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SPECIAL FEATURE REVIEW

From bedside to bench: how existing therapies inform the relationship between Epstein–Barr virus and multiple sclerosis

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Abstract

Therapy for relapsing–remitting multiple sclerosis (MS) has advanced dramatically despite incomplete understanding of the cause of the condition. Current treatment involves inducing broad effects on immune cell populations with consequent off-target side effects, and no treatment can completely prevent disability progression. Further therapeutic advancement will require a better understanding of the pathobiology of MS. Interest in the role of Epstein–Barr virus (EBV) in multiple sclerosis has intensified based on strong epidemiological evidence of an association between EBV seroprevalence and MS. Hypotheses proposed to explain the biological relationship between EBV and MS include molecular mimicry, EBV immortalised autoreactive B cells and infection of glial cells by EBV. Examining the interaction between EBV and immunotherapies that have demonstrated efficacy in MS offers clues to the validity of these hypotheses. The efficacy of B cell depleting therapies could be consistent with a hypothesis that EBV-infected B cells drive MS; however, loss of T cell control of B cells does not exacerbate MS. A number of MS therapies invoke change in EBV-specific T cell populations, but pathogenic EBV-specific T cells with cross-reactivity to CNS antigen have not been identified. Immune reconstitution therapies induce EBV viraemia and expansion of EBV-specific T cell clones, but this does not correlate with relapse. Much remains unknown regarding the role of EBV in MS pathogenesis. We discuss future translational research that could fill important knowledge gaps.

Keywords: disease-modifying therapy, Epstein–Barr virus, multiple sclerosis, translational immunology

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disorder of the central nervous system (CNS). Most people with MS (PwMS) initially experience a relapsing–remitting course. Despite decades of research, the activation and regulation of autoreactive immune dysfunction presumed to underly episodic neuroinflammation is poorly understood. The introduction of increasingly effective therapies has led to significant improvement in the lives of many PwMS.[1] Even with access to these therapies, PwMS may experience relapses and progressive neurological disability. Medications also carry the risk of treatment-related complications, including serious illness and death. In the absence of a detailed understanding of the immunopathogenesis of MS, available treatment strategies are either globally immunosuppressive or prevent immune surveillance of the CNS with consequent off-target side effects. A clearer understanding of the immunopathogenesis of MS would enable the development of more specific treatment strategies.

Epidemiological research has identified several risk factors for the development of MS. However, the mechanism by which these risk factors trigger pathogenic change in the adaptive immune system is incompletely understood. It remains unclear whether all cases of MS share the same aetiology, or whether it represents a common pathology that has a variety of triggers. Epstein–Barr virus (EBV) is a B cell lymphotropic virus, which can evade the immune system by establishing latent infection in memory B cells. Interest in the relationship between EBV in and MS is accelerating, in part as a result of mounting epidemiological data, which show a strong correlation between EBV seropositivity and multiple sclerosis. However, the role of EBV in MS pathogenesis remains unclear. EBV infection is ubiquitous in human populations, but MS is rare. Therefore, EBV infection is not sufficient to cause MS. If EBV is either a risk factor or necessary part of MS development, understanding its role in pathogenesis is vital. Translational research focussing on the potential pathogenic role of EBV in MS has involved controversy because of variable results across studies and differing interpretation of data. Other challenges include barriers to direct assessment of the CNS compartment and the lack of an authentic animal model of MS.

Examining mechanisms of disease-modifying therapies (DMTs) allows us to identify shifts in the immune system, which correlate with meaningful improvement in MS disease activity in humans. The mechanisms of immunosuppressive agents can offer insight into the underlying biology that sustains the immune attack on the CNS but are less useful in understanding the way that immune attack is first triggered. By contrast, immune reconstitution therapies involve the rebuilding of a new T and B cell receptor repertoire with the same genetic background and thus offer a unique opportunity to examine how the immune system is triggered to attack the CNS in MS. Further, several immunomodulatory therapies exacerbate MS and reviewing their action also provides useful clues.

This review examines the ways in which EBV is hypothesised to play a role in MS pathogenesis and outlines how our understanding of the molecular basis of disease-modifying therapies can potentially corroborate or refute those potential mechanisms. We also explore advances in immunophenotyping that may help to delineate the role of EBV in MS biology.

THE EPIDEMIOLOGICAL ASSOCIATION BETWEEN EBV AND MS

Interest in the role of EBV in MS pathogenesis is built upon the well-established relationship between EBV seropositivity and MS. A meta-analysis of studies published prior to December 2018 calculated an odds ratio of 3.9 for EBV seropositivity across PwMS compared with controls. However, the authors noted significant data heterogeneity and evidence of bias towards publication of studies showing an association between MS and EBV.[2]

Explanations for the association between MS and EBV include the following:

- EBV exposure is obligatory in the pathogenesis of MS; all people with MS have been infected with EBV.
- EBV is a risk factor but is not essential for the pathogenesis of MS; disease heterogeneity is present.
- The association between EBV serostatus and MS disease does not reflect causation and is related to another factor or factors.

Model 1: EBV is obligatory in the pathogenesis of MS

The prevalence of EBV seropositivity is influenced by detection method, with the use of immunofluorescence and/or testing for multiple antibodies achieving higher sensitivity.[3] Some studies have found near-universal EBV seropositivity in adult MS cohorts when high-sensitivity detection methods are used. EBV seroprevalence was 100% in a German study of 901 PwMS, which used multiple modalities to test for EBV. Seroprevalence in the control population was also very high (> 98% after age 45) but was lower than in the MS population.[3]

Model 2: EBV is a risk factor for MS but is not obligatory

A prospective study of US military personnel found that of 801 people who developed MS, only one remained EBV seronegative. Of the 35 people who were EBV seronegative at baseline and later developed MS, all but one seroconverted prior to MS diagnosis. However, prospective MRI data were not routinely collected prior to clinical diagnosis of MS, so the time of onset of MS inflammation (as opposed to first clinical attack) is not definitively known in these patients. 3.2% of controls remained EBV seronegative. The odds ratio for positive EBV serology and MS was 32.[4] The fact that one person who developed MS remained seronegative for EBV argues against the hypothesis that EBV infection is obligatory. The authors discuss the possibility that EBV may be the cause of MS in most, but not all, cases. The possibility of misdiagnosis is also considered, but detailed clinical information about cases is not available.[4]

Studies of EBV seroprevalence in children with MS offer important insights because prevalence of infection is lower than in adults. In a meta-analysis, 85% of children with MS were seropositive compared with 51% of controls. Many of the included studies involved testing for multiple EBV antibodies.[2] In most of the studies, EBV antibody status was determined after MS diagnosis, with an interval of several years in some children.[5–7] These studies support an association between EBV seropositivity and multiple sclerosis but argue against EBV exposure being a necessary factor.

Model 3: The association between EBV and MS is not causative

An alternative hypothesis is that the same immune system characteristics which predispose people to MS increase the likelihood of manifesting with positive serological tests following EBV infection. The fact that no single serological test has 100% sensitivity indicates variation in antibody response after EBV infection. In addition, individuals who are EBV seronegative but have EBV-specific T cell responses are described.[8] Whether or not this variation correlates with MS is not known. In one study, PwMS were found to have higher anti EBNA-1 IgG and anti-VCA IgG titres than healthy controls.[9] There was, however, no association between these antibody titres and EBV DNA copy number in the blood.[9] This suggests that the difference in antibody titre across PwMS and healthy controls may be related to immune system characteristics. The relationship between genetic factors and EBV seroprevalence is difficult to study because of the scarcity of EBV seronegative adults.[10] However, EBV seropositivity has been associated with a number of MHC class I and II alleles and with polymorphisms in the IL-10 gene.[10]

The variation in EBV seropositivity rate across different epidemiological studies leaves room for debate about the relationship between EBV and MS, particularly regarding whether EBV infection is present in all PwMS prior to the onset of symptoms. Establishing the true epidemiological relationship between EBV infection and MS onset is important when evaluating potential pathogenic mechanisms. For example, if EBV is obligatory in the pathogenesis of MS, then a biological mechanism by which EBV causes MS should be identifiable in all people.

MECHANISMS BY WHICH EBV MIGHT CONTRIBUTE TO MS PATHOGENESIS

The potential role of EBV in MS can be conceptualised as either a disease 'trigger' or 'driver'.[11] If EBV is a 'trigger' of disease, the mechanism may involve molecular mimicry between EBV antigens and CNS protein. Models of EBV as a driver of disease include infection of CNS tissue and the autoreactive B cell hypothesis.

Molecular mimicry hypothesis

Molecular mimicry involves the production of antibodies and/or T cell clones in response to an exogenous antigen, which then recognise self-antigen and induce immune attack of host tissue.[12] Models of molecular mimicry to EBV involving both antibodies and T cells have been suggested in MS. These models, along with potential interplay with DMTs, are depicted in Figure 1.

Antibody-mediated molecular mimicry. Antibodies which cross-react to EBNA-1 and GlialCAM (an adhesion molecule expressed on CNS glial cells) have recently been identified in 20–25% of people in an MS cohort.[13] Prior to the description of GlialCAM, other antigens proposed to be the target of molecular mimicry have included alpha B-Crystallin, anoctamin 2 and myelin basic protein.[14,15] Many of these antigens have been identified *in vitro* and/or in animal models, without translation to MS disease *in vivo*.[14]

Guillain–Barre syndrome (GBS) is a prototypical example of an antibody-mediated autoimmune disease caused by molecular mimicry. Several pathogenic antibodies have been identified, some of which associate with distinct disease

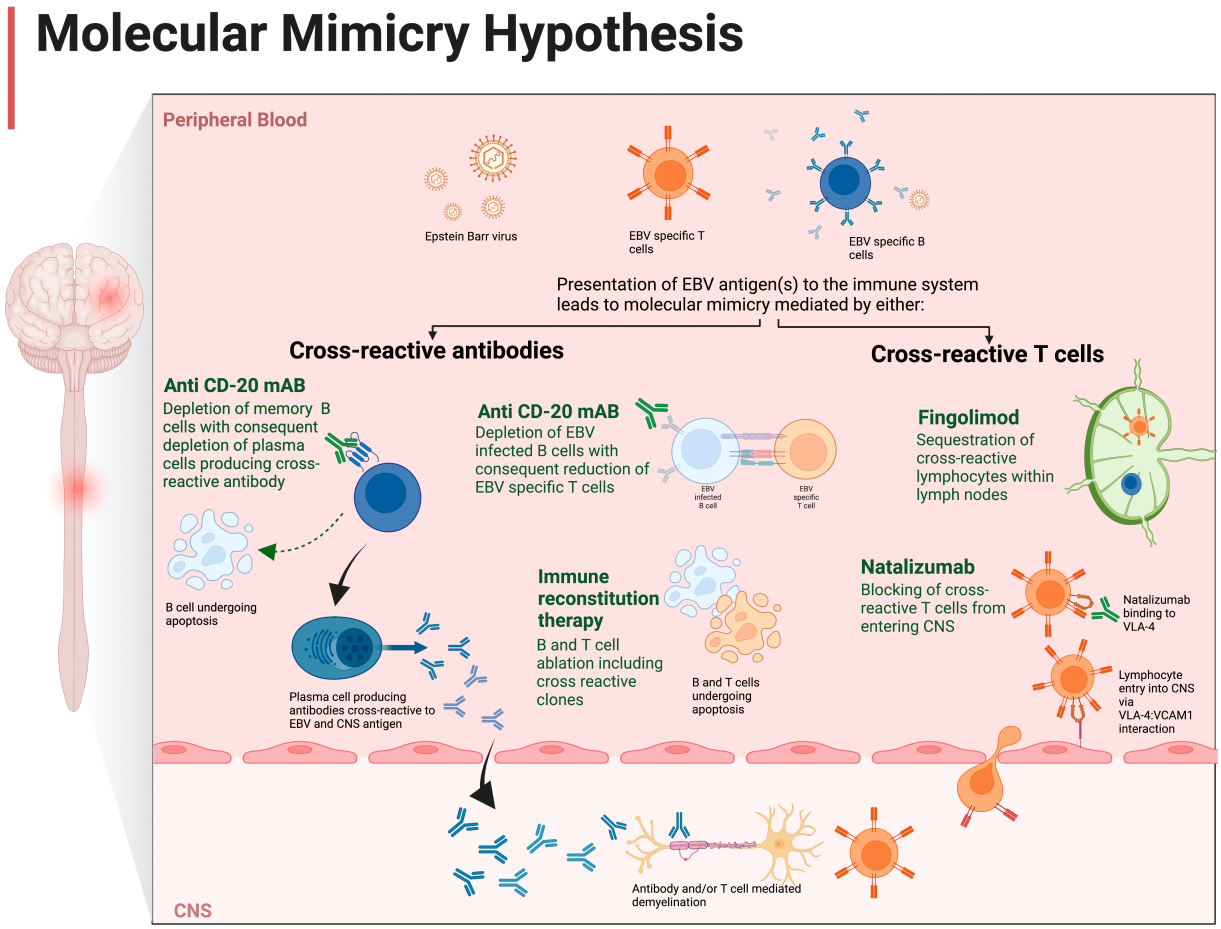

Figure 1. MS therapies and the Molecular Mimicry Hypothesis. Overview of the molecular mimicry hypothesis (black text): As a consequence of the interaction between EBV and the adaptive immune system either EBV-specific T cells (orange) or EBV-specific antibodies (blue) are produced, some of which are cross-reactive to CNS antigen. Within the CNS immune-mediated demyelination occurs, mediated by either T cells or antibodies cross-reactive to both EBV and CNS antigens. Proposed mechanism of MS therapies (green text): Anti-CD20 mAbs deplete B cells, with consequent depletion of antibody-producing plasma cells. Anti-CD20 mAbs also cause reduction of EBV-specific T cells by reducing antigen presentation by EBV-infected B cells. Immune reconstitution therapies ablate both B and T cell populations. Fingolimod sequesters both B and T cells within lymph nodes. Natalizumab prevents T cells from entering the CNS by preventing VLA-4 (very late antigen-4/ Integrin $\alpha 4\beta 1$) from binding to VCAM1 (vascular cell adhesion protein 1). This image was created with Biorender.[101]

phenotypes. These antibodies can be reliably identified across different cohorts with GBS and can be used to induce disease in animal models.[12,16] These features of reproducibility and pathogenicity have not been clearly demonstrated for any of the potential molecular mimicry targets in multiple sclerosis. Moreover, GBS is a self-limiting monophasic illness, in which recovery is the rule and is accelerated by immunotherapy.[17] By contrast, MS typically runs a relapsing–remitting course with a significant number of people entering a progressive phase of disease.

T cell-mediated molecular mimicry. Multiple sclerosis is often described as a 'T cell mediated' disease based on its pathological features and some similarities with animal models of CNS autoimmune inflammation. Consequently, there has been interest in a potential role of T cells with cross-reactivity to EBV and CNS antigen. EBV-specific $CD8^+$ T cells are enriched in the CSF compared with the blood in PwMS. This is not seen in other inflammatory neurological diseases.[18] A study examining peripheral blood T cell receptor (TCR) sequences found that PwMS had a higher proportion of EBV-specific sequences than controls. The range of these sequences was broad. At least five different TCR sequences were more common in PwMS than controls, corresponding to several different lytic and latent phase proteins.[19] TCR sequences specific to other viruses were found at similar frequency in PwMS than controls. This is consistent with other work demonstrating altered T cell responses to a range of lytic and latent EBV proteins in PwMS. The broad range of T cell responses argues against a single molecular mimicry event consistent across all PwMS. The authors comment that more work needs to be done to determine whether these changes are a cause or effect of MS pathology, or an unrelated epiphenomenon.[19] No studies have identified $CD8^+$ T cells with cross-reactivity to EBV and CNS antigen. Cross-reactive $CD4^+$ T cells have been identified in PwMS, but the same cell populations were also identified in healthy controls.[20]

Autoreactive B cells

Professor Michael Pender first introduced the hypothesis that EBV-infected autoreactive B cells are involved in MS pathogenesis in 2003.[21] In that hypothesis, it is proposed that stochastic EBV infection prevents autoreactive B cells from dying via apoptosis. EBV infection causes persistence of CNS-specific autoreactive B cells, which in turn promotes the survival of autoreactive T cells via co-stimulatory survival signals. Autoantibodies and T cell-mediated damage cause tissue destruction.[9] B cell aggregates form follicles with germinal centres in the meninges. These propagate autoreactive B cells, which contribute to inflammation and neurodegeneration in the cortex.[22] The autoreactive B cell hypothesis is depicted in Figure 2. Potential interactions between EBV-infected autoreactive B cells and DMTs are also shown.

A 2007 study reported the presence of EBV-infected B cells in MS lesions, including the presence of meningeal B cell aggregates.[23] However, subsequent work, including reanalysis of the same tissue samples by other authors and analysis of other cohorts, has failed to detect EBV in MS lesions.[24–26] More recent autopsy studies have reported an increased presence of EBV-infected B cells in MS brains compared with controls.[27,28] However, total CD20 B cells and plasma cells were more prevalent in the brain parenchyma, reflecting the inflammatory nature of multiple sclerosis.[27] Treatment status is not reported in these studies. The association between immunosuppression and EBV viral load raises the question as to whether this finding might reflect the effect of treatment or inherent immune dysregulation in MS. Defective cytotoxic $CD8^+$ T cell control of EBV-infected B cells has been hypothesised to underly the development and persistence of autoreactive B cells.[9] Studies of EBV-specific T cell immunity in MS have yielded inconsistent results.[9] Moreover, genetic immunodeficiencies which cause specific vulnerability to EBV are not associated with an elevated risk of multiple sclerosis.[29]

The autoreactive B cell hypothesis has also been proposed to drive systemic autoimmune diseases.[21] Elevated titres of one or more EBV antibodies have been identified in systemic lupus erythematosus, Sjogren's syndrome and rheumatoid arthritis.[30–32] As with MS, it is unclear whether differences in these antibody results implicate a role of EBV in autoimmune disease, or alternatively reflect differences in antibody production in patients with dysregulated immune systems. Even though single-nucleotide polymorphisms shared across MS and other

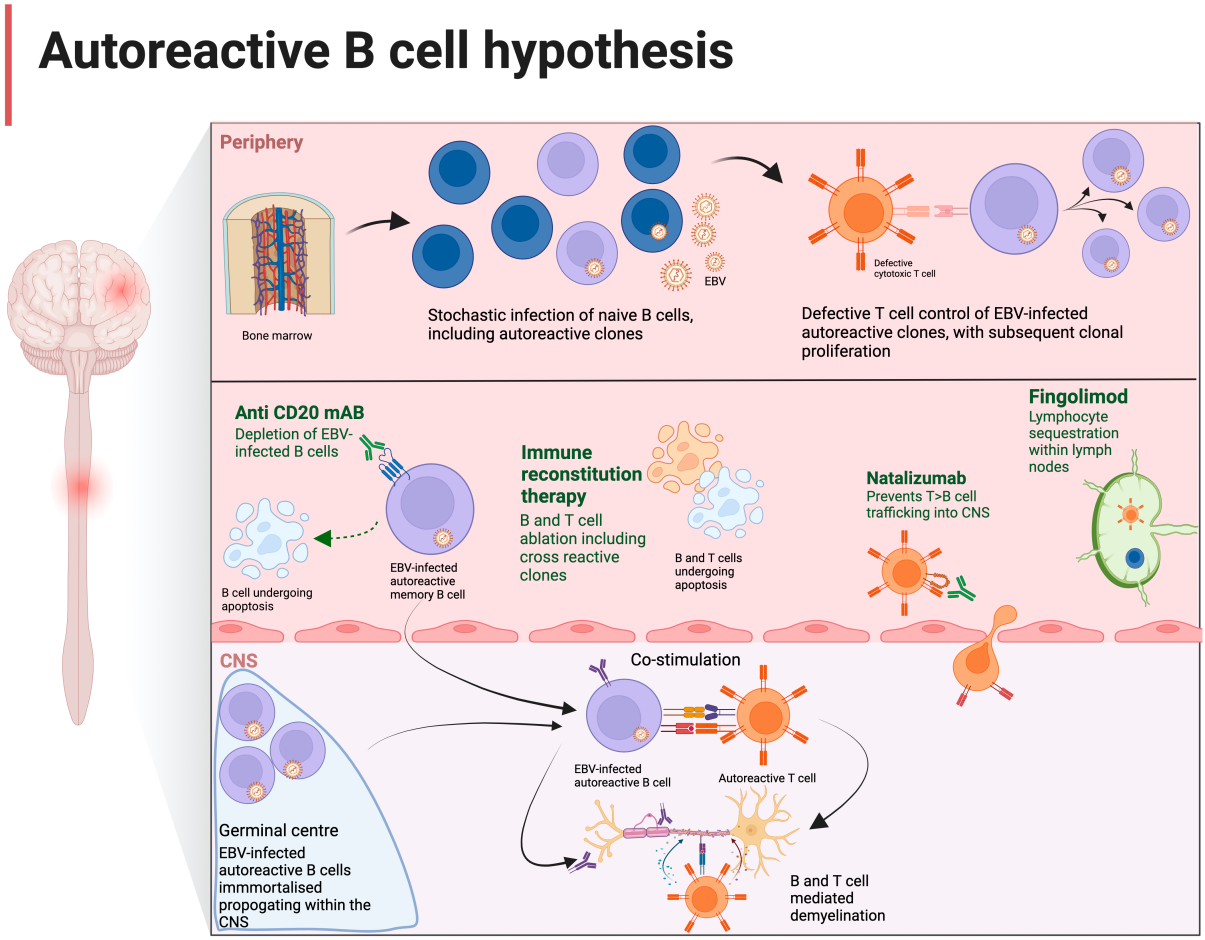

Figure 2. MS therapies and the Autoreactive B cell Hypothesis. Overview of the autoreactive B cell hypothesis (black text): Immature B cells are generated in the bone marrow and enter the circulation. Stochastic VDJ recombination leads to the generation of both non-self-antigen-specific clones (blue) and autoreactive B cell clones (purple). EBV infects a proportion of both nonself and autoreactive B cell clones. In PwMS, T cell-mediated killing of EBV-infected autoreactive B cells is ineffective. EBV-infected autoreactive B cells then travel into the CNS where they interact with T cells. Both autoreactive B and T cells contribute to immune-mediated demyelination. Proposed mechanism of action of MS therapies (green): Anti-CD20 monoclonal antibodies (mAb) cause EBV-infected autoreactive B cells to die via apoptosis. Immune reconstitution therapies ablate both B and T cell populations, with consequent removal of autoreactive B cell clones. Natalizumab prevents both T and B cells from entering the CNS, with greater suppression of T cell trafficking. Fingolimod sequesters T and B cells within lymph nodes. This image was created with Biorender.[101]

autoimmune disorders can be identified in genome-wide association studies (GWAS), clinical overlap is not a common phenomenon (although case reports do exist).[33] This argues against a shared immune response to EBV infection as the cause of these conditions.

Infection of central nervous system glial cells

Multiple sclerosis has classically been considered an 'outside in disease', in which a dysregulated immune system attacks normal brain tissue. Evidence from GWAS that link MS almost entirely to immune system genes and not those associated with glial cells or neurons supports this model. Some authors have proposed that MS may instead be an 'inside out disease', in which oligodendrocyte degeneration triggers a secondary immune response. Potential causes of oligodendrocyte loss include toxic exposure and viral infection.[34] Pathological evidence to support EBV infection of CNS glial cells is limited. A 2018 brain-bank study reported staining for EBV-encoded RNAs (EB-ER) in a minority of astrocytes and microglia in from postmortem MS specimens.

What MS therapies can tell us about the role of EBV in MS: The bottom line

- High efficacy therapies for MS either deplete B cell populations or sequester them outside the CNS.
- Medications effective for MS have not been consistently shown to reduce either EBV-specific antibodies or T cells. There is no evidence that people with MS have EBV-specific T cells with cross-reactivity to CNS antigen that are not found in controls.
- The effectiveness of therapies which deplete memory B cells has been hypothesised to be related to reduction in EBV infected autoreactive B cells. However, how these peripherally acting medications would affect cells immortalised within the CNS is less clear.
- EBV viraemia following immune reconstitution therapy does not appear to trigger relapse of inflammatory disease.

Figure 3. Key points summarising how the mechanism of MS therapies can inform the relationship between EBV and MS.

Immunohistochemistry staining for the expression of EBV proteins in those cells was not reported.[28] EBV has been reported to infect astrocyte and glioblastoma cell lines *in vitro*.[35,36] Infection led to the expression of some EBV proteins but did not significantly change the overall transcription profile of cell lines.[35] There is no conclusive evidence of EBV-infected glial cells expressing EBV latency programmes.[37]

A number of neurotropic viruses cause encephalitis because of direct infection of neural tissue. Encephalitis may also occur in the setting of systemic inflammation in response to viral illness, without viral infection of CNS tissue.[38] EBV encephalitis is a monophasic illness, which occurs in the setting of acute infection, usually in children or immunosuppressed adults.[39] EBV DNA is detectable in the CSF and is not present after neurological symptoms improve. IgM reactive with EBV viral capsid antigen is detected in the CSF of affected individuals, consistent with acute EBV infection.[40] There are no pathological data to suggest that EBV encephalitis involves viral infection of neurons or glia. Histology shows a mixed lymphoid population with admixed macrophages. EBER-positive cells are seen. These features are similar to CNS post-transplant lymphoproliferative disorder (PTLD).[41] There is more to be learnt about EBV encephalitis, but no strong indication that EBV is neurotropic, causes demyelination or precedes MS.

In summary

The pathogenesis of multiple sclerosis involves the complex interplay between genetic susceptibility and environmental exposures. Robust translational data delineating a clear mechanism by which EBV might interact with these factors to cause MS are lacking (Figure 3).

HOW MS THERAPIES INFORM THE EBV DEBATE

The mechanisms of action of DMTs used to treat MS are summarised in Table 1. The effects of DMTs on peripheral and CNS B cells, and on markers of EBV infection, are summarised in Table 2.

B cell depletion

B cell depletion via anti-CD20 monoclonal antibodies (examples listed in Table 1) is highly effective in treating the inflammatory component of MS. This does not indicate that MS is necessarily antibody mediated. Anti-CD20 treatment is effective in the absence of changes in serum immunoglobulin (Ig) levels or the reduction or disappearance of oligoclonal bands in the CSF.[42] These findings are not surprising given that CD20 is downregulated on long-lived plasma cells, which are the source of most serum immunoglobulin, and monoclonal antibody therapies such as ocrelizumab have poor CNS penetrance. The conclusion that antibodies themselves are not responsible for the inflammatory component of MS is also supported by the relative ineffectiveness of plasmapheresis in the treatment of MS.[43] Plasmapheresis is a vital acute treatment in antibody-mediated neurological diseases, including those with intrathecal antibody production such as NMDA receptor encephalitis and neuromyelitis optica.[44,45]

B cells also have a critical role in antigen presentation to T cells.[46] Reduction of cross-reactive EBV T cell clones has been proposed to be the

Table 1. Mechanism of action and efficacy of immunosuppressive disease-modifying therapies for multiple sclerosis

Medication	Mechanism of action	Efficacy in preventing inflammatory MS: Relative rate of relapse vs placebo. Data from icer.org[78]
Anti-CD20 monoclonal antibodies *Rituximab*: chimeric anti-CD20 IgG mAb *Ocrelizumab*: recombinant humanised anti-CD20 IgG mAb *Ofatumumab*: fully human anti-CD20 mAb	Severe depletion of cells which carry CD20 surface marker. CD20 is expressed on most B cells and some T cells. CD20 is not expressed on pre-B cells and plasma cells.	Rituximab: 0.51 (0.27–0.93) Ocrelizumab: 0.35 (0.27–0.44)
Alpha-4-integrin antagonist *Natalizumab*	Blocks a-4-integrin, a leucocyte surface marker which aids transfer across the blood–brain barrier. Prevents lymphocyte trafficking into the central nervous system.	0.31 (0.25–0.4)
Sphingosine-1-phosphate receptor modulators *Fingolimod* *Siponimod* *Ozanimod*	Sequesters lymphocytes within lymph nodes. Causes peripheral lymphopenia. Altered T cell inflammatory phenotype.[79] Possible direct effect on CNS tissue.[80]	Fingolimod: 0.46 (0.39–0.55)
Fumaric acid derivatives *Dimethyl Fumarate* *Diroximel fumarate*	Multiaction immunomodulatory mechanism.[81] Mechanism involves activation of Nrf2 pathway and inhibition of NFkB. Causes peripheral lymphopenia.	Dimethyl fumarate: 0.53 (0.43–0.63)
Teriflunomide (TER)	Inhibits pyrimidine synthesis by inhibiting dihydroorotate dehydrogenase. Decreases proliferation of dividing cells, particularly lymphocytes.	TER 14 mg 0.67 (0.56–0.79) TER 7 mg 0.77 (0.75–0.93)
Interferon-B (IFN B) *Interferon beta-1a* *Interferon beta-1b*	Multifactorial anti-inflammatory effects[43]	IFN B-1a 44 µg 0.64 (0.54–0.73) IFN B-1b 250 µg 0.65 (0.55–0.77) IFN b-1a 22 µg 0.7 (0.55–0.85) IFN b-ac 30 µg 0.83 (0.74–0.94)
Glatiramer acetate (GA)	Random amino acid copolymer. Mechanism of immunomodulatory effect is not fully understood	GA 20 mg 0.63 (0.55–0.71) GA 40 mg 0.67 (0.52–0.86) vs placebo

Immune reconstitution therapy

Therapy	Mechanism of action	Efficacy: Relative relapse rate vs placebo data from various sources
Cladribine	Deoxyadenosine analogue. Impairs purine salvage pathway—impairs DNA synthesis and repair. Causes peripheral lymphopenia.[43]	0.43 (0.37–0.51) vs placebo over 2 years 3.5 mg kg^{-1}[82]
Alemtuzumab *Anti-CD52 monoclonal antibody*	Depletion of cells which carry CD52 surface marker. CD52 is present on all mature lymphocytes (both B and T cells).	0.28 (0.22–0.35) vs placebo[78]
Autologous haematopoietic stem cell transplantation	Multiple conditioning chemotherapy regimens used. Commonly cyclophosphamide-ATG and BEAM-ATG. Chemotherapy causes severe depletion of circulating mononuclear cells with subsequent reintroduction of autologous haematopoietic stem cells.	No data on efficacy vs placebo

Table 2. Effect of MS therapies on B cells and measures of EBV infection

Agents	Effect on total B and memory B cells in the peripheral circulation	Effect on total B and memory B cells in the CNS	Effect on measures of EBV infection
Monoclonal antibodies (mAb)			
Anti-CD20 monoclonal antibodies *Ocrelizumab*: humanised anti-CD20 IgG mAb *Rituximab*: anti-CD20 chimeric IgG mAb *Ofatumumab*: fully human anti-CD20 mAb	Severe reduction in absolute $CD19^+$ B cell count, including memory B cells. Preservation of pre-B cells and plasma cells.[83] Memory B cell population takes longer to repopulate than overall $CD19^+$ B cell population.[83]	*Rituximab*: $CD19^+$ B cells reduced to 10% of pretreatment level in people with RRMS.[42] *Ocrelizumab*: $CD19^+$B cells reduced to less than 10% of pretreatment level.[49]	*Ocrelizumab*: Reduced $CD8^+$ T cell responses to EBV-infected lymphoblastic cell lines *in vitro*.[47] *Ocrelizumab*: No change to the frequency of EBV-specific TCR sequences in peripheral blood.[19]
Alpha-4-integrin antagonist *Natalizumab*	Increase in absolute number of $CD19^+$ B cells, including memory B cells.[84]	Warnke *et al*. 2015: Reduction $CD19^+$ B cells as a proportion of all lymphocytes; 0.4% compared to 2.4% in untreated PwMS.[85] Stuve *et al*. 2006: Reduction in CSF $CD19^+$ B cells and $CD138^+$ plasma cells relative to pretreatment level (no absolute values given).[86] Cuculiza Henriksen *et al*. 2021: Reduction in $CD38^-$ memory B cells as a proportion of total B cells. No change in proportion of $CD38^+$ memory B cells.[84] Kowarik *et al*. 2021: 3-fold reduction in memory B cells in CSF, driven by >10-fold drop in one of four patients.[87]	No change in EBNA-1 titre over time.[88,89] Increase in EBV-specific TCR sequences in peripheral blood.[19] Decrease in the proportion of $CD8^+$ T cells which respond to lytic EBV antigens compared with untreated patients with active MS. Increase in the proportion of $CD8^+$ T cells which respond to latent EBV antigens compared with untreated patients with active MS.[90]
Oral therapies			
Sphingosine-1-phosphate receptor agonists *Fingolimod* *Siponimod* *Ozanimod*	*Fingolimod*: Significant reduction in absolute peripheral mononuclear cell count. Reduction in proportion of PBMCs which are $CD19^+$ B cells.[91] Reduction in memory B cells as a proportion of total B cells.[83]	*Fingolimod*: Total number of B cells in CNS unchanged but an increase in Bregs.[43] *Fingolimod*: No change in total B cell or memory B cell count.[87]	No data
Fumaric acid derivatives *Dimethyl Fumarate* *Diroximel fumarate*	Reduction in absolute memory B cell number in RRMS.[92,93] Reduction in memory B cells as a proportion of $CD19^+$ B cells[94] although other studies have found no change.[95]	No change in absolute or relative $CD19^+$ B cell count in CSF relative to placebo in PPMS.[96]	No data
Teriflunomide	Reduction in absolute $CD19^+$ B cell count	No data	Inhibits cellular proliferation/promotes apoptosis of EBV-transformed lymphoblastoid cell lines *in vitro*.[98]

(Continued)

Table 2. Continued.

Agents	Effect on total B and memory B cells in the peripheral circulation	Effect on total B and memory B cells in the CNS	Effect on measures of EBV infection
	No significant change in proportion of B cells which are memory B cells.[97]		
Injectable therapies			
Interferon-B *Interferon beta-1a* *Interferon beta-1b*	Reduction in absolute memory B cell numbers. Reduction of $CD27^+$ memory B cells as a proportion of $CD19^+$ B cells and as a proportion of total PBMCs.[63]	No data	Altered peripheral $CD4^+$ T cell responses to EBNA-1.[64] No change in EBNA-1 IgG titre.[89]
Glatiramer acetate	Decrease in total circulating memory B cells and plasmablasts.[43]		
Immune reconstitution therapies			
Cladribine	Decreased absolute B cell count to 10–15% of baseline after first cycle.[99] Decreased proportion of memory B cells relative to total B cell population.[62] Long-term depletion of memory B cells.[43]	Cladribine penetrates the blood–brain barrier[43] No data on CSF B cell numbers	No data
Alemtuzumab	Severe reduction in absolute B cell numbers. Early hyperproliferation of immature B cells. Slower repopulation of memory B cells.[43]	Decrease in absolute $CD19^+$ B cell number[100]	EBV viraemia is common after Alemtuzumab treatment as part of allogenic HSCT.[57] No data for PwMS treated with Alemtuzumab monotherapy.
AHSCT	Transient depletion of $CD19^+$ B cells, with recovery 3 months after AHSCT. Slower recovery of memory B cells with reduced repertoire diversity persisting at 1 year after transplantation.[56]	No data	EBV viraemia is common after AHSCT for MS.[55] Increase in proportion of $CD8^+$ T cell clones which are specific to EBV.[54]

mechanism by which B cell therapy is effective. *In vitro* work by Pham *et al*. found that T cell proliferative responses to lymphoblastic cell lines and EBV virions were reduced in peripheral blood from PwMS treated with ocrelizumab. This has been suggested to support molecular mimicry, with the hypothesis that MS is caused by cross-reactive T cells which arise in response to EBV and that depleting EBV-infected B cells prevents ongoing molecular mimicry events. However, T cell responses to EBV in ocrelizumab-treated PwMS remained robust in this study, and the range in measured responses was very broad.[47] There was no treatment control group, such as people with other autoimmune diseases treated with anti-CD20 therapy. Reduced T cell responses to EBV antigens are expected given the B cell-depleting mechanism of ocrelizumab and is unlikely to be specific to PwMS. More recently, Schneider-Hohendorf *et al*. found that treatment with anti-CD20 monoclonal antibodies did not change the frequency of EBV-specific $CD8^+$ TCR sequences in the peripheral blood.[19] TCR specificity was defined based on published databases, and private EBV-specific clones could not be identified. Changes in EBV-specific T cell populations are relevant only if they contain clones with cross-reactivity to CNS antigen, which is yet to be demonstrated.

Proponents of the autoreactive B cell hypothesis have suggested that the effectiveness of B cell depleting agents arises as a result of reduction in EBV-infected memory B cells within the CNS. This is the mechanism by which B cell depleting agents are effective in PTLD.[48] The argument that depletion of EBV-driven memory B cells is key to the efficacy of B cell depleting agents is not specific to the autoreactive B cell hypothesis and also applies to other theories about the way in which the continued presence of EBV might drive MS.[48] As summarised in Table 2, most MS therapies deplete memory B cells in peripheral blood to some degree. However, relative depletion of memory B cells across agents is difficult to quantify, and many studies report changes in B cell numbers as a proportion relative to other lymphocyte subsets rather than reporting absolute counts. Data specific to treatment effect on memory B cell numbers in the CNS are not available for all therapies.

Anti-CD20 monoclonal antibodies have poor CNS penetrance but do reduce CNS B cell numbers.[42,49] This suggests that the mechanism of action involves a reduction in peripheral B cells, which transit into the CNS.[50] It is not clear what effect peripheral B cell depletion would have on the hypothesised immortalised autoreactive B cells, which propagate autonomously within the CNS.

Lymphocyte sequestration

The efficacy of lymphocyte sequestration via alpha-4 integrin inhibition and sphingosine-1-phosphate receptor modulation is thought to involve inhibition of T cell trafficking into the CNS, although the antigen specificity of presumed pathogenic T cells is not defined. If the pathogenesis of MS involves T cell-mediated molecular mimicry, such therapies would be expected to be efficacious. Natalizumab is a monoclonal antibody to alpha-4 integrin, a cell surface receptor required for lymphocyte trafficking across the blood–brain barrier. Longitudinal analysis of antigen-specific TCR sequences in the peripheral blood of Natalizumab-treated PwMS found an increase in EBV-specific sequences, with no corresponding increase in TCR sequences specific to other viruses. Data on CSF TCR sequences were not available. The authors postulate that Natalizumab treatment may further enrich EBV-specific T cells in the blood of Natalizumab-treated PwMS by preventing T cell entry into the CNS but allowing exit back into the periphery.[19] This finding is of particular interest as cessation of Natalizumab is associated with a risk of severe rebound CNS inflammation.[51]

Natalizumab treatment prevents B and T lymphocytes from crossing the blood–brain barrier but does not affect B cells already contained within the CNS. The reduction in CNS T cell surveillance in Natalizumab-treated PwMS could allow the expansion of hypothesised EBV-infected autoreactive B cells. There is no suggestion that such B cell expansion occurs. Cases of CNS lymphoma in association with Natalizumab therapy are rare, and unlike other immunosuppressants, Natalizumab appears to be associated with EBV-negative CNS lymphoma.[52] However, quantifying the severity of natalizumab-induced T cell deficiency within CNS parenchyma, and establishing whether the extent of such deficiency should theoretically allow autoreactive B cell expansion, is not possible. Until CNS-resident EBV-infected autoreactive B cells are identified, any proposed interaction with therapies that deplete CNS T cells remains speculative.

Immune reconstitution therapies

Immune reconstitution therapies (IRTs) offer long-term remission from the inflammatory component

of MS, with a durable effect that exceeds the period of lymphopenia. Overall clinical efficacy is high, but recurrent clinical and/or radiological relapse does occur in a proportion of PwMS. This provides a useful lens by which to examine how CNS inflammation might be triggered. AHSCT involves chemo-ablation of the mature immune system, with subsequent reintroduction of the patient's own haematopoietic stem cells that then reconstitute the immune system. Alemtuzumab is an anti-CD52 antibody, which causes profound depletion of both B and T cells. Cladribine is a synthetic deoxyadenosine analogue that interferes with DNA synthesis. It is preferentially activated in lymphocytes, causing targeted depletion with B cells being depleted to a greater degree than T cells.[53] All three treatments involve regeneration of a mature immune system without alteration of the immune-genetic haplotype. During immune reconstitution, the T cell repertoire is re-established via the stochastic generation of naive T cell clones, thymic selection based on the pattern of reactivity to self-antigen and self-MHC, and subsequent extra-thymic clonal expansion driven by reactivity to antigen.[54]

Epstein–Barr virus viraemia and an increase in EBV-specific T cells are seen following immune reconstitution therapy. The London study group demonstrated detectable viraemia in 80% of PwMS between day 23 and 46 post-AHSCT.[55] A Swiss study identified viraemia in 2 of 20 PwMS undergoing AHSCT for MS; however, 90% of the cohort had been treated with B cell therapy prior to transplant, which may have contributed to the low rate of post-transplant viraemia.[56] EBV DNAemia is common in patients treated with Alemtuzumab as a conditioning agent during allogenic HSCT.[57] No data are available for treatment with Alemtuzumab alone, or with Cladribine.

T cell receptor repertoire profiling post-AHSCT shows the expansion of public TCR clones specific to common viruses, including EBV, but rates of inflammatory relapse are low.[54] This demonstrates that repeat exposure to EBV, including the expansion of EBV-specific T cell clones, is not sufficient to trigger relapse of disease. These data, like those from anti-CD20 treatment noted above, suggest a lack of correlation between the presence and frequency of EBV-specific TCRs in peripheral blood and treatment effect. The efficacy of IRTs despite the continued presence of EBV does not necessarily rule out EBV as an antigenic trigger in MS given the stochastic nature of T cell generation.

There are no data on the number or proportion of EBV-infected B cells following AHSCT for MS. In the allogeneic HSCT setting (for haematological malignancy), EBV DNAemia and impaired T cell immunity post-transplant resulted in a significant increase in the number and proportion of latently infected CD27 memory B cells, suggesting that high-level EBV reactivation actually drives the expansion of latently infected lymphoblasts in the peripheral blood.[58] Examining whether this phenomenon occurs in PwMS undergoing autologous transplant, and whether there is any correlation with inflammatory relapse, would be valuable. EBV-driven lymphoproliferative disease post-Alemtuzumab monotherapy for other autoimmune disease has been described, and EBV hepatitis has been reported in PwMS treated with Alemtuzumab.[59,60] There is no literature reporting a correlation between EBV reactivation following Alemtuzumab and MS relapse. Similarly, there is no suggestion of MS relapse in the two patients reported to have developed PTLD following AHSCT for MS.[61]

Cladribine causes moderate lymphocyte depletion. In the CLARITY phase III study, total lymphocyte count decreased by 42% after the first cycle and 58% after the second cycle. Lymphocytes recovered to the normal range in 86% of people within 12 months.[53] Despite this, Cladribine can have a long-lasting impact on MS disease. There is disproportionally severe depletion of memory B cells compared with other B cell populations after Cladribine treatment.[62] No studies have examined the impact of Cladribine on EBV-infected memory B cells or EBV DNAemia.

Interferons

Interferons are cytokines that induce the expression of immune effector genes with broad immunomodulatory effects, including antiviral properties. Interferon beta, a type I interferon, can be produced by many different cell types in response to infection or damage. Interferon gamma, a type two interferon, is largely a product of immune cells. Both have been trialled therapeutically in MS, with differing results.

Interferon beta was the first DMT to become available for MS. It has modest efficacy in preventing relapses, and its use has declined as higher efficacy therapies have become available. Interferon beta therapy is associated with a reduction in peripheral memory B cell numbers.[63]

Interferon beta does not cross the blood–brain barrier, and its effect on B cells within the CNS is unclear.[50] *In vitro* measurement of the $CD4^+$ T cell proliferative response to EBNA-1 is reduced in PwMS treated with interferon beta.[64] This analysis excluded people who had experienced relapses or progression despite treatment with interferon and those who were seronegative for EBV.[64] By contrast, interferon gamma is associated with exacerbation of multiple sclerosis.[65,66] *In vitro* work has demonstrated that interferon gamma causes the upregulation of MHC-I receptors on mouse oligodendrocyte progenitor cells, with subsequent cell death.[67] The pleiotropic effects of these cytokines, along with the complex and bidirectional relationship between EBV-infected B cells and cytokine signalling, make hypothesising a potential relationship between interferons, EBV and MS disease difficult.

Medications that exacerbate MS

Several immunomodulatory medications have been shown to exacerbate the inflammatory component of multiple sclerosis. Lenercept is a recombinant fusion protein, which interacts with the TNF receptor. Its use was associated with an increased relapse rate in a randomised controlled trial examining its potential use in MS.[68] The reason that TNF-α inhibitors exacerbate MS is unclear. They do not cross the blood–brain barrier. TNF alpha plays a central role in controlling pathogens in the periphery. Interestingly, there are a number of case reports of EBV-related lymphoma occurring in people treated with TNF-α inhibitors. However, multiple studies examining the relationship between TNF-α treatment and EBV viral load have not found an association.[69,70] A case–control study of patients treated with TNF-α inhibitors for other autoimmune conditions reported an increased rate of both nondemyelinating and demyelinating inflammatory events in the CNS.[71] This suggests that the relationship between TNF-α inhibitors and neuroinflammation is not specific to multiple sclerosis.

Atacicept is a recombinant fusion protein which binds two cytokines: B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL). Atacicept causes peripheral depletion of mature B cells and plasma cells, but memory B cells are not depleted.[72] Randomised controlled trials of Atacicept demonstrated an increased rate of inflammatory demyelination in separate cohorts with MS and optic neuritis.[72,73] Several authors have suggested that the increased relapse rate seen in Atacicept may be because it fails to deplete memory B cell populations, hence would be unlikely to reduce EBV-infected B cell load.[37,74] However, in the absence of direct data on EBV viral load or EBV-infected B cell populations in people treated with Atacicept, this remains speculative.[37]

THE NEXT GENERATION: HOW THE '-OMICS' ERA MAY ANSWER OUTSTANDING QUESTIONS

The presence of EBV in the CNS

In vivo evidence of the 'autoreactive B cell hypothesis' requires the confirmation of EBV gene latency antigen within the CNS. The gold standard EBV DNA fluorescence *in situ* hybridisation (ISH) detects viral genomes regardless of latency state yet is notoriously challenging in a virus designed to escape immune detection through persistence with minimal genome replication. EBV typically downregulates latent antigen expression with only noncoding EB-ERs detectable in the latency 0 stage. Detection of EBV-encoded RNAs (EB-ER ISH) has been employed to infer the presence of EBV in CNS infiltrating B cells, astrocytes and microglia in previous publications. More recent studies have adopted enhanced PCR techniques to support the presence of EBV genes in a small cohort of postmortem snap-frozen brain tissues.[75] It is believed that evolution of sample processing, with a focus on meningeal preservation, will further increase the yield of CNS EBV detection.

The bottom line

It is likely that advances in molecular biology will allow a better characterisation of EBV presence and gene expression in postmortem tissue. Whether this is unique to PwMS will determine the significance of this finding.

Latent EBV infection and anti-EBV immunity

In blood, detection of B cells latently infected with EBV is also difficult. It has been estimated that 10–460 per 10^7 B cells can be detected with DNA PCR following *in vitro* T cell depletion, resulting in a laborious workflow for analysis of these cells. Next-generation sequencing has accelerated, by orders of

magnitude, the breadth and depth of cellular profiling. Targeted technologies can assess a preselected set of molecular dimensions (preselected genes for mRNA expression studies and protein-level detection) using molecular 'baits' to profile genes or proteins at a single-cell resolution. Microfluidic approaches enable highly quantitative assays for single-cell (sc) gene expression, which can now be combined with B (and T) cell receptor profiles. However, the low frequency of latently infected B cells poses a significant limitation to this approach. Enriching the analysis to settings where viral escape is probable, for example in the lymphopenic environment is likely to enhance the yield of profiling EBV-infected B cells in PwMS. Historically, antiviral T cell responses have been measured by the production of a particular cytokine, such as IFN-γ in response to antigen. However, this technique does not enable the isolation of cells for subsequent assays. Identification of such lymphocytes after antigenic stimulation through cell surface markers (e.g. CD25 (IL-2Rα) and CD134 (OX40) for $CD4^+$ T cells) can enable FACS isolation of viral-specific T cells for downstream sequencing.

The bottom line

Novel approaches for the isolation of viral-specific T cells, exploiting situations where virally infected cells may be enriched, and the expansion of sc RNA sequencing will enable rigorous profiling of EBV immunity in future. Determining how this differs in MS and non-MS individuals will be the next vital step in understanding the relationship between EBV and MS.

Individual EBV responses and the development of MS

Genome-wide association studies (GWAS) have identified more than 200 common genetic variants (SNPs) linked with MS, the majority encoding for cytokines, cytokine receptors, transcription factors and co-stimulatory molecules of the adaptive immune system.[76,77] Notably, most DNA variants identified in GWAS are found in regions that do not encode for genes. It has recently been confirmed that these SNPs exert their effects by altering gene expression through a mechanism termed 'expression Quantitative Trait Loci' (eQTLs). Study of eQTLs has identified unique effects on gene expression of *EAF2* in B cells in PwMS, which has been postulated to explain variability in host–pathogen interactions and MS risk.

The bottom line

We are increasingly able to describe the relationship between population genetics in specific diseases and the translational effect on immunity. In appropriate cohort studies, this will enable us to describe genetic and molecular underpinnings of diseases like MS and explain why common viruses such as EBV may induce disease in certain individuals only.

CONCLUSION

Understanding the cause of multiple sclerosis is vital to improving the lives of people who are affected by this potentially disabling disease. Without a clear understanding of the underlying pathobiology, we cannot create targeted treatments that offer disease remission without a high burden of off-target side effects. Interest in the role of EBV in MS results, in part, because of the potential to offer targeted treatment such as EBV-specific chimeric T lymphocyte therapy. There is also hope of disease prevention via vaccination, which is of particular interest to PwMS because relatives of affected individuals have a significantly increased risk of developing disease. There is an urgent need to confirm that an epidemiological association between EBV infection and MS is mechanistically relevant to disease pathogenesis.

Contemporary highly effective MS immunotherapies either deplete lymphocytes or sequester them in the peripheral immune compartment. Therefore, the therapeutic effect could relate to either an antibody or T cell-mediated molecular mimicry mechanism. It is attractive to speculate that B cell depleting therapies diminish the EBV reservoir and potentially delete proposed autoreactive B cells. However, there is currently no evidence that this specifically relates to the depletion of EBV-infected autoreactive B cells in the CNS. Immune reconstitution therapies can be highly effective in providing durable freedom from inflammatory relapse despite the continued presence of EBV. Hypotheses for the mechanism by which EBV might cause MS need to explain this phenomenon.

How might we discover the answer to this vital question? EBV is a human-specific virus that does not naturally infect mice or nonhuman primates and animal models of MS have significant limitations. Most adults are infected with EBV, and EBV-positive PwMS have usually been infected years before the onset of disease. This makes studying potential EBV-driven immune changes that associate with subsequent development of MS challenging. This paper has focussed on the potential role of EBV in the pathogenesis of inflammatory MS. Progressive disease can occur despite suppression of inflammation, at least as far as can be ascertained by measuring clinical relapses or new MRI lesions. Addressing whether EBV infection has any role in indolent progressive disease is another key area of investigation. Employing our 'bedside' experience to translational research can then set the stage for new treatment, which targets the underlying molecular pathways which cause MS.

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AUTHOR CONTRIBUTIONS

Zoe Anne Dyer: Conceptualization; writing – original draft; writing – review and editing. **David Tscharke:** Conceptualization; supervision; writing – review and editing. **Ian Sutton:** Conceptualization; supervision; writing – review and editing. **Jennifer Massey:** Conceptualization; supervision; writing – review and editing.

CONFLICT OF INTEREST

Jennifer Massey and Ian Sutton have received honoraria from Biogen, Roche, Sanofi Genzyme, Merck and Teva.

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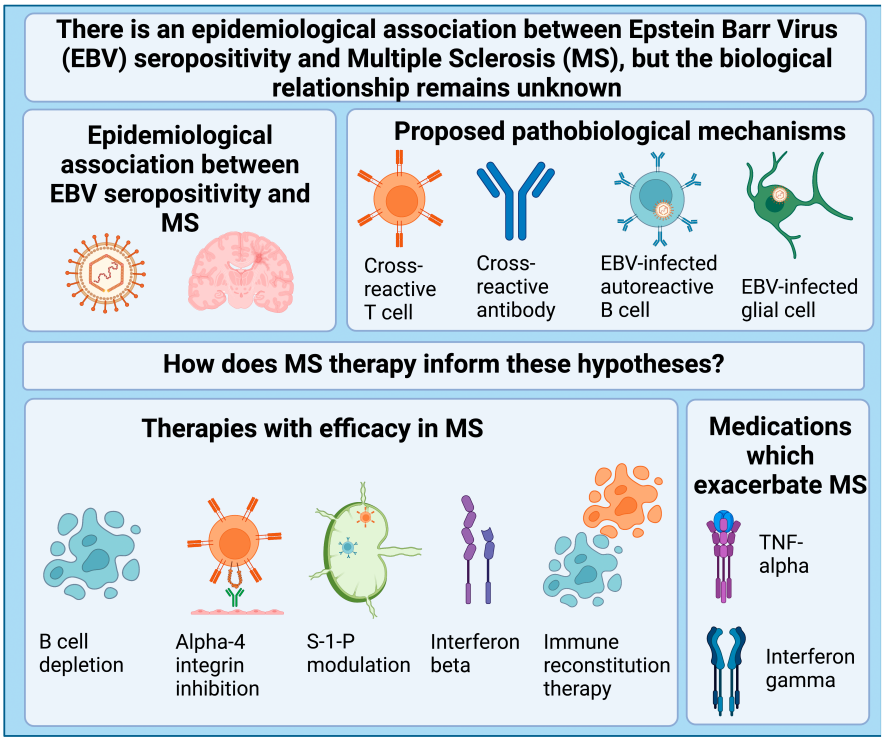

Epidemiological evidence supports a potential role for Epstein–Barr Virus (EBV) in multiple sclerosis (MS) pathogenesis. Several hypotheses propose differing roles for EBV in establishing disease, but none have been proven to date. Investigating the effect of immunosuppressive and immune-modulatory therapies used in MS on immune responses to EBV, EBV reactivation and EBV load offers useful insights into the validity of these hypotheses.