELL THERAPIES IN MS

Success in therapeutics with targeting CD20 on B cells has raised interest in other mechanisms to target B cells, but the results have not always been as expected. Early on, the drug atacicept was tried in two studies, one in optic neuritis patients in hope of preventing MS development and the second in MS patients in a phase 2 trial. Atacicept is a human recombinant fusion protein that comprises the binding portion of a receptor for both B-Lymphocyte Stimulator (B-LyS) and A PRoliferation-Inducing Ligand (APRIL), two important factors supporting B cell maturation and survival. Unexpectedly atacicept led to more attacks (47). These ill-fated trials may in fact point to the importance of memory B cells in MS activity because, while inhibiting late state B cells and plasma cells, atacicept selectively spares memory B cells (48).

Bruton's tyrosine kinase (BTK) is a cytoplasmic enzyme important for B cell signaling; inhibition of BTK results in B cell inhibition (49). A phase 2 placebo-controlled trial in RMS patients of varying doses of the oral BTK inhibitor evobrutinib showed fewer CELS at the higher doses compared to placebo (50). Currently, this and several other BTK inhibitors are being studied in MS patients. Early reports suggest moderate efficacy of BTK inhibitors that is not as profound as that seen with anti-CD20 mAb therapies.

CONCLUSIONS

In summary, eliminating circulating $CD20^+$ B cells leads to a profound reduction in MS clinical and MRI activity in RMS patients. B cells likely contribute to MS pathogenesis in several ways, including their enhancement of T cell activation and proliferation. B cells are critical for capturing and presenting low concentration antigens, such as myelin proteins to T cells.

B cells also likely contribute to MS pathogenesis by direct and indirect production of pro-inflammatory cytokines and chemokines. Elimination of pro-inflammatory $CD20^+$ T cells may also play a role. The mechanism by which B cells contribute to MS activity appears to be independent of their role in Ab production. Collecting longer-term safety data will be important to determine the safety of using these therapies chronically. Studies to determine exactly how B cell depletion inhibits MS activity will undoubtedly lead to better understanding of MS pathogenesis.

AUTHOR CONTRIBUTIONS

CR analyzed the clinical trials data and safety information and drafted the manuscript on these topics. AC analyzed the mechanisms of action data and drafted the manuscript, revised the draft of clinical trials and safety data, and approved the final draft. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Role of B Cells in Primary Progressive Multiple Sclerosis

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The success of ocrelizumab in reducing confirmed disability accumulation in primary progressive multiple sclerosis (PPMS) via CD20-targeted depletion implicates B cells as causal agents in the pathogenesis of PPMS. This review explores the possible mechanisms by which B cells contribute to disease progression in PPMS, specifically exploring cytokine production, antigen presentation, and antibody synthesis. B cells may contribute to disease progression in PPMS through cytokine production, specifically GM-CSF and IL-6, which can drive naïve T-cell differentiation into pro-inflammatory Th1/Th17 cells. B cell production of the cytokine LT-α may induce follicular dendritic cell production of CXCL13 and lead indirectly to T and B cell infiltration into the CNS. In contrast, production of IL-10 by B cells likely induces an anti-inflammatory effect that may play a role in reducing neuroinflammation in PPMS. Therefore, reduced production of IL-10 may contribute to disease worsening. B cells are also capable of potent antigen presentation and may induce pro-inflammatory T-cell differentiation via cognate interactions. B cells may also contribute to disease activity via antibody synthesis, although it's unlikely the benefit of ocrelizumab in PPMS occurs via antibody decrement. Finally, various B cell subsets likely promulgate pro- or anti-inflammatory effects in MS.

Keywords: B cell, multiple sclerosis, immune pathogenesis, inflammation, primary progressive multiple sclerosis

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INTRODUCTION

Multiple Sclerosis (MS) is the most prevalent chronic demyelinating disorder of the central nervous system (CNS) affecting more than 2 million people worldwide and over 700,000 people in the United States (1). There are multiple different subtypes of MS. Most common is the relapsing remitting MS (RRMS) subtype that affects the vast majority of MS patients. Approximately 85–90% of patients present with RRMS (2), which is characterized by relapsing and then remitting neurological deficits without progressive disability between relapses. In later stages, RRMS patients may exhibit ongoing worsening without obvious remission, termed secondary progressive MS (SPMS). Roughly 36–60% of patients who first develop RRMS will go on to develop SPMS, on average 10 years after disease onset (3, 4). A less common subtype, primary progressive MS (PPMS), is characterized by gradual worsening of neurological function from disease onset without evidence

of remission. Approximately 10–15% of patients with MS have PPMS (2). Of all the MS subtypes, PPMS has the worse prognosis, with patients reaching much higher levels of disability compared to patients with RRMS and SPMS (5). The pathophysiologic mechanisms leading to these distinct clinical phenotypes in MS subtypes is an area of ongoing research. The pathological hallmarks of MS are inflammation, demyelination, remyelination, and neurodegeneration occurring either focally or diffusely in the brain and spinal cord (6). These features are present in all MS subtypes, although in PPMS and SPMS there is a predominance of diffuse low level inflammation, slowly expanding pre-existing lesions, and a more intact blood brain barrier when compared to RRMS (7).

B cells have been implicated in the pathology of MS through the presence and diagnostic significance of oligoclonal bands (8–11), an increased concentration of unique B cells subsets in the periphery and CNS of MS patients (12–15), and the formation of CNS ectopic lymphoid follicles (16–18). B cells may contribute to disease progression in PPMS through cytokine production, antigen presentation and antibody synthesis. A summary of the mechanism of action of B cells in the immunopathogenesis of PPMS is shown in **Figure 1**. Further, the effect of B cells in MS is likely subset-dependent with some B cells exerting an anti-inflammatory effect (19–21), while others a pro-inflammatory effect (22, 23). The influence of various B cell subgroups in MS is supported by clinical trial data, which demonstrates a reduction in relapses in RRMS patients treated with anti-CD20 antibodies (24) and an increased relapse rate after depletion of plasma cells and late stage B cells (23). In PPMS, the success of ocrelizumab in reducing disability progression is likely a result of selective depletion of pro-inflammatory B cell subsets in PPMS patients with MRI evidence of clinically significant ongoing inflammation

PROGRESSIVE MS PATHOLOGY AND CLINICAL CHARACTERISTICS

The pathology of PPMS and SPMS are characterized by widespread diffuse inflammation with slowly expanding lesions, abundant cortical demyelination, brain atrophy, and lymphocyte infiltration and microglial activation in normal appearing white matter (25). In contrast, RRMS is typified by new and active focal inflammatory demyelinating lesions in the CNS white matter. The pathogenic mechanisms underlying PPMS and SPMS are incompletely understood and it remains unclear whether these disease subtypes are caused by similar or unique pathogenic mechanisms (26). Increasing recognition that relapses and MRI-identified lesion activity also occur in some patients with PPMS and SPMS, typically in the early stages of the disease, led to a modification of the phenotypic categories of progressive multiple sclerosis (27). Recent guidelines for diagnosing PPMS and SPMS now include two qualifiers: (1) with or without disease activity, defined by MRI or clinical evidence of inflammatory lesions or relapses; and (2) with or without progression, defined as gradual worsening disability independent of relapses (27). There are multiple areas of clinical and pathological overlap between the different disease subtypes which have led researchers to hypothesize that two distinct yet related pathophysiologic mechanisms are driving the phenotypic differences seen in these subtypes of MS (28, 29). More specifically, one emerging concept is that relapses and remissions, characteristic of RRMS, are caused by an inflammatory process driven by autoreactive effector T cells, while progressive accumulation of disability without remission, characteristic of SPMS and PPMS, is the result of a neurodegenerative process driven by dysfunction of the innate immune system and B cells (30).

There are now over 20 FDA approved disease modifying agents for MS, with one designated by the FDA as an effective option for PPMS. Ocrelizumab is the only FDA approved medication for PPMS, having been approved in early 2017 (31). Ocrelizumab is a monoclonal antibody that targets CD20, a cell marker found principally on B cells (24). The mechanism of action of ocrelizumab is considered to be mainly anti-inflammatory via selective depletion of B cells. In a randomized double-blinded, placebo-controlled trial in patients with PPMS, ocrelizumab reduced confirmed disability as defined by slowed advancement in the expanded disability status scale (EDSS) (31). Prior to this, numerous other immune-targeting therapeutic drugs approved to treat RRMS had been trialed in PPMS without success. Interferon β-1a (32) and β-1b (33), fingolimod (34), rituximab (35) and glatiramer acetate (36) were ineffective at reducing disability accumulation in PPMS. Dronabinol (37) and high dose biotin (38) were also trialed in PPMS and SPMS with the hope that these drugs would promote neuroprotection and repair. Dronabinol showed no significant change in disease worsening, whereas high dose biotin demonstrated disability improvement in 12.6% of patients compared with 0% of the placebo arm in a randomized double-blinded placebo controlled study (38). However, a phase III clinical trial of high dose biotin in the treatment of PPMS and SPMS demonstrated that high dose biotin failed to meet its primary and secondary endpoints, including improvement of disability or progression of disability (39). Research into the mechanisms by which biotin may exert a beneficial effect in progressive MS is ongoing (40). Teriflunomide (41), natalizumab (42), alemtuzumab (43), mitoxantrone (44), and hematopoietic bone marrow transplantation (45, 46) have also been found to alter B cells in MS patients but have not been tested in large scale clinical trials for PPMS. It is not currently known whether PPMS is pathogenetically distinct from RRMS and SPMS but the clinical success of Ocrelizumab in PPMS, viewed in the context of the failure of other disease-modifying therapies, implies a difference in the disease mechanism of PPMS. The mechanism or mechanisms by which B cell depletion produces a therapeutic effect in PPMS will be explored herein.

EVIDENCE FOR A PATHOGENIC ROLE OF B CELLS IN PPMS

Oligoclonal Bands

The presence of unique oligoclonal bands (OCBs) and increased intrathecal IgG synthesis by antigen experienced B cells has long been recognized as a component of MS (47). OCBs are

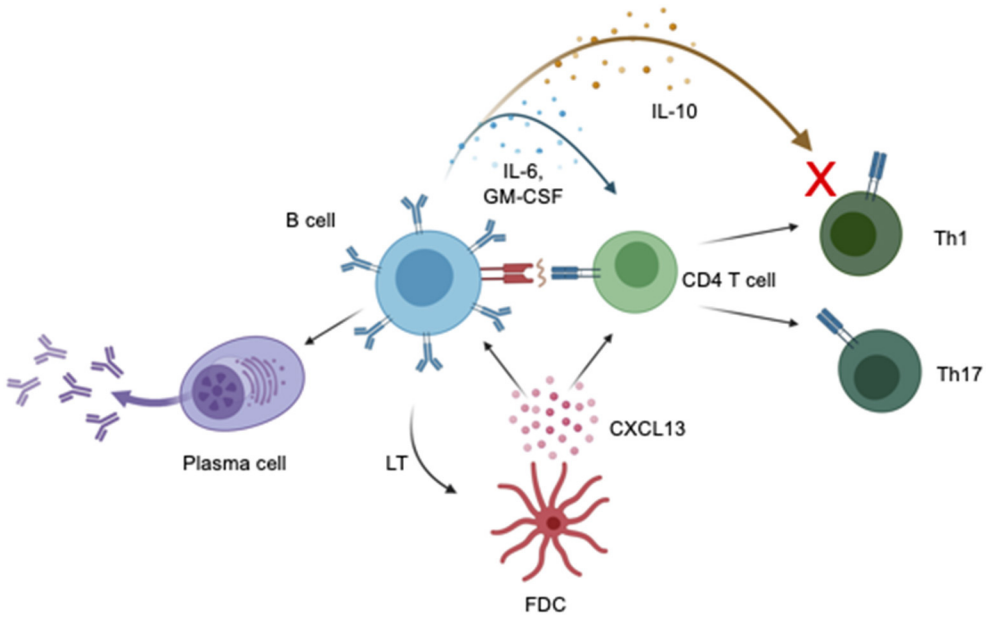

FIGURE 1 | Impact of B cells on PPMS pathogenesis. Production of cytokines influences the function of CD4 T cells, including promoting and suppressing inflammation. Production of the cytokines IL-6 and GM-CSF can induce differentiation of CD4 T cells into Th1 and Th17 T cells which can then cause CNS damage. The cytokine IL-10 is believed to decrease activity of Th1 effector T cells and reduce neuroinflammation in EAE and MS. Decreased IL-10 production by B cells may result in increased neuroinflammation in MS. B cells induce T cell activation and differentiation into pro-inflammatory T cell subsets via antigen presentation via the tri-molecular complex of MHCII, antigen, and T cell receptor. B cells are capable of differentiating into antibody secreting cells which produce antibodies capable of directly damaging the CNS. Binding of lymphotoxin (LT) by follicular dendritic cells induces secretion of CXCL13 which may serve as a chemoattractant for B cells and T cells, increasing lymphocyte infiltration into the CNS. Created with BioRender.com.

detectable in ∼95% of patients upon first presentation who subsequently go on to develop MS. Further, OCBs may have prognostic value in determining the likelihood of progression from CIS to multiple sclerosis and disability accumulation (9–11). Preferential expression of variable gene segments in isolated CNS immunoglobulin from patients with MS indicate that immunoglobulins present in the CNS have undergone affinity maturation likely driven by the presence of a specific antigen (48, 49). MS patients with OCBs have a more aggressive disease course than MS patients without OCBs (50). Moreover, in patients with progressive disease, oligoclonal IgM bands in the CSF are linked to faster progression into SPMS (51, 52) and with active inflammation in PPMS (53). These findings indicate a potential pathogenic role for intrathecal immunoglobulins in MS.

B Cells in the CSF and Peripheral Blood

In healthy patients, B cells are hardly detectable in the CSF, whereas in MS the mean frequency of B cells among CSF leukocytes is about 5% (12, 13). In contrast to B cells in the periphery, most B cells in the CNS are memory B cells, identified by surface expression of CD27. In patients with RRMS, elevated features of neurodegeneration, as revealed by MRI, correlate with increased numbers of peripheral B cells and a higher proportion of activated B cells (14). Patients with SPMS have greater numbers of specific B cell populations in their peripheral blood, notably DC-SIGN$^+$ B cells and CD83$^+$ B cells (54), which correlate with disease progression. Another study found that CD19$^+$ B lymphocytes expressing TNFα in the periphery are increased in patients with PPMS compared to patients with SPMS, RRMS, or healthy controls (15). CD19$^+$ B lymphocytes from RRMS and SPMS patients display hyper-phosphorylation of p65 (55), but this hyperactivity has not been confirmed in PPMS. In addition, anti-inflammatory "regulatory B cells", which produce IL-13, IL-10, and TGF-β, are reduced in all subtypes of MS compared to healthy controls. Multiple studies have reported flow cytometric characterization of the phenotype of both CSF and peripheral immune cells that offer insight into the possible underlying mechanisms leading to B cell proliferation and activation in MS. Overall, the presence of increased numbers of activated B cells in the CSF and periphery of patients with PPMS and SPMS, the lack of regulatory B cells in all forms of MS, and the correlation of increased B cell populations with disease progression in patients with SPMS indicate a unique role for B cells in the pathology of progressive MS.

Ectopic Lymphoid Follicles

The CNS is separated physically from the peripheral circulation by the blood brain barrier (BBB), which compartmentalizes the CNS and restricts leukocyte migration into the brain and spinal cord. Historically, the CNS was believed to be an immune-privileged site, but recent evidence has demonstrated a steady trafficking of memory T cells between the periphery and the CNS (56). It is hypothesized that memory T cells enter the CSF using specific adhesion molecules, chemokines, and chemokine receptors and enter the CSF through the epithelium of the choroid plexus (56). These memory T cells then circulate through

the CSF and interact with CNS myeloid antigen presenting cells (APCs) within the subarachnoid space surrounding the leptomeninges where they may propagate an immune response. Diverse immune cell infiltrates have been observed in the leptomeninges in patients with RRMS, SPMS, and PPMS (17, 18). Numerous studies have linked these immune cell infiltrates to demyelination and neuronal degeneration in the adjacent cortex (18, 57) leading researchers to postulate that leptomeningeal inflammation is a potential driver of disease progression in MS (16). The spectrum of leptomeningeal inflammation exhibits significant variability, ranging from disorganized collections of immune cells in some patients to well-organized collections of immune cells with many features similar to lymphoid tissue in others (58). These well-organized immune cell structures have been termed ectopic lymphoid follicle-like structures (ELFs) and are characterized by separate B and T cell regions, a network of follicular dendritic cells, plasma cells, and proliferating B cells with evidence of ongoing germinal center reactions (59). While the true incidence and significance of these ELFs in MS patients is still under heavy scrutiny, they are not uncommon in progressive forms of MS. One autopsy study found evidence of ELFs in the meninges in up to 40% of patients with SPMS, but not in RRMS or PPMS (59). Further autopsy series of patients with MS have supported the presence of meningeal ELFs in patients with SPMS (17). Notably, the presence of ELFs is linked with increased cortical demyelination (17).

Further studies looking exclusively at PPMS found no proof of ELFs but instead evidence of more widespread disorganized leptomeningeal inflammation (16). In an autopsy series of 26 patients with PPMS, formal organization of ELFs was not detected; however, a subset of PPMS patients had extensive meningeal immune cell infiltration, consisting of both B and T cells. Patients with evidence of widespread leptomeningeal inflammation had a more severe clinical course and increased cortical demyelination. Further investigation corroborated these findings and demonstrated the presence of ELFs in patients with SPMS and generalized leptomeningeal inflammation in patients with PPMS and RRMS (60). Interestingly, in progressive patients, leptomeningeal inflammation is only present in patients with pathologically active disease defined as the presence of classically active or slowly expanding lesions at the time of autopsy. Patients with pathologically inactive plaques do not display features of leptomeningeal inflammation (60). The makeup of the leptomeningeal immune cell infiltrate varies by disease subtype, with an increased prevalence of plasma cells in patients with either PPMS or SPMS. Additionally, progressive patients with pathologically inactive disease have levels of overall leptomeningeal inflammation similar to those of healthy controls but still have a modest but significantly higher number of plasma cells and overall B cells (60). Leptomeningeal inflammation, given that it is more prevalent in the subset of patients with PPMS who had active disease and can be visualized on MRI (60, 61), may serve as a potential biomarker to identify patients with PPMS who may benefit most from B cell therapy.

Given the correlation between both ELFs in SPMS and widespread disorganized leptomeningeal inflammation in PPMS with adjacent cortical pathology, it is possible that leptomeningeal inflammation is an independent driver of disability, particularly in progressive MS (16, 17). However, the specific role of leptomeningeal inflammation in MS pathogenesis remains an area of active debate. Some studies have described extensive subpial demyelination in patients with PPMS and SPMS without convincing evidence of ELFs or B cell infiltration (62). This seems to indicate that leptomeningeal inflammation with ELFs or B cells may not be needed for cortical demyelination observed in these patients. Additionally, given that most research data on leptomeningeal inflammation in MS comes from autopsy series, the possibility that the leptomeningeal inflammation is a secondary response to primary cortical demyelination rather than a causative factor remains.

MECHANISM OF ACTION OF B CELL MEDIATED DISEASE PROGRESSION IN PPMS

Antibody Production

The presence of unique oligoclonal bands in the CSF of MS patients led to the hypothesis that B cells could be contributing directly to MS pathogenesis via autoantibody mediated CNS tissue damage (47). This idea is supported by the presence of CNS B cell clonal populations in patients with MS that demonstrate evidence of somatic hypermutation and antigen driven affinity maturation (48, 49). Additionally, plasma cells isolated from the CSF of MS patients produce antibodies that make up oligoclonal bands (63). Compared to RRMS patients, SPMS and PPMS patients have higher amounts of plasma cells in perivascular and meningeal immune cell infiltrates indicating a unique role of plasma cells in progressive disease (60). Early studies exploring the role of antibodies in MS pathogenesis demonstrated antibodies bound to disintegrating myelin in acute MS lesions at autopsy and in the marmoset model of EAE (64). Immunoglobulins bound to myelin could induce tissue damage via complement activation (65), activation of microglia/macrophages via activating Fc receptors (66), disturbance of oligodendrocyte physiology (67), or by proteolytic activity on myelin basic protein (68). Additionally, the number of antibody-secreting plasma cells increases with age in patients with PPMS and SPMS (60). Overall, these data indicate that CNS plasma cell antibody production could be playing a role in PPMS disease progression.

It should be emphasized that no specific self-antigen has yet been identified that has consistently been verified as an autoantibody target in MS (69). Evidence supporting intrathecal antibody-mediated injury derives from a study involving adoptive transfer of Ig from the CSF of PPMS patients to naïve mice. These mice succumbed to motor deficits paralleled by CNS pathology, including demyelination and axonal loss within the spinal cord (70). Many potential self-antigens have been implicated by the presence of specific autoantibodies in patients with PPMS. Candidate targets for auto-antibodies in PPMS include anti-neurofilament light (71), anti-ganglioside GM3 (72), and anti-SPAG16 (54). However, these antibodies have not been

reliably detected in large populations of PPMS patients, nor has a causal mechanism of injury been well-established. In a study of patients with all subtypes of MS, antibodies specific to KIR4.1 (an ATP-sensitive inward rectifying potassium channel expressed found primarily on glial cells) were found in roughly half of the subjects. However, the presence of anti-KIR4.1 antibodies did not correspond to a specific MS phenotype (73) and subsequent studies have failed to reproduce these findings (74). Overall, while many autoantibodies have been identified in patients with PPMS, no specific autoantibody has been reliably linked to CNS damage.

Clinical data from anti-CD20 treatment of patients with MS argues against a link between treatment benefit and antibody production. B cells down-regulate CD20 expression as they develop into plasma cells and thus mature plasma cells secreting antibodies do not express CD20 (75). Therefore, plasma cells are not directly targeted by ocrelizumab or rituximab and anti-CD20 therapies are unlikely to have a direct impact on intrathecal antibody levels, at least in the short term. This is supported by a lack of measurable change in total serum antibody levels in MS patients treated with rituximab, even in those patients experiencing clinical benefit (76). Additional clinical studies specifically evaluating rituximab's effect on antibody levels have confirmed that rituximab does not change peripheral antibody levels (77). Further, CSF IgG levels, IgG index and oligoclonal band numbers are also unchanged in patients with RRMS treated with rituximab, even in the presence of depleted CSF B and T cells (78). Given that anti-CD20 therapy depletes the vast majority of plasma cell precursor cells, it's possible that long-term CD20-targeted B cell depletion therapy may impact plasma cells in treated patients and thereby alter antibody levels, but antibody modulation does not appear to contribute to the clinical benefit seen shortly after treatment in MS.

Cytokine Production

B cells exert both pro-inflammatory and anti-inflammatory effects depending on distinct cytokine production (79). B cells are capable of controlling the polarization of effector T cell responses and the formation of memory T cells through cytokine secretion (79). A subset of B cells exhibits anti-inflammatory properties through the secretion of IL-10, TGF-β and IL-35. These unique B cells are identified by CD markers CD19 and CD138 and have been termed "regulatory B cells" due to their hypothesized role in the production of these anti-inflammatory cytokines (79, 80). B cells also produce cytokines that induce T-cell differentiation toward Th1, Th2, or Th17 subtypes (81) and exert an anti-inflammatory role in mouse models of autoimmunity (80).

Patients with RRMS and SPMS have a dysregulated cytokine network, specifically demonstrating a decrease in the anti-inflammatory cytokine IL-10 (82). B cells (particularly memory B cells) isolated from individuals with RRMS and SPMS can also be activated to produce abnormally high amounts of the cytokines TNF-α, LT-α, IL-6, and GM-CSF (82, 83). A study on the peripheral blood of MS patients demonstrated that peripheral pro-inflammatory B cells, defined by the cell surface marker CD19 and by secretion of the cytokine TNF-α, are significantly increased in all subtypes of MS, particularly those with PPMS (15). Additionally, peripheral B regulatory cells, identified by the cell surface marker CD19 and secretion of the cytokines IL-10 and TGF-β, are reduced in all subtypes of MS, particularly those with PPMS. The overproduction or underproduction of specific cytokines by B cells could play a causal role in the pathogenesis of PPMS.

B Cell Production of LTα

LTα is secreted by B and T cells and binding of membrane bound LTα to follicular dendritic cells induces CXCL13 production (84). CXCL13 is a ligand that binds to the chemokine receptor CXCR5, which is expressed on virtually all B cells, a subset of T cells, and transiently on T cells upon activation (85, 86). CXCL13 is presumed to be a potent chemoattractant that plays a causative role in T and B cell CNS infiltration and lesion formation in MS (87) and is locally produced in active demyelinating MS lesions (87). Elevated CSF CXCL13 also correlates with an increased risk of relapse and unfavorable prognosis in patients with RRMS (88). Elevated levels of CSF CXCL13 increase the likelihood of conversion of CIS to MS (88). In patients with RRMS treated with rituximab, decreased levels of the chemokine CXCL13 correlate with decreased levels of T cells (89). This led study researchers to hypothesize that B cell depletion induces secondary T cell depletion through reduced LT-α-mediated follicular dendritic cell production of CXCL13. Analysis of CSF cytokines has also demonstrated an increase in CXCL13 in patients with PPMS compared to healthy control (90). Additionally, in patients with PPMS, CSF CXCL13 was found to correlate with CSF B and T cell levels (91) and higher amounts of CXCL13 were found in patients with disease activity compared to those without (92). Overall, these data suggest a possible pathogenic role for B cells in PPMS via LT-α and CXCL13, which may be mitigated by anti-CD20 therapies.

B Cell Production of IL-6

Murine EAE is a commonly used animal model that has been used to decipher the immunopathogenic mechanisms of MS and devise novel therapies (93). EAE is induced by immunizing mice with CNS tissue or myelin peptides in the presence of an adjuvant or by the adoptive transfer of encephalitogenic T cells into naïve mice. Different strains of mice will exhibit different pathology after induction of disease. The SJL/J mouse strain typically demonstrates a relapsing remitting form of demyelinating disease when immunized, whereas C57BL/6 mice display a monophasic or chronic progressive demyelinating disease (94). The latter is considered a suitable model for studying the demyelination and axonal damage present in PPMS and SPMS, although notable differences between murine and human MS disease pathology have raised obvious limitations for the interpretation of EAE results (94).

B cells from mice with EAE produce more IL-6 than naive mice and treatment with monoclonal anti-CD20 antibodies leads to normalized B cell production of IL-6 (95). Genetic deletion of IL-6 exclusively in B-cells during EAE demonstrates a more indolent course compared to control mice without B cell IL-6 deletion (95). In co-culture, B cells enhance Th1 and Th17 T cell responses to fungal infection *in vitro*, partly through IL-6 signaling (96). Additionally, analysis of CSF

from patients with PPMS and RRMS revealed that patients with PPMS have significantly higher levels of intrathecal IL-6 production (97). Recent clinical data demonstrates that treatment of PPMS patients with ocrelizumab leads to a reduction in B cell production of IL-6 which correlates with a shift in T cells to a more anti-inflammatory phenotype (98). The concordance of animal and human studies with clinical data in PPMS patients treated with ocrelizumab offers strong evidence for a role of IL-6 in the pathogenesis of PPMS. Taken together, these findings indicate that B cell production of IL-6 could exert inflammatory damage in PPMS by skewing T cells toward a pro-inflammatory phenotype.

B Cell Production of IL-10

IL-10 is a potent immunoregulatory molecule that is dysregulated in several autoimmune diseases, such as inflammatory bowel disease, rheumatoid arthritis and systemic lupus erythematous (99). Selective genetic deletion of IL-10 in B cells during EAE results in a non-remitting disease course believed to be driven by increased Th1 cell activity (100), supporting an IL-10-mediated anti-inflammatory effect of B cells. Disease is suppressed in EAE mice that received IL-10-producing B cells (101). A distinct subpopulation of B cells, termed B10 cells, potentially function as negative regulators of inflammation and autoimmunity (80). B10 cells have been isolated in the peripheral blood of patients with PPMS, RRMS, and SPMS (102) leading to the hypothesis that deficient functioning of this B cell population may be driving MS pathogenesis (82). What remains unclear is the role of B10 cells in progressive disease; a specific function of B10 cells (or lack thereof) has not been detailed in studies on PPMS to date. Given that the evidence for the anti-inflammatory role of B cell derived IL-10 in PPMS comes primarily from animal studies, it remains to be seen whether these findings will be observed in patients with PPMS and therefore it's specific role in the pathogenesis of PPMS remains unclear.

Reconstitution of Anti-inflammatory B Cell Population

In addition to the immediate effects of anti-CD20 therapies on patients with PPMS there is also the potential for more long-lasting effects from treatment, specifically through reconstitution of an anti-inflammatory B cell population that may further modulate disease progression and/or activity. Treatment of RRMS patients with rituximab leads to reconstitution of B cells producing lower levels of GM-CSF and higher levels of IL-10 (83). This suggests a durable effect of rituximab on the immunologic underpinnings of MS pathogenic processes. It remains to be seen whether such anti-inflammatory B cell reconstitution occurs in PPMS patients treated with ocrelizumab.

Antigen Presentation to T Cells

B cells are extremely potent APCs for T cells. They selectively internalize antigen bound to surface immunoglobin and then present this to T cells via MHC II molecules. The antigen concentration necessary for selective internalization and presentation by B cells are 103- to 104-fold lower than those required for presentation by monocytes (103) which potentially makes B cells a necessary APC for T cell activation when antigen levels are low (104). B cells are also more effective APCs when they recognize the same antigen as T cells (103).

The relevance of B cell antigen presentation to MS pathogenesis was initially explored in EAE mouse models. Mice with selective deficiency of MHC II molecules on B cells are resistant to EAE (105). In contrast, mice selectively expressing MHC II only on MOG specific B cells and no other APCs are susceptible to EAE (105). This suggests a causal role of B cells in MS pathogenesis through a mechanism of antigen presentation enhanced by a cognate antigen between B and T cells. In a study exploring the role of B cells in mice with EAE induced by recombinant MOG protein, which produces what is considered a "B cell dependent" EAE mouse model, anti-CD20 treatment reduces Th1 and Th17 subsets significantly more than in the EAE model induced by immunization with MOG peptide residues 35–55 (106). This indicates that B cells, via antigen presentation, may induce a pro-inflammatory polarization with an increase in Th1 and Th17 subsets.

The antigen presentation function of B cells has been explored further in recent human studies. *In vitro* T cell proliferation was found to be increased in RRMS patients with the HLA-DR15$^+$ risk haplotype compared to those RRMS patients without the risk haplotype (107). Given that the HLA-DR15 gene encodes a distinct MHC II, this data led to the hypothesis that the increased risk of MS with this haplotype is a direct consequence of antigen presentation by B cells. The study further explored the pathogenicity of the HLA-DR15$^+$ haplotype and found that *in vitro* proliferation of T cells was dependent on co-culturing with B cells. When HLA-DR expression by B cells was inhibited by ibrutinib, T cell proliferation was decreased, implying an HLA-DR dependent mechanism of T cell activation by B cells. Additionally, in RRMS patients treated with rituximab, *ex vivo* proliferation and production of pro-inflammatory cytokines by T cells was substantially reduced. The addition of autologous CD20$^+$ B cells obtained pre-treatment with rituximab was found to restore CD4$^+$ T cell proliferation. Memory B cells, specifically un-switched memory B cells, were the B cell population most strongly correlated with T cell proliferation (107). A recent pathological study demonstrated that PPMS patients had higher amounts of B cells within their CNS lesions compared to patients with RRMS (108). Additionally, lower amounts of B cells within these lesions was correlated with decreased CNS T cell infiltration a better clinical outcome (108). Overall, these data indicate that B cell modulation of T cells via antigen presentation is a likely contributor to MS pathogenesis with memory B cells implicated as the B cell population contributing most to T cell proliferation via antigen presentation. Current research studies have consisted almost exclusively of animal studies and human studies in RRMS and therefore it remains to be seen whether these findings can be replicated in PPMS.

The Pro-inflammatory and Anti-inflammatory Role of B Cells

The clinical success of ocrelizumab viewed alongside research indicating both pro- and anti-inflammatory effects of distinct

B cell populations and cytokines indicates a multi-faceted role of B cells in inflammation. This idea is supported by the pro-inflammatory effects of atacicept in MS, an anti-inflammatory drug previously trialed to treat RRMS (23).

Atacicept is a human recombinant fusion protein that binds to the receptor for both BLyS (B-Lymphocyte Stimulator) and APRIL (A PRoliferation-Inducing Ligand) acting as an antagonist to these ligands and inhibiting receptor activation. These two cytokines are important for B-cell maturation, function, and survival. Atacicept has selective effects on B cells, depleting plasma cells and late stage B cells while sparing B-cell progenitor cells and memory B cells (109). Atacicept is the only immunotherapy for MS whose mechanism of action leads to relative sparing of memory B cells (22).

In a randomized double-blind, placebo-controlled trial of atacicept in patients with RRMS, patients who received atacicept had a higher annualized relapse rate compared to those receiving placebo (23). For this reason, the trial was suspended early and has led to the hypothesis that atacicept's depletion of plasma cells and relative sparing of memory B cells implies that plasma cells mainly function as anti-inflammatory cells while memory B cells are pro-inflammatory in MS (13, 110).

This hypothesis is supported by further data highlighting these distinct functions of plasma cells and memory B cells. In an EAE mouse model, plasma cells from the gut were found to play an anti-inflammatory role on neuroinflammation in EAE through the secretion of IL-10 (19). This fits with previously mentioned data regarding the anti-inflammatory effects of IL-10 in EAE (101) and implicates plasma cells as the B cell subtype responsible for IL-10 secretion. Additionally, immunoglobulin produced by intrathecal plasma cells in progressive multiple sclerosis may have a direct anti-inflammatory effect by binding to inhibitory Fc receptors (111). Oligodendrocyte-specific Igs might also promote remyelination (112). In contrast, memory B cells are likely pro-inflammatory and recent research indicates that *ex vivo* memory B cells play a prominent role in inducing $CD4^+$ self-reactivity, likely through a mechanism of antigen presentation (107).

Clinical evidence demonstrating that atacicept increases the rate of MS relapses, taken in conjunction with additional findings suggesting an anti-inflammatory role of plasma cells and a pro-inflammatory role of memory B cells, indicates that B cells can have both a pro and anti-inflammatory effect in MS depending on their specific clonal subset, causing either disease mitigation or progression, respectively.

OCRELIZUMAB IN PPMS

The success of ocrelizumab at reducing disability in PPMS, in the context of previous failures of other anti-inflammatory drugs approved for RRMS, in particular the anti-CD20 monoclonal antibody rituximab, raises important questions about the specific mechanisms by which ocrelizumab exerts its therapeutic benefit. One hypothesis put forth regarding the success of ocrelizumab and failure of rituximab derives from phenotypic differences in the types of PPMS patients enrolled in each study. Rituximab and ocrelizumab are both CD20 monoclonal antibodies. CD20 is a cell surface marker expressed on most B cell subsets with the exception of early pro-B cells, late stage plasmablasts and terminally differentiated plasma cells (113). In clinical trials, ocrelizumab, but not rituximab, significantly reduced disability progression in PPMS patients (31, 35). In the OLYMPUS trial involving treatment of PPMS patients with rituximab, no significant reduction in disease progression was observed overall (35). However, a subgroup analysis revealed that younger age (<51) and the presence of a gadolinium enhancing lesions on MRI (≥1 gadolinium enhancing lesion at baseline) were predictive of treatment responsiveness (35). In particular, patients who had these characteristics in the placebo arm were 3 times more likely to have clinical disease progression compared to the same demographic of patients treated with rituximab (35). The subsequent ORATORIO trial of ocrelizumab in PPMS was designed with recruitment directed at relatively younger participants (mean age 44.6 years; maximum age 55 years), with shorter disease durations (mean 6.4 years; maximum 15 years), and included a relatively high proportion of participants with gadolinium enhancing lesions at baseline (26%) (31). For comparison, in previous PPMS clinical trials with rituximab, fingolimod, and glatiramer acetate, the percentage of participants with any baseline gadolinium enhancement was 24.5, 13, and 14%, respectively (34–36). In ORATORIO, the subgroup of patients with gadolinium-enhancing lesions at baseline had a greater reduction in risk of disease progression (although the difference was not significant) for those with enhancing lesions (hazard ratio 0.65 [95% CI 0.40–1.06]) vs. for those without enhancing lesions (0.84 [0.62–1.13]) (114). These differences in the patient populations in each study have led to speculation that there are a subset of patients with PPMS, specifically young patients with evidence of active inflammation, who preferentially benefit from B cell depletion therapy due to removal of a B cell-mediated inflammatory effect (115).

A recent retrospective study examined the off-label use of rituximab in the treatment of PPMS and found that 41.5% of PPMS patients treated with rituximab had significant disease progression after 3 years (116). The patients had a higher degree of inflammation prior to treatment as demonstrated by the presence of gadolinium enhancing lesions in 50% of the patients on their baseline brain MRI (116). In contrast, ORATORIO demonstrated a 32.9% incidence of disease progression at 12 weeks with a 26% incidence of gadolinium enhancing lesions on baseline brain MRI (31). The off-label rituximab study had numerous limitations including a retrospective design, which prevented the researchers from including a control group, and a relatively low amount of PPMS patients (43 total) (116). Additionally, it is unclear the specific criteria that led to the off-label use of rituximab and it is likely that the patients were selected for treatment due to rapid disease progression which may have led to a bias selection of patients with a more aggressive form of PPMS. Nevertheless, the study suggests that a significant amount of PPMS patients, despite having evidence of inflammation on their brain MRI, will continue to progress after B-cell depletion with rituximab.

Functional differences in the antibody structure of ocrelizumab compared to rituximab may lead to more favorable

safety and tolerability profiles but are unlikely to significantly change the levels of circulating B cells in the periphery and CNS of treated PPMS patients. Rituximab is a chimeric antibody with an Fab domain derived from mouse protein, whereas ocrelizumab is exclusively derived from human protein (117). Compared to rituximab, ocrelizumab has a structurally distinct Fc region domain that binds with higher affinity to natural killer cells. This difference leads to relatively stronger antibody-dependent cell cytotoxicity and relatively weaker complement-dependent cytotoxicity for ocrelizumab compared to rituximab (24). This relative decrease in complement-dependent cytotoxicity is hypothesized to reduce the rate of adverse effects by reducing rates of systemic complement mediated cytokine release (118). Additionally, ocrelizumab has a distinct Fab binding domain that alters its binding affinity to CD20 (119). This difference in epitope binding affinity is unlikely to translate to increased depletion of circulating B cells with ocrelizumab compared to rituximab given that PPMS patients treated with rituximab had near-complete depletion of circulating B cells, defined as a >95% decrease of $CD19^+$ B cells, from week 2 to 96 after rituximab treatment (35). Additionally, in RRMS patients treated with rituximab, CSF B cells were reduced by 90% at 24 weeks post-treatment with rituximab (78). Another study examining the efficacy of dual intravenous and intrathecal rituximab for depleting CNS B cells in patients with SPMS found that peripheral B cells were reliably depleted but CSF B cells were incompletely and transiently depleted (120). While it's possible that ocrelizumab may deplete CSF B cells more effectively than rituximab, given that ocrelizumab is administered intravenously it is unlikely to achieve the CNS penetration necessary to outperform intrathecal rituximab administration.

CONCLUSION

Ocrelizumab is the first and only FDA approved disease-modifying therapy for patients with PPMS. The characteristics of patients treated in ORATORIO indicate that ocrelizumab likely exerts an anti-inflammatory effect with the most pronounced benefit occurring in younger PPMS patients with a high propensity for disease activity (114). This idea is supported in the rituximab clinical trial in PPMS that showed benefit to a subgroup of younger patients with gadolinium enhancing lesions on MRI (35). Ocrelizumab likely induces an anti-inflammatory effect primarily through abrogating B cell functions, such as cytokine production and antigen presentation. B cells exhibit a spectrum of activity in MS with memory B cells playing a pro-inflammatory role and a subset of B cell lineage cells, such as segments of plasmablasts/plasma cells contributing to the suppression of inflammation. Cytokines produced by B cells, including LT-α, IL-6, and GM-CSF, have been implicated as drivers of the pro-inflammatory effects in MS via T-cell differentiation from naïve T cells into inflammatory Th1/Th17 cells as well as via indirect myeloid cell stimulation of T cells. In contrast, production of IL-10 by B cells may cause an anti-inflammatory effect in PPMS. However, there is currently a lack of clinical human studies to definitively support or refute this claim. B cell antigen presentation also likely plays a prominent role in driving T cell activity by inducing naïve T cell differentiation to Th1/Th17 and driving MS pathogenesis. Ocrelizumab is unlikely to exert benefit in MS through antibody decrement given that immunoglobulin levels remain elevated despite B and T cell depletion in the presence of a treatment benefit. It is unclear if PPMS patients treated with ocrelizumab will experience reconstitution of anti-inflammatory B cells after therapy in a similar way to RRMS patients treated with rituximab (83).

Altogether, the above data indicates that ocrelizumab likely reduces disease progression in PPMS by reducing inflammation. This mechanism of action represents a continuation of the therapeutic paradigm used to treat RRMS in which the primary treatment modality involves drugs that work via reducing inflammation. The benefit of ocrelizumab but the failure of multiple other RRMS anti-inflammatory drugs, in conjunction with the phenotypic differences in PPMS compared to RRMS, has important implications about disease pathogenesis and treatment. Clinical trial data indicates that there is likely a subset of patients with PPMS, typically younger, newly diagnosed patients with gadolinium enhancing lesions on MRI, who have active inflammation contributing to their progressive disability who would benefit from a high potency anti-inflammatory medication. These qualitative differences in subgroups of PPMS patients have implications for the way we classify patients with PPMS. The recent revisions to the classification of MS to include new qualifiers for active disease and presence of progression represents an effort to further delineate PPMS into more clinically useful groups (27). Clinical trials examining the effect of anti-inflammatory treatments on PPMS in patients with or without active disease and with or without progression would shed light on further clinically meaningful phenotypic differences within the PPMS subtype. Leptomeningeal inflammation, given that it is more prevalent in the subset of patients with PPMS who had active disease and that it can be visualized on MRI (60, 61), may serve as a potential biomarker to identify patients with PPMS who may benefit most from B cell therapy. The clinical data also implies that for the majority of patients with PPMS, specifically those older patients without evidence of active disease, further anti-inflammatory treatment is unlikely to influence disease progression. Dedicated research in patients with PPMS without evidence of active inflammation and refinement of MS animal models of neurodegeneration in the absence of inflammation may help elucidate the non-inflammatory, neurodegenerative processes contributing to PPMS disease progression.

Broadening our understanding of disease pathogenesis in PPMS and harnessing that knowledge to develop new and effective treatments represents the next frontier in MS research. This goal carries with it unique challenges given the reduced prevalence of PPMS compared to RRMS and SPMS, making clinical trial recruitment more difficult. Additionally, the EAE mouse model, the most widely studied animal model for MS, is of questionable utility in PPMS given the lack of progressive MS pathologic features (94). Dedicated clinical studies of progressive disease, expanded and novel animal models for progressive disease, and shifting treatment paradigms will hopefully lead to future breakthroughs for patients with PPMS.

AUTHOR CONTRIBUTIONS

JH drafted the manuscript. JH, RA, NM, and GW provided the content and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Memory B Cells in Multiple Sclerosis: Emerging Players in Disease Pathogenesis

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Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system. Once thought to be primarily driven by T cells, B cells are emerging as central players in MS immunopathogenesis. Interest in multiple B cell phenotypes in MS expanded following the efficacy of B cell-depleting agents targeting CD20 in relapsing-remitting MS and inflammatory primary progressive MS patients. Interestingly, these therapies primarily target non-antibody secreting cells. Emerging studies seek to explore B cell functions beyond antibody-mediated roles, including cytokine production, antigen presentation, and ectopic follicle-like aggregate formation. Importantly, memory B cells (Bmem) are rising as a key B cell phenotype to investigate in MS due to their antigen-experience, increased lifespan, and rapid response to stimulation. Bmem display diverse effector functions including cytokine production, antigen presentation, and serving as antigen-experienced precursors to antibody-secreting cells. In this review, we explore the cellular and molecular processes involved in Bmem development, Bmem phenotypes, and effector functions. We then examine how these concepts may be applied to the potential role(s) of Bmem in MS pathogenesis. We investigate Bmem both within the periphery and inside the CNS compartment, focusing on Bmem phenotypes and proposed functions in MS and its animal models. Finally, we review how current immunomodulatory therapies, including B cell-directed therapies and other immunomodulatory therapies, modify Bmem and how this knowledge may be harnessed to direct therapeutic strategies in MS.

Keywords: memory B cells, multiple sclerosis, neuroinflammation, B cells, multiple sclerosis-drug therapy

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INTRODUCTION

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS), with a highly variable and unpredictable disease course that can manifest as a variety of physical and cognitive symptoms. Although cellular inflammation in MS has historically focused on one key player in adaptive immunity, T cells, B cells are now recognized as central mediators in MS pathogenesis. B cell antibody-mediated immunity has been implicated in MS pathogenesis since the discovery of elevated CSF IgG in 1942 (1). Subsequently, in 1959 oligoclonal bands (OCBs) in the cerebrospinal fluid (CSF) were identified (2) and, to date, OCBs remain a diagnostic hallmark in MS (3). OCB presence indicates niches of clonally-related antibody-secreting cells (ASC), including

plasmablasts and plasma cells, within the CNS. Since the discovery of OCBs in MS, researchers have dedicated intense focus towards identifying the antigenic targets of ASC in the CNS compartment. However, in contrast to CNS neuroinflammatory diseases such as neuromyelitis optica, with clear autoantibody targets (aquaporin-4), probing antibody specificity in MS has not revealed consistent targets (4, 5), with some studies implicating diverse CNS self-antigens (6, 7) and viral antigens (8). The role of ASCs and OCBs in MS still remains elusive, with suggested involvement in pro-inflammatory functions, including autoantibody production, antibody- or complement-dependent cellular cytotoxicity, and opsonization, or anti-inflammatory functions, including production of the anti-inflammatory cytokine IL-10 (9, 10).

Further interest in the role of non-ASC B cells as key players in the MS immunopathogenesis followed the relatively recent success of B cell depletion therapies targeting CD20. These therapies, including rituximab (11, 12), ocrelizumab (13), and ofatumumab (14) reduced new inflammatory lesions and relapses despite the sparing of most ASCs, i.e. $CD20^-$ plasma cells and some plasmablasts. These novel findings fueled considerable interest in examining the phenotype and function of non-ASC B cells in MS. Current research seeks to explore B cell function in MS beyond antibody-dependent roles to define antibody-independent mechanisms, including antigen presentation, cytokine production, and ectopic lymphoid follicle-like structures. Among non-ASC B cell subtypes, increased attention has been directed towards the role of memory B cells (Bmem) in regulating immune processes in MS. Bmem have several unique features, including increased longevity, the capacity to rapidly respond to re-exposure to antigen, and the ability to serve as direct antigen-experienced precursors to antibody-secreting cells. Due to the relatively recent interest in Bmem, our knowledge regarding the exact functions of Bmem in MS is expanding. This review aims to explore our current understanding of this key component of immunological memory in MS and its animal models.

In the first part of this review, we summarize the current knowledge regarding Bmem development, trafficking, phenotypes, and function during homeostasis and inflammatory conditions, providing a basis for understanding the mechanisms in which Bmem may contribute to MS and are targeted by immunomodulatory therapies.

In the second part of this review, we describe Bmem in MS and its animal models reviewing phenotypes and putative functions, and finally, we examine the effectiveness of current therapeutic approaches in targeting Bmem.

Bmem DEVELOPMENT

A key player in immunological memory, Bmem can be defined as a B cell that has encountered antigen and remains in a quiescent state until re-exposed to antigen, at which point the cell rapidly responds to the second challenge. Upon first pathogen encounter, the majority of Bmem are derived from germinal center (GC) reactions. GCs are specialized structures within secondary lymphoid tissue (SLT) where mature, antigen-experienced B cells undergo cognate interactions with T cells, proliferate, undergo somatic hypermutation to increase B cell receptor (BCR) affinity for antigen, perform immunoglobulin (Ig) isotype switching, and are selected based on affinity for a specific antigenic target. Select GC B cells ultimately differentiate to produce antigen-specific, isotype-switched ASC or Bmem. Though GC B cells serve as the precursor for both ASC and Bmem, the mechanisms regulating Bmem versus ASC differentiation remain poorly understood. Numerous factors have been proposed to contribute to Bmem formation, but no "master regulator" for Bmem differentiation has been identified. Animal models have suggested the transcription factor BACH2 selects GC B cells with intermediate affinity to differentiate into Bmem (15). Additionally, Bmem generation is associated with an increased expression of factors including ZBTB32 (16), KLF2 (17, 18), ABF-1 (19), STAT5, BCL-6 (20, 21), and SKI (21), which, in general, repress differentiation to an ASC phenotype. Cytokines, including IL-24 (22) and IL-9 (17) can enhance Bmem formation. Moreover, *in vitro*, IL-2, IL-10, and CD40L were demonstrated to be involved in differentiating GC B cells to a Bmem phenotype (23). Outside of GC, a small proportion of antigen-experienced B cells may additionally be selected for based on low affinity to form Bmem in an early wave prior to GC formation (24, 25). GC-independent isotype-unswitched (IgM) or –switched (IgG) Bmem exhibit low affinity due to unmutated Ig variable genes (26). In humans, few Bmem lack somatic mutations for antigen (27), suggesting most Bmem are GC-derived. Following Bmem formation, these cells may reside in survival niches including SLT such as the spleen (28) for years in a resting state independent of antigen; however, these niches are localized near areas of antigen encounter (29). Bmem are also observed in the tonsils and the bone marrow and may enter into circulation to patrol at low levels (28). Bmem express higher levels of the adhesion molecules LFA-1 and VLA-4 compared to naive B cells, with VLA-4 primarily mediating Bmem retention in SLT (30). *In vitro*, Bmem migrate towards CXCL12 (23, 31), CCL19, and CXCL13 (23, 32) suggesting these chemokines may be involved in movement within the SLT and trafficking to survival niches or sites of inflammation. If the humoral immunity generated from long-lived plasma cells residing in the bone marrow is not sufficient to eliminate pathogens, Bmem become actively involved in the inflammatory response. Upon re-exposure to antigen, Bmem will generate a more rapid and potent antigen-specific response relative to naïve B cells (33).

Bmem PHENOTYPES

In humans, Bmem are conventionally identified by the expression of tumor necrosis factor superfamily member CD27, a protein regulating entry into plasma cell lineage and properties associated with Bmem including isotype switching and Ig variable gene mutation (34, 35). However, CD27 is not exclusive to Bmem and is likely a marker of GC and post-GC

activation as CD27 is also expressed on GC B cells and post-GC B cells, including ASC (**Figure 1**). Thus, CD27 expression should be coupled with low levels of CD23 (36) and the lack of expression of the ASC marker CD138 (syndecan-1) to identify Bmem in humans. Further inclusion of specific patterns of CD38 (37), CD21 (38), CD24 (39), CD19 (40), B220 (41), FCRL4 (FcRH4) (38, 42) and CD25 (43, 44) can delineate heterogeneous Bmem populations (**Figure 1**). Thus far, the main populations of $CD19^{+}CD27^{+}CD138^{-}$ Bmem present in peripheral blood and bone marrow include three isotype-unswitched Bmem phenotypes, including $IgM^{+}IgD^{+}$, IgM^{-} IgD^{+}, $IgM^{+}IgD^{-}$ (IgM-only memory cells), and isotype-switched IgM-IgD- phenotypes, including IgG, IgA, or IgE+ Bmem. Bmem are typically isotype-switched and primarily express IgG subclasses. IgG+ Bmem comprise 15-20% of peripheral blood B cells, including predominately IgG_1, IgG_2, IgG_3 subclasses (45). Among IgG Bmem, it should be noted that a small proportion of "atypical" IgG Bmem may lack CD27 (38, 45, 46). Isotype-switched IgA Bmem comprise around 10% of B cells in peripheral blood and are generally implicated in mucosa-associated tissues (45) while IgE Bmem involved in allergic responses are rarely detectable in humans and mice and their development and lifespan is poorly understood (45). Among isotype-unswitched phenotypes, IgM and IgD-expressing Bmem, including IgM^{+} IgD^{+} (15% of B cells), IgM^{-} IgD^{+} (1%), or $IgM^{+}IgD^{-}$ (5%) may be found within the blood or bone marrow (34, 47, 48).

In rodent models, Bmem identification is hampered by the low frequency of Bmem (49) and the lack of CD27 expression among Bmem (50). Further definitive Bmem markers in mice have remained elusive. Exploration of novel Bmem markers in mice have relied on several methods including 1) boosting Bmem frequencies using antigen-based cell enrichment protocols (51, 52), 2) protein immunization in BCR transgenic mice with a fixed BCR specificity (29), 3) adoptive transfer of antigen-specific B cells (53), or 4) genetic tagging of activation-induced cytidine deaminase (AID), an enzyme essential for isotype switching and somatic hypermutation identifying GC-derived B cells including Bmem and ASC (33). Murine studies have proposed at least 10 Bmem subsets utilizing Ig isotyping combined with surface expression of CD80 (49, 54, 55), PDL2 (54, 55), CD73 (55, 56), CD38 (57). However, these markers may be expressed on other murine B cell subtypes, so a diverse panel of surface markers is necessary for identifying Bmem (**Figure 2**). For isotype-switched Bmem, IgG surface (IgGs) versus intracellular (IgGi) expression (58, 59) in combination with CD138 or Blimp-1 (60, 61) may be used to distinguish ASC ($IgGi^{hi/+}$, $igGs^{low}$, $CD138^{+}$, $Blimp\text{-}1^{+}$) (62) and Bmem ($IgGi^{low}$, $IgGs^{hi/+}$, $CD138^{-}$, $Blimp\text{-}1^{-}$). Moreover, similar to assaying human Bmem, *in vitro* stimulation using polyclonal activators (i.e. CpG DNA, R848 TLR7/8 agonist) to convert Bmem into ASC, combined with a conventional Enzyme-linked ImmunoSPOT (ELISPOT) assay, may be used to quantify Bmem and determine antigen specificity and Ig isotype in mice (63–65).

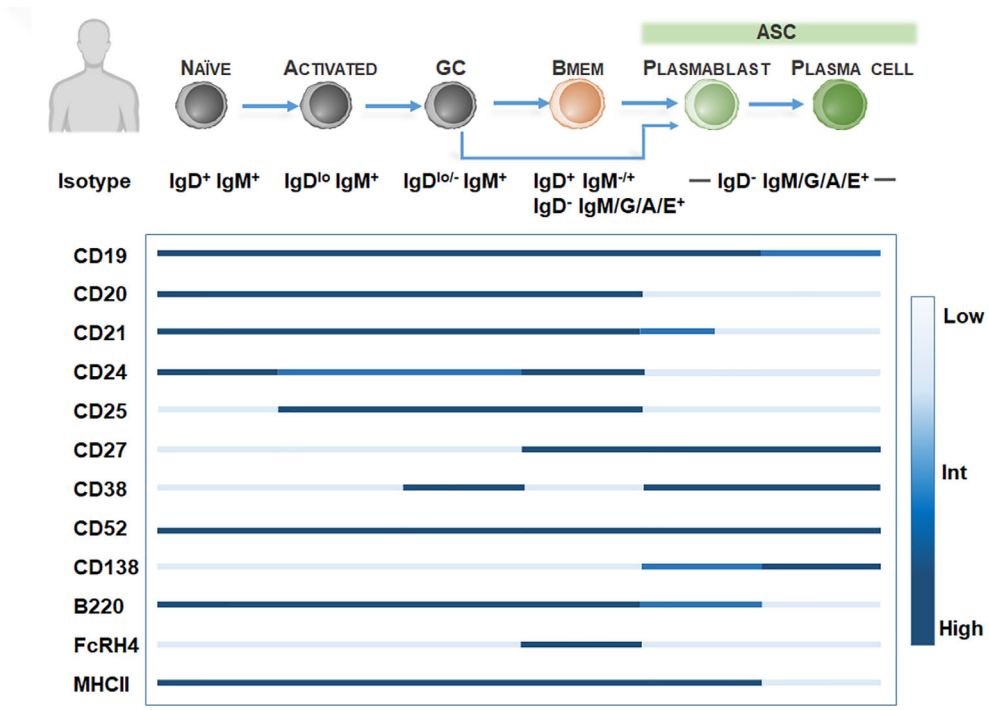

FIGURE 1 | Bmem phenotyping markers in humans. Overview of B cell differentiation in humans, focusing on naïve B cell to ASC phenotypes, with BCR isotype and surface marker expression. Surface marker levels simplified to highlight relative expression, including low, intermediate, or high levels.

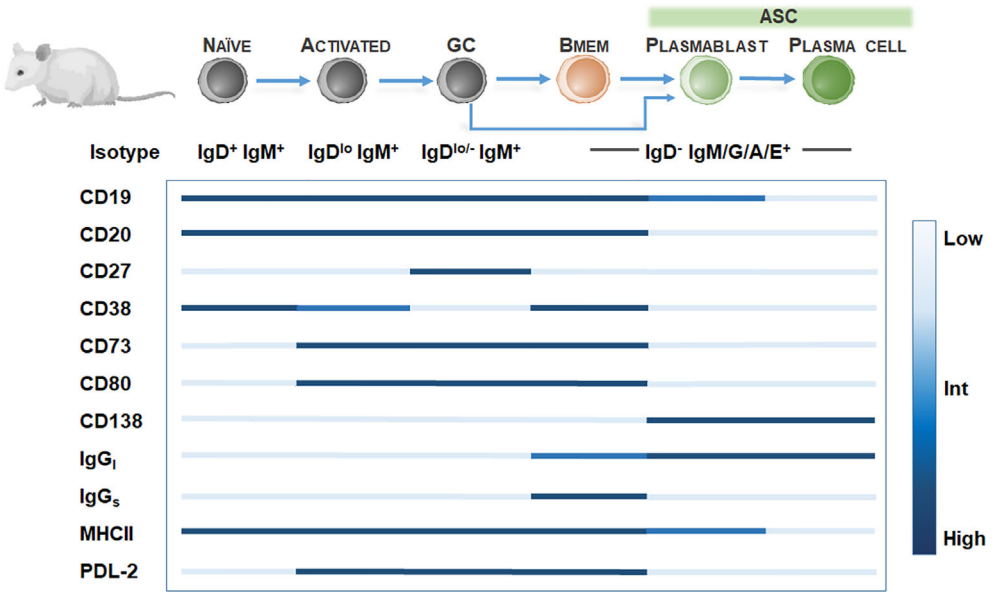

FIGURE 2 | Bmem phenotyping markers in murine models. Overview of B cell differentiation in mice, focusing on naïve B cell to ASC phenotypes, with BCR isotype and surface marker expression. Surface marker levels simplified to highlight relative expression, including low, intermediate, or high levels.

Bmem FUNCTION

Compared to naïve mature B cells, Bmem display several distinctive features. Bmem have enhanced longevity and can survive for years and perhaps for the lifetime of the host independent of antigen (66, 67). In comparison, naïve mature B cells have a lifespan of weeks (68). Furthermore, since most Bmem are GC-derived, Bmem are generally clonally expanded, isotype-switched, and have undergone somatic hypermutation of Ig variable genes to increase antigen affinity. Unlike naïve or activated mature B cells, Bmem are able to rapidly proliferate and differentiate into ASC with minimal stimulation requirements, including re-exposure to low levels of antigen (69, 70), T cell help (71–73), or polyclonal stimulation (73, 74). Bmem enter cell cycle, differentiate into ASC, and potentially re-seed GC quicker than mature B cells (75, 76). These advantages are likely due to a combination of factors, including reduced quiescence factors (Kruppel-like factor 4 and 9; PLZF) (77), higher expression of co-stimulatory molecules (CD80, CD86) (78, 79), CD27 (50), IL21R (80), SLAM (signaling lymphocytic activation molecule) (79), TLR7/9 (81), and anti-apoptotic molecules (BCL2) (82). Once activated, Bmem can follow two paths: 1) rapidly differentiating into ASC or 2) re-entering into secondary GC reactions to undergo further affinity maturation and isotype-switching. In murine studies, IgG Bmem show a greater proclivity to differentiate into ASC, while IgM Bmem are often selected for re-entry in GC reactions (33, 51). Bmem differentiating to ASC can contribute to the rapid and copious production of high affinity antibodies to supplement antibody produced by terminally differentiated plasma cells residing in niches, such as the bone marrow. In addition to rapid differentiation to ASC, Bmem are potent antigen-presenting cells (APCs), expressing MHCII (83) that enables not only the efficient recognition of antigen, but the ability to process antigen for presentation to activate other immune cells, including T cells (84). Finally, Bmem produce a wide array of cytokines including TNF (85, 86), GM-CSF (86), IL-6 (86, 87), lymphotoxin (LT) (85), and IL-10 (85).

Bmem IN MULTIPLE SCLEROSIS

In MS, B cells are located within multiple compartments in the CNS, including the CSF, parenchyma, and meninges. However, studies exploring Bmem in MS have primarily focused on the peripheral blood and CSF, with few studies examining Bmem localization in the parenchyma and meninges. Among these studies, there are notable discrepancies in defining Bmem, with the majority of studies defining Bmem based exclusively on CD27 expression. Therefore, for each mentioned study, the surface markers utilized to define Bmem will be noted.

Phenotype, Trafficking, and Localization

In MS, Bmem frequencies are elevated in the CSF compared to peripheral blood (88, 89) and Bmem comprise the majority of B cells populating the CSF (90, 91) ($CD27^+$ IgD^- (88, 91); CD19+CD27+ (89); CD27+ CD138- (90); CD19+ CD27+ IgD- and IgD+). In contrast to the peripheral blood, the proportion of $CD19^+$ B cells among total lymphocytes is significantly lower in the CSF (91). However, the proportion of class-switched B cells, including isotype-switched Bmem, among CD19+ B cells is enriched in the CSF (91). Further studies have confirmed the

majority of Bmem populating the CSF display an isotype-switched phenotype (71%; $CD19^+$ $CD27^+$ IgD^- IgM^-) (92). In agreement with these findings, B cells populating the CSF, including Bmem, bear extensive somatic mutations and exhibit clonal expansion (88). Conversely, in a recent pre-print, Bmem in peripheral blood from MS patients displayed an Ig isotype distribution of 50% IgM, 30% IgA, and 20% IgG (93). In MS patients, ASC populating the CSF exhibit a selective enrichment towards the IgG1 allotype G1m1 compared to the peripheral blood (94). In a recent pre-print, Bmem in the intrathecal compartment did not exhibit the same dominance towards the G1m1 allotype constant region polymorphism, suggesting certain B cell-lineages may preferentially differentiate (95). To date, it remains unclear if skewed Ig allotypes influence MS risk and phenotype (96, 97).

Bmem are not restricted to the CSF compartment, and Bmem ($CD27^+$) are found within the brain parenchyma (98, 99). Furthermore, B cells recovered from MS plaques display mutations and clonal expansion (100, 101), suggesting primarily differentiated B cells (Bmem/ASC) occupy the parenchymal space, similar to the CSF. It has been suggested that BCR mutations and clonal expansion may be acquired in the CNS compartment (89), possibly aided by inflammatory aggregates in the brain meninges mimicking some features of ectopic lymphoid follicles (102). In a recent pre-print, extensive clonal connections were found among Bmem and ASC in the CSF compartment (95). Clonal connections between Bmem and ASC were also found to span different isotypes, including IgM/IgG1, IgG1/IgG2, and IgM/IgA1. These findings suggest ASC and Bmem share a common origin, although it remains unclear whether these clonal similarities originate in the periphery or the intrathecal compartment. At least a proportion of B cells appear to undergo an active exchange between the periphery and CNS in MS, with $CD27^+$ IgD- B cells sharing similar repertoires between the peripheral blood and CSF (91, 103). Moreover, Stern et al. demonstrated the B cell clonal families observed in MS brain tissue were frequently derived from founders in the deep cervical lymph nodes (104). Regardless of the mechanism promoting Bmem persistence in the CNS, the exact chemokines initiating and/or sustaining Bmem trafficking to the CNS compartment in MS remain to be determined. Several chemokine receptors including CXCR4 (105), CXCR5 (91), CXCR3 (95), CCR1, CCR2 and CCR4 (88) have been implicated in trafficking and are upregulated on CSF B cells compared to paired-peripheral blood. Adhesion molecules regulating Bmem entry into the CNS meninges and parenchymal compartments are less clearly understood. VLA-4 has been implicated in aiding B cell transmigration in ex vivo culture studies (106) and murine studies (107), though these studies have examined global B cell migration and further studies are required to determine whether VLA-4 is essential for Bmem transmigration.

Function

Antibody Production and Antigen Specificity

Tracking Bmem conversion into ASC to investigate antibody production and specificity *in vivo* remains challenging and often requires specialized murine models. Alternatively, *in vitro*, Bmem can be stimulated to convert into ASC utilizing polyclonal activators specifically triggering Bmem differentiation, including the TLR7/8 agonist R848 (108, 109). Bmem may subsequently be quantified and Ig isotype and antibody production may be evaluated. Limited studies exist examining Bmem conversion to ASC and antibody production in MS. Hohmann et al. isolated B cells from the peripheral blood of MS patients and compared IgG antibodies produced by ASCs or Bmem-derived ASCs, i.e. B cells *in vitro* stimulated using R848 and IL-2 by ELISPOT (110). Bmem-derived ASCs generated larger spot size compared to ASCs, suggesting enhanced IgG secretion from Bmem-derived ASCs.

B cell antigen specificity in MS has remained unclear and is documented as heterogeneous, with antibody targets ranging from self-antigens to viral antigens. With regards to Bmem, there have been few studies on this topic. Hohmann et al. exclusively examined reactivity to normal human brain lysates (110). Among 15 of the 30 relapsing-remitting MS (RRMS) patients tested, brain-reactive Bmem-derived ASC were present in the peripheral blood. In some patients, brain-reactive Bmem were present in relapse and remission, while other patients displayed brain-reactive Bmem in the relapse only. The presence of brain-reactive B cells, including Bmem, predicted relapse. Brain-reactive B cells were not observed in the peripheral blood of healthy donors or other neurological disease controls (111).

Antigen Presentation

Bmem are conventionally regarded as potent APCs. In MS, CSF Bmem ($CD27^+$ IgD^-) display upregulated expression of two co-stimulatory molecules key in antigen presenting functions, CD80 and CD86, compared to naïve B cells (88). Although this is a well-known feature of Bmem regardless of disease pathogenesis, this finding suggests Bmem in the CSF of MS patients also display an enhanced ability to engage with immune cells, including T cells. In alignment with these findings, *ex vivo* Bmem ($CD19^+$ $CD27^+$) isolated from RRMS patients elicited autologous CD4 T cell proliferation in the presence of antigens including, tetanus toxoid, myelin basic protein (MBP), and myelin oligodendrocyte protein (MOG) (112). Moreover, Bmem isolated from some RRMS patients are capable of activating CD4 T helper (Th) cells in the presence of myelin antigens *in vitro*, inducing T cell proliferation and IFNγ production (112). Furthermore, the *in vitro* spontaneous proliferation of Th1 cells observed in patients carrying the risk allele HLA-DR15 was found to be mediated by Bmem ($CD27^+$) with high MHCII surface receptor HLA-DR expression (113).

Cytokine Production

B cells, including Bmem, in MS patients may exhibit a propensity towards a dysregulated cytokine network. An increased frequency of Bmem ($CD27^+$) producing GM-CSF was observed in the peripheral blood obtained from MS patients compared to healthy controls (86). Furthermore, *in vitro* stimulated B cells isolated from the peripheral blood of RRMS and SPMS patients exhibit a decreased production of the anti-inflammatory

cytokine IL-10 compared to healthy controls, while LT and TNF levels were comparable (85). Further studies demonstrated stimulated Bmem ($CD19^+$ $CD27^+$) obtained from RRMS patients produce elevated LT and lower IL-10 than naïve B cells (112). However, Bmem isolated from healthy donors produced comparable levels of both cytokines. *In vitro* stimulated Bmem obtained from healthy donors also exhibited lower levels of IL-10 production compared to naïve B cells (85), thus, low levels of IL-10 production seems to be typical Bmem feature regardless of disease pathogenesis. The reduced IL-10 production by B cells observed in RRMS and SPMS patients may therefore be attributed to another B cell phenotype, including IL-10-producing regulatory B cells or ASC (9, 114).

Associations With Clinical Disease

Recent studies have sought to investigate the association of Bmem with clinical outcomes in MS. In RRMS patients, an increased $CD5^+$ Bmem subpopulation was associated with the remitting stage compared to the relapsing stage (115). Furthermore, Nissimov et al. demonstrated elevated peripheral blood Bmem frequencies were associated with a lower expanded disability status scale score (116). Conversely, Comabella et al. determined that increases in isotype-unswitched and -switched Bmem ($CD19^+$ $CD27^+$ IgD^+ or IgD^-) in the peripheral blood from RRMS patients were associated with an MRI phenotype with high neurodegeneration, defined by increased contrast-enhancing lesions and non-enhancing black holes on T1-weighted images, and decreased brain parenchymal fraction (117). Bmem populations also differ in peripheral blood obtained from pediatric and adult MS patients (118). In pediatric MS, Bmem ($CD20^+$ $CD27^+$) are elevated in the peripheral blood compared to healthy children and adolescents. In contrast to adult MS patients who display elevated isotype-switched Bmem ($CD20^+$ $CD27^+$ IgD^-) and plasma cells in peripheral blood, non-switched Bmem ($CD20^+$ $CD27^+$ IgD^+) and plasmablasts were increased in frequency in pediatric MS patients.

Bmem IN ANIMAL MODELS OF MS

Murine models of MS generally have been limited in exploring Bmem due to the lack of conventional Bmem markers, the low quantity of Bmem (25), the shifted surface expression of proposed murine markers on Bmem isolated from CNS compartment (63), and the time-consuming methods utilized to isolate Bmem and quantify by *in vitro* stimulation assays (64, 65). In this section, we will review data on Bmem obtained from pre-clinical models of MS, including two viral models of demyelination, mouse hepatitis virus (MHV) and Theiler's murine encephalomyelitis virus (TMEV), and the autoimmune model, experimental autoimmune encephalomyelitis (EAE).

Viral Models of Demyelination

Viral immune-mediated demyelination models emulating features of MS, including MHV (coronavirus family) and TMEV (picornavirus family), require B cell and antibody responses for viral control (119, 120) and recruit diverse B cell subtypes CNS (59, 121). There is also evidence for B cell involvement in demyelination and clinical disability (122–125).

Intracerebral MHV infection, including the A59 and JHM strains, induces an acute inflammatory demyelinating disease, with prominent B cell CNS infiltration mimicking the acute inflammatory stages of MS. In MHV models, Bmem are present in the CNS parenchyma as evaluated by flow cytometry (59), genetic tagging of AID-expressing B cells (126), and *in vitro* stimulation and evaluation *via* ELISPOT assays (63). Among total CNS-infiltrating Bmem ($CD19^+$, $CD138^-$, IgD^-, IgG2a/b surface+, IgG2a/b intracellular^{low}) the majority comprise an IgG2a/2b isotype-switched phenotype. ELISPOT analysis of *in vitro* stimulated Bmem determined that ASC and Bmem are initially recruited to the CNS (brain/spinal cord) with similar kinetics, but during the chronic phase of infection (day 35 post infection-p.i.), virus-specific IgG ASC persisted at higher frequencies than IgG Bmem in the spinal cord, the predominant site of inflammation and demyelination (63). ELISPOT analyses revealed that antibody production levels were similar between ASC and Bmem-derived ASC in both brain and spinal cord tissues. Gene expression analysis of chemokine receptors on CNS-infiltrating Bmem ($CD19^+$ IgD^- $CD138^-$) revealed highly upregulated expression of CXCR3 and CCR7, with moderate expression of CXCR4 and CXCR5 (59). Compared to ASC ($CD138^+$), Bmem expressed higher levels of CCR7 and CXCR5, with similar expression of CXCR4, and lower expression of CXCR3. These results suggest multiple chemokine receptors may be simultaneously regulated on Bmem to direct recruitment. AID-genetically tagged Bmem and ASC were continually recruited from the periphery to the CNS concurrent with GC maturation (126). Moreover, once recruited to the CNS, there was no evidence of AID mRNA expression among Bmem, suggesting these cells were not undergoing somatic hypermutation or isotype switching in the CNS compartment during chronic infection (59). It still remains unclear whether Bmem are required for sustaining the local antibody production responsible for controlling viral recrudescence. Future studies are also required to determine if Bmem contribute to antibody-independent functions, including local cytokine production and antigen presentation.

In the chronic progressive demyelinating disease model, TMEV-induced demyelinating disease (TMEV-IDD), intracranial infection with TMEV mimics several neurodegenerative and clinical features of progressive MS (127). In chronic disease (day 120 p.i.) a phase of accumulating disability, Bmem (IgG^+ $CD138^-$) were identified in spinal cord tissue (121). Although the function of Bmem in TMEV-IDD remains to be determined, B cell depletion therapy (anti-CD20) targeting non-ASC B cells, including Bmem, exacerbated microglial activation, increased T cell infiltration, demyelination, and axonal damage (123).

Autoimmune Models

Although a wide array of EAE models exist, the most commonly utilized EAE models emulate the acute or relapsing/remitting stages

of MS (128) and are induced independent of B cells (128–130). Due to the limited B cell involvement in these models, including the MOG_{35-55} peptide model induced in C57BL/6 mice, the role of Bmem in EAE models remains relatively unexplored.

Several therapeutic interventions targeting B cell subtypes including Bmem may provide insights into Bmem function in EAE autoimmune models of MS. In anti-CD20 studies in EAE, clinical disease is suppressed in murine MOG_{35-55} (131, 132) and marmoset EAE models (133, 134). CD20 depletion was also found to ablate IL-6 producing B cells (131), including Bmem. In a T-independent protein immunization murine model (TNP-LPS) anti-CD20 administration depleted existing and adoptively transferred Bmem (135). Mice deficient in B cell maturation antigen (BCMA), an important receptor for B cell-activating factor (BAFF) and a proliferating inducing ligand (APRIL) regulating ASC differentiation and survival, showed exacerbated EAE disease severity (136). *In vitro*, BCMA expression directly inhibited Bmem expansion and anti-inflammatory cytokine production, suggesting BCMA deficient mice may show increased proportions of Bmem. Together, these studies suggest Bmem may contribute to EAE pathogenesis. However, other therapeutic interventions have suggested Bmem may play a dispensable or, perhaps, beneficial role in EAE pathogenesis. Atacicept, a TACI fusion protein that inhibits the B cell survival factors B lymphocyte stimulator (BlyS) and APRIL, spares B cell progenitors and Bmem (137). Atacicept's use has been explored in both the B cell-dependent recombinant human MOG_{1-125} (rhMOG) and B cell-independent MOG_{35-55} models. In both models, prophylactic treatment resulted in reduced B cell infiltration into the CNS, delayed disease onset, and attenuated disease severity (138). In addition, a key cytokine promoting Bmem survival, IL-15, was found to be enhanced in a murine lupus model following TACI-IgG treatment (139).

Altogether, further studies are required to determine Bmem function in EAE models of MS as anti-CD20 therapies, atacicept, and BCMA deficiency all affect multiple B cell subsets. Following the success of B cell-depleting therapies in MS, increasingly studies are utilizing B cell-dependent EAE models, including rhMOG EAE and EAE induced in IgH^{MOG} transgenic mice where 30% of B cells are specific for MOG (140). Future studies utilizing these models may pinpoint the exact Bmem phenotypes and Bmem functions involved in autoimmune models of MS.

MS IMMUNOMODULATORY THERAPIES AND THE EFFECT ON Bmem

B Cell-Directed Immunomodulatory Therapies

B cell depletion therapies targeting CD20, including rituximab, ocrelizumab, and ofatumumab, deplete all B cells except ASC and pro-B cells (141) (**Figure 1**; **Table 1**) and have shown significant efficacy in reducing clinical relapse rates and new lesion formation in RRMS patients (11, 196). Additionally, in young, inflammatory primary progressive MS (PPMS) patients, ocrelizumab has been shown to reduce clinical disease progression and brain atrophy (197). Following anti-CD20 therapies, B cells including Bmem are significantly decreased in the peripheral blood of MS patients (142, 146) (**Table 1**), with dramatic peripheral B cell depletion still evident by 6 months post-treatment. In rituximab-treated patients, a reduction in CSF B cells was also observed in RRMS patients (147, 148), while PPMS patients were only shown to exhibit a moderate reduction (149). In RRMS patients, rituximab treatment was shown to normalize the ratio of GM-CSF to IL-10 producing B cells in the peripheral blood (86). Eight-to-24 months post-treatment, reappearing peripheral blood B cells were strongly diminished in memory B cells (116).

Further B cell-directed therapies have sought to target a more diverse range of B cell phenotypes. Inebilizumab (MEDI-551), an anti-CD19 monoclonal antibody targets pro-B cells through memory B cells, plasmablasts, and some plasma cells (155, 198). In contrast to CD20 which is also expressed on a subpopulation of $CD4^+$ T cells, CD19 is exclusively expressed on B cells (198). Similar to anti-CD20 directed therapies, treatment in RRMS patients results in reduced peripheral B cells (156, 157) and decreased gadolinium-enhancing lesions (157). B cell immunomodulatory therapies targeting B cell survival factors have shown contrasting effects on clinical outcomes. Atacicept treatment in RRMS patients resulted in an increased annualized relapse rate and unaltered gadolinium-enhancing lesions leading to the early termination of the phase II clinical trial (199). In rheumatoid arthritis patients, atacicept treatment led to an increase Bmem numbers in the peripheral blood (152), confirming previous studies that Bmem are spared (137). Similarly, tabalumab, an anti-BAFF monoclonal antibody which blocks immature B cells, mature B cells, and ASC survival, also fails to deplete Bmem (153, 154). Bmem were increased in the peripheral blood (154) and no reduction in gadolinium-enhancing lesions was observed in RRMS patients (200). The findings of unchanged or worse clinical outcomes in atacicept and tabalumab may be due to the minimal effect on Bmem (152, 201), although further studies are required.

Recently, the landscape of MS therapies targeting B cells has expanded to include Bruton's tyrosine kinase (BTK) inhibitors. BTK is a critical enzyme for signaling through the BCR, FcγR, and GM-CSF receptor and is therefore involved in both adaptive and innate immune responses (160, 202). BTK inhibition affects myeloid cells, including microglia (203), and other hematopoietic lineage cells with exception to T cells, plasma cells, and natural killer cells (161). As small molecules, many BTK inhibitors also rapidly penetrate the blood-brain barrier (202, 203). The BTK inhibitors evobrutinib, tolebrutinib, fenebrutinib, orelabrutinib, and B11091 are currently in clinical development for relapsing and progressive forms of MS (**Table 1**). In clinical trials, BTK inhibitors were shown to reduce gadolinium-enhancing lesions (204) and new or enlarging T2 hypointense lesions (205), but did not reduce annualized relapse rates or disease progression in RRMS

TABLE 1 | Immunomodulatory MS treatment effects on Bmem and B cell function.

MS treatment	Target	Target cells/pathways	Bmem phenotypic markers	Memory B cells in blood	B cells in CNS compartment	Effects on B cell function	Outcome	FDA approval/ clinical trial phase
Immunomodulatory: B cell-directed								
Rituximab	Chimeric mAb Anti-CD20	-Expressed on all B cells, but terminally differentiated plasma cells (141) -Some T cells express CD20 (142, 143) -Greater CDC than ADCC (144)	CD19+, CD27+, IgD- (142) CD19+ (145),CD27+ (86)	Decreased (142, 146)	RRMS: CSF CD19+ B cells decreased (147, 148) PPMS: Moderate reduction in CSF B cells compared to PB (149)	RRMS: Ratio of GM-CSF to IL-10 producing B cells in PB normalized (86)	RRMS: patients: -Reductions in new brain MRI lesions -Reduced clinical relapse rates	Phase II
Ocrelizumab	Humanized IgG1 Anti-CD20	-Expressed on all B cells, but terminally differentiated plasma cells -Some T cells express CD20 (143) -Greater ADCC than CDC (144)	N/A	Decreased total CD19+ B cells (150)	Decreased CD19+ B cells (151)	N/A	RRMS: -reduced gd-enhancing lesions and new lesion formation -reduced clinical relapses PPMS: -clinical progression reduced -reduction whole brain atrophy and WM lesion volume	FDA approved: RRMS and PPMS
Ofatumumab	Fully humanized IgG1 Anti-CD20	Expressed on all B cells, but terminally differentiated plasma cells -Some T cells express CD20 (143) -Greater CDC than ADCC activity (144)	N/A	Decreased total CD19+ B cells (145)	N/A	N/A	RRMS: -reduction in number of new gd+ lesions	Phase 2b
Atacicept	Fully human recombinant TACI fusion protein	-Blocks mature B cells and plasma cell survival -Memory B cells spared (137)	Rheumatoid arthritis: CD19+, CD20+, CD27+, CD38– (152)	-Increase in Rheumatoid arthritis patients (152)	N/A	N/A	RRMS: -Annualized relapse rates increased compared to placebo -Similar gd-enhancing lesions	Phase II -Early termination
Tabalumab	Fully humanized IgG4 mAb anti-BAFF (membrane bound and soluble)	Blocks immature/ transitional B cells, naïve/ mature B cells and plasma cell survival (153, 154)	CD19+, CD27+, IgD- (154) CD19+, CD27+, IgD + (154)	-Increase (154)	N/A	N/A	RRMS: -No reduction in gd-enhancing lesions	Phase II
Inebilizumab MEDI-551	Humanized IgG1 mAb Anti-CD19 -Afucosylated IgG Fc region enhances ADCC (155, 156)	Targets pro-B cells through memory B cells, plasmablasts, and some plasma cells (155, 156)	N/A	-Total CD20 + (156, 157), and PC gene phenotype reduced (157)	N/A	N/A	RRMS: -Reduction in new gd-enhancing lesions over 24 weeks	Phase I

(Continued)

TABLE 1 | Continued

MS treatment	Target	Target cells/pathways	Bmem phenotypic markers	Memory B cells in blood	B cells in CNS compartment	Effects on B cell function	Outcome	FDA approval/ clinical trial phase
BTK inhibitors: -Evobrutinib -Tolebrutinib -Fenebrutinib -Orelabrutinib -BIIB091	BTK binding mechanism (158): Evobrutinib: Covalent, irreversible (159) Tolebrutinib: Covalent, irreversible Fenebrutinib: Non-covalent, reversible Orelabrutinib: Covalent, irreversible BIIB091: Non-colavent, reversible	B cells, myeloid cells, and hematopoietic cell lineages (160) except for T cells, plasma cells, and NK cells (161)	Evobrutinib: CD19+ CD20+ IgD- CD27+ CD38- (162)	Evobrutinib: No reduction in peripheral blood Bmem over 48 weeks (162)	N/A	Evobrutinib: Reduced CXCR3+ Bmem migration across human brain endothelial cells *in vitro* (163)	Evobrutinib: RRMS -Reduced gd-enhancing lesions -No effect on annualized relapse rates or disability progression Tolebrutinib: RRMS Reduced new gad-enhancing lesions -Reduced new or enlarging T2 hypointense lesions	Evobrutinib: Phase 3 Tolebrutinib: Phase 3 Fenebrutinib: Phase 3 Orelabrutinib: Phase 2 BIIB091: Phase 1
Immunomodulatory								
IFN-β therapies	Binds to Interferon α/β receptor (IFNAR)	Widespread reduction in cellular and molecular pro-inflammatory mediators and an increase in anti-inflammatory mediators (164)	CD19+, CD27+, CD38-, IgM-IgD- (165); CD27+, IgD-; and CD27 +, IgD+ (166)	Decreased (165) Decreased (166)	N/A	-Decreased MHCII on B cells (167) -Reduced CD80+ (168) and CD40+ (169) B cells -Increased IL-10 production by *in vitro* stimulated B cells (168, 170)	RRMS: -Reduced relapses -Reduced MRI lesion activity -Reduced brain atrophy -Increased time to reach CDMS -Reduced risk of sustained disability progression	FDA approved: RRMS
Glatiramer acetate	Synthetic polypeptide mixture resembling myelin basic protein	Widespread effects on innate and adaptive immunity; suppression of pro-inflammatory mediators; increase in anti-inflammatory mediators (171)	CD27+, IgD- and CD27+, IgD+ (172)	Decreased (172)	(148)	-Reduced CD69, CD25, CD95 expression; decreased TNFα production; increased IL-10 production (173)	RRMS -Reduced relapses -Increased proportion of relapse free patients -reduction in gd-enhancing lesions and new lesions	FDA approved: RRMS
Cladribine	Synthetic chlorinated deoxyadenosine analog	Preferential depletion of T and B lymphocytes (174)	CD19+, CD27+, IgD-, IgM (175)	Decreased (175)	N/A	N/A	RRMS: -Reduced clinical relapse -increased proportion of relapse-free patients -increased proportion patients free from 3 month confirmed disability progression -reduced gd-	FDA approved: RRMS

(Continued)

TABLE 1 | Continued

MS treatment	Target	Target cells/pathways	Bmem phenotypic markers	Memory B cells in blood	B cells in CNS compartment	Effects on B cell function	Outcome	FDA approval/ clinical trial phase
							enhancing lesions and active T2 lesions	
Fingolimod	Structural analog to sphingosine	S1P receptor expressing lymphocytes	CD19+, IgD+, CD27+; CD19+, IgD-, CD27+; CD19+, CD20+, CD27+ (93, 176) CD19+, CD27+, CD38int/high (177) CD27var, CD38- (178)	Decreased (177, 178)	No change in CSF B cell percentage (93, 179)	Impaired CSF B cell clonal expansion (93) -Reduced activation of memory b cells (177)	RRMS: -Reduced number and volume of gd-enhancing lesions -Reduced new and enlarging T2 lesions -Reduced relapse rate -Increased percentage of relapse-free patients -Delayed disability progression	FDA approved: RRMS
Dimethyl fumarate	Fumaric acid ester	Widespread anti-inflammatory properties, including shift from Th1 to Th2 profile (180)	CD27+ (181, 182) CD27var, CD38- (178) CD27+, IgA or IgG+ class-switched Bmem; CD27+, IgM+ unswitched-Bmem (183)	Decreased (178, 181, 182) Class-switched and unswitched both reduced (183)	Decreased (184)	-Reduction in GM-CSF, TNF-alpha, IL-6 producing B cells (181, 183) -reducing phosphorylation of STAT5/6 and NFκB in surviving B cells (183) -IL-10 production by B cells intact (182)	RRMS: -Number of gd + lesions reduced -Reduced new or enlarging T2 lesions and new T1 hyopintensities -Improved annualized relapse rate -Reduced risk of disability progression	FDA approved: RRMS
Teriflunomide	Active metabolite of leflunomide	Rapidly proliferating cells, including T and B cells *via* inhibition of *de novo* pyrimidine synthesis (185)	CD19+, CD27dim/+, CD38dim (186)	B cells reduced (185), but no change in Bmem percentages (186)	N/A	-Inhibits B cell proliferation (187)	RRMS -Reduced annualized relapse rate -Fewer patients experience 3 month sustained disability worsening -More patients relapse free -Reduced MRI total lesion volume and gd-enhancing lesions	FDA approved: RRMS
Mitoxantrone	synthetic anthracenedione derivative	Immunosuppressive including B cell, T helper and T cytotoxic lymphocytes (188, 189)	CD19+, CD27+ (85)	Decreased (85)	N/A	No effect of B cell proliferation (188) -Preferential death of CD27+ B cells *vs* CD27- B cells	RRMS: -Reduced proportion of patients with confirmed	FDA approved: RRMS SPMS

(Continued)

TABLE 1 | Continued

MS treatment	Target	Target cells/pathways	Bmem phenotypic markers	Memory B cells in blood	B cells in CNS compartment	Effects on B cell function	Outcome	FDA approval/ clinical trial phase
						B cells show decrease in lymphotoxin and TNF-α production (85) Increased IL-10 *in vitro* (85)	progression over 2 years -prolongs time to first treated relapse SPMS: -delayed progression -Reduced new T2 lesions	
Alemtuzumab	Humanized mAb IgGk anti-CD52	-High levels on T and B cells -Lower levels on NK cells, monocytes, DCs, macrophages, and eosinophils -Relative sparing of Tregs and little/no expression on neutrophils, plasma cells, hematopoietic precursor cells (190, 191)	CD19+, CD27+ (192)	Decreased (192)	N/A	N/A	RRMS: -Reduced annualized relapse rate *vs* subcutaneous IFNβ-1a -Six-month sustained accumulation of disability reduced -Improvement of EDSS -Increased patients free from any clinical/MRI disease activity	FDA approved: RRMS
Natalizumab	humanized IgG1 mAb to $\alpha_4\beta_1$ integrin	All leukocytes except neutrophils (193, 194)	CD19, +, CD27+, IgD+ (193) CD27var, CD38- (178)	Increased (178, 193)	Decreased B cell percentages (179); Bmem and plasmablasts (93)	- increased CD95+ B cells, increased MHCII+ B cells, increased CD40+ b cell percentage, and increases TNF and IL-6 in *in vitro* stimulated B cells (178)	RRMS: -Reduced annualized relapse rate Reduced risk of sustained disability worsening at 2 years -Decreased gd-enhancing lesions and new/enlarging T2-hypointense lesions	FDA approved: RRMS
Daclizumab	Humanized IgG1 mAb to CD25	Primarily CD4 T cells, but also activated CD8 T cells, dendritic cells, NK cells, and activated B cells and Bmem (195)	CD19+, CD27+	Decreased	N/A	N/A	RRMS: -Reduced annualized relapse rate -Reduced contrast-enhancing lesions and new/enlarging T2 lesions -Improved clinical rating scales	FDA approved: RRMS

ADCC, antibody-dependent cytotoxicity; CDC, complement-dependent cytotoxicity; CSF, cerebrospinal fluid; EDSS, Expanded Disability Status Scale; gd, gadolinium; mAb, monoclonal antibody; N/A, not available; PB, peripheral blood; PPMS, primary-progressive multiple sclerosis; RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; var, variable.

TABLE 2 | Bmem in MS: Unresolved questions.

What mechanisms promote Bmem trafficking to the CNS? Adhesion molecules, chemokines etc.
Do Bmem participate in meningeal inflammation?
Do Bmem play a significant role in sustaining local ASC/Ab in the CNS?
What is the antigen specificity of Bmem recruited to the CNS? Is it the antigen diversity similar to CSF Abs?
Are Bmem pro-inflammatory, anti-inflammatory, or do Bmem play a pleiotropic role in MS?
Do Bmem phenotypes, kinetics, and functions differ by MS disease phenotype?
How do Bmem interact with other immune cells and CNS resident cells within the CNS?
How can Bmem be utilized to monitor and optimize therapeutic effects?

patients (204). Preliminary studies monitoring peripheral blood B cells in evobrutinib-treated RRMS and SPMS patients revealed no clinically relevant changes in the number of total B cells or Bmem over the 48 week treatment period (162). However, *in vitro* assays demonstrated an alteration in Bmem function, with reduced CXCR3+ Bmem migration across human brain endothelial cells (206).

Other Immunomodulatory Therapies

Numerous immunomodulatory therapies utilized in MS have also been observed to affect Bmem. Although not traditionally viewed as modulating the B cell compartment, these therapies can have direct or indirect effects on Bmem survival and function. Interferon (IFN)-β, glatiramer acetate, fingolimod, dimethyl fumarate, and mitoxantrone all reduce Bmem numbers in peripheral blood and alter global B cell function following therapeutic treatment (**Table 1**). Peripheral blood B cells obtained from IFN-β-treated patients exhibit reductions in MHCII expression (167), reduced co-stimulatory molecules CD80 (168) and CD40 (169), and an increase in IL-10 production (168, 170), suggesting a shift in the overall B cell profile to an anti-inflammatory state. IFN-β treatment was also found to increase Bmem apoptosis (115). Glatiramer acetate-treated MS patients also show alterations in B cell function, resulting in reduced activation markers (CD69, CD95), decreased TNF production, and increased IL-10 production (173). Fingolimod, which targets SIP receptor-expressing lymphocytes such as T cells and B cells results in impaired CSF B cell clonal expansion (93), including Bmem, and reduced Bmem activation in peripheral blood from MS patients (177). Dimethyl fumarate treatment results in similar modulation reducing B cell activation (183) and the production of the pro-inflammatory cytokines GM-CSF, TNF, and IL-6 (181, 183), while IL-10 production is unaltered (182). Mitoxantrone treatment, immunosuppressive to T cells and B cells, does not affect B cell proliferation (188), but results in the preferential death of CD27-expressing B cells and a shift to an anti-inflammatory state, with reduced LT and TNF production, and increased IL-10 production *in vitro* (85). Conversely, natalizumab, which blocks leukocyte $\alpha_4\beta_1$-mediated entry into the CNS, results in a 2.4-fold increase in Bmem in the peripheral blood (178, 193), but a reduction of Bmem in the CSF (93). In contrast to the aforementioned therapies, B cell activation (CD95, CD40, MHCII expression) and TNF and IL-6 production was increased in the peripheral blood of natalizumab-treated MS patients (178). Multiple other immunomodulatory therapies which have shown to be effective in improving clinical outcomes in RRMS patients, including cladribine, teriflunomide, daclizumab, and alemtuzumab all decrease peripheral Bmem numbers (**Table 1**), though findings related to the functional changes in B cells following therapeutic treatment remain to be determined.

Bmem and Tailoring Therapeutic Treatment

Bmem in peripheral blood may prove useful for monitoring therapeutic effects in MS. In one study, Novi et al. utilized a Bmem-based reinfusion protocol for rituximab administration. Bmem monitoring ($CD19^+$ $CD27^+$ PBMCs) was used to orchestrate rituximab reinfusion, leading to a reduced number of reinfusions while still reducing disease activity (146). This study highlights the potential role for monitoring Bmem to tailor immunomodulatory treatments in MS. Future studies may also investigate the utility of monitoring Bmem in peripheral blood to predict response to therapy, including B cell depletion, in MS. Bmem monitoring in peripheral blood is a currently utilized strategy for predicting response to B cell depletion therapies in several autoimmune diseases implicating B cells including Sjogren's syndrome, system lupus erythematosus, and rheumatoid arthritis (207–209).

Altogether, future studies are required to determine the exact effects on Bmem function following immunomodulatory treatment, including whether Bmem are central to the efficacy of disease-modifying therapies, and whether Bmem monitoring can be used to "personalize" immunotherapy.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The cause of MS is unknown but growing evidence suggests multiple B cell phenotypes are central players in MS pathogenesis. In MS, Bmem in both the peripheral and CNS compartments are increasingly being explored to define the exact relationship with disease development and progression. Important observations highlighted in the current review include the presence of Bmem alterations in both the peripheral blood and CNS compartments in MS; evidence for potential roles in antibody production, antigen presentation, and cytokine production (**Figure 3**); and effective targeting of Bmem using currently available immunomodulatory therapies. Future studies should aim to address several key unresolved questions to provide more in-depth insights regarding Bmem in MS (**Table 2**), including trafficking mechanisms, action within the CNS compartment, functional relevance in MS immunopathogenesis, and defining associations with clinical

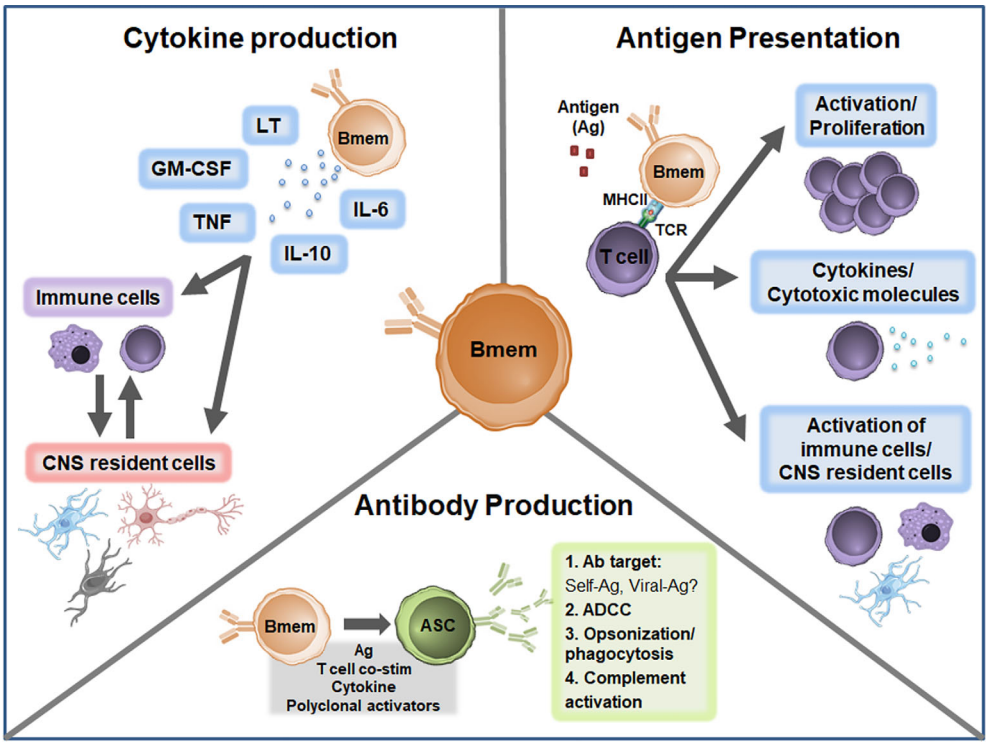

FIGURE 3 | Proposed Bmem functions in MS. Canonical Bmem functions include cytokine production, antigen presentation, and antibody production. Bmem may produce a variety of pro-inflammatory and anti-inflammatory cytokines in MS, including lymphotoxin (LT), GM-CSF, TNF, IL-10, and IL-6. The production of these cytokines may 1) modulate the inflammatory function of immune cells, including monocytes and T cells in the periphery or CNS or 2) may alter the function and survival of CNS resident cells, including neurons, astrocytes, microglia, and oligodendrocytes. Bmem-derived cytokines may also modulate the interactions between CNS-localized immune cells and CNS resident cells. Bmem are potent antigen presenting cells (APCs) and upon uptake and presentation of antigen (Ag) may interact with other immune cells, including T cells, to enhance cell proliferation and effector functions. For example, following T cell-Bmem interaction, activated T cells may engage in cytokine production or cytotoxic molecule secretion. Bmem antigen presentation to T cells may also modify T cell engagement with other immune cells in the periphery or CNS and interaction with CNS resident cells. Bmem can differentiate into ASC following stimulation, including re-exposure to antigen, T cell co-stimulation (co-stim), cytokine stimulation, or T cell-independent polyclonal stimulation. Upon differentiation, Bmem-derived ASC may be involved in sustaining antibody responses in the CNS compartment. Bmem-derived ASC may also contribute to several antibody-dependent functions implicated in MS, including targeting self or viral antigens, antibody-dependent cellular cytotoxicity (ADCC), opsonization/phagocytosis, and complement engagement.

outcomes. These insights may help to guide therapeutic strategies to develop novel agents specific for Bmem and tailor current therapeutic treatment regimens.

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KD and AP outlined the subject for the review. KD reviewed the literature, drafted the figures and tables, and wrote the manuscript. AP and FG edited and revised the manuscript. All authors contributed to the article and approved the submitted version.

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