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THEORETICAL ARTICLE

Epstein–Barr virus infection, B-cell dysfunction and other risk factors converge in gut-associated lymphoid tissue to drive the immunopathogenesis of multiple sclerosis: a hypothesis

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Abstract

Multiple sclerosis is associated with Epstein–Barr virus (EBV) infection, B-cell dysfunction, gut dysbiosis, and environmental and genetic risk factors, including female sex. A disease model incorporating all these factors remains elusive. Here, we hypothesise that EBV-infected memory B cells (MBCs) migrate to gut-associated lymphoid tissue (GALT) through EBV-induced expression of LPAM-1, where they are subsequently activated by gut microbes and/or their products resulting in EBV reactivation and compartmentalised anti-EBV immune responses. These responses involve marginal zone (MZ) B cells that activate $CD4^+$ T-cell responses, via HLA-DRB1, which promote downstream B-cell differentiation towards $CD11c^+$/T-bet^+ MBCs, as well as conventional MBCs. Intrinsic expression of low-affinity B-cell receptors (BCRs) by MZ B cells and $CD11c^+$/T-bet^+ MBCs promotes polyreactive BCR/antibody responses against EBV proteins (e.g. EBNA-1) that cross-react with central nervous system (CNS) autoantigens (e.g. GlialCAM). EBV protein/autoantigen-specific $CD11c^+$/T-bet^+ MBCs migrate to the meningeal immune system and CNS, facilitated by their expression of CXCR3, and induce cytotoxic $CD8^+$ T-cell responses against CNS autoantigens amplified by BAFF, released from EBV-infected MBCs. An increased abundance of circulating IgA^+ MBCs, observed in MS patients, might also reflect GALT-derived immune responses, including disease-enhancing IgA antibody responses against EBV and gut microbiota-specific regulatory IgA^+ plasma cells. Female sex increases MZ B-cell and $CD11c^+$/T-bet^+ MBC activity while environmental risk factors affect gut dysbiosis. Thus, EBV infection, B-cell dysfunction and other risk factors converge in GALT to generate aberrant B-cell responses that drive pathogenic T-cell responses in the CNS.

Keywords: $CD11c^+$/T-bet^+ memory B cells, Epstein–Barr virus, gut-associated lymphoid tissue, marginal zone B cells, multiple sclerosis

INTRODUCTION

Multiple sclerosis (MS) is an immune-mediated inflammatory disease of the central nervous system (CNS) that results in demyelination of neurons and neurological disability. Multiple risk factors for developing MS have been identified,[1,2] including female sex; genetic variations impacting the function of the immune system,[3] particularly alleles of genes encoding human leucocyte antigens (HLAs) of which HLA-DRB1*1501 is the strongest genetic risk factor[4]; chronic infection with Epstein–Barr virus (EBV)[5]; variation of the gut microbiome; low ultraviolet radiation (UVR) exposure; low vitamin D levels; and smoking cigarettes. While past research on the immunopathology of MS has focussed on $CD4^+$ T cells, there is growing awareness that $CD8^+$ T cells and B cells, and the effect of EBV infection on these cells, are central to the immunopathology underlying MS.[6] We have examined various aspects of B-cell dysfunction[7–10] and the relationship of gut microbiome-derived short-chain fatty acids (SCFAs) with immune dysfunction[11] in patients with early MS and identified abnormalities that led to the hypothesis proposed here; that EBV infection, B-cell dysfunction and other risk factors converge in gut-associated lymphoid tissue (GALT) to generate aberrant B-cell responses that drive pathogenic T-cell responses in the CNS (Figure 1).

Recently, there have been several important developments in understanding the relationship between EBV infection and the immunopathogenesis of MS.[12–14] However, the hypothesis presented here, coupled with published evidence from this and other laboratories, provides a deeper and clearer insight into the mechanisms by which EBV infection may drive MS development.

EBV-INDUCED DYSFUNCTION OF MEMORY B CELLS IS CENTRAL TO THE IMMUNOPATHOGENESIS OF MS

The peri-vascular and parenchymal inflammatory infiltrates associated with demyelination in brains of MS patients are characterised by an abundance of $CD8^+$ T cells, which predominantly have characteristics of resident memory T (Trm) cells, and B cells.[6,15,16] While uncertainty remains about interactions between $CD8^+$ T cells and B cells,[6] B cells clearly play a fundamental role in the inflammatory process. This became apparent over 40 years ago with the description of oligoclonal immunoglobulins in the CSF of MS patients. More recently, the contribution of B cells has been demonstrated by the pronounced therapeutic effect of B-cell depletion therapies.[17] Most recently, it has been reported that CSF oligoclonal immunoglobulins are enriched for antibodies reactive with EBV proteins, particularly EBV nuclear antigen-1 (EBNA-1), reflecting the expansion of oligoclonal B cells producing an antibody response against EBNA-1.[12] Because of antigenic cross-reactivity, some of these antibodies also recognise GlialCAM, a cell adhesion molecule expressed by astrocytes and oligodendrocytes in the CNS, in a subset of patients. Furthermore, plasma levels of IgG antibodies to GlialCAM were significantly higher in three independent groups of MS patients compared with controls.[12] These findings provide a mechanistic explanation for the numerous reports linking MS with EBV infection,[5] culminating in recent reports that serological evidence of EBV infection is present in 100% of MS patients[18] and that EBV infection acquired in late adolescence or early adulthood is associated with a 32-fold increased risk of developing MS.[13] Furthermore, the particular significance of antibodies to EBNA-1 in MS immunopathogenesis is illustrated by reports that high serum levels of anti-EBNA-1 IgG antibodies are not only associated with an increased risk of MS but synergise with other risk factors to increase that risk and predict conversion from clinically isolated syndrome suggestive of MS (CIS) to MS, whereas antibodies to other EBV proteins do not.[19–21]

Epstein–Barr virus is a human gamma-herpesvirus that is transmitted from one person to another primarily by saliva and infects both epithelial cells and B cells, the latter via CD21 (complement receptor 2). Following primary EBV infection, a reservoir of latently infected B cells is established in lymphoid tissue, particularly in the oro-pharynx,[22] from where EBV-infected B cells may migrate to GALT and associated lymph nodes.[23] This migration is facilitated by EBV-induced expression of the 'gut homing molecule' LPAM-1 (integrin $\alpha4/\beta7$) (Figure 1). EBV primarily establishes persistent infection of IgD^-CD27^+ B cells (conventional memory B cells [cMBCs]). However, EBV DNA is also detectable to a lesser degree in $CD27^+IgD^+$ B cells,[24] which form the major component of the IgM^+ MBC subpopulation and exhibit characteristics of circulating marginal

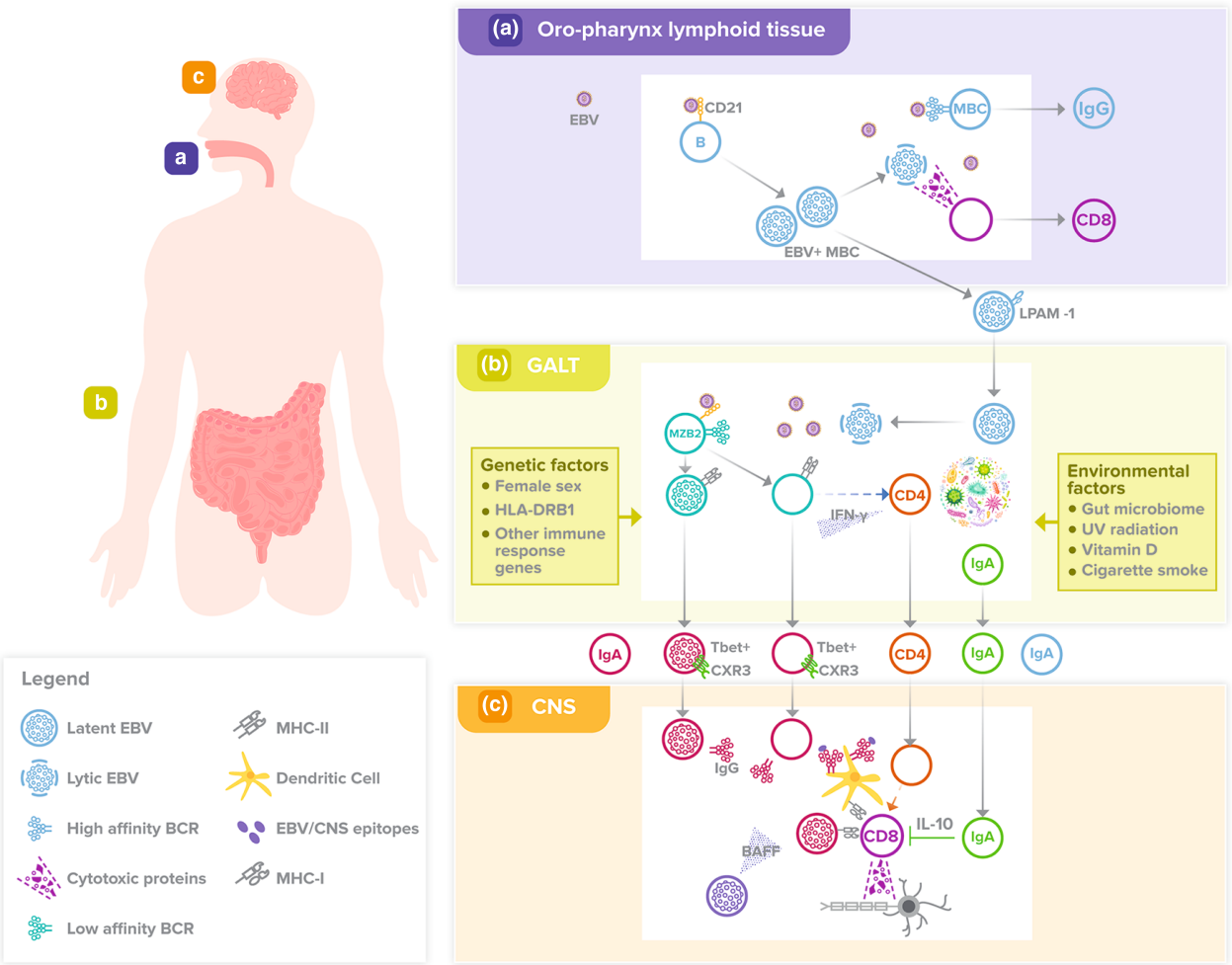

Figure 1. Proposed model of MS disease pathogenesis. **(a)** During primary EBV infection, a reservoir of EBV-infected MBCs is established in lymphoid tissue, particularly in the oro-pharynx. Reactivation of latent EBV infection in MBCs responding to oropharyngeal infections leads to lytic EBV infection, which induces an EBV-specific $CD8^+$ T-cell and antibody response by circulating T cells and B cells expressing high affinity BCRs. Active EBV infection in MBCs during primary EBV infection, and possibly during subsequent reactivations of infection, induces LPAM-1 expression on EBV-infected MBCs and their migration to GALT. **(b)** Episodic reactivation of latent EBV infection in MBCs residing in GALT by gut microbes via BCRs and/or pathogen recognition receptors and/or SCFAs leads to lytic EBV infection, which induces a compartmentalised EBV-specific immune response. This response includes MZB2 cells, some of which may also become infected by EBV. Low-affinity BCRs on MZB2 cells promote an antibody response against EBV proteins, such as EBNA-1, that is polyreactive and cross-reacts with CNS autoantigens, such as GlialCAM. $CD4^+$ T cells are activated by MBZ2 cells via HLA-DRB1 and 'autoproliferate', including in blood, as well as migrate to the CNS. These $CD4^+$ T cells produce IFN-γ, which facilitates a $CD11c^+$/T-bet^+ MBC differentiation pathway amongst both EBV-infected and -uninfected EBV/autoantigen-specific MZB2 cells. Gut microbiota-specific IgA^+ B cells and/or plasma cells are also produced in GALT and migrate to the CNS. In addition, IgA^+ cMBCs and T-bet^+ MBCs accumulate in blood, possibly as a result of repeated EBV reactivation in GALT, and may generate EBV-specific IgA antibody responses that interfere with EBV-specific IgG antibody responses. The major genetic and environmental factors associated with an increased risk of developing MS affect these processes in GALT. **(c)** Both EBV-infected and -uninfected EBV protein/CNS autoantigen-specific $CD11c^+$/T-bet^+ IgG^+ MBCs migrate to the meningeal immune system within the dura, and subsequently to the CNS, facilitated by cell-surface expression of CXCR3. At sites of inflammation in the CNS, these B cells mediate a B-cell/antibody response against EBV proteins (e.g. EBNA-1) and CNS autoantigens (e.g. GlialCAM). This drives an autoantigen-specific $CD8^+$ T-cell response by $CD11c^+$/T-bet^+ IgG^+ MBCs acting as APCs themselves and/or by generating production of IgG antibodies that form immune complexes with autoantigens and deliver them to cDCs, which cross-present antigens to, and activate, $CD8^+$ T cells. Autoantigen-specific $CD8^+$ T cells mediate a cytotoxic cellular immune response against antigens of myelin and/or glial cells. These activities are augmented by BAFF produced by EBV-infected $CD11c^+$/T-bet^+ MBCs or cMBCs or both. Gut microbe-specific IgA^+ plasma cells may modulate $CD8^+$ T-cell activation via IL-10.

zone (MZ) B cells.[25] Infection of B cells by EBV leads to the production of various EBV proteins, including the cell-surface molecules latent membrane protein (LMP) 1 and 2, as well as viral transcription factors, such as EBNA-1. Together, these molecules modulate B-cell function to keep the infected MBC alive and evade innate and adaptive immune responses.[26] Such immune evasion strategies first become apparent during primary EBV infection when EBV-induced B-cell dysfunction causes a decreased abundance of circulating MBCs and impaired EBV-specific neutralising antibody responses.[27] This appears to reflect impaired B-cell proliferation and survival associated with increased expression of Fas (CD95) on B cells and increased plasma levels of Fas ligand, as well as increased plasma levels of B-cell-activating factor (BAFF) but decreased levels of a proliferation-inducing ligand (APRIL), associated with reciprocal changes in B-cell expression of their receptors.

Approximately 95% of adults are infected by EBV with the majority of infections occurring during childhood. The risk of MS is highest in individuals who experience symptomatic primary EBV infection, usually presenting as infectious mononucleosis (IM), in adolescence or early adulthood.[28] IM is not observed in younger children,[29,30] possibly reflecting differences in the immune system associated with young age, such as Th2-skewedness, more abundant regulatory T cells and a smaller pool of memory T cells, rather than differences in plasma EBV DNA loads, which are comparable in children with primary EBV infection and IM patients.[30,31] As tonsillar B cells of IM patients strongly express the β7 integrin component of LPAM-1, it has been proposed that EBV establishes a reservoir of latently infected B cells in GALT during primary infection to evade EBV-specific $CD8^+$ T-cell responses.[23] In chronic EBV infection, EBV-specific circulating $CD8^+$ memory T ($CD8^+$ Tm) cells and $CD8^+$ Trm cells normally control EBV replication in epithelial cells and MBCs of the oropharynx.[32] By contrast, EBV-specific $CD8^+$ T-cell responses may be less effective in GALT because gut $CD8^+$ T cells are predominantly intra-epithelial and lamina propia $CD8^+$ Trm cells.[31,33] In addition, MS patients exhibit progressively less effective circulating $CD8^+$ Tm cell responses against EBV-infected B cells, related to a decreased abundance and exhaustion of EBV-specific $CD8^+$ Tm cells. This is associated with skewing of the EBV-specific immune response towards latent phase antigens and increased production of IgG antibodies to EBNA-1.[34]

DISEASE-ASSOCIATED MBCS IN MS PATIENTS EXHIBIT HETEROGENEOUS IMMUNOPHENOTYPIC AND FUNCTIONAL CHARACTERISTICS

A large proportion of B cells infiltrating the brain of MS patients are $CD27^+$ MBCs and express the EBV proteins LMP-1 and -2 on their surface.[35] These cells also produce BAFF, likely as a result of the transcriptional regulation by LMP-1.[36] Analyses of CSF cells from MS patients have provided some insight into the derivation of these B cells. An unsupervised analysis of cells detected by mass cytometry demonstrated a $CD27^+$ B-cell population that was clearly associated with MS, although represented $< 1.5\%$ of total cells.[37] Notably, these B cells expressed the chemokine receptor CXCR3, which facilitates cell trafficking to tissues and is only expressed by about one-third of circulating MBCs.[38] In a separate study, analysis of single-cell transcriptomes and immunoglobulin sequences in defined B-cell subpopulations demonstrated that clonally expanded, somatically hypermutated $IgG1^+$ and IgM^+ B cells were associated with inflammation, blood–brain barrier breakdown and intrathecal immunoglobulin synthesis.[39] Furthermore, when compared with blood, transcripts upregulated in CSF switched MBCs (IgD^-CD27^+) and plasmablasts included those for the Th-1-associated transcription factor T-bet, as well as CXCR3, providing further evidence that disease-associated MBCs might differ from the majority of circulating MBCs. In addition, somatically hypermutated (antigen experienced) IgM^+ B cells had a phenotype resembling MZ B cells. Together, these observations provided evidence that MBCs associated with CNS immunopathology in MS might, wholly or in part, differ from cMBCs.

One of the first abnormalities of circulating B cells recognised in MS patients was expansion of age-associated B cells (ABCs).[40] While ABCs were initially considered to be activated and senescent MBCs, it is now clear that they represent a distinct subpopulation of MBCs, commonly referred to as 'atypical MBCs', which exhibit phenotypic and functional characteristics that distinguish them from cMBCs. These characteristics include activation via TLR7 and 9 as well as B-cell

receptors (BCRs) and differentiation under the influence of T cells and Th1-associated cytokines, such as IFN-γ.[41–43] In addition, they express a distinctive pattern of molecules that determine their functional characteristics, particularly T-bet and the integrin molecule CD11c (integrin alpha X).[41,44,45] Furthermore, transcriptome analysis of 'atypical MBCs' has demonstrated high expression of CD18 (integrin β2).[42] The CD11c/CD18 complex is a major adhesion molecule on antigen presenting cells (APCs), particularly conventional dendritic cells (cDCs).[46] 'Atypical MBCs' express high levels of CXCR3 in health and disease,[47] reflecting the regulation of CXCR3 expression by T-bet,[48] and low levels of CD21, while CD27 expression is variable.[41] Thus, whereas $CD21^{-}$/T-bet^{+} MBCs usually exhibit a double negative (DN; $CD27^{-}$/IgD^{-}) phenotype, differentiation towards plasma cells is associated with high CD27 expression.[49] Several other names are used to describe B-cell subpopulations that are identical to, or substantially overlap with, 'atypical MBCs'[50–54] (Table 1), including $CD11c^{+}$/T-bet^{+} MBCs which, in our opinion, best describes these cells with regard to important functional characteristics and is how we will refer to them moving forward.

Production of $CD11c^{+}$/T-bet^{+} MBCs is part of the normal B-cell response to infection by various pathogens, most comprehensively described for viruses and *Plasmodium sp*., and to viral vaccines.[41,55,56] Furthermore, data from studies in mice suggest that $CD11c^{+}$/T-bet^{+} MBCs contribute to the control, as opposed to prevention, of virus infections, including infection by EBV-like gamma-herpesvirus 68.[57,58] In view of the T-cell dependence of $CD11c^{+}$/T-bet^{+} MBC differentiation,[43] the functional effects of these cells likely occur in concert with those of effector T cells, particularly $CD8^{+}$ T cells. There is also accumulating evidence indicating that $CD11c^{+}$/T-bet^{+} MBCs contribute to autoantibody responses and that activation of these cells via TLR7 (the gene of which is on the X chromosome) may in part explain the female predominance of some autoimmune diseases.[52,59] Indeed, ABCs were first observed in aged female mice predisposed to autoimmune disease.[59] Furthermore, B-cell-specific depletion of T-bet in a mouse model of SLE not only resulted in decreased disease manifestations but also resulted in reduced levels of germinal centre B cells and plasmablasts, suggesting that T-bet^{+} B cells may be precursors of autoantibody-producing plasmablasts. B cell-specific T-bet depletion was also associated with decreased T-cell activation.[60]

Although circulating $CD11c^{+}$/T-bet^{+} MBCs are increased in some MS patients,[40] their contribution to MS immunopathology has been unclear. Recently, van Langelaar *et al*.[61] demonstrated that patients with advanced MS exhibited an accumulation of $CXCR3^{+}$ B cells in the CSF, meninges and brain and that circulating $CXCR3^{+}$ B cells expressed T-bet, the level of which correlated with the level of CXCR3 expression. These CXCR3(T-$bet)^{+}$ B cells also expressed IgG. CXCR3(T-$bet)^{+}$ IgG^{+} B cells were less abundant in blood than the CNS but their abundance increased after natalizumab therapy, which blocks α4 integrin and prevents migration of cells expressing this molecule, providing evidence that CXCR3(T-$bet)^{+}$ IgG^{+} B cells migrate from blood to the CNS of MS patients. The high transmigration capacity of CXCR3(T-$bet)^{+}$ IgG^{+} B cells was confirmed by *ex vivo* studies.[61] In addition, *ex-vivo* cell culture studies demonstrated that T-cell-derived IFN-γ increased the expression of T-bet in B cells of MS patients to a greater degree than in controls and that this correlated strongly with CXCR3 expression.[61] Together, these findings

Table 1. Various names used to describe $CD11c^{+}$/T-bet^{+} memory B cells

Name	Context in which described
Age-associated B cells	Originally described in aged mice and subsequently shown to be increased in autoimmune disease in mice and humans[45]
Tissue-like MBCs	HIV-1 infection[50]
Atypical MBCs	Chronic infectious diseases, including HIV-1 infection, malaria and tuberculosis[51]
Double negative type 2 (DN2) B cells	Systemic lupus erythematosus (SLE)[52]
$CD21^{low}$ B cells	Common variable immunodeficiency disorder[53]
$CD11c^{+}$, T-bet^{+} or $CD11c^{+}$/T-bet^{+} MBCs	Autoimmune diseases, including SLE, rheumatoid arthritis and systemic sclerosis, as well as infectious diseases, including HIV-1 infection and malaria[54]

provided compelling evidence that CD11c$^+$/T-bet$^+$ MBCs are more abundant than normal in the CNS of MS patients, to where they have migrated from blood facilitated by their expression of CXCR3.

Preliminary evidence suggests that CXCR3 (T-bet)$^+$ IgG$^+$ B cells are induced by reactivation of EBV infection, as EBV DNA load correlated with the frequency of circulating CXCR3$^+$ cMBCs in MS patients who had received an autologous haematopoietic stem cell transplant, which increases the risk of EBV reactivation.[62] Supportive evidence is provided by a study of the single-cell transcriptome of primary B cells infected with EBV *ex vivo*, which demonstrated that EBV infection simulates antigen-induced activation and differentiation of B cells, including production of 'atypical MBCs' expressing CXCR3, FcRL4 and T-bet.[63] In mice, CD11c$^+$ B cells exhibit potent APC activity for T cells, associated with higher expression of HLA-DR and the costimulatory molecules CD80 and CD86 as well as more stable interactions with T cells, compared with follicular B cells.[64] Therefore, CD11c$^+$/T-bet$^+$ MBCs may be involved in MS pathogenesis through increased numbers related to EBV replication, increased migratory capacity to the CNS via CXCR3 expression and by acting as APCs for pathogenic T cells (Figure 1).

While most B cells in the meninges, peri-vascular spaces and brain parenchyma adjacent to areas of demyelination are IgG$^+$, IgA$^+$ cells are also present and predominantly have the characteristics of plasma cells.[65] These cells presumably produce the IgA that increases in CSF during relapses of MS.[65] Probstel *et al*.[65] also reported that a subset of these cells are gut microbiota-specific IgA$^+$ plasma cells that express IL-10 and might play an immunoregulatory role in MS.

REACTIVATION OF EBV IN GALT MAY INDUCE PATHOGENIC MBC RESPONSES

As evidence mounts that CD11c$^+$/T-bet$^+$ MBCs may be important contributors to MS immunopathology in the CNS, evidence is also emerging that B-cell dysfunction induced by EBV infection may arise in GALT. Within GALT and associated mesenteric lymph nodes,[23] EBV-infected MBCs are exposed to microbial SCFAs, which have been shown to exert stimulatory and inhibitory effects on EBV replication,[66] as well as to antigenic stimulation by gut microbes via BCR-dependent and -independent mechanisms.[67] We propose that MBC activation and corresponding reactivation of EBV infection induces compartmentalised immune responses against EBV. The GALT consists of multifollicular lymphoid tissues, such as Peyer's patches of the small intestine, isolated lymphoid follicles of the small and large intestines, the appendix, caecal and colonic patches and rectal lymphoid tissues.[67] These lymphoid tissues contain diverse immune cells involved in the initation and propagation of immune responses, including CD4$^+$ T cells, IgM$^+$ MBCs such as MZ B cells, as well as both IgA$^+$ and IgG$^+$ MBCs. IgA$^+$ MBCs are particularly prominent because of the importance of IgA antibodies in regulating microbial growth in the intestines. Furthermore, IgA$^+$ B cells and/or plasma cells derived from GALT are an important component of the gut-meningeal immune axis that protects the CNS from gut-derived infections.[68,69] MZ B cells, which differentiate from IgM^{hi} T2 transitional B cells, circulate between the spleen MZ and GALT, where they occupy a micro-anatomical niche independently of cMBCs.[67,70] Two subpopulations of MZ B cells have recently been defined in GALT and spleen, referred to as MZB1 and MZB2 cells.[71] MZB2 cells exhibit characteristics that suggest they contribute to antiviral responses and, importantly, express HLA-DRB1 in contrast to MZB1 cells.[71] DN MBCs have also been demonstrated in GALT but it is unclear whether they are CD11c$^+$/T-bet$^+$ MBCs.[71] Studies undertaken in mice and humans have demonstrated that CD11c$^+$/T-bet$^+$ MBCs migrate to MZ regions of the spleen after interacting with T follicular helper (Tfh) cells in lymphoid follicles[44,72] and it is possible that, like MZ B cells, they also migrate to GALT. In support of this proposal is the finding that rotavirus infection of the gastro-intestinal tract induces circulating antigen-specific MBCs that are skewed towards CD27$^-$ IgG$^+$ MBCs, as well as MZ B cells.[73,74]

To gain further insight into circulating MBC dysfunction in patients with early MS, we investigated the characteristics of MBCs expressing BCRs with different immunoglobulin isotypes.[10] We demonstrated an increased abundance of IgA$^+$ MBCs, which has been confirmed in an independent study of patients with more advanced MS.[75] The IgA$^+$ MBCs in our study

consisted of one major and two smaller subpopulations that were all more abundant compared with controls. One of the smaller subpopulations consisted of $CD27^{-}$ IgA^{+} MBCs, which are enriched amongst gut bacteria-specific B cells,[76] and may be precursors of the gut microbiota-specific IgA^{+} plasma cells proposed to exert an immunoregulatory role in MS patients.[65] The major subpopulation had characteristics of cMBCs ($CD27^{+}$/$CD24^{hi}$) while the other smaller subpopulation consisted of T-bet^{+}/$CD21^{low}$ IgA^{+} MBCs. The increased abundance of these subpopulations also suggested an accumulation of MBCs from mucosal B-cell responses, possibly from repeated episodes of EBV reactivation. In support of this proposal are reports that patients with naso-pharyngeal carcinoma (NPC), a classical EBV-induced cancer of mucosal tissue, exhibit an increased frequency of IgA^{+} B cells in the tumor microenvironment and blood,[77,78] although a direct relationship remains to be established. Furthermore, a strong relationship between high serum levels of IgA antibodies to EBV proteins and both the development of, and a poor outcome from, NPC has been reported.[79,80] Preliminary evidence suggests that IgA antibodies to EBV proteins may have a pathogenic effect in EBV-associated diseases, including interference with antibody-dependent cellular cytotoxicity (ADCC) of EBV-infected cells mediated by IgG antibodies.[79,81] Similar mechanisms have been reported to explain the adverse effect of IgA anti-HIV-1 gp120 on ADCC responses in people with HIV-1 infection.[82] The circulating IgA^{+} MBCs that are more abundant in MS patients might therefore be part of separate immune responses against gut microbiota or EBV that have opposite effects on disease pathogenesis (Figure 1). It is possible that the increased abundance of circulating IgA^{+} MBCs in EBV-associated diseases is an effect of EBV infection itself, as expression of EBV LMP-1 on B cells induces class switch recombination (CSR) of immunoglobulin heavy chain (IGHC) gene DNA, potentially through increased expression of BAFF and APRIL.[36]

In addition to the increased abundance of circulating IgA^{+} MBC, further evidence that B-cell dysfunction in patients with early MS arises in GALT is provided by our observations on abnormalities of circulating MZ B cells. First, female patients exhibited decreased expression of the inhibitory Fcγ receptor (FcγR) FcγRIIb on circulating MZ B cells, as well as on naïve B cells, which was associated with serological evidence of EBV reactivation.[9] Second, EBV reactivation was associated with an increased abundance of circulating T-bet^{+}/$CD21^{low}$ IgM^{+} MBCs and $CXCR3^{+}$ IgM^{+} MBCs as well as increased expression of HLA-DR on IgM^{+} MBC in all patients.[10] Based on these findings and those of multiple studies in mice and humans implicating MZ B cells in producing autoantibody responses,[83] we propose that MZ B-cell activation and dysfunction associated with EBV reactivation in GALT may be determinants of pathogenic B-cell responses in MS (Figure 1). MZ B cells express BCRs with low affinity for antigens,[84] which would confer greater polyreactivity with antigens.[85] While this may be advantageous in 'frontline' B-cell responses against pathogens, including gut microbes, it increases the likelihood of BCR and antibody cross-reactivity with autoantigens and induction of autoantibody responses.[86] Indeed, monoclonal IgG antibodies to HIV-1 gp140 derived from intestinal B cells of people with HIV infection exhibit low affinity, high polyreactivity and cross-reactivity with autoantigens.[87] Impaired regulation of MZ B-cell activation via decreased FcγRIIB expression in female patients with MS would compound these abnormalities by impairing peripheral tolerance mechanisms.[88] In addition, murine T-bet^{+} IgM^{+} MBCs, which also express CXCR3, generate multilineage effector B cells[89] and maintain long-term antibody responses.[90] It is therefore possible that the circulating T-bet^{+}/$CD21^{low}$ and $CXCR3^{+}$ IgM^{+} MBCs we observed to be associated with EBV reactivation are precursors of the CXCR3 $(T\text{-}bet)^{+}$ IgG^{+} B cells implicated in the immunopathogenesis of brain lesions in advanced MS[61] and of the circulating T-bet^{+}/$CD21^{low}$ IgA^{+} MBCs that we reported are increased in patients with early MS.[10] However, further studies are required to experimentally establish such a link. Infection of $CD11c^{+}$/T-bet^{+} MBCs by EBV is likely to occur at the MZ B-cell stage because these cells express large amounts of CD21.[83] Notably, CpG DNA ligation of TLR9 induces terminal differentiation of MZ B cells into autoantibody-producing cells,[91] which may contribute to EBV-induced MZ B-cell dysfunction. Therefore, we propose that MZ B cells expressing polyreactive BCRs are primed by the microbiome and/or lytic EBV infection in the GALT and differentiate into $CD11c^{+}$/T-bet^{+} MBCs.

EBV-INDUCED ACTIVATION AND DYSFUNCTION OF B CELLS IN GALT MAY INDUCE B-CELL RESPONSES THAT FACILITATE PATHOGENIC CD8$^+$ T-CELL RESPONSES IN THE CNS

While debate continues about the role of CD8$^+$ T cells in CNS lesions of MS patients,[92] compelling arguments have been made that they mediate glial cell damage and/or demyelination, although uncertainty remains concerning their antigen reactivity.[6] Whether the CD8$^+$ T-cell response is primarily against neurons, other CNS cell-types, or against EBV-infected cells, with bystander damage to neurons, remains uncertain. Indeed, there has been a robust debate as to whether or not EBV proteins or genomes are detectable in brain lesions of MS patients.[93] In our view, there is convincing evidence of EBV in inflammatory cells, including B cells,[94,95] as well as evidence that CD8$^+$ T cells reactive with multiple lytic and latent phase proteins of EBV and exhibiting a cytotoxic phenotype are co-located with B cells in CNS lesions of patients with advanced MS.[94] However, it could not be determined whether these CD8$^+$ T cells were EBNA-1-specific because only the most immunodominant EBNAs (3A and 3C) were included in the pentamer probes used. Nevertheless, the demonstration of cross-reactive B-cell responses against EBNA-1 and GlialCAM in patients with MS[12] provides support for a hypothesis that EBV-infected MBCs, including CD11c$^+$/T-bet$^+$ MBCs, generate B-cell responses against both EBV proteins and glial cell and/or myelin proteins that could drive pathogenic CD8$^+$ T-cell responses. Thus, EBV-infected B cells are capable of cross-presenting antigens to CD8$^+$ T cells, facilitated by increased expression of class I and II MHC molecules and the costimulatory molecules CD40, CD80 and CD86, as well as activation of the cross-presentation machinery.[96] This process is likely more robust in CD11c$^+$/T-bet$^+$ MBCs because they express molecules typically found on APCs (CD11c/CD18) and exhibit potent APC activity in mice.[64] In addition, IgG antibodies to antigens, such as EBNA-1/GlialCAM, might enhance CD8$^+$ T-cell responses against these molecules, and possibly other CNS autoantigens through epitope spreading,[97] via cDCs. As exemplified by HIV-1[98,99] and murine cytomegalovirus[100] infections, antibodies can act synergistically with CD8$^+$ T cells in immune responses against viruses, which is likely to occur via binding of antigen–antibody complexes to FcγRs on cDCs and cross-presentation of antigens to CD8$^+$ T cells.[101,102] The latter mechanism would likely be augmented by an IgG antibody response generated from CD11c$^+$/T-bet$^+$ MBCs as they are skewed towards IgG3, as well as IgG1, production.[103–105] IgG3 binds more avidly to cell-surface FcγRs than other IgG subclasses[106] and also elicits an antiviral response by binding to the intra-cellular Fc receptor TRIM21,[107] which when bound by immune complexes in DCs, promotes antigen cross-presentation and stimulation of CD8$^+$ T cells.[108] Furthermore, in murine models of SLE, CD11c$^+$/T-bet$^+$ MBCs cause aberrant differentiation of Tfh cells resulting in impaired affinity maturation of antibody responses.[109] Our observation that serum levels of IgG2, which is encoded by the third IGHC gene block (IgG2, IgG4 and IgA) and a product of high levels of CSR in MBCs,[105] are decreased in patients with early MS[7] raises the possibility that expansion of CD11c$^+$/T-bet$^+$ MBCs may also impair Tfh cell regulation of B-cell CSR in patients with MS. Together, these effects of CD11c$^+$/T-bet$^+$ MBCs might explain our observations that higher serum IgG3 levels predict the rate of progression of early MS[7] and that the abundance of IgG3$^+$ MBC is increased in some MS patients.[110]

Another mechanism by which MBCs might activate T cells was proposed by Jelcic *et al.*,[111] who demonstrated that circulating MBCs from patients with MS drive the proliferation of autologous CD4$^+$ T cells (referred to as autoproliferation). This mechanism was HLA-DR-dependent and most notable in patients carrying HLA-DR15. 'Autoproliferative' CD4$^+$ T cells exhibited a Th1 phenotype, including production of IFN-γ, and it was proposed that they contribute to autoimmune responses in the CNS. Notably, the degree of CD4$^+$ T-cell 'autoproliferation' positively correlated with the frequency of circulating unswitched MBCs only, implying that CD4$^+$ T-cell proliferation was driven by IgM$^+$ MBCs, which are predominantly MZ B cells. Furthermore, the same research group demonstrated that CD4$^+$ T-cell responses against self-peptides, presented by HLA-DR15 on B cells, may be cross-reactive with EBV peptides.[112] Given that MZ B cells can potently stimulate CD4$^+$ T cells,[113] our observations that reactivation of EBV infection in patients with early MS is associated with increased expression of HLA-DR on IgM$^+$ MBC[10] and decreased expression

of FcγRIIb on circulating MZ B cells in females[9] raises the possibility that EBV-induced activation and/or dysregulation of MZ B cells may contribute to $CD4^+$ T-cell 'autoproliferation'. Furthermore, IFN-γ produced by 'autoproliferative' $CD4^+$ T cells might promote a pathway of $CD11c^+$/T-bet^+ MBC differentiation from MZ B cells[43] (Figure 1). It should be noted that 'autoproliferative' $CD4^+$ T cells were most frequent during disease remission but this likely reflects the 'brain-homing' characteristics of these cells during disease relapse.[111]

EBV-INDUCED DYSREGULATION OF THE BAFF/BAFF-R PATHWAY MAY CONTRIBUTE TO PATHOGENIC B-CELL RESPONSES THAT DRIVE $CD8^+$ T-CELL RESPONSES

Primary EBV infection is associated with increased production of BAFF and decreased expression of BAFF-R on B cells,[27] likely reflecting the effect of LMP-1 on BAFF production by B cells[36] and BAFF-R shedding resulting from BAFF ligation.[114] Our analysis of circulating MBCs in patients with early MS demonstrated decreased BAFF-R expression on multiple B-cell subpopulations and was related to serum BAFF levels.[10] EBV-induced dysregulation of the BAFF/BAFF-R pathway may therefore persist in early MS, although we did not observe a relationship between BAFF-R expression and EBV reactivation.[10] The role that the BAFF/BAFF-R pathway plays in the immunopathogenesis MS is unclear, as highlighted by the failure of BAFF inhibitor therapy to prevent relapses of MS in clinical trials.[115] However, there is robust evidence that CSF BAFF levels and CSF/serum BAFF indices are decreased in MS[37,116] and that CSF/serum BAFF indices correlate inversely with intrathecal IgG synthesis,[117] suggesting BAFF utilisation by CNS and/or meningeal B cells. Evidence from other diseases, such as malaria,[118] suggests that the effect of BAFF on CNS and/or meningeal B cells may impact $CD11c^+$/T-bet^+ MBCs to a greater degree than cMBCs. In controlled human malaria infection, an increased abundance of circulating $CD11c^+$/T-bet^+ MBCs, increased plasma BAFF levels and reduced expression of BAFF-R on B cells were observed and BAFF levels correlated with proliferation of $CD11c^+$/T-bet^+ MBCs but not cMBCs.[118] In addition, in patients with SLE, inhibition of BAFF activity by belimumab, a monoclonal antibody to BAFF (also known as Blys), led to a decline in circulating $CD11c^+$/T-bet^+ MBCs (defined as IgD^-/$CD27^-$ or $CD11c^+$/$CD21^-$), as well as naïve B cells but not cMBCs (IgD^-/$CD27^+$).[119] Interestingly, murine T-bet^+ IgM^+ MBCs express larger amounts of BAFF-R compared with follicular B cells.[89] Given that BAFF enhances the APC activity of B cells,[120] it is possible that BAFF produced by EBV-infected MBCs[35] or astrocytes[121] augments the effects of pathogenic B cells on $CD8^+$ T-cell responses in the CNS (Figure 1). The effect of belimumab therapy is currently being evaluated in patients with MS (ClinicalTrials.gov identifier: NCT04767698).

THE INCREASED RISK OF MS DEVELOPMENT IN FEMALES MAY BE RELATED TO SEX-SPECIFIC B-CELL RESPONSES AGAINST EBV

MS is 2–3 times more common in females than in males.[122,123] It is well-established that females respond more strongly to viral infections than males and this also applies to EBV, as we have recently reviewed.[124] As a result, EBNA-1-specific IgG antibody levels are generally higher in females than in males[125,126] and this likely contributes to some of the increased risk of MS in females. Sex-related differences in the function of $CD11c^+$/T-bet^+ MBCs and MZ B cells might also contribute. Data from animal models suggest that $CD11c^+$/T-bet^+ MBCs are more prominent in females than in males, particularly during ageing when only female mice display an expansion of these cells.[59] A recent study has further identified that $CD11c^+$/T-bet^+ MBCs are functionally different in females compared with males, particularly in their ability to mount an interferon gene signature, and this difference was related to the development of SLE in female mice.[127] The mechanism by which $CD11c^+$/T-bet^+ MBCs are expanded in females remains unclear but likely relates to TLR7 signalling being a driving pathway for $CD11c^+$/T-bet^+ MBC induction and TLR7 expression being higher in females because of deficient X chromosome inactivation.[45] Support for this argument is provided by the recent report that *TLR7* gain-of-function mutations are a cause of SLE.[128] The location of *CXCR3* on the X chromosome and its susceptibility to X chromosome inactivation escape[129] may also result in higher CXCR3 expression on $CD11c^+$/T-bet^+ MBCs in females. In our studies, low expression of FcγRIIb was only observed on MZ and naive B cells in females.[9]

Healthy females have lower expression of FcγRIIb on B cells than males[130] and oestrogens increase the abundance of MZ B cells.[83] Hyper-responsiveness and increased abundance of MZ B cells might therefore contribute to dysregulation of downstream B-cell subsets following class switching and female predisposition to autoimmune diseases, including MS.

POTENTIAL EFFECTS OF CHANGES IN GUT MICROBIOME, LOW SUN EXPOSURE, LOW VITAMIN D LEVELS AND CIGARETTE SMOKING ON EBV-INDUCED B-CELL DYSFUNCTION IN GALT

Environmental risk factors for MS, such as an altered gut microbiome, low sun exposure, low vitamin D levels and cigarette smoking must also be considered in a hypothesis that implicates EBV-infected MBC within GALT in the immunopathogenesis of MS (Figure 1). Here, these risk factors will be considered as potential regulators of EBV infection and immune cell interactions in GALT.

Gut microbiota

The gut microbiota plays a homeostatic role in regulating intestinal barrier integrity and cellular interactions in GALT. Soluble products of gut microbes, particularly SCFAs, may alter B-cell activity[131,132] and contribute to modulating other immune cells in GALT.[133,134] This laboratory has shown, like others,[135] that serum propionate levels are lower in patients with CIS or MS than in healthy controls.[11] Furthermore, serum levels of propionate positively correlated with the frequencies of circulating Tfh and T follicular regulatory cells. Serum levels of butyrate correlated positively with frequencies of IL-10-producing B cells but negatively with frequencies of cMBCs, while acetate levels correlated negatively with TNF production by polyclonally-activated circulating total B cells. Thus, levels of serum SCFAs were associated with changes in circulating immune cells that can be implicated in regulation of immune cell function in GALT and the development of MS. Furthermore, the findings of other investigators suggest that there is a link between changes in the gut microbiota and dysregulation of MZ B cells associated with autoimmunity.[83]

Antigenic cross-reactivity between gut microbes and autoantigens has been proposed as a mechanism of inducing autoreactive T cells in several autoimmune diseases.[136] In addition, SCFAs produced by gut microbes may exert stimulatory or inhibitory effects on EBV reactivation in GALT MBCs, particularly in the colon where acetate, propionate and butyrate are produced in large amounts by bacteria.[66] However, the relationship between plasma levels of SCFAs and their abundance in the colon, and the effect of relative amounts of each SCFA on EBV reactivation, require further investigations.

Low sunlight exposure

A latitude gradient for MS risk has been recognised for decades, with a higher prevalence of MS recorded in populations living at higher latitudes.[137] A recent study has also suggested a relationship between low sun exposure and MS severity.[138] Multiple mechanisms of immune suppression after skin exposure to sunlight and its component ultraviolet B (UVB) radiation have been proposed and many altered immune cell types and soluble mediators have been described as potential contributors to systemic immunosuppression.[139–141] A recent study by our group suggested that narrowband UVB phototherapy primed the participant's B cells to be less responsive to activation through TLR7.[8] Direct induction of type I IFNs through sun exposure could be another mechanism of UV-induced immune modulation of MS.[138]

In a recent report, low sun exposure acted synergistically with high EBNA-1 antibody levels in its association to increased MS risk[142] supporting the submitted hypothesis. The authors proposed that the two risk factors for MS (EBV infection and low sun exposure) re-enforced the actions of each other. In addition, the proportion of EBV positive individuals is positively associated with latitude independently of MS status.[143]

Exposure of skin to suberythemal narrowband UVB phototherapy can also modulate the gut microbiome.[144] In healthy individuals receiving three exposures to narrowband UVB radiation over a single week, there was increased diversity of the gut microbiome and the relative abundance of Firmicutes and Proteobacteria increased in their faecal samples; the Bacteroidetes phyla were reduced by UVB.[144] In mice chronically exposed to suberythemal

broadband UVR, similar faecal changes in Firmicutes and Bacteroidetes have been reported.[145]

The impact of these UVR-induced changes has been difficult to interpret for patients with MS, for whom there have been multiple varied and confusing disease-associated changes reported in their microbiome.[11] In the context of gut microbiota, families within Firmicutes, such as Lachnospiraceae and Ruminococcaceae, are generally considered health promoting.[146] Despite indirect evidence, further studies are required to determine whether any UVB radiation-induced changes to the microbiome of MS patients might regulate reactivation of EBV infection and/or EBV-specific immune responses and EBV-induced immune dysregulation in the GALT.

Low vitamin D levels

Low vitamin D levels are a risk factor for MS.[147] However, it is much debated whether they are merely a biomarker of low sun exposure or the causal agent in the development of immune-driven diseases associated with low sun exposure. Supplementation studies of vitamin D for patients with MS have cast doubt on the role of reduced vitamin D levels *per se*[147] but more studies are required. Of interest, reduced serum levels of EBNA-1 antibodies have been reported in vitamin D-supplemented MS patients.[148,149] Although the active form of vitamin D ($1,25(OH)_2$ vitamin D) has proven direct effects on immune cells *in vitro*,[150] in studies of mice, vitamin D obtained by diet induced a different gut microbiome to that measured in those obtaining their vitamin D by broadband UVR exposure.[145,151] By contrast, there was evidence that UVR-induced vitamin D was responsible for microbiome changes in individuals receiving UVB phototherapy.[144] It remains possible that UVR regulates the gut microbiome, and we propose immune cell reactivity in the GALT, by multiple pathways that may be dependent on, and independent of, vitamin D actions.

Cigarette smoke

Smoking cigarettes is a well-established risk factor for MS.[152] Recently, the impact of smoking on EBV reactivation and circulating levels of anti-EBNA IgG and IgA has been investigated.[153] These and other studies[154] suggest that smoking may promote EBV reactivation and increase serum EBNA-1 IgG antibody levels. Furthermore, a history of IM and smoking appears to interact to increase the risk of MS.[153] Cigarette smoking also affects the composition of the gut microbiome[155] and may interact with other factors affecting the gut microbiome in MS patients. Smoking induces an expansion of circulating cMBCs, including IgA^+ cells,[156,157] but this effect may be transient because circulating B-cell profiles are not different in smokers and non-smokers.[157] Expression of CD11c or T-bet by B cells does not differ in smokers compared with non-smokers.[158]

CONCLUSIONS AND FUTURE PERSPECTIVES

Our hypothesis brings together the major genetic and environmental risk factors for developing MS into a single disease model (Figure 1) and proposes mechanisms to explain altered B-cell phenotypes, the production of B-cell and antibody responses against EBV proteins that cross-react with CNS autoantigens, the contribution of $CD4^+$ T cells, and the concurrence of $CD8^+$ T cells and B cells in CNS inflammatory lesions. It also opens new avenues of research on MS immunopathogenesis and therapy. As shown in Table 2, all three types of MBCs likely contribute to the immunopathogenesis of MS, but through different mechanisms. As all express CD20, monoclonal anti-CD20 antibody therapies would deplete all subpopulations. We suggest that investigation of EBV-specific immune responses arising in GALT may be as enlightening as investigations of gut microbiota-specific immune responses have been, specifically those involving MZ B cells, $CD11c^+$/T-bet^+ IgG^+ MBCs and IgA^+ MBCs. Furthermore, the possibility that all GALT-associated B cells contribute to the gut-meningeal immune axis[69] in health and disease should be considered. Such investigations may lead to more targeted therapies for modulating B-cell dysfunction. For example, therapeutic inhibition of adenosine receptor 2a on $CD11c^+$/T-bet^+ MBCs[159] is potentially a means of decreasing pathogenic B-cell responses without impairing global B-cell function. Future investigations of the gut microbiota and/or SCFAs in studies of MS pathogenesis should include analyses of the relationship with EBV reactivation and activation of circulating MBCs, particularly MZ B cells. Such investigations may identify new biomarkers to assess interactions of EBV with B cells and reveal

Table 2. Major subpopulations of human circulating memory B cells, highlighting important functional characteristics, proposed mechanisms of involvement in the immunopathogenesis of multiple sclerosis and known effects of MS therapies on them

	Marginal zone B cells	$CD11c^+$/T-bet^+ MBCs	Conventional MBCs
Distinguishing and important immunophenotypes	$CD19^+$, $CD20^+$, $CD27^+$, IgD^{low}, IgM^{hi}	$CD19^+$, $CD20^+$, $CD27^{+/-}$, IgD^-, $CXCR3^+$, $CD11c^+$	$CD19^+$, $CD20^+$, $CD27^+$, IgD^-
Subpopulations	MZB1 and MZB2	IgG^+, IgA^+, (IgM-only)	IgM-only, IgG^+, IgA^+
Primary functions	Initiating B-cell and antibody responses ('first responders') Long-term Bcell memory	Controlling infections in concert with effector T cells (mainly established for viruses and *Plasmodium sp.*)	Preventing infections through memory responses that produce plasmablasts and long-lived plasma cells and antibodies
Important functional characteristics	Microbe recognition by low-affinity BCRs and various pathogen recognition molecules APC function for $CD4^+$ T cells via HLA molecules MBZ2 cells express HLA-DBR1 and exhibit antiviral characteristics	Microbe recognition by BCRs and TLR7/9 Probable APCs for T cells, involving CD11c/CD18 Antibody responses skewed towards IgG3, enhancing cDC activation and antigen presentation to $CD8^+$ T cells	Microbe recognition by high affinity BCRs Production of antibodies with high affinity for antigens and functional diversity of Fc regions through immunoglobulin isotype switching of B cells
Proposed involvement in the immunopathogenesis of MS	Initiate a B-cell response against EBV proteins, such as EBNA-1, in GALT that is cross-reactive with autoantigens, such as GlialCAM Possibly activate 'autoproliferative' $CD4^+$ T cells, as well as pathogenic B cells in CSF	EBNA-1/GlialCAM-specific IgG^+ cells: • Differentiate from MZB2 cells, under the influence of IFN-γ from $CD4^+$ T cells, and migrate to the CNS facilitated by CXCR3 expression • Induce a cytotoxic $CD8^+$ T-cell response against glial cells and/or myelin in the CNS by acting as APCs and/or eliciting antibody-dependent activation of cDCs • Possibly produce BAFF in CNS IgA^+ cells possibly produce IgA anti-EBV that interferes with IgG antibodies.	EBV-infected cells possibly produce BAFF in CNS IgA^+ cells possibly produce IgA anti-EBV that interferes with IgG antibodies.
Demonstrated effect of MS therapies[1,160–162]	Depletion by anti-CD52 (ATB), anti-CD20 (OLB), IFN-β and Cladribine[a]	Inhibition of adhesion to receptors in CNS by anti-α4-integrin (NLB) Depletion by MS therapies?[b]	Inhibition of adhesion to receptors in CNS and possibly GALT by anti-α4-integrin (NLB) Depletion by anti-CD52 (ATB) anti-CD20 (OLB), IFN-β, DMF, GTA, Cladribine and Fingolimod

ATB, alemtuzumab; DMF, dimethyl fumarate; GTA, glatiramer acetate; NLB, natalizumab; OLB, ocrelizumab.

[a]Based on data for 'unswitched' $CD27^+$ B cells.

[b]Insufficient information on depletion of this subpopulation by MS therapies but probable that anti-CD20 (OLB), at least, does this.

novel therapeutic targets to limit EBV-associated immunopathology in the CNS.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Jonatan Leffler: Investigation; writing – review and editing. **Stephanie Trend:** Investigation; writing – review and editing. **Prue H Hart:** Funding acquisition; investigation; project administration; supervision; writing – review and editing. **Martyn A French:** Conceptualization; project administration; supervision; writing – original draft; writing – review and editing.

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