ANTIBODY-INDEPENDENT FUNCTIONS OF B CELLS IN MULTIPLE SCLEROSIS

LIZA RIJVERS • 2021

ANTILICHAAM-ONAFHANKELIJKE FUNCTIES VAN B CELLEN IN MULTIPELE SCLEROSE

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Antibody-Independent Functions of B cells in Multiple Sclerosis

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General Introduction

GENERAL INTRODUCTION

1. MULTIPLE SCLEROSIS

Multiple Sclerosis (MS) is a chronic immune-mediated disease of the central nervous system (CNS) with inflammation, demyelination and neurodegeneration as hallmarks of the pathology [1-3]. Diagnosis is based on the presence of CNS lesions that are disseminated in time and space. The clinical manifestations and course of the disease are very heterogeneous. In approximately 85% of the patients, the initial phase of the disease course is characterized by a first neurological attack, i.e. clinically isolated syndrome (CIS). More than half of CIS patients encounter subsequent episodes of attacks (relapses) that usually last for days or weeks, i.e. relapsing-remitting MS (RRMS). Over time, up to 90% of RRMS patients develop permanent neurological deficits where progression of clinical disability becomes more prominent, i.e. secondary progressive MS (SPMS). In 15% of MS patients, the disease is already progressive from the time of onset and is called primary progressive MS (PPMS). The activity and progression of the disease is clinically assessed using magnetic resonance imaging (MRI) and standardized measures for disability such as Expanded Disability Status Scale (EDSS) scores. The different clinical courses of MS are summarized in Figure 1.

MS is a widely studied neurological disease in terms of epidemiology and is the primary cause of non-traumatic disability in young adults. MS is mainly found in individuals of northern European ancestry, with a prevalence estimated between ~1 per 400-1,000 individuals in Western countries. Approximately 2.3 million people worldwide suffer from MS and it is associated with a high economic burden (between 23.100-50.500 euros per person with MS per year in The Netherlands). MS disease onset is typically between 20 and 35 years of age and has a female to male ratio of 2.5 [4-6].

1.1 Pathology

The hallmark of MS pathology is the formation of lesions (focal plaques) within the CNS, which are areas of demyelination that are located around postcapillary venules. Lesions are found in both the white- and grey matter of the brain, but also in the spinal cord and optic nerve [7-10]. Another known, but incompletely understood feature of MS is breakdown of the blood-brain barrier (BBB) [11]. It is thought to involve pro-inflammatory cytokines and chemokines produced by CNS-resident cells, endothelial cells and infiltrating leukocytes [12, 13]. The increased recruitment of activated leukocytes such as macrophages and lymphocytes due to a disrupted BBB causes inflammation and demyelination, followed by oligodendrocyte loss, reactive gliosis and neuro-axonal damage [13,

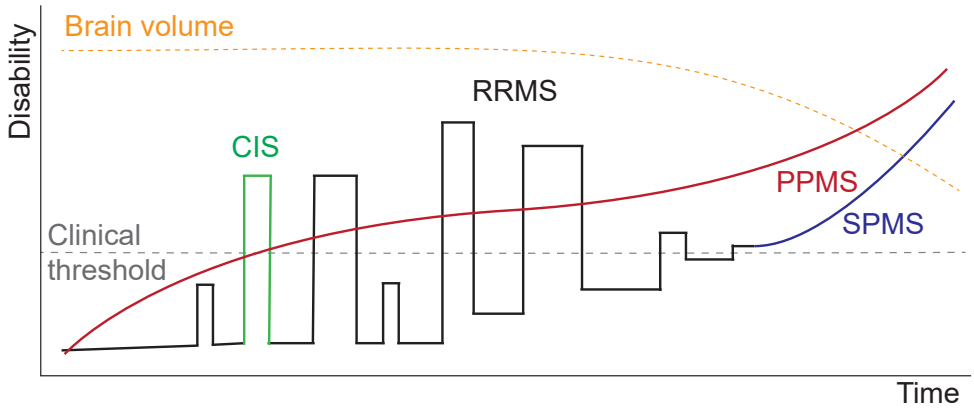

Figure 1. The heterogeneous clinical course of MS.

Clinically isolated syndrome (CIS) is the first clinical presentation of MS. After a period of subsequent neurological attacks, the majority of relapsing-remitting MS (RRMS) patients will develop secondary progressive disease (SPMS). A small number of MS patients have progressive disease right from the start of the course, which is termed primary progressive MS (PPMS).

14]. Following demyelination, myelin sheaths are often regenerated (i.e. remyelination), leading to clinical recovery after a relapse [15]. However, remyelination is a limited process; it depends on age, disease duration, lesion location, axonal integrity and the presence of oligodendrocyte progenitor cells [16]. This remyelination process is more efficient early in the disease course and at a younger age [17]. Remyelination failure contributes to irreversible neurodegeneration [18], which occurs already in the earliest phases of the disease and is most likely the cause of permanent clinical disability later in the disease [19].

In the progressive phase, CNS inflammation is compartmentalized and originally thought to be purely mediated by CNS-resident cells [20]. However, recent evidence shows that also CNS-resident lymphocytes play a role by slowly accumulating in the connective tissue spaces of the brain, such as the perivascular Virchow Robin spaces and meninges [21-23]. Within the meninges, these cells form follicle-like structures that partly resemble tertiary lymphoid follicles and where local reactivation of lymphocytes might occur to drive progressive disease [24]. These structures are found in approximately 40% of SPMS patients and link to a more severe clinical course, shorter disease duration and earlier death [25, 26]. Although neurodegeneration is most likely the result of ongoing inflammation and demyelination in the CNS (outside-in hypothesis), there are some indications that neurodegeneration can also be the cause of these processes (inside-out hypothesis) [16, 19].

2. ETIOLOGY OF MS

2.1. Life style and environmental factors

One of the most well established risk factors is Epstein-Barr virus (EBV) infection in adolescence and early adulthood, causing infectious mononucleosis in 30-40% of EBV-infected individuals [27]. Almost all MS patients are seropositive for EBV, while this is the case for 90-95% of healthy individuals. However, individuals with MS have higher levels of antibodies against EBV nuclear antigen 1 (EBNA1) [28]. Evidence for this association mostly comes from epidemiological studies and it remains incompletely understood how EBV is involved in the pathogenesis of MS [29-33]. Another important risk factor is the lack of sun exposure, correlated to low vitamin D levels. Low vitamin D levels are associated with an increased risk of MS and a higher disease activity. Although the mechanism of how vitamin D is involved in MS pathogenesis is unknown, it has an important immune regulatory role [34, 35]. Smoking and obesity are two other risk factors associated with MS. The risk of smoking is dose-dependent, and links to faster disability progression [36]. Not only active, but also passive smoking has been reported to be associated with disease onset [34]. Furthermore, obesity during adolescence is associated with a 2-fold increased risk of MS and a worse clinical outcome during disease [37]. Obesity is associated with low-grade inflammation, with increased pro-inflammatory mediators are produced in fat tissue [38], possibly contributing to MS pathology. Other less-established environmental risk factors are excessive alcohol or caffeine consumption and shift work [34].

Pregnancy is a natural modifier of MS disease activity. Pregnant women with MS are protected from relapses during the third trimester, while relapse risk is significantly increased after delivery [39-41]. During these time periods, there are enormous fluctuations in hormones such as estradiol, estriol and progesterone, probably affecting immune mechanisms that contribute to relapse occurrence. Adaptations in maternal immune tolerance are required for tolerating the fetus during pregnancy. Placenta-derived hormones such as estriol and progesterone can directly affect immune cell function. It has been suggested that estriol exhibits dose-dependent effects on immune cells, where low levels promote and high levels inhibit cell-mediated immunity [42]. How pregnancy influences immune subsets to modulate neuro-inflammatory activity in patients with MS remains a question to be solved.

2.2 Genetic risk factors

The proof for genetic predisposition to MS comes from monozygotic and dizygotic twin studies. The risk in monozygotic twins is 35% compared with 6% in dizygotic twins and 3% in normal siblings. Additionally, according to several family studies, 15% to 20% of people with MS have a relative with the disease. The major genetic risk factor in MS

is the human leukocyte antigen (HLA) class II locus [34, 43]. More than 30 independent genetic associations within the extended HLA region have been identified [44]. Individuals who are homozygous carrier of the major HLA-DRB1*1501 allele have an OR exceeding 6.0 for developing MS and that is associated with younger age at onset [45]. Next to HLA-DRB1*15:01, HLA-DRB1 has multiple other associations with MS, with four amino acid changes capturing most of them [46]. HLA-DRB1*15:01 is often linked to a haplotype consisting of both DQA1*01:02 and DQB1*06:02 alleles. Furthermore, the presence of HLA class I allele HLA-A*02:01 is protective for MS [47]. HLA genes are highly polymorphic and encode for molecules regulating the adaptive immune system (discussed in more detail below). Their contribution to and regulation during the pathogenesis of MS is poorly understood [48].

Interestingly, there is considerable interaction between the HLA-DRB1*1501 allele and various environmental factors such as EBV and vitamin D. The association of both infectious mononucleosis (caused by EBV infection) and anti-EBNA1 antibody levels with MS risk is increased in the presence of HLA-DRB1*1501 [49]. Furthermore, there is a vitamin D responsive element (VDRE) zone in the promotor region of the HLA-DRB1*1501 gene, which seems to regulate its expression [50]. SNPs in genes involved in vitamin D metabolism have also been identified to be associated with MS, and VDRE sites are enriched close to or in other MS-associated genes [47, 51].

Genome wide association studies (GWAS) have identified 200 genetic risk variants outside the HLA locus for MS [52]. Identification of the causal genes and their interplay is warranted to understand the functional pathways and mechanisms involved in the pathogenesis. Most of these variants encode for molecules involved in the immune system and are associated with several other autoimmune disorders, such as type 1 diabetes, SLE and rheumatoid arthritis. No genetic overlap is seen with typical neurodegenerative diseases and only a few variants have a function in the CNS, supporting the fact that MS has an immune-mediated onset [53, 54]. A large part of these risk variants influence promotor and enhancer regions of genes regulating the function of B and T lymphocytes [53], which are cells of the adaptive immune system.

3. ADAPTIVE IMMUNE SYSTEM

Adaptive immunity is also known as acquired or specific immunity, which is required for generating so-called 'memory' after a first encounter with a pathogen, such as a virus or bacteria. This makes sure that the same pathogen is efficiently recognized and cleared after subsequent encounters. B lymphocytes specifically recognize extracellular antigens to which antibodies are produced, while T lymphocytes respond to intracellular antigens

that are processed and presented by HLA molecules. Antibodies produced by B cells neutralize microorganisms by blocking the surface of the pathogen, form immune complexes with microorganisms that are detected by other immune cells, or facilitate cell lysis by activating the complement system. In general, $CD8^+$ cytotoxic T lymphocytes (CTLs) detect endogenously derived (i.e. viral or tumor) antigens presented on HLA class I to directly kill their target cell. $CD4^+$ T helper (T_H) lymphocytes are activated by exogenously derived antigens presented on HLA class II to enhance the effector function of other immune cells, including B lymphocytes and CTLs. To generate an adaptive immune response in secondary lymphoid organs such as the lymph node, lymphocytes first have to undergo several selection and developmental processes in the bone marrow (both B and T cells) and thymus (T cells). During these processes, unique B- and T-cell receptors are formed and tested to be able to specifically react to pathogens and not, for example, self-antigens.

Originally, MS was mainly considered a T cell-driven autoimmune disease, in which pro-inflammatory T cells enter the CNS and recognize self-antigens such as myelin. This was mainly concluded from studies in the animal model of MS, experimental autoimmune encephalomyelitis. However, this T-cell centric view did not adequately explain responses to therapy [55]. Results from new anti-CD20 therapy now also puts B cells forward as key players in the pathogenesis of MS. In particular, this indicates that B cells already have a disease-inducing role in the periphery, likely as the result of defects in their selection and development (see also 4.1). Furthermore, B cells are also involved in sustaining local inflammation in the more progressive phase of the disease (see section 4.3).

3.1. B-cell development

Within the bone marrow, $CD34^+$ stem cells differentiate into pro- and pre-B cells that form immature B cells. During these first steps of differentiation, the B cell receptor (BCR) is generated and edited to increase specificity against non-self but prevent reactivity against self [56, 57]. First, at the pro-B cell stage, the BCR heavy chain is produced by recombination of so-called V(D)J gene segments. After developing into pre-B cells, the heavy chain will be presented on the cell surface in conjunction with a surrogate light chain, which enables selection of productive heavy chains. B cells without a productive heavy chain are removed from the repertoire. B cells with a self-reactive BCR are removed by central tolerance checkpoints. This mechanism keeps self-reactive B cells in check through apoptosis, BCR editing or anergy. Next, the light chain is rearranged and subsequently expressed on the surface of immature B cells. These immature B cells exit the bone marrow and enter the circulation as new emigrant/transitional B cells. Because BCR rearrangement is a random process and despite central tolerance checkpoints, approximately 25% of the circulating B cell populations are still able to recognize self-antigens [58].

Transitional B cells constitute 5-10% of the total peripheral B-cell pool in healthy adults and are unresponsive to BCR stimulation. Transitional B cells enter the spleen where a crucial cell-fate decision oriented toward either a T-cell independent (TI) or a T-cell dependent (TD) response occurs. They either enter the marginal zone (MZ) and develop into IgM^+IgD^+ natural effector memory B cells or short-lived IgM^+ plasma cells [59-61] in a T cell-independent manner via extensive BCR cross-linking or activation of Toll-like receptors (TLR) and other innate receptors [62-64], or exit the spleen as naive mature B cells for further T-cell dependent differentiation.

These naive mature B cells enter the follicle of secondary lymphoid organs and are able to respond to antigens via their BCR. To become fully activated, B cells need a second and third signal [65], which is provided by costimulatory molecules and cytokines expressed by the interacting T_H cells at the follicular border. An important costimulatory interaction is between CD40 on the B cell and CD40 ligand (CD40L) on the T_H cell. This interaction is not only important for the activation of B cells, but is next to HLA class II/T-cell receptor (TCR) interaction essential for inducing peripheral B-cell tolerance. This second tolerance checkpoint suppresses or eliminates most of the remaining self-reactive B cells via similar processes as in the bone marrow [66-68]. Moreover, inducible ICOSL expression on B cells promote the interaction between B- and T cells via the surface receptor ICOS on T cells [69, 70]. ICOS-ICOSL interaction results in the production of IL-21 and IL-4 by T_{FH} cells, necessary for B cell differentiation and survival [70]. Another important co-stimulatory interaction is between the B7 molecules (CD80/86) on the B cell and their T cell-activating binding partner CD28, inducing T cells to proliferate and produce cytokines such as IL-2, IFN-γ, TNF-α and GM-CSF, but not IL-4 [71, 72].

After the initial interaction with T_H cells, naive mature B cells either differentiate into IgM^+ short-lived plasmablasts or enter active sites in the follicles called germinal centers (GCs). Within GCs, B cells undergo somatic hypermutations and interact with T_{FH} cells and follicular dendritic cells to further mature into memory B cells or long-lived plasma cells [73]. Memory B cells have the capacity to self-renew, but may also form plasmablasts as a precursor for antibody-secreting plasma cells. Memory B cells exit the germinal centers to reside in the marginal zone or enter the circulation. Plasmablasts also exit germinal centers and migrate to the bone marrow or other tissues to become long-lived plasma cells [74, 75] (Figure 2).

The initial GC responses generate IgM-only memory B cells, which can undergo subsequent class-switching by rearranging the constant region of the heavy chain to form other isotypes [76-78]. Four different IgG (IgG1-4) and two different IgA (IgA1-2) subclasses are known [79]. Although class-switching does not alter the antigen specificity, it does influence the effector function of B cells [80]. All IgG subclasses are involved in pathogen neutralization, however, only IgG1 and IgG3 are potent activators of the complement system

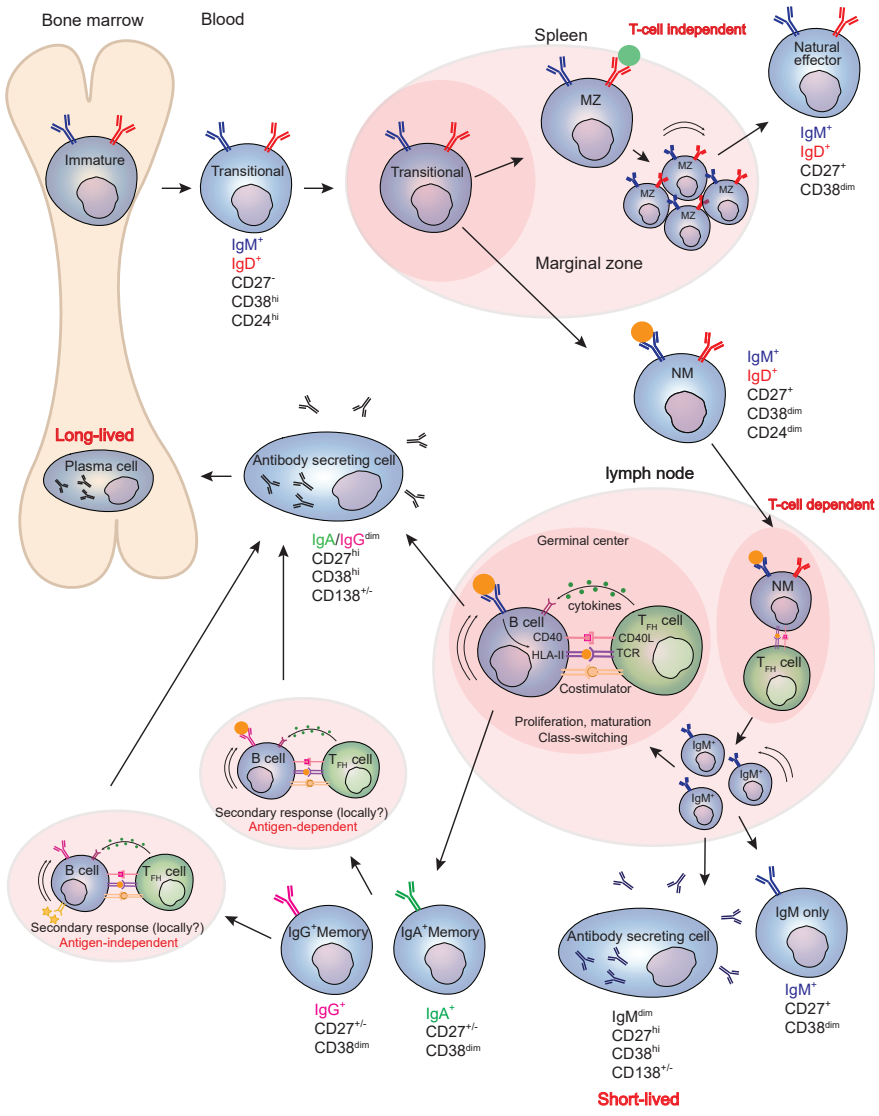

Figure 2. Peripheral B-cell development.

Immature B cells are formed in the bone marrow and enter the circulation as transitional B cells (IgM^+IgD^+). These transitional B cells traffic to the spleen, where they either proliferate in the marginal zone (MZ) and become IgM^+IgD^+ natural effector B cells, or exit the spleen as naive mature (NM) B cells. When these cells encounter an antigen in the lymph node, a primary immune response is generated resulting in the formation of IgM^+ memory B cells. These cells enter the circulation either as IgM-only B cells or short-lived plasmablasts, or undergo class-switching within germinal centers (GC). A GC response is dependent on the interaction of B cells with follicular T helper (T_{FH}) cells via HLA-II antigen presentation, co-stimulation and cytokine production. GC B cells exit the lymph nodes as switched memory B cell ($IgA^+/IgG^+/IgE^+$) or antibody-secreting cells. These antibody-secreting cells re-enter the bone marrow (and possibly also inflamed tissues) to become long-lived plasma cells.

and inducers of antibody-dependent cell-mediated cytotoxicity [81]. IgM is primarily involved in complement activation [82]. Furthermore, while IgA1 is the predominant subclass found in mucosal tissues and serum regulating the first and second line of defense against invading pathogens, IgA2 is commonly found in the lower intestinal tract regulating local microflora [83, 84]. Finally, IgE is the last possible isotype and is the mediator of allergic responses and parasitic infections [85].

3.2. B-cell antigen processing and presentation

A unique feature of B cells is their capacity to specifically recognize, take up and process antigens via the BCR for HLA-II-mediated presentation to $CD4^+$ T_H cells. The efficiency of this pathway is important for determining which antigen-specific B and T_H cells will develop into effector subsets and contribute to an adaptive immune response. After recognition by the BCR, antigens are taken up, processed and presented on HLA-II molecules to T cells [86]. Specific antigen recognition mediates BCR oligomerization and downstream signaling that promotes dynamic actin cytoskeletal rearrangements. This enables efficient BCR-antigen uptake and internalization into endosomal compartments. The interaction between the BCR and its cognate antigen additionally triggers the biogenesis of MHC class II-containing antigen loading compartments (MIICs), in which both the antigen and the accessory molecules such as HLA class II molecules and proteases assemble [87-89]. To reach these compartments, HLA class II molecules that are synthesized in the endoplasmic reticulum (ER) need to bind to a specific chaperone termed the invariant chain (CD74 or Ii). This binding is required for correct folding of the HLA class II molecules, preventing unwanted early antigen binding, and directing HLA class II molecules to the endo-lysosomal system [90]. The cytoplasmic tail of Ii contains two di-leucine sorting motifs, resulting in the transport of HLA-II molecules to the MIICs, either directly from the trans-Golgi network or indirectly via the plasma membrane [91]. This depends on the binding of adaptor protein AP1 (trans-Golgi network adaptor) and AP2 (plasma membrane adaptor) to these Ii sorting motifs [92, 93]. Ii also promotes the interaction with myosin II, which is required for the assembly of HLA class II and BCR-antigen complexes in MIICs. For efficient HLA class II peptide loading in these compartments, both the antigen and Ii need to be cleaved by specific proteases, such as cysteine protease cathepsin S [94], signal peptide peptidase-like 2a (SPPL2a) [95] and IFN-γ-inducible lysosomal thiol reductase (GILT) [96]. The cleavage of Ii leaves a small remnant peptide bound to the binding groove, termed class-II associated invariant chain peptide (CLIP). CLIP is then exchanged for an antigenic peptide via a process catalyzed by HLA-II chaperones HLA-DM and HLA-DO [97]. This cleavage also removes the endosomal retention motif of Ii, enabling HLA class II/peptide complexes to be exported to the plasma membrane and presented to $CD4^+$ T cells (Figure 3). Alternatively, 2-5% of CD74 is expressed at the cell surface independently of HLA class

II, functioning as a high-affinity receptor for the macrophage migration inhibitory factor (MIF) [98-100]. MIF is a pro-inflammatory cytokine that has been shown to control B-cell proliferation, migration and survival in mice [98, 101].

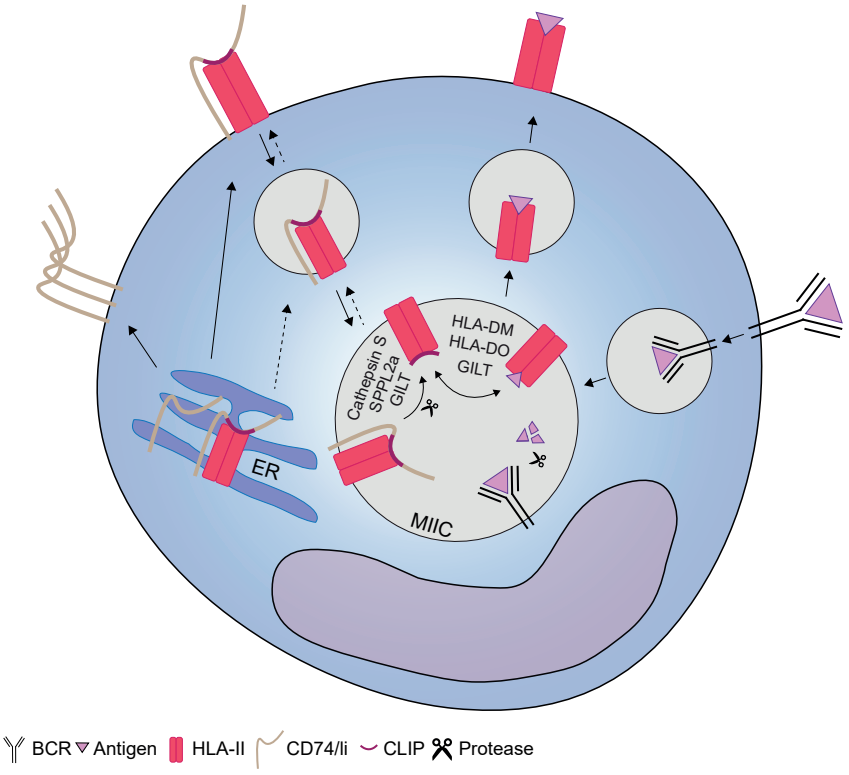

Figure 3. BCR-mediated antigen presentation pathway.
After antigen recognition via the BCR, the BCR-antigen complex is internalized and transported into MIICs. At the same time, a HLA-II molecule is formed in the endoplasmic reticulum (ER) and needs to bind to the invariant chain (Ii or CD74) in order to stabilize and enter these same compartments. Once HLA-II/Ii complexes have arrived in the MIIC, proteases such as cathepsin S, SPPL2a and GILT are required for the cleavage of Ii into CLIP and antigens into peptides. CLIP remains bound to the HLA-II peptide-binding groove and is exchanged for an antigenic peptide, a process regulated by chaperones HLA-DM and HLA-DO as well as GILT. After peptide loading, the HLA-II/peptide complex is transported to the plasma membrane and presented to a $CD4^+$ T cell.

4. B CELLS AND MS

4.1. Defects in peripheral B-cell development

Even in healthy individuals, a minority of self-reactive B cells survive both central and peripheral tolerance checkpoints during development and remain present in the

circulation [58]. In most autoimmune diseases, both central and peripheral B-cell tolerance checkpoints are impaired [67, 102, 103]. Interestingly, in MS patients, only peripheral tolerance is defective, resulting in more naive self-reactive B cells with an activated phenotype in the circulation [67, 104]. In this regard, it may be that the defect in peripheral tolerance in MS is a consequence of regulatory T cell (T_{REG}) dysfunction. T_{REGs} are essential for the maintenance of peripheral tolerance. Dysfunction of the suppressing capacity of the T_{REGs} is associated with several autoimmune diseases including MS [105]. Both the frequency and regulatory function of T_{REGs} have been reported to be lower in MS patients than in healthy individuals [106, 107]. However, a B-cell intrinsic defect underlying the escape from peripheral tolerance cannot be excluded.

After these initial tolerance checkpoints, receptor editing of memory B cells, by inducing somatic hyper mutations, can result in more self-reactive clones than the ancestor memory B cells [108]. Additionally, loss of anergy might result in reactivation of self-reactive B cell clones. The important characteristic of anergic B cells is the abrogation of their BCR signaling capacities [109, 110], therefore they are not responding to self-antigen and do not undergo rapid apoptosis, but persist in secondary lymphoid tissues [111, 112]. Although most anergic B cells are a result of arrest at the developmental stage, displaying a transitional phenotype, B-cell anergy can also be induced in the periphery via chronic antigen stimulation. Self-antigens or persistent infections are able to chronically induce the BCR, resulting in an unresponsive ('exhausted') B cell [113, 114]. Despite their inability to signal via the BCR, anergic B cells retain the ability to present antigen and respond to T_H cell-derived stimuli [109, 115]. Self-tolerance within the T cell compartment ensures that these anergic B cells do not receive activating signals. However, if self-reactive BCR cross-react with a certain pathogen, there may be loss of anergy resulting in the activation of B-cell populations capable of driving autoimmunity [108, 109].

4.2. T-cell activation by antigen presenting B cells

The striking effect of anti-CD20 therapies to limit new MS relapses has shifted the framework of MS immunopathogenesis. The contribution of B cells to CNS inflammatory disease activity have been linked to the antibody-independent functions of B cells, as part of cascades of immunological interactions in the periphery that mediate disease activity. B-cell depletion therapy significantly reduce pro-inflammatory T_H-cell responses in MS, both *ex vivo* and *in vivo* [116]. Furthermore, MHC class II expression on murine B cells was reported to be indispensable for the onset of experimental autoimmune encephalomyelitis (EAE), a mouse model of MS [117, 118]. Moreover, CD40L expression is required to induce costimulatory activity on antigen presenting cells for *in vivo* activation of $CD4^+$ T cells in EAE [119]. The *in silico* evidence that autoimmunity-associated HLA class II molecules have an altered peptide-binding groove [46, 120], together with the potential role of several minor risk

variants in the HLA class II pathway, insinuates that antigens are differently processed and presented by B cells [121, 122]. This is supported by the increased ability of memory B cells to trigger CNS-infiltrating T_H cells in MS patients carrying *HLA-DRB1*1501* [122]. Next to the genetic variants directly within the MHC region, GWAS studies have identified multiple SNPs that might be involved in the regulation of antigen presentation [44].

Furthermore, B cells from MS patients have an altered capacity to produce pro-inflammatory cytokines such as IL-6, GM-CSF, TNFα and LTα and are deficient in the production of regulatory cytokine IL-10 [123-126]. Interestingly, this abnormal cytokine response of B cells from MS patients is most prominent when these cells were activated in the context of IFN-γ or in the presence of TLR9 stimulation [127]. This suggests that infections that generate a T_H1 host response, including bacteria and viruses, could trigger abnormal pro-inflammatory B cell cytokine responses in MS patients. This aberrant cytokine profile of B cells from MS patients can induce abnormal effector T-cell responses through TNFα and IL-6 as well as pro-inflammatory myeloid cell responses. Strikingly, anti-CD20 therapies reduces the pro-inflammatory responses of T_H1 and T_H17 cells as well as myeloid cells in the periphery of MS patients [123, 126, 127].

Although the antibody-independent function of the B cell is mostly linked to their role in the periphery, local interactions within the CNS between B- and T cells also probably contribute to the disease. Cerebrospinal fluid (CSF) and CNS B cells of MS patients express elevated levels of HLA class II and T-cell co-stimulatory B7 molecules [128-130]. It can therefore be expected that infiltrating B cells also re-activate local T cells to mediate MS pathology (Figure 4).

4.3. Enhanced recruitment of B cells to the CNS

The BBB is dysfunctional during the early phase of MS, resulting in local recruitment of pathogenic immune cells including B cells [131]. Differences in expression of chemokine receptors, integrins and pro-inflammatory cytokines by infiltrating memory B cells and plasma cells mediate their trafficking in and out of the CNS [132, 133], but also their local organization and impact (Figure 4). Chemokines expressed in the CNS might contribute to the migration of lymphocytes across the BBB by acting as chemoattractants and sustaining ongoing inflammation; i.e. elevated levels of CXCL10, CXCL12, CXCL13 and MIF were found in the CSF of MS patients [134]. Hence, chemokine receptor profiles of B cells are a determining factor for which functional B cell subsets will be attracted to the inflamed CNS. For example, migration of memory B cells and plasma cells may be facilitated by the expression of chemokine receptors such as CCR6, CXCR3 and CXCR4 [135]. Furthermore, high levels of integrin α4β1 (VLA-4) allow B cells to bind to VCAM-1 on brain endothelial cells, contributing to the migration of B cells across the BBB [136]. This is supported by the reduced B-cell infiltration into the CNS and disease susceptibility in EAE mice when

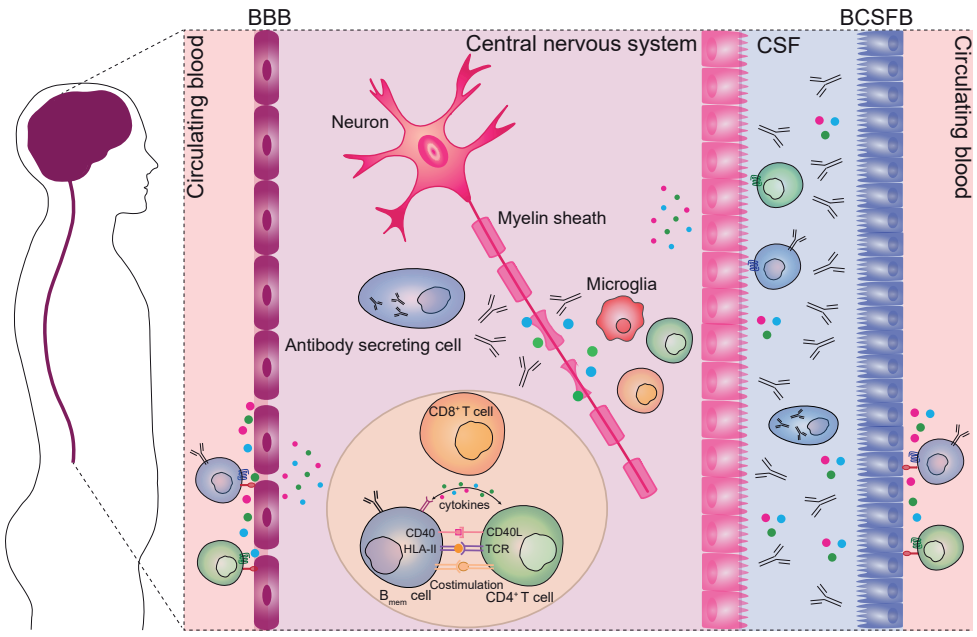

Figure 4. Recruitment and impact of B cells in the CNS of MS patients.
Both memory B and T cells are able to migrate through the blood-brain barrier (BBB) or via the blood-CSF barrier (BCSFB) through the expression of chemokine receptors, adhesion molecules and pro-inflammatory cytokines. After entering the CNS, B and T cells probably interact in perivascular spaces and meningeal follicle-like structures, resulting in re-activation, clonal expansion and eventually local inflammation and demyelination.

VLA-4 is inhibited [137]. Additional studies in EAE demonstrate that MIF antibody inhibition reduces vascular cell adhesion protein 1 (VCAM-1) expression on the BBB and results in decreased recruitment of inflammatory cells, suggesting that MIF plays a role in the homing and transmigration of CNS-specific B and T cells [138]. Furthermore, activated leukocyte cell adhesion molecule (ALCAM) is a cell adhesion molecule that is expressed by B cell and drives their migration across multiple CNS barriers [139]. Moreover, ALCAM expressing B cells are increased in both peripheral blood and brain lesions of MS patients [140].

Within the CNS of MS patients, B cells have been found to accumulate in active white matter lesions and the meninges, the membrane covering the brain and spinal cord [20]. They can be detected in both early and late stages of MS, but are most abundant in patients with RRMS [20, 141]. B cells are enriched in perivascular lesions and only rarely in the subarachnoid space [13, 24, 141]. Within the meninges, B cell-rich follicle-like structures localize next to cortical lesions, presumably mediating progressive loss of neurological function in MS [21, 142]. This meningeal inflammation is associated with a more aggressive MS course and a more severe cortical pathology involving microglial activation and neuronal

loss [21, 22]. Although ectopic follicle-like structures have only been described in SPMS patients, meningeal aggregates of B cells can also be found in patients with RRMS and PPMS [143, 144]. These B cell-rich structures share some features with tertiary lymphoid follicles seen in other organ-specific chronic and acute inflammatory conditions [145]. They not only recapitulate the cellular and structural organization of secondary lymphoid organs, but also support the function of germinal centers. They retain the necessary molecular machinery to support B cell differentiation and proliferation including class-switching and antibody diversification [145]. However, many of the regulatory mechanisms that govern tolerance in secondary lymphoid organs are not seen in these autoimmunity-associated tertiary lymphoid follicles [146]. This allows the entry of autoreactive B cells and their differentiation into plasma cells that potentially release disease-specific auto-antibodies, which has already been shown in rheumatoid arthritis [147] and Sjögren's syndrome [148]. Another possible role for B cells in these tertiary lymphoid follicles is to reactivate pro-inflammatory T cells that contribute to MS pathology [122].

4.4. Increased antibody production in the CNS

In contrast to healthy individuals, high antibody levels are found in the CSF of MS patients. These CSF antibodies include characteristic oligoclonal bands (OCBs), as seen in CSF electrophoresis of isoelectric focusing. CSF OCBs are used as a diagnostic tool in MS. IgG OCBs can be found in 90% of MS patients, while IgM OCBs are present in approximately 30-40% of MS patients and have been associated with more active disease [149, 150]. The pathogenic role of antibodies in the CNS and the relevant antigen specificities of the B cells involved in MS remain unclear. Owing to the demyelinating nature of the disease, myelin proteins such as MOG, MBP and proteolipid protein have been extensively investigated as target antigens of B cells in MS patients. Despite extensive investigation, studies have yielded mixed results regarding the specificity of B cells and antibodies and might actually identify patients who do not have MS [151-153]. Additionally, studies demonstrated CSF antibody reactivity against various viral and self-antigens, indicating that the B-cell response might not be directed against a single epitope, antigen or cell type and is highly diverse between MS patients [154-156].

The presence of elevated immunoglobulin synthesis rates in the CSF of MS suggests that immunoglobulin is being produced locally within the CNS (intrathecal production). This intrathecal production of immunoglobulin is supported by somatic hypermutation analysis of B cells and plasma cells, which had demonstrated a restricted number of expanded clones within the CNS of people with MS [157-160]. These clones are shared between different CNS compartments, i.e. CSF, meninges and parenchyma, and similar to those in the periphery [161-165], indicating that there is trafficking of B cells in and out of the CNS.

5. SCOPE OF THIS THESIS

The strong and rapid beneficial clinical effects of anti-CD20 treatment in MS patients have revealed that B cells serve as antigen-presenting rather than antibody-producing cells in the periphery. B-cell depletion therapy does not remove antibody-producing plasma cells but significantly reduces pro-inflammatory T_H-cell responses in MS. However, the exact underlying antibody-independent functions of B cells in MS are poorly understood. It is likely that during MS pathogenesis, B cells not only trigger, but also receive signals back from T_H cells to develop into CNS-infiltrating pathogenic B cell subsets (Figure 5). In this thesis, we aimed to uncover genes and pathways that are functionally altered in human B cells and potentially contribute to MS.

In **chapter 2,** we assessed the triggers, development and CNS infiltration capacity of pathogenic T-bet$^+$CXCR3$^+$ B cells in MS patients. We made use of blood, CSF, meningeal and brain tissues from MS patients to study the phenotype of CNS-infiltrating B cells. Similar analysis was performed for memory B cells trapped in the blood of anti-α4-integrin antibody (natalizumab)-treated MS patients. Both IFNγR-/TLR9-mediated GC-like differentiation and BBB transmigration of T-bet$^+$CXCR3$^+$ B cells were assessed *in vitro*.

Since little is known about how B-cell intrinsic HLA class II expression is regulated during MS disease onset, we studied the impact of autoimmunity-associated risk locus *CLEC16A* on the B cell antigen processing and presentation pathway in MS in **chapter 3**.

In **chapter 4,** we studied the MIF pathway as an underlying molecular mechanism of B-cell survival, migration and chronic inflammation in early MS. MIF associates with chronic inflammation and B-cell survival in mice and is upregulated in the CNS of MS patients. MIF utilizes as a receptor CD74, a molecule also involved in antigen presentation. How MIF regulates immune subsets to promote disease activity in MS was not studied before.

Pregnancy is a natural modifier of disease activity in MS patients, but the underlying mechanisms remain elusive. In **chapter 5,** we studied the effect of pregnancy on B-cell differentiation in MS patients both *ex vivo* and *in vitro.*

In **chapter 6**, we discussed how the interaction of B cells with T cells is possibly affected by intrinsic and extrinsic factors and drives the infiltration of pathogenic subsets into the CNS of MS patients.

Finally, our findings on the role of B cells in MS are summarized, put in context and discussed in **chapter 7**.

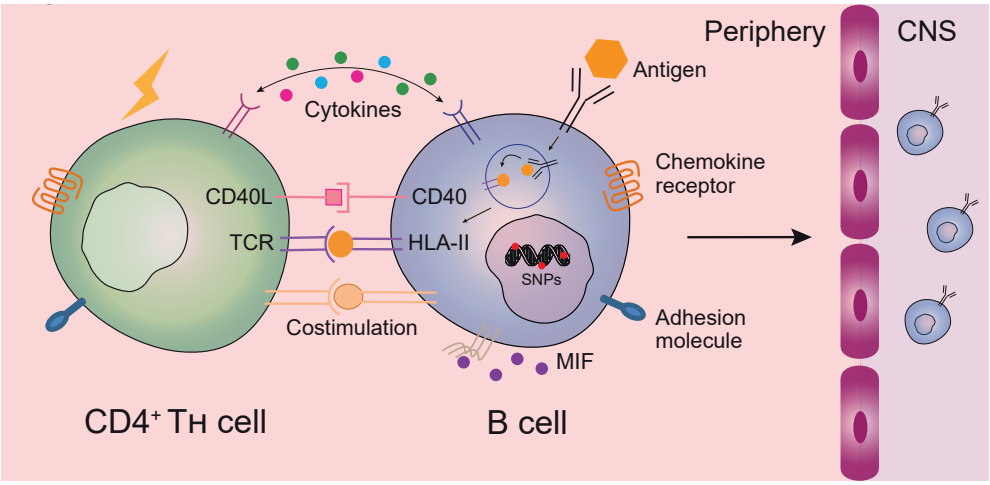

Figure 5. Peripheral B- and T-cell interaction as underlying mechanism in MS .
Pathogenic B cells interact with CD4+ T effector cells in secondary lymph nodes to drive their development and recruitment to the CNS. This interaction depends on the potential of B cells to internalize, process and present antigen to and respond to costimulatory and cytokine signals derived from CD4+ T_H cells. In MS, the antigen-presenting and CNS-infiltrating capacity of B cells is probably influenced by genetic and environmental risk factors.

REFERENCES

1 **Lublin, F. D., Reingold, S. C., Cohen, J. A., Cutter, G. R., Sorensen, P. S., Thompson, A. J., Wolinsky, J. S., et al.,** Defining the clinical course of multiple sclerosis: the 2013 revisions. *Neurology* 2014. **83**: 278-286.

2 **Krieger, S. C., Cook, K., De Nino, S. and Fletcher, M.,** The topographical model of multiple sclerosis: A dynamic visualization of disease course. *Neurol Neuroimmunol Neuroinflamm* 2016. **3**: e279.

3 **Filippi, M., Bar-Or, A., Piehl, F., Preziosa, P., Solari, A., Vukusic, S. and Rocca, M. A.,** Multiple sclerosis. *Nat Rev Dis Primers* 2018. **4**: 43.

4 **Collaborators, G. B. D. M. S.,** Global, regional, and national burden of multiple sclerosis 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol* 2019. **18**: 269-285.

5 **Kobelt, G., Eriksson, J., Phillips, G. and Berg, J.,** The burden of multiple sclerosis 2015: Methods of data collection, assessment and analysis of costs, quality of life and symptoms. *Mult Scler* 2017. **23**: 4-16.

6 **Uitdehaag, B., Kobelt, G., Berg, J., Capsa, D., Dalen, J. and European Multiple Sclerosis, P.,** New insights into the burden and costs of multiple sclerosis in Europe: Results for the Netherlands. *Mult Scler* 2017. **23**: 117-129.

7 **Gilmore, C. P., Donaldson, I., Bo, L., Owens, T., Lowe, J. and Evangelou, N.,** Regional variations in the extent and pattern of grey matter demyelination in multiple sclerosis: a comparison between the cerebral cortex, cerebellar cortex, deep grey matter nuclei and the spinal cord. *J Neurol Neurosurg Psychiatry* 2009. **80**: 182-187.

8 **Green, A. J., McQuaid, S., Hauser, S. L., Allen, I. V. and Lyness, R.,** Ocular pathology in multiple sclerosis: retinal atrophy and inflammation irrespective of disease duration. *Brain* 2010. **133**: 1591-1601.

9 **Petrova, N., Carassiti, D., Altmann, D. R., Baker, D. and Schmierer, K.,** Axonal loss in the multiple sclerosis spinal cord revisited. *Brain Pathol* 2018. **28**: 334-348.

10 **Calabrese, M., Magliozzi, R., Ciccarelli, O., Geurts, J. J., Reynolds, R. and Martin, R.,** Exploring the origins of grey matter damage in multiple sclerosis. *Nat Rev Neurosci* 2015. **16**: 147-158.

11 **Minagar, A. and Alexander, J. S.,** Blood-brain barrier disruption in multiple sclerosis. *Mult Scler* 2003. **9**: 540-549.

12 **Ortiz, G. G., Pacheco-Moises, F. P., Macias-Islas, M. A., Flores-Alvarado, L. J., Mireles-Ramirez, M. A., Gonzalez-Renovato, E. D., Hernandez-Navarro, V. E., et al.,** Role of the blood-brain barrier in multiple sclerosis. *Arch Med Res* 2014. **45**: 687-697.

13 **Lucchinetti, C., Bruck, W., Parisi, J., Scheithauer, B., Rodriguez, M. and Lassmann, H.,** Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol* 2000. **47**: 707-717.

14 **Frohman, E. M., Racke, M. K. and Raine, C. S.,** Multiple sclerosis--the plaque and its pathogenesis. *N Engl J Med* 2006. **354**: 942-955.

15 **Patrikios, P., Stadelmann, C., Kutzelnigg, A., Rauschka, H., Schmidbauer, M., Laursen, H., Sorensen, P. S., et al.,** Remyelination is extensive in a subset of multiple sclerosis patients. *Brain* 2006. **129**: 3165-3172.

16 **Lassmann, H.,** Multiple Sclerosis Pathology. *Cold Spring Harb Perspect Med* 2018. **8**.

17 **Goldschmidt, T., Antel, J., Konig, F. B., Bruck, W. and Kuhlmann, T.,** Remyelination capacity of the MS brain decreases with disease chronicity. *Neurology* 2009. **72**: 1914-1921.

18 **Irvine, K. A. and Blakemore, W. F.,** Remyelination protects axons from demyelination-associated axon degeneration. *Brain* 2008. **131**: 1464-1477.

19 **Mahad, D. H., Trapp, B. D. and Lassmann, H.,** Pathological mechanisms in progressive multiple sclerosis. *Lancet Neurol* 2015. **14**: 183-193.

20 **Machado-Santos, J., Saji, E., Troscher, A. R., Paunovic, M., Liblau, R., Gabriely, G., Bien, C. G., et al.,** The compartmentalized inflammatory response in the multiple sclerosis brain is composed of tissue-resident CD8+ T lymphocytes and B cells. *Brain* 2018. **141**: 2066-2082.

21 **Magliozzi, R., Howell, O., Vora, A., Serafini, B., Nicholas, R., Puopolo, M., Reynolds, R., et al.,** Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. *Brain* 2007. **130**: 1089-1104.

22 **Magliozzi, R., Howell, O. W., Reeves, C., Roncaroli, F., Nicholas, R., Serafini, B., Aloisi, F., et al.,** A Gradient of neuronal loss and meningeal inflammation in multiple sclerosis. *Ann Neurol* 2010. **68**: 477-493.

23 **Esiri, M. M. and Gay, D.,** Immunological and neuropathological significance of the Virchow-Robin space. *J Neurol Sci* 1990. **100**: 3-8.

24 **Serafini, B., Rosicarelli, B., Magliozzi, R., Stigliano, E. and Aloisi, F.,** Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. *Brain Pathol* 2004. **14**: 164-174.

25 **Makshakov, G., Magonov, E., Totolyan, N., Nazarov, V., Lapin, S., Mazing, A., Verbitskaya, E., et al.,** Leptomeningeal Contrast Enhancement Is Associated with Disability Progression and Grey Matter Atrophy in Multiple Sclerosis. *Neurol Res Int* 2017. **2017**: 8652463.

26 **Zivadinov, R., Ramasamy, D. P., Vaneckova, M., Gandhi, S., Chandra, A., Hagemeier, J., Bergsland, N., et al.,** Leptomeningeal contrast enhancement is associated with progression of cortical atrophy in MS: A retrospective, pilot, observational longitudinal study. *Mult Scler* 2017. **23**: 1336-1345.

27 **Fleisher, G., Henle, W., Henle, G., Lennette, E. T. and Biggar, R. J.,** Primary infection with Epstein-Barr virus in infants in the United States: clinical and serologic observations. *J Infect Dis* 1979. **139**: 553-558.

28 **Sundstrom, P., Nystrom, M., Ruuth, K. and Lundgren, E.,** Antibodies to specific EBNA-1 domains and HLA DRB1*1501 interact as risk factors for multiple sclerosis. *J Neuroimmunol* 2009. **215**: 102-107.

29 **Ascherio, A. and Munger, K. L.,** Epstein-barr virus infection and multiple sclerosis: a review. *J Neuroimmune Pharmacol* 2010. **5**: 271-277.

30 **Lunemann, J. D., Kamradt, T., Martin, R. and Munz, C.,** Epstein-barr virus: environmental trigger of multiple sclerosis? *J Virol* 2007. **81**: 6777-6784.

31 **Levin, L. I., Munger, K. L., Rubertone, M. V., Peck, C. A., Lennette, E. T., Spiegelman, D. and Ascherio, A.,** Temporal relationship between elevation of epstein-barr virus antibody titers and initial onset of neurological symptoms in multiple sclerosis. *JAMA* 2005. **293**: 2496-2500.

32 **Wandinger, K., Jabs, W., Siekhaus, A., Bubel, S., Trillenberg, P., Wagner, H., Wessel, K., et al.,** Association between clinical disease activity and Epstein-Barr virus reactivation in MS. *Neurology* 2000. **55**: 178-184.

33 **Owens, G. P. and Bennett, J. L.,** Trigger, pathogen, or bystander: the complex nexus linking Epstein- Barr virus and multiple sclerosis. *Mult Scler* 2012. **18**: 1204-1208.

34 **Olsson, T., Barcellos, L. F. and Alfredsson, L.,** Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis. *Nat Rev Neurol* 2017. **13**: 25-36.

35 **Pierrot-Deseilligny, C. and Souberbielle, J. C.,** Vitamin D and multiple sclerosis: An update. *Mult Scler Relat Disord* 2017. **14**: 35-45.

36 **Healy, B. C., Ali, E. N., Guttmann, C. R., Chitnis, T., Glanz, B. I., Buckle, G., Houtchens, M., et al.,** Smoking and disease progression in multiple sclerosis. *Arch Neurol* 2009. **66**: 858-864.

37 **Hedstrom, A. K., Lima Bomfim, I., Barcellos, L., Gianfrancesco, M., Schaefer, C., Kockum, I., Olsson, T., et al.,** Interaction between adolescent obesity and HLA risk genes in the etiology of multiple sclerosis. *Neurology* 2014. **82**: 865-872.

38 **Lumeng, C. N., Bodzin, J. L. and Saltiel, A. R.,** Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* 2007. **117**: 175-184.

39 **Confavreux, C., Hutchinson, M., Hours, M. M., Cortinovis-Tourniaire, P. and Moreau, T.,** Rate of pregnancy-related relapse in multiple sclerosis. Pregnancy in Multiple Sclerosis Group. *N Engl J Med* 1998. **339**: 285-291.

40 **Runmarker, B. and Andersen, O.,** Pregnancy is associated with a lower risk of onset and a better prognosis in multiple sclerosis. *Brain* 1995. **118 (Pt 1)**: 253-261.

41 **Paavilainen, T., Kurki, T., Parkkola, R., Farkkila, M., Salonen, O., Dastidar, P., Elovaara, I., et al.,** Magnetic resonance imaging of the brain used to detect early post-partum activation of multiple sclerosis. *Eur J Neurol* 2007. **14**: 1216-1221.

42 **Gilmore, W., Weiner, L. P. and Correale, J.,** Effect of estradiol on cytokine secretion by proteolipid protein-specific T cell clones isolated from multiple sclerosis patients and normal control subjects. *J Immunol* 1997. **158**: 446-451.

43 **Compston, A. and Coles, A.,** Multiple sclerosis. *Lancet* 2002. **359**: 1221-1231.

44 **International Multiple Sclerosis Genetics, C.,** Multiple sclerosis genomic map implicates peripheral immune cells and microglia in susceptibility. *Science* 2019. **365**.

45 **Isobe, N., Keshavan, A., Gourraud, P. A., Zhu, A. H., Datta, E., Schlaeger, R., Caillier, S. J., et al.,** Association of HLA Genetic Risk Burden With Disease Phenotypes in Multiple Sclerosis. *JAMA Neurol* 2016. **73**: 795-802.

46 **Patsopoulos, N. A., Barcellos, L. F., Hintzen, R. Q., Schaefer, C., van Duijn, C. M., Noble, J. A., Raj, T., et al.,** Fine-mapping the genetic association of the major histocompatibility complex in multiple sclerosis: HLA and non-HLA effects. *PLoS Genet* 2013. **9**: e1003926.

47 **International Multiple Sclerosis Genetics, C., Wellcome Trust Case Control, C., Sawcer, S., Hellenthal, G., Pirinen, M., Spencer, C. C., Patsopoulos, N. A., et al.,** Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* 2011. **476**: 214-219.

48 **Ramagopalan, S. V., Knight, J. C. and Ebers, G. C.,** Multiple sclerosis and the major histocompatibility complex. *Curr Opin Neurol* 2009. **22**: 219-225.

49 **Sundqvist, E., Sundstrom, P., Linden, M., Hedstrom, A. K., Aloisi, F., Hillert, J., Kockum, I., et al.,** Epstein-Barr virus and multiple sclerosis: interaction with HLA. *Genes Immun* 2012. **13**: 14-20.

50 **Ramagopalan, S. V., Maugeri, N. J., Handunnetthi, L., Lincoln, M. R., Orton, S. M., Dyment, D. A., Deluca, G. C., et al.,** Expression of the multiple sclerosis-associated MHC class II Allele HLA-DRB1*1501 is regulated by vitamin D. *PLoS Genet* 2009. **5**: e1000369.

51 **Zhuang, J. C., Huang, Z. Y., Zhao, G. X., Yu, H., Li, Z. X. and Wu, Z. Y.,** Variants of CYP27B1 are associated with both multiple sclerosis and neuromyelitis optica patients in Han Chinese population. *Gene* 2015. **557**: 236-239.

52 Multiple sclerosis genomic map implicates peripheral immune cells and microglia in susceptibility. *Science* 2019. **365**: eaav7188.

53 **Farh, K. K., Marson, A., Zhu, J., Kleinewietfeld, M., Housley, W. J., Beik, S., Shoresh, N., et al.,** Genetic and epigenetic fine mapping of causal autoimmune disease variants. *Nature* 2015. **518**: 337-343.

54 **Patsopoulos, N. A.,** Genetics of Multiple Sclerosis: An Overview and New Directions. *Cold Spring Harb Perspect Med* 2018. **8**.

55 **Baker, D., Marta, M., Pryce, G., Giovannoni, G. and Schmierer, K.,** Memory B Cells are Major Targets for Effective Immunotherapy in Relapsing Multiple Sclerosis. *EBioMedicine* 2017. **16**: 41-50.

56 **Hardy, R. R. and Hayakawa, K.,** B cell development pathways. *Annu Rev Immunol* 2001. **19**: 595-621.

57 **Vale, A. M., Nobrega, A. and Schroeder, H. W., Jr.,** The role of evolutionarily conserved germ-line DH sequence in B-1 cell development and natural antibody production. *Ann N Y Acad Sci* 2015. **1362**: 48-56.

58 **Meffre, E. and Wardemann, H.,** B-cell tolerance checkpoints in health and autoimmunity. *Curr Opin Immunol* 2008. **20**: 632-638.

59 **Spencer, J., Perry, M. E. and Dunn-Walters, D. K.,** Human marginal-zone B cells. *Immunol Today* 1998. **19**: 421-426.

60 **Martin, F. and Kearney, J. F.,** Marginal-zone B cells. *Nat Rev Immunol* 2002. **2**: 323-335.

61 **Berkowska, M. A., Driessen, G. J., Bikos, V., Grosserichter-Wagener, C., Stamatopoulos, K., Cerutti, A., He, B., et al.,** Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. *Blood* 2011. **118**: 2150-2158.

62 **Mond, J. J., Vos, Q., Lees, A. and Snapper, C. M.,** T cell independent antigens. *Curr Opin Immunol* 1995. **7**: 349-354.

63 **Weller, S., Faili, A., Garcia, C., Braun, M. C., Le Deist, F. F., de Saint Basile, G. G., Hermine, O., et al.,** CD40-CD40L independent Ig gene hypermutation suggests a second B cell diversification pathway in humans. *Proc Natl Acad Sci U S A* 2001. **98**: 1166-1170.

64 **Delgado, M. F., Coviello, S., Monsalvo, A. C., Melendi, G. A., Hernandez, J. Z., Batalle, J. P., Diaz, L., et al.,** Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. *Nat Med* 2009. **15**: 34-41.

65 **Bretscher, P. and Cohn, M.,** A theory of self-nonself discrimination. *Science* 1970. **169**: 1042-1049.

66 **Herve, M., Isnardi, I., Ng, Y. S., Bussel, J. B., Ochs, H. D., Cunningham-Rundles, C. and Meffre, E.,** CD40 ligand and MHC class II expression are essential for human peripheral B cell tolerance. *J Exp Med* 2007. **204**: 1583-1593.

67 **Kinnunen, T., Chamberlain, N., Morbach, H., Choi, J., Kim, S., Craft, J., Mayer, L., et al.,** Accumulation of peripheral autoreactive B cells in the absence of functional human regulatory T cells. *Blood* 2013. **121**: 1595-1603.

68 **Meffre, E.,** The establishment of early B cell tolerance in humans: lessons from primary immunodeficiency diseases. *Ann N Y Acad Sci* 2011. **1246**: 1-10.

69 **Liu, D., Xu, H., Shih, C., Wan, Z., Ma, X., Ma, W., Luo, D., et al.,** T-B-cell entanglement and ICOSL-driven feed-forward regulation of germinal centre reaction. *Nature* 2015. **517**: 214-218.

70 **Shulman, Z., Gitlin, A. D., Weinstein, J. S., Lainez, B., Esplugues, E., Flavell, R. A., Craft, J. E., et al.,** Dynamic signaling by T follicular helper cells during germinal center B cell selection. *Science* 2014. **345**: 1058-1062.

71 **Bar-Or, A., Oliveira, E. M., Anderson, D. E., Krieger, J. I., Duddy, M., O'Connor, K. C. and Hafler, D. A.,** Immunological memory: contribution of memory B cells expressing costimulatory molecules in the resting state. *J Immunol* 2001. **167**: 5669-5677.

72 **Gimmi, C. D., Freeman, G. J., Gribben, J. G., Sugita, K., Freedman, A. S., Morimoto, C. and Nadler, L. M.,** B-cell surface antigen B7 provides a costimulatory signal that induces T cells to proliferate and secrete interleukin 2. *Proc Natl Acad Sci U S A* 1991. **88**: 6575-6579.

73 **MacLennan, I. C.,** Germinal centers. *Annu Rev Immunol* 1994. **12**: 117-139.

74 **Odendahl, M., Mei, H., Hoyer, B. F., Jacobi, A. M., Hansen, A., Muehlinghaus, G., Berek, C., et al.,** Generation of migratory antigen-specific plasma blasts and mobilization of resident plasma cells in a secondary immune response. *Blood* 2005. **105**: 1614-1621.

75 **Perez-Andres, M., Paiva, B., Nieto, W. G., Caraux, A., Schmitz, A., Almeida, J., Vogt, R. F., Jr., et al.,** Human peripheral blood B-cell compartments: a crossroad in B-cell traffic. *Cytometry B Clin Cytom* 2010. **78 Suppl 1**: S47-60.

76 **Dogan, I., Bertocci, B., Vilmont, V., Delbos, F., Megret, J., Storck, S., Reynaud, C. A., et al.,** Multiple layers of B cell memory with different effector functions. *Nat Immunol* 2009. **10**: 1292-1299.

77 **Seifert, M. and Kuppers, R.,** Molecular footprints of a germinal center derivation of human IgM+(IgD+) CD27+ B cells and the dynamics of memory B cell generation. *J Exp Med* 2009. **206**: 2659-2669.

78 **Bende, R. J., van Maldegem, F., Triesscheijn, M., Wormhoudt, T. A., Guijt, R. and van Noesel, C. J.,** Germinal centers in human lymph nodes contain reactivated memory B cells. *J Exp Med* 2007. **204**: 2655-2665.

79 **Stavnezer, J.,** Molecular processes that regulate class switching. *Curr Top Microbiol Immunol* 2000. **245**: 127-168.

80 **Muramatsu, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinkai, Y. and Honjo, T.,** Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 2000. **102**: 553-563.

81 **Bruggemann, M., Williams, G. T., Bindon, C. I., Clark, M. R., Walker, M. R., Jefferis, R., Waldmann, H., et al.,** Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. *J Exp Med* 1987. **166**: 1351-1361.

82 **Brewer, J. W., Randall, T. D., Parkhouse, R. M. and Corley, R. B.,** Mechanism and subcellular localization of secretory IgM polymer assembly. *J Biol Chem* 1994. **269**: 17338-17348.

83 **Brandtzaeg, P. and Johansen, F. E.,** Mucosal B cells: phenotypic characteristics, transcriptional regulation, and homing properties. *Immunol Rev* 2005. **206**: 32-63.

84 **Woof, J. M. and Kerr, M. A.,** IgA function--variations on a theme. *Immunology* 2004. **113**: 175-177.

85 **Kawakami, T. and Galli, S. J.,** Regulation of mast-cell and basophil function and survival by IgE. *Nat Rev Immunol* 2002. **2**: 773-786.

86 **Yuseff, M. I. and Lennon-Dumenil, A. M.,** B Cells use Conserved Polarity Cues to Regulate Their Antigen Processing and Presentation Functions. *Front Immunol* 2015. **6**: 251.

87 **Cheng, P. C., Steele, C. R., Gu, L., Song, W. and Pierce, S. K.,** MHC class II antigen processing in B cells: accelerated intracellular targeting of antigens. *J Immunol* 1999. **162**: 7171-7180.

88 **Forquet, F., Barois, N., Machy, P., Trucy, J., Zimmermann, V. S., Leserman, L. and Davoust, J.,** Presentation of antigens internalized through the B cell receptor requires newly synthesized MHC class II molecules. *J Immunol* 1999. **162**: 3408-3416.

89 **Lankar, D., Vincent-Schneider, H., Briken, V., Yokozeki, T., Raposo, G. and Bonnerot, C.,** Dynamics of major histocompatibility complex class II compartments during B cell receptor-mediated cell activation. *J Exp Med* 2002. **195**: 461-472.

90 **Wolf, P. R. and Ploegh, H. L.,** How MHC class II molecules acquire peptide cargo: biosynthesis and trafficking through the endocytic pathway. *Annu Rev Cell Dev Biol* 1995. **11**: 267-306.

91 **Landsverk, O. J., Bakke, O. and Gregers, T. F.,** MHC II and the endocytic pathway: regulation by invariant chain. *Scand J Immunol* 2009. **70**: 184-193.

92 **Hofmann, M. W., Honing, S., Rodionov, D., Dobberstein, B., von Figura, K. and Bakke, O.,** The leucine-based sorting motifs in the cytoplasmic domain of the invariant chain are recognized by the clathrin adaptors AP1 and AP2 and their medium chains. *J Biol Chem* 1999. **274**: 36153-36158.

93 **Neefjes, J., Jongsma, M. L., Paul, P. and Bakke, O.,** Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol* 2011. **11**: 823-836.

94 **Villadangos, J. A., Bryant, R. A., Deussing, J., Driessen, C., Lennon-Dumenil, A. M., Riese, R. J., Roth, W., et al.,** Proteases involved in MHC class II antigen presentation. *Immunol Rev* 1999. **172**: 109-120.

95 **Schneppenheim, J., Dressel, R., Huttl, S., Lullmann-Rauch, R., Engelke, M., Dittmann, K., Wienands, J., et al.,** The intramembrane protease SPPL2a promotes B cell development and controls endosomal traffic by cleavage of the invariant chain. *J Exp Med* 2013. **210**: 41-58.

96 **Hastings, K. T.,** GILT: Shaping the MHC Class II-Restricted Peptidome and CD4(+) T Cell-Mediated Immunity. *Front Immunol* 2013. **4**: 429.

97 **Watts, C.,** Antigen processing in the endocytic compartment. *Curr Opin Immunol* 2001. **13**: 26-31.

98 **Leng, L., Metz, C. N., Fang, Y., Xu, J., Donnelly, S., Baugh, J., Delohery, T., et al.,** MIF signal transduction initiated by binding to CD74. *J Exp Med* 2003. **197**: 1467-1476.

99 **Wilson, K. M., Labeta, M. O., Pawelec, G. and Fernandez, N.,** Cell-surface expression of human histocompatibility leucocyte antigen (HLA) class II-associated invariant chain (CD74) does not always correlate with cell-surface expression of HLA class II molecules. *Immunology* 1993. **79**: 331-335.

100 **Wraight, C. J., van Endert, P., Moller, P., Lipp, J., Ling, N. R., MacLennan, I. C., Koch, N., et al.,** Human major histocompatibility complex class II invariant chain is expressed on the cell surface. *J Biol Chem* 1990. **265**: 5787-5792.

101 **Mitchell, R. A., Liao, H., Chesney, J., Fingerle-Rowson, G., Baugh, J., David, J. and Bucala, R.,** Macrophage migration inhibitory factor (MIF) sustains macrophage proinflammatory function by inhibiting p53: regulatory role in the innate immune response. *Proc Natl Acad Sci U S A* 2002. **99**: 345-350.

102 **Yurasov, S., Wardemann, H., Hammersen, J., Tsuiji, M., Meffre, E., Pascual, V. and Nussenzweig, M. C.,** Defective B cell tolerance checkpoints in systemic lupus erythematosus. *J Exp Med* 2005. **201**: 703-711.

103 **Samuels, J., Ng, Y. S., Coupillaud, C., Paget, D. and Meffre, E.,** Impaired early B cell tolerance in patients with rheumatoid arthritis. *J Exp Med* 2005. **201**: 1659-1667.

104 **Kinnunen, T., Chamberlain, N., Morbach, H., Cantaert, T., Lynch, M., Preston-Hurlburt, P., Herold, K. C., et al.,** Specific peripheral B cell tolerance defects in patients with multiple sclerosis. *J Clin Invest* 2013. **123**: 2737-2741.

105 **Huan, J., Culbertson, N., Spencer, L., Bartholomew, R., Burrows, G. G., Chou, Y. K., Bourdette, D., et al.,** Decreased FOXP3 levels in multiple sclerosis patients. *Journal of Neuroscience Research* 2005. **81**: 45-52.

106 **Dhaeze, T., Peelen, E., Hombrouck, A., Peeters, L., Van Wijmeersch, B., Lemkens, N., Lemkens, P., et al.,** Circulating Follicular Regulatory T Cells Are Defective in Multiple Sclerosis. *J Immunol* 2015. **195**: 832-840.

107 **Viglietta, V., Baecher-Allan, C., Weiner, H. L. and Hafler, D. A.,** Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med* 2004. **199**: 971-979.

108 **Brink, R. and Phan, T. G.,** Self-Reactive B Cells in the Germinal Center Reaction. *Annu Rev Immunol* 2018. **36**: 339-357.

109 **Cooke, M. P., Heath, A. W., Shokat, K. M., Zeng, Y., Finkelman, F. D., Linsley, P. S., Howard, M., et al.,** Immunoglobulin signal transduction guides the specificity of B cell-T cell interactions and is blocked in tolerant self-reactive B cells. *J Exp Med* 1994. **179**: 425-438.

110 **Healy, J. I., Dolmetsch, R. E., Timmerman, L. A., Cyster, J. G., Thomas, M. L., Crabtree, G. R., Lewis, R. S., et al.,** Different nuclear signals are activated by the B cell receptor during positive versus negative signaling. *Immunity* 1997. **6**: 419-428.

111 **Fulcher, D. A. and Basten, A.,** Reduced life span of anergic self-reactive B cells in a double-transgenic model. *J Exp Med* 1994. **179**: 125-134.

112 **Phan, T. G., Amesbury, M., Gardam, S., Crosbie, J., Hasbold, J., Hodgkin, P. D., Basten, A., et al.,** B cell receptor-independent stimuli trigger immunoglobulin (Ig) class switch recombination and production of IgG autoantibodies by anergic self-reactive B cells. *J Exp Med* 2003. **197**: 845-860.

113 **Andrews, S. F. and Wilson, P. C.,** The anergic B cell. *Blood* 2010. **115**: 4976-4978.

114 **Yarkoni, Y., Getahun, A. and Cambier, J. C.,** Molecular underpinning of B-cell anergy. *Immunol Rev* 2010. **237**: 249-263.

115 **Eris, J. M., Basten, A., Brink, R., Doherty, K., Kehry, M. R. and Hodgkin, P. D.,** Anergic self-reactive B cells present self antigen and respond normally to CD40-dependent T-cell signals but are defective in antigen-receptor-mediated functions. *Proc Natl Acad Sci U S A* 1994. **91**: 4392-4396.

116 **Hauser, S. L., Waubant, E., Arnold, D. L., Vollmer, T., Antel, J., Fox, R. J., Bar-Or, A., et al.,** B-Cell Depletion with Rituximab in Relapsing–Remitting Multiple Sclerosis. *New England Journal of Medicine* 2008. **358**: 676-688.

117 **Molnarfi, N., Schulze-Topphoff, U., Weber, M. S., Patarroyo, J. C., Prod'homme, T., Varrin-Doyer, M., Shetty, A., et al.,** MHC class II–dependent B cell APC function is required for induction of CNS autoimmunity independent of myelin-specific antibodies. *The Journal of Experimental Medicine* 2013. **210**: 2921-2937.

118 **Parker Harp, C. R., Archambault, A. S., Sim, J., Ferris, S. T., Mikesell, R. J., Koni, P. A., Shimoda, M., et al.,** B cell antigen presentation is sufficient to drive neuroinflammation in an animal model of multiple sclerosis. *J Immunol* 2015. **194**: 5077-5084.

119 **Grewal, I. S., Foellmer, H. G., Grewal, K. D., Xu, J., Hardardottir, F., Baron, J. L., Janeway, C. A., Jr., et al.,** Requirement for CD40 ligand in costimulation induction, T cell activation, and experimental allergic encephalomyelitis. *Science* 1996. **273**: 1864-1867.

120 **Raychaudhuri, S., Sandor, C., Stahl, E. A., Freudenberg, J., Lee, H. S., Jia, X., Alfredsson, L., et al.,** Five amino acids in three HLA proteins explain most of the association between MHC and seropositive rheumatoid arthritis. *Nat Genet* 2012. **44**: 291-296.

121 **Morandi, E., Jagessar, S. A., 't Hart, B. A. and Gran, B.,** EBV Infection Empowers Human B Cells for Autoimmunity: Role of Autophagy and Relevance to Multiple Sclerosis. *The Journal of Immunology* 2017: ji1700178.

122 **Jelcic, I., Al Nimer, F., Wang, J., Lentsch, V., Planas, R., Jelcic, I., Madjovski, A., et al.,** Memory B Cells Activate Brain-Homing, Autoreactive CD4+ T Cells in Multiple Sclerosis. *Cell* 2018. **175**: 85-100.e123.

123 **Bar-Or, A., Fawaz, L., Fan, B., Darlington, P. J., Rieger, A., Ghorayeb, C., Calabresi, P. A., et al.,** Abnormal B-cell cytokine responses a trigger of T-cell-mediated disease in MS? *Ann Neurol* 2010. **67**: 452-461.

124 **Barr, T. A., Shen, P., Brown, S., Lampropoulou, V., Roch, T., Lawrie, S., Fan, B., et al.,** B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. *J Exp Med* 2012. **209**: 1001-1010.

125 **Li, R., Rezk, A., Healy, L. M., Muirhead, G., Prat, A., Gommerman, J. L., Bar-Or, A., et al.,** Cytokine-Defined B Cell Responses as Therapeutic Targets in Multiple Sclerosis. *Front Immunol* 2015. **6**: 626.

126 **Li, R., Rezk, A., Miyazaki, Y., Hilgenberg, E., Touil, H., Shen, P., Moore, C. S., et al.,** Proinflammatory GM-CSF-producing B cells in multiple sclerosis and B cell depletion therapy. *Sci Transl Med* 2015. **7**: 310ra166.

127 **Bar-Or, A., Fawaz, L., Fan, B., Darlington, P. J., Rieger, A., Ghorayeb, C., Calabresi, P. A., et al.,** Abnormal B-cell cytokine responses a trigger of T-cell–mediated disease in MS? *Annals of Neurology* 2010. **67**: 452-461.

128 **Genc, K., Dona, D. L. and Reder, A. T.,** Increased CD80(+) B cells in active multiple sclerosis and reversal by interferon beta-1b therapy. *J Clin Invest* 1997. **99**: 2664-2671.

129 **Comabella, M., Canto, E., Nurtdinov, R., Rio, J., Villar, L. M., Picon, C., Castillo, J., et al.,** MRI phenotypes with high neurodegeneration are associated with peripheral blood B-cell changes. *Hum Mol Genet* 2016. **25**: 308-316.

130 **Fraussen, J., Claes, N., Van Wijmeersch, B., van Horssen, J., Stinissen, P., Hupperts, R. and Somers, V.,** B cells of multiple sclerosis patients induce autoreactive proinflammatory T cell responses. *Clin Immunol* 2016. **173**: 124-132.

131 **Dendrou, C. A., Fugger, L. and Friese, M. A.,** Immunopathology of multiple sclerosis. *Nature Reviews Immunology* 2015. **15**: 545.

132 **Reboldi, A., Coisne, C., Baumjohann, D., Benvenuto, F., Bottinelli, D., Lira, S., Uccelli, A., et al.,** C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat Immunol* 2009. **10**: 514-523.

133 **Filippi, M., Bar-Or, A., Piehl, F., Preziosa, P., Solari, A., Vukusic, S. and Rocca, M. A.,** Multiple sclerosis. *Nature Reviews Disease Primers* 2018. **4**: 43.

134 **Khaibullin, T., Ivanova, V., Martynova, E., Cherepnev, G., Khabirov, F., Granatov, E., Rizvanov, A., et al.,** Elevated Levels of Proinflammatory Cytokines in Cerebrospinal Fluid of Multiple Sclerosis Patients. *Front Immunol* 2017. **8**: 531.

135 **Subileau, E. A., Rezaie, P., Davies, H. A., Colyer, F. M., Greenwood, J., Male, D. K. and Romero, I. A.,** Expression of chemokines and their receptors by human brain endothelium: implications for multiple sclerosis. *J Neuropathol Exp Neurol* 2009. **68**: 227-240.

136 **Elices, M. J., Osborn, L., Takada, Y., Crouse, C., Luhowskyj, S., Hemler, M. E. and Lobb, R. R.,** VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/ Fibronectin binding site. *Cell* 1990. **60**: 577-584.

137 **Lehmann-Horn, K., Sagan, S. A., Bernard, C. C., Sobel, R. A. and Zamvil, S. S.,** B-cell very late antigen-4 deficiency reduces leukocyte recruitment and susceptibility to central nervous system autoimmunity. *Ann Neurol* 2015. **77**: 902-908.

138 **Denkinger, C. M., Denkinger, M., Kort, J. J., Metz, C. and Forsthuber, T. G.,** In vivo blockade of macrophage migration inhibitory factor ameliorates acute experimental autoimmune encephalomyelitis by impairing the homing of encephalitogenic T cells to the central nervous system. *J Immunol* 2003. **170**: 1274-1282.

139 **Cayrol, R., Wosik, K., Berard, J. L., Dodelet-Devillers, A., Ifergan, I., Kebir, H., Haqqani, A. S., et al.,** Activated leukocyte cell adhesion molecule promotes leukocyte trafficking into the central nervous system. *Nat Immunol* 2008. **9**: 137-145.

140 **Michel, L., Grasmuck, C., Charabati, M., Lecuyer, M. A., Zandee, S., Dhaeze, T., Alvarez, J. I., et al.,** Activated leukocyte cell adhesion molecule regulates B lymphocyte migration across central nervous system barriers. *Sci Transl Med* 2019. **11**.

141 **Frischer, J. M., Bramow, S., Dal-Bianco, A., Lucchinetti, C. F., Rauschka, H., Schmidbauer, M., Laursen, H., et al.,** The relation between inflammation and neurodegeneration in multiple sclerosis brains. *Brain* 2009. **132**: 1175-1189.

142 **Howell, O. W., Reeves, C. A., Nicholas, R., Carassiti, D., Radotra, B., Gentleman, S. M., Serafini, B., et al.,** Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis. *Brain* 2011. **134**: 2755-2771.

143 **Choi, S. R., Howell, O. W., Carassiti, D., Magliozzi, R., Gveric, D., Muraro, P. A., Nicholas, R., et al.,** Meningeal inflammation plays a role in the pathology of primary progressive multiple sclerosis. *Brain* 2012. **135**: 2925-2937.

144 **Lucchinetti, C. F., Popescu, B. F., Bunyan, R. F., Moll, N. M., Roemer, S. F., Lassmann, H., Bruck, W., et al.,** Inflammatory cortical demyelination in early multiple sclerosis. *N Engl J Med* 2011. **365**: 2188-2197.

145 **Pitzalis, C., Jones, G. W., Bombardieri, M. and Jones, S. A.,** Ectopic lymphoid-like structures in infection, cancer and autoimmunity. *Nat Rev Immunol* 2014. **14**: 447-462.

146 **Le Pottier, L., Devauchelle, V., Fautrel, A., Daridon, C., Saraux, A., Youinou, P. and Pers, J. O.,** Ectopic germinal centers are rare in Sjogren's syndrome salivary glands and do not exclude autoreactive B cells. *J Immunol* 2009. **182**: 3540-3547.

147 **Humby, F., Bombardieri, M., Manzo, A., Kelly, S., Blades, M. C., Kirkham, B., Spencer, J., et al.,** Ectopic lymphoid structures support ongoing production of class-switched autoantibodies in rheumatoid synovium. *PLoS Med* 2009. **6**: e1.

148 **Salomonsson, S., Jonsson, M. V., Skarstein, K., Brokstad, K. A., Hjelmstrom, P., Wahren-Herlenius, M. and Jonsson, R.,** Cellular basis of ectopic germinal center formation and autoantibody production in the target organ of patients with Sjogren's syndrome. *Arthritis Rheum* 2003. **48**: 3187-3201.

149 **Villar, L. M., Masjuan, J., Sadaba, M. C., Gonzalez-Porque, P., Plaza, J., Bootello, A. and Alvarez-Cermeno, J. C.,** Early differential diagnosis of multiple sclerosis using a new oligoclonal band test. *Arch Neurol* 2005. **62**: 574-577.

150 **Villar, L. M., Sadaba, M. C., Roldan, E., Masjuan, J., Gonzalez-Porque, P., Villarrubia, N., Espino, M., et al.,** Intrathecal synthesis of oligoclonal IgM against myelin lipids predicts an aggressive disease course in MS. *J Clin Invest* 2005. **115**: 187-194.

151 **Kuhle, J., Pohl, C., Mehling, M., Edan, G., Freedman, M. S., Hartung, H. P., Polman, C. H., et al.,** Lack of association between antimyelin antibodies and progression to multiple sclerosis. *N Engl J Med* 2007. **356**: 371-378.

152 **Ketelslegers, I. A., Van Pelt, D. E., Bryde, S., Neuteboom, R. F., Catsman-Berrevoets, C. E., Hamann, D. and Hintzen, R. Q.,** Anti-MOG antibodies plead against MS diagnosis in an Acquired Demyelinating Syndromes cohort. *Mult Scler* 2015. **21**: 1513-1520.

153 **Sabatino, J. J., Jr., Probstel, A. K. and Zamvil, S. S.,** B cells in autoimmune and neurodegenerative central nervous system diseases. *Nat Rev Neurosci* 2019. **20**: 728-745.

154 **Reiber, H., Ungefehr, S. and Jacobi, C.,** The intrathecal, polyspecific and oligoclonal immune response in multiple sclerosis. *Mult Scler* 1998. **4**: 111-117.

155 **Jarius, S., Eichhorn, P., Franciotta, D., Petereit, H. F., Akman-Demir, G., Wick, M. and Wildemann, B.,** The MRZ reaction as a highly specific marker of multiple sclerosis: re-evaluation and structured review of the literature. *J Neurol* 2017. **264**: 453-466.

156 **Brandle, S. M., Obermeier, B., Senel, M., Bruder, J., Mentele, R., Khademi, M., Olsson, T., et al.,** Distinct oligoclonal band antibodies in multiple sclerosis recognize ubiquitous self-proteins. *Proc Natl Acad Sci U S A* 2016. **113**: 7864-7869.

157 **Qin, Y., Duquette, P., Zhang, Y., Talbot, P., Poole, R. and Antel, J.,** Clonal expansion and somatic hypermutation of V(H) genes of B cells from cerebrospinal fluid in multiple sclerosis. *J Clin Invest* 1998. **102**: 1045-1050.

158 **Qin, Y., Duquette, P., Zhang, Y., Olek, M., Da, R. R., Richardson, J., Antel, J. P., et al.,** Intrathecal B-cell clonal expansion, an early sign of humoral immunity, in the cerebrospinal fluid of patients with clinically isolated syndrome suggestive of multiple sclerosis. *Lab Invest* 2003. **83**: 1081-1088.

159 **Baranzini, S. E., Jeong, M. C., Butunoi, C., Murray, R. S., Bernard, C. C. and Oksenberg, J. R.,** B cell repertoire diversity and clonal expansion in multiple sclerosis brain lesions. *J Immunol* 1999. **163**: 5133-5144.

160 **Colombo, M., Dono, M., Gazzola, P., Roncella, S., Valetto, A., Chiorazzi, N., Mancardi, G. L., et al.,** Accumulation of clonally related B lymphocytes in the cerebrospinal fluid of multiple sclerosis patients. *J Immunol* 2000. **164**: 2782-2789.

161 **Eggers, E. L., Michel, B. A., Wu, H., Wang, S. Z., Bevan, C. J., Abounasr, A., Pierson, N. S., et al.,** Clonal relationships of CSF B cells in treatment-naive multiple sclerosis patients. *JCI Insight* 2017. **2**.

162 **Lovato, L., Willis, S. N., Rodig, S. J., Caron, T., Almendinger, S. E., Howell, O. W., Reynolds, R., et al.,** Related B cell clones populate the meninges and parenchyma of patients with multiple sclerosis. *Brain : a journal of neurology* 2011. **134**: 534-541.

163 **Palanichamy, A., Apeltsin, L., Kuo, T. C., Sirota, M., Wang, S., Pitts, S. J., Sundar, P. D., et al.,** Immunoglobulin class-switched B cells form an active immune axis between CNS and periphery in multiple sclerosis. *Sci Transl Med* 2014. **6**: 248ra106.

164 **Stern, J. N., Yaari, G., Vander Heiden, J. A., Church, G., Donahue, W. F., Hintzen, R. Q., Huttner, A. J., et al.,** B cells populating the multiple sclerosis brain mature in the draining cervical lymph nodes. *Sci Transl Med* 2014. **6**: 248ra107.

165 **von Budingen, H. C., Kuo, T. C., Sirota, M., van Belle, C. J., Apeltsin, L., Glanville, J., Cree, B. A., et al.,** B cell exchange across the blood-brain barrier in multiple sclerosis. *J Clin Invest* 2012. **122**: 4533-4543.

2

Induction of brain-infiltrating T-bet-expressing B cells in multiple sclerosis

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ABSTRACT

Objective: Results from anti-CD20 therapies demonstrate that B- and T-cell interaction is a major driver of multiple sclerosis (MS). The local presence of B-cell follicle-like structures and oligoclonal bands in MS patients indicate that certain B cells infiltrate the CNS to mediate pathology. Which peripheral triggers underlie the development of CNS-infiltrating B cells is not fully understood.

Methods: *Ex vivo* flow cytometry was used to assess chemokine receptor profiles of B cells in blood, CSF, meningeal and brain tissues of MS patients (*n*=10). Similar analyses were performed for distinct memory subsets in the blood of untreated and natalizumab-treated MS patients (*n*=38). To assess T-bet(CXCR3)$^{+}$ B-cell differentiation, we cultured B cells from MS patients (*n*=21) and healthy individuals (n=34) under T helper 1 and TLR9-inducing conditions. Their CNS transmigration capacity was confirmed using brain endothelial monolayers.

Results: CXC chemokine receptor 3 (CXCR3)-expressing B cells were enriched in different CNS compartments of MS patients. Treatment with clinically effective drug natalizumab prevented the recruitment of CXCR3^{high} IgG1^{+} subsets, corresponding to their increased ability to cross CNS barriers *in vitro*. Blocking of IFN-γ reduced the transmigration potential and antigen-presenting cell function of these cells. IFN-γ-induced B cells from MS patients showed increased T-bet expression and plasmablast development. Additional TLR9 triggering further upregulated T-bet and CXCR3, and was essential for IgG1 switching.

Interpretation: This study demonstrates that T-bet^{high} IgG1^{+} B cells are triggered by IFN-γ and TLR9 signals, likely contributing to enhanced CXCR3-mediated recruitment and local reactivity in the CNS of MS patients.

INTRODUCTION

B cells are one of the main contributors to chronic autoimmune pathology in multiple sclerosis (MS), as supported by results from large genome-wide association studies [1]. B-cell repertoires in the central nervous system (CNS) and the periphery are closely connected, suggesting that disease-relevant B-cell networks interact at both sides of the blood-brain barrier [2-5]. There is evidence that the beneficial effects of anti-CD20 monoclonal antibody therapy are related to the ablation of functional B cells interacting with T cells [6, 7]. The meninges of MS patients contain tertiary lymphoid structures that are filled with B and T cells, close to cortical lesions [8]. This strongly suggests that B- and T-cell interaction is a major event in triggering and sustaining inflammation in the CNS.

In MS, autoreactive (naive) B cells escape peripheral selection and probably receive specific signals from $CD4^+$ T cells within secondary lymphoid organs to differentiate into memory populations before entering the CNS [5, 9, 10]. The presence of oligoclonal bands (OCB) in the cerebrospinal fluid (CSF) of MS patients implies that these memory B cells undergo local reactivation (with the help of $CD4^+$ T cells) to further develop into immunoglobulin (Ig)-producing plasmablasts and plasma cells [8, 11]. Although memory B cells have been recently shown to promote the differentiation of CNS-infiltrating $CD4^+$ T cells in MS, little is known about how and which functional B-cell subsets are triggered in the periphery to infiltrate the CNS and contribute to MS pathology.

In mice, the T helper 1 (Th1) cytokine interferon-gamma (IFN-γ) induces the interaction between autoreactive B cells and $CD4^+$ T cells to form tertiary lymphoid structures and promote systemic autoimmune diseases such as systemic lupus erythematosus (SLE) [12]. In these cases, IFN-γ induces the expression of the T-box transcription factor T-bet, resulting in enhanced Ig class switching and CXC chemokine receptor 3 (CXCR3) expression in murine B cells [13, 14]. Interestingly, B cell-intrinsic T-bet expression associates with increased pathogenic responses [14, 15] and is induced by systemic infections [16], a major environmental trigger in MS [17]. Toll-like receptor 9 (TLR9), which binds to pathogen-related CpG-DNA, integrates with the B-cell receptor (BCR), CD40 and cytokine signals to stimulate T-bet^+ B-cell development [18, 19]. Additionally, B cells from MS patients were previously reported to exhibit an enhanced pro-inflammatory phenotype when activated with IFN-γ and TLR9 ligand CpG-DNA [7].

Here, we aimed to explore the CNS transmigration capacity of T-bet(CXCR3)-expressing B cells and which peripheral triggers are involved in the development of such populations in MS patients. We explored the recruitment of human $CXCR3^+$ B cells to the CNS both *ex vivo* and *in vitro*. Furthermore, the susceptibility of blood-derived B cells from MS patients and healthy individuals to T-bet-inducing stimuli and how this influences their

differentiation into CXCR3$^+$ memory subsets was determined using different T cell-based culture systems.

MATERIALS AND METHODS

Patients

Post-mortem CSF, meninges, brain tissues and blood samples were freshly obtained from MS brain donors (Netherlands Brain Bank, Amsterdam, The Netherlands). The main cause of death was legally granted euthanasia (8 of 10 donors). The two other donors died from pneumonia or MS. These tissues had a very short post-mortem delay of 8.92 hours [interquartile range (IQR): 8.50 – 9.50 hours] and pH of the CSF was 6.59 [IQR: 6.44 – 6.87]. All other MS patients and healthy controls were included at Erasmus MC (Rotterdam, The Netherlands), which is a national tertiary referral center for MS patients (MS Center ErasMS). Patients and controls were age- and gender-matched per study group. Patient characteristics are summarized in Table 1. Primary material was obtained between 2007 and

Table 1. Characteristics of patients and controls used in this study.

Cohorts	Subject, *n*	Gender, female *n* (%)	Age in years median (IQR)[A]	Disease duration in months, median (IQR)[B]
***Ex vivo* B cells, CNS vs blood**				
MS	10	9 (90%)	52 (50 - 65)	NA
***Ex vivo* B cells, blood subsets**				
HC	10	7 (70%)	47 (32 - 54	NA
MS, no Tx	10	7 (70%)	45 (43 - 53)	48 (24 - 120)
MS, NAT Tx				
First cohort	10	7 (70%)	40 (29 - 46)[C]	90 (31 - 124)
Second cohort	9	6 (66%)	36 (26 - 43)[C]	46 (41 - 130)
Third cohort[D]	9	5 (56%)	28 (21 - 43)[C]	28 (19 – 41)
***In vitro*-stimulated B cells**				
HC				
Total	10	8 (80%)	44 (32 - 56)	NA
Naive	8	5 (63%)	39 (27 - 50)	NA
MS, no Tx				
Total	9	7 (80%)	41 (34 - 56)	36 (36 – 73)
Naive	12	8 (67%)	38 (28 - 42)	4 (3 – 15)

[A] At time of sampling
[B] Time from MS diagnosis to sampling
[C] At time of pre-treatment sampling
[D] IgG subclass instead of total IgG analysis

MS = multiple sclerosis; HC = healthy control; NAT = natalizumab, Tx = treatment; IQR = interquartile range; NA = not applicable

2018. All patients gave written informed consent and study protocols were approved by the medical ethics committee of the Erasmus MC (Rotterdam) and VUmc (Amsterdam, The Netherlands).

Mononuclear cell isolation from blood and CNS compartments

Peripheral blood mononuclear cells (PBMCs) were isolated according to the manufacturer's instructions from blood of MS patients and matched controls with the use of vacutainer CPT® tubes containing sodium heparin (BD Biosciences, Erembodegem, Belgium). PBMCs were frozen and stored in liquid nitrogen until use as previously described [20]. Mononuclear cells were isolated from buffy coats using Ficoll-Paque Plus (GE Healthcare, Freiburg, Germany) and density gradient centrifugation. Blood and CSF samples from MS brain donors were acquired post-mortem through heart puncture and ventricle drainage, respectively [20]. Heart blood mononuclear cells were isolated as described for buffy coat material. Collection tubes with CSF were centrifuged for 10 min at 500g. CSF and blood mononuclear cell fractions were resuspended in RPMI 1640 (Lonza, Verviers, Belgium) containing 10% heat inactivated human AB serum (Sanquin, Rotterdam, The Netherlands) and 1% penicillin/streptomycin (Lonza) and left to rest at 37°C until further use. Meninges were washed in phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) 3 times, cut into pieces and incubated with liberase (Roche Applied Science, Penzberg, Germany) for 1 hour at 37°C. After which the meninges were filtered through a cell strainer (45 µm) and cells were washed using Ficoll-Paque Plus (GE Healthcare). Single cell suspensions from the meninges were resuspended in PBS containing 0.1% BSA until further use. Brain tissue samples were processed and single-cell suspensions were obtained as previously reported [21].

Antibodies and flow cytometry

Multicolor flow cytometric analysis was performed using fluorochrome-labeled monoclonal anti-human antibodies (mAbs; Table 2). PBMCs were stained extracellularly for 30 minutes at 4°C. Cultured B cells were stained with a fixable viability stain (FVS 700) for 15 min at 4°C and subsequently stained for either extracellular only or both extracellular and intracellular markers. For intracellular staining, cells were fixed with 2% paraformaldehyde (Merck, Schipol-Rijk, The Netherlands) and permeabilized with PBS pH7.4 containing 0.3% BSA and 0.5% saponin (Sigma-Aldrich, Saint-Louis, MO) and stained with T-bet for 60 min at 4°C. All measurements were conducted with an LSRII-Fortessa flow cytometer and analyzed using FACS Diva software, version 8.0.1 (both BD Biosciences). *Ex vivo* Th17.1 (IFN-γ^{high}IL-17^{low}) and Th17 (IFN-γ^{neg}) cells in blood were defined as CCR6^{+}CXCR3^{+}CCR4^{-} (Th17.1) and CCR6^{+}CXCR3^{-}CCR4^{+} (Th17), described recently [20].

Table 2. Monoclonal anti-human antibodies used for FACS.

Antibody marker	Fluorochrome	Clone	Company
CD3	AF700	SK7	Biolegend[A]
CD3	BV785	SK7	BD Biosciences
CD4	BV510	OKT4	Biolegend[A]
CD19	BV785	HIB19	BD Biosciences
CD20	AF700	2H7	BD Biosciences
CD21	BV711	B-Ly4	BD Biosciences
CD27	BV421	M-T271	BD Biosciences
CD38	PE-Cy7 and PerCP-Cy5.5	HIT2	Biolegend[A]
CD49d (VLA-4)	APC	9F10	BD Biosciences
CCR4	PE-Cy7	L291H4	Biolegend[A]
CCR6	PE	G024E3	Biolegend[A]
CXCR3	BV605 and APC	G025H7	Biolegend[A]
CXCR5	APC-R700	RF8B2	BD Biosciences
IgA	FITC	IS11-8E10	Miltenyi Biotech[B]
IgD	PE and PE-CF594	IA6-2	BD Biosciences
IgG	ACP-H7	G18-145	BD Biosciences
IgG1	PE	HP6001	Southern Biotech[C]
IgG2	AF488	HP6002	Southern Biotech[C]
IgM	BV510	MHM-88	Biolegend[A]
T-bet	PE-Cy7	4B10	Biolegend[A]
Fixable viability dye (FVS 700)	AF700		BD Biosciences

[A] Biolegend, London, UK
[B] Miltenyi Biotech, Leiden, The Netherlands
[C] Southern Biotech via ITK diagnostics, Uithoorn, The Netherlands

Human B-cell migration assays

FACS-sorted $CD27^-$ and $CD27^+$ memory $CD19^+$ B cells from buffy coat-derived PBMCs were placed on 96 wells permeable transwell plates with a 0.3 µm pore size (2×10^5 cells/well; Corning, Amsterdam, The Netherlands). B-cell migration towards medium or CXC chemokine ligand (CXCL)10 (900 ng/ml; R&D Systems, Abingdon, UK) was analyzed after 3h in 37°C. In addition, 2,5-5×10^5 memory B cells were placed on confluent monolayers of human brain endothelial cells (hCMEC/D3) on 5 µm pore size transwell plates (Corning) coated with collagen and migration was analyzed after 5 hours [22]. Percentages of memory B-cell subsets were compared before and after transmigration using flow cytometry.

Antigen-primed, autologous B- and Th-cell co-cultures

BCR-mediated uptake and presentation of *Salmonella typhimurium (S. typhimurium)* SL1344 was used as a model for antigen presentation, as previously demonstrated [23]. mAb anti-human IgG (MH16-1, Sanquin, Amsterdam, The Netherlands) was mixed with mAb against *S. typhimurium* lipopolysaccharide (LPS) (1E6, Invitrogen, Paisley, UK) and rat

anti-mouse IgG1 antibody (RM161.1, Sanquin) to generate BCR-LPS tetrameric antibody complexes. Exponentially grown bacteria were washed twice with PBS, incubated with BCR-LPS tetrameric antibody complexes for 30 min at room temperature, and washed twice to remove unbound antibodies. B cells were incubated with viable anti-IgG coated *S. typhimurium* [23] at 20 bacteria per cell for 45 min at 37°C without antibiotics. Next, cells were washed 3 times and cultured for 60 min in media containing 100 µg/ml gentamicin (Invitrogen) to eliminate non-phagocytosed bacteria. B cells were co-cultured in RPMI supplemented with 5% fetal calf serum, 1% (100 U/ml) penicillin, 1% (100 µg/ml) streptamycin (Lonza), 1% (2mM) ultraglutamine (Lonza), 0.1% (50 µM) beta-mercaptoethanol (Sigma Aldrich), 0.1% (20 µg/ml) apotransferrin (depleted for human IgG with protein-G sepharose; Sigma Aldrich) (further referred to as B-cell medium) and 10 µg/ml gentamicin together with autologous $CD4^+$ T cells (MACS sorted). B cells (1 x 10^5) and T cells (0.5 x 10^5) were cultured in 200 µl at 37°C in the presence of 5% CO_2 in 96-well round-bottom plates (Greiner Bio-One; Alphen Aan Den Rijn, The Netherlands) for 6 days. Cultures were performed in the presence of recombinant interleukin (IL)-21 (50 ng/ml; Thermo Fisher Scientific, Landsmeer, The Netherlands), recombinant IL-2 (50 IU/ml, Miltenyi Biotec), and with or without an anti-IFN-γ blocking antibody (MD-1, 10 µg/ml, U-CyTech Biosciences, Utrecht, The Netherlands).

IL-21/3T3-CD40L assay for human B-cell differentiation

To mimic B-cell differentiation *in vitro*, murine NIH3T3 fibroblasts expressing human CD40L (3T3-CD40L) [23] were irradiated at 30 Gy using a RS320 X-ray (Beckhoff, Eindhoven, The Netherlands), taken up in B-cell medium and seeded on flat bottom 96-wells plates (10x10^3 cells per well; Greiner Bio-One) to allow adherence overnight. $CD19^+$ (total) B cells were isolated from buffy coat-derived PBMCs using CD19 microbeads and the autoMACS Pro Separator (both Miltenyi Biotec). Total, naive ($CD38^{-/dim}CD27^-IgG^-IgA^-$) and memory ($CD38^{-/dim}CD27^+IgG^+$) B cells were isolated from healthy and MS blood using a BD FACSAria™ III cell sorter. These fractions were resuspended in B-cell medium and 20-25 x 10^3 cells were co-cultured with irradiated 3T3-CD40L cells and stimulated with a combination of IL-21 (50 ng/ml; Thermo Fisher), IFN-γ (50 ng/ml; Peprotech/bio-connect, Huissen, The Netherlands) and CpG-ODN (2006-G5; 10 µg/ml; InvivoGen/bio-connect) at 37°C and 5% CO_2. After 6 and 11 days of culture, supernatants were collected and stored at -80°C until use for enzyme-linked immunosorbent assay (ELISA). The cells were stained and assessed using flow cytometry as described above.

IgG1 ELISA

Nunc MaxiSorp plates (Sanbio BV) were coated overnight with anti-human IgG1 monoclonal capture antibody (1 µg/ml; clone MH161-1, Sanquin, Amsterdam, The Netherlands)

in PBS. After washing with PBS-0.02% Tween-20, the supernatants from *in vitro* B-cell cultures (described above) were diluted in high performance ELISA buffer (HPE; Sanquin) and incubated for 60 min. Subsequently, plates were washed and incubated for 60 min with anti-human IgG conjugated with horseradish peroxidase, a monoclonal detection antibody (1 µg/ml; clone MH16-1, Sanquin). After washing, the ELISA was developed with MQ containing 0.11M sodium-acetate (pH 5.5), 100 µg/ml tetramethylbenzidine and 0.003% (v/v) H_2O_2 (all from Merck). The reaction was stopped by addition of 2M H_2SO_4 (Merck). Optical densities at 450nm were measured with a Biotek Synergy2. Background readings at 540nm were subtracted. Results were related to a titration curve of a serum sample of a healthy donor in each plate.

Statistical analyses

All data sets were analyzed with Graphpad Prism Software, version 7 (GraphPad Software Inc., San Diego, CA) and compared using two sided Mann-Whitney U tests, Wilcoxon matched-pairs signed rank test, 1 or 2-way ANOVA with Tukey's post hoc test, Friedman paired with Dunn's post hoc test and Spearman correlation coefficients (as indicated in each figure legend). Experimental data are depicted as the mean ± SEM. Prior to statistical analyses, data sets were tested for normal distribution. *p* values less than 0.05 were considered significant.

RESULTS

CXCR3-expressing B cells are selectively enriched in distinct CNS compartments of MS patients

Enhanced chemotaxis is one of the key mechanisms by which B cells can enter distinct CNS compartments of MS patients [11]. Production of the chemo-attractants CXCL10, CXCL13 and CCL20 in the CNS has been associated with B-cell recruitment, distribution and reactivity in MS [24-26]. We compared the presence of B cells that express the chemokine receptors that correspond to these ligands, $CXCR3^+$ (CXCL10), $CXCR5^+$ (CXCL13) and $CCR6^+$ (CCL20) between paired blood, CSF, meningeal and brain tissues from 10 MS patients (Table 1). To realize this, single-cell suspensions were obtained from autopsied brain compartments using a standardized protocol [21]. From these fractions, viable $CD45^+CD3^-CD19^+$ B cells were gated and analyzed for chemokine receptor expression using flow cytometry (Fig 1A). We were able to measure sufficient numbers of viable B cells from each compartment for all donors (mean [range], blood: 21,509 [610-98,562], CSF: 12,629 [50-59,499], meninges: 13,819 [91-36,644] and brain tissue: 2,889 [26-18,050]. The frequencies of $CXCR3^+$, and not $CXCR5^+$ or $CCR6^+$ B cells were strongly increased in *ex*

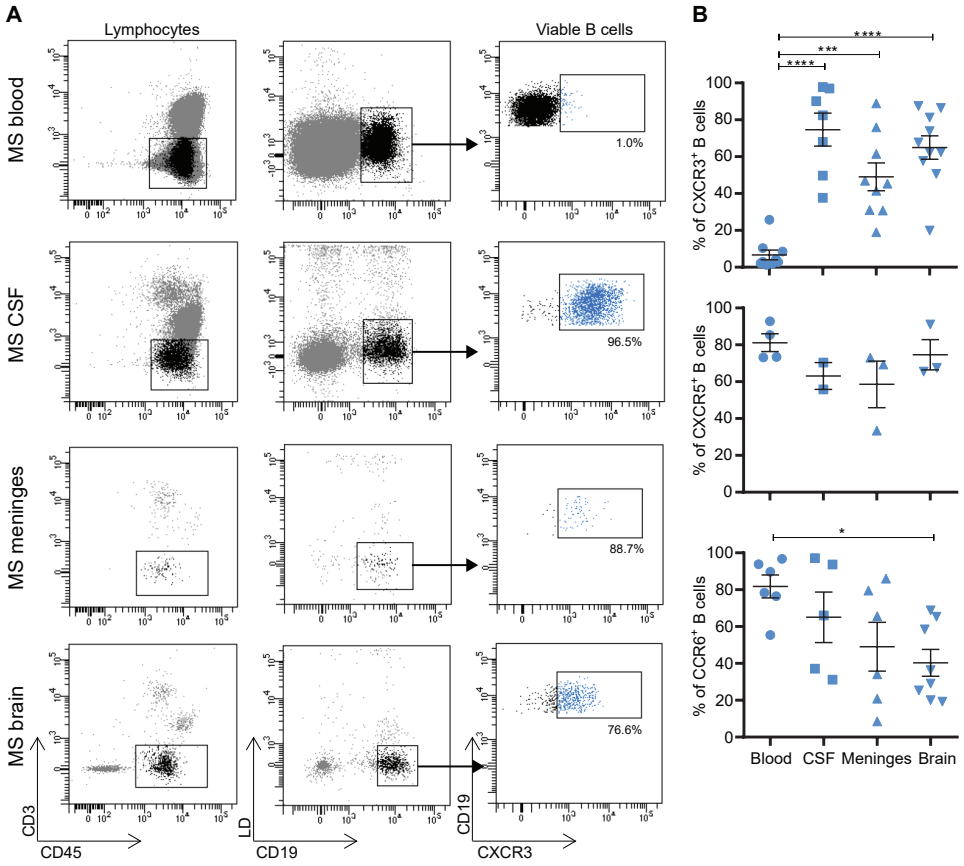


Figure 1. CXCR3⁺ B cells are abundant in the CNS compared to blood of MS patients.

(**A**) Representative FACS plots and gating of CXCR3-expressing CD19⁺ B cells within viable CD45⁺CD3⁻ lymphocyte fractions derived from the blood, CSF, meninges and brain tissue of an MS patient. (**B**) Frequencies of CXCR3⁺, CXCR5⁺ and CCR6⁺ B cells in distinct paired compartments from MS patients. For blood, CSF and meningeal samples each dot represents a different patient. A total of 10 brain tissues from 7 different MS patients were used for the analysis of CXCR3⁺ B cells. Any samples with less than 25 viable B cells were excluded from these analyses. Data are presented as the mean ± SEM. ****$p < 0.0001$, ***$p < 0.001$, and *$p < 0.05$. The p values for **B** were calculated by a 1-way ANOVA test.

vivo cell suspensions from MS brain tissues ($p < 0.0001$), meninges ($p = 0.0003$) and CSF ($p < 0.0001$) compared to blood (Fig 1B). Also CXCR3-expressing T cells, including Th17.1, were enriched in the CNS compartments of these donors (data not shown), supporting our recent observations [20].

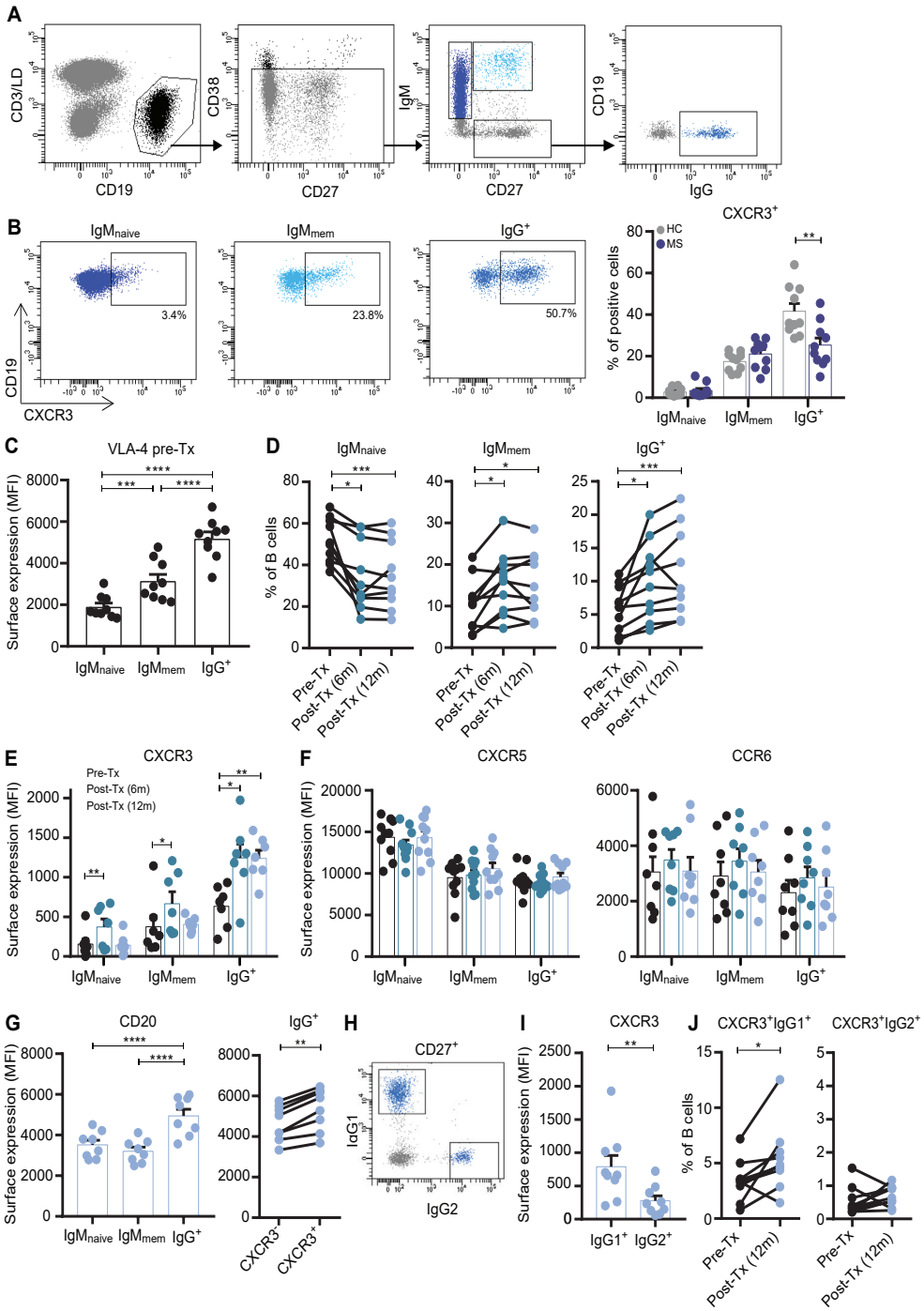
Reduced frequencies of CXCR3$^+$IgG(1)$^+$ B cells in the blood of MS patients are abrogated after natalizumab treatment

To determine how CXCR3 is involved in the local attraction of different B-cell populations in MS, we assessed the proportions of CXCR3-expressing naive ($CD27^-IgM^+$; IgM_{naive}) and both IgM^+ memory ($CD27^+IgM^+$; IgM_{mem}) and IgG^+ memory ($CD27^+IgG^+$) B cells in the peripheral blood of untreated MS patients ($n = 10$) and age- and gender-matched healthy controls (HC, $n = 10$; Table 1 and Fig 2A). CXCR3- expressing IgG^+ cells were reduced ($p = 0.007$), while no differences were seen for IgM_{naive} and IgM_{mem} cells in MS versus HC blood (Fig 2B).

To address this potential migration of $CXCR3^+IgG^+$ memory B cells into the CNS, we analyzed the distribution of these B-cell subsets in the blood of MS patients treated with the anti-α4β1 integrin (VLA-4) antibody natalizumab (Table 1), a drug that effectively reduces MS disease activity by blocking lymphocyte recruitment to the CNS [27]. VLA-4 was most abundantly expressed on blood IgG^+ B cells from MS patients prior to natalizumab treatment (Fig 2C). Elevated frequencies of both IgM_{mem} (pre-treatment versus 6m post-treatment $p = 0.042$ and 12m post-treatment $p = 0.011$) and IgG^+ (pre-treatment versus 6m post-treatment $p = 0.022$ and 12m post-treatment $p < 0.001$) B cells were found in blood of MS patients both 6- and 12-months post- versus pre-treatment (Fig 2D). However, only IgG^+ and not IgM_{naive} or IgM_{mem} B cells from MS patients treated with natalizumab for 12 months, showed increased expression levels of CXCR3 ($p < 0.01$; Fig 2E), and not CXCR5 or CCR6 (Fig 2F). These findings were validated in a second cohort of nine MS patients treated with natalizumab (Table1; data not shown). Notably, CD20 expression levels were

Figure 2 (see right page). Reduced frequencies and natalizumab-mediated accumulation of CXCR3$^+$IgG(1)$^+$ B cells in MS blood.

(**A**) FACS gating strategy used to define IgM_{naive} ($CD27^-IgM^+$), IgM_{mem} ($CD27^+IgM^+$) and IgG^+ ($CD27^+IgG^+$) B-cell subsets. (**B**) Gating and quantification of CXCR3-expressing IgM_{naive}, IgM_{mem} and IgG^+ B-cell frequencies in the blood of untreated MS patients ($n = 10$; dark blue dots) and both age-/gender-matched healthy controls ($n = 10$; grey dots, see Table 1). (**C**) VLA-4 surface expression on IgM_{naive}, IgM_{mem} and IgG^+ B cells from blood of MS patients before natalizumab treatment ($n = 9$). (**D**) The percentage of IgM_{naive}, IgM_{mem} and IgG^+ B cells in MS blood before (black dots) and both 6 months (marine blue dots) and 12 months (light blue dots) after natalizumab treatment (paired samples; $n = 10$; see Table 1). Surface expression levels of (**E**) CXCR3, (**F**) CXCR5 and CCR6 on IgM_{naive}, IgM_{mem} and IgG^+ B cells in pre- and post-natalizumab-treated MS patient blood ($n = 7$-10). (**G**) CD20 expression on IgM_{naive}, IgM_{mem} and IgG^+ B cells as well as paired $CXCR3^-$ and $CXCR3^+$ IgG^+ populations in blood of MS patients treated with natalizumab for 12 months ($n = 8$). (**H-J**) Gating example and quantifications of $IgG1^+$ and $IgG2^+$ B cells expressing CXCR3 in MS patients treated with natalizumab for 12 months ($n = 9$). Data are presented as the mean ± SEM. $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$. The p values were calculated by Mann-Whitney U (**B**), 2-way ANOVA (**C** and **G**), Friedman paired (**D-F**) and Wilcoxon matched-pairs signed rank (**G, I** and **J**) tests.


A
CD3/LD
CD19
CD38
CD27
IgM
CD27
CD19
IgG
B
IgMnaive
IgMmem
IgG+
3.4%
23.8%
50.7%
CD19
CXCR3
CXCR3+
HC
MS
% of positive cells
C
VLA-4 pre-Tx
Surface expression (MFI)
D
% of B cells
Pre-Tx
Post-Tx (6m)
Post-Tx (12m)
E
CXCR3
F
CXCR5
CCR6
G
CD20
CXCR3-
CXCR3+
H
CD27+
IgG1
IgG2
I
J
CXCR3+IgG1+
CXCR3+IgG2+

increased on IgG$^+$ B cells and higher on CXCR3$^+$ compared to CXCR3$^-$ counterparts in the blood of natalizumab-treated MS patients (Fig 2G).

Since intrathecally synthesized OCBs are restricted to the IgG1 subclass in the CSF of MS patients [28], we also analyzed IgG1$^+$ B cells for their frequencies and CXCR3 expression in the blood of a third cohort of natalizumab-treated MS patients (Fig 2H). IgG1$^+$ B cells not only expressed higher levels of CXCR3 (p = 0.007; Fig 2I), but also showed increased frequencies in post-treatment samples (p = 0.027; Fig 2J) compared to IgG2$^+$ B cells. The selective accumulation of CXCR3^{high} IgG(1)$^+$ B cells in the blood of natalizumab-treated patients underlines the potency of this subset to transmigrate into the CNS to mediate MS disease activity.

CXCR3$^+$IgG1$^+$ B cells have an enhanced capacity to transmigrate across the blood-brain barrier in vitro

To functionally test the transmigration potential of CXCR3$^+$IgG1$^+$ B cells into the CNS, we sorted memory B cells from the blood and assessed in vitro migration of subsets towards CXCL10. Fractions of CXCR3-expressing IgM$_{mem}$, IgG1$^+$ and IgG2$^+$ B cells were assessed within the total memory pool before and after migration through transwell filters. In contrast to IgM$_{mem}$ and IgG2$^+$ populations, IgG1$^+$ B cells showed prominent recruitment to CXCL10 ($p < 0.0001$ before versus after migration; Fig 3A and B). This was not seen using medium only (Fig 3B). To mimic B-cell transmigration across the blood-CNS barrier, these experiments were repeated using cultured confluent monolayers of human brain endothelial cells (BEC) [22]. We found a similar CXCL10-mediated migratory advantage of IgG1$^+$ B cells ($p < 0.0001$; Fig 3A and C), which is consistent with the abundance of CXCR3 on IgG1$^+$ compared to IgM$_{mem}$ and IgG2$^+$ B cells in MS patients (Fig 2G-I). These data demonstrate that CXCR3^{high} IgG1$^+$ B cells in the blood have a heightened ability to infiltrate the CNS, probably accounting for the local IgG1 subclass restriction of OCB in MS patients [28].

IFN-γ promotes CXCR3 expression and CD4$^+$ T-cell activation by human T-bet$^+$ B cells under T_{FH}1-like culture conditions

In MS blood, the proportion of CXCR3$^+$IgG$^+$ B cells correlated to Th17.1 (IFN-γ^{high}IL-17^{low}; r = 0.566, p = 0.0003) and not to Th17 (IFN-γ^{neg}) cells (Fig 4A) [20]. Th cell-derived IFN-γ is known as a central driver of autoreactive B cells in mice [10] and also induces CXCR3 expression on human memory B cells [29]. Therefore, we aimed to better understand how the differentiation and function of human CXCR3$^+$ memory B cells is influenced by IFN-γ before entering the CNS. To address this, we mimicked the effects of IFN-γ-producing T follicular helper (T_{FH}) cells on B-cell subsets *in vitro*. First, an IL-21-based human B- and T-cell co-culture system was used to assess whether Th1-derived IFN-γ influenced CXCR3 expression on IgG$^+$ B cells in an antigen-specific manner. Since antigen uptake by B cells is limited

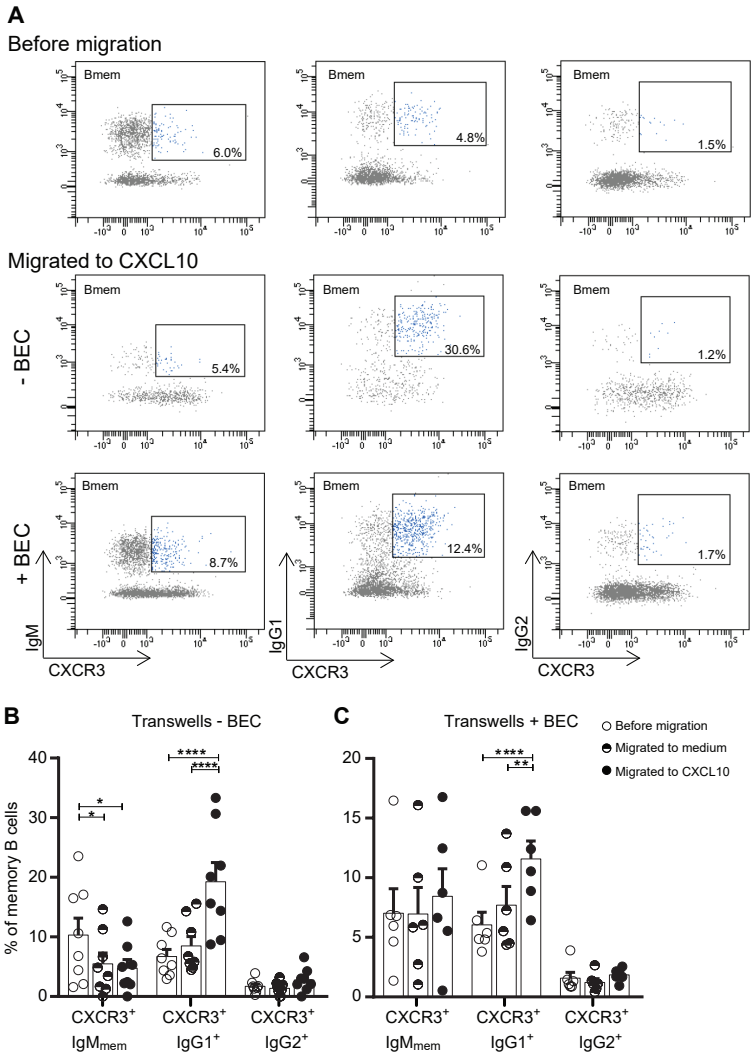


Figure 3. Enhanced migration of CXCR3⁺IgG1⁺ B cells across transwell filters and human brain endothelial monolayers *in vitro*.

Sorted memory B cells from healthy donor blood were assessed for selective *in vitro* transmigration towards CXCL10. (**A**) Representative FACS plots and (**B**, **C**) quantifications of viable CXCR3-expressing IgM_{mem}, $IgG1^+$ and $IgG2^+$ B-cells migrating across transwell filters with and without confluent monolayers of human brain endothelial cells (BEC). Percentages of subsets within the total memory pool were compared before and after migration, both to medium and CXCL10 (-BEC, $n = 8$; +BEC, $n = 6$). These experiments were performed in duplicate for each donor for which the average is shown. Data are presented as the mean ± SEM. ****$p < 0.0001$, **$p < 0.01$ and *$p < 0.05$. The p values were calculated by 2-way ANOVA (**B, C**).

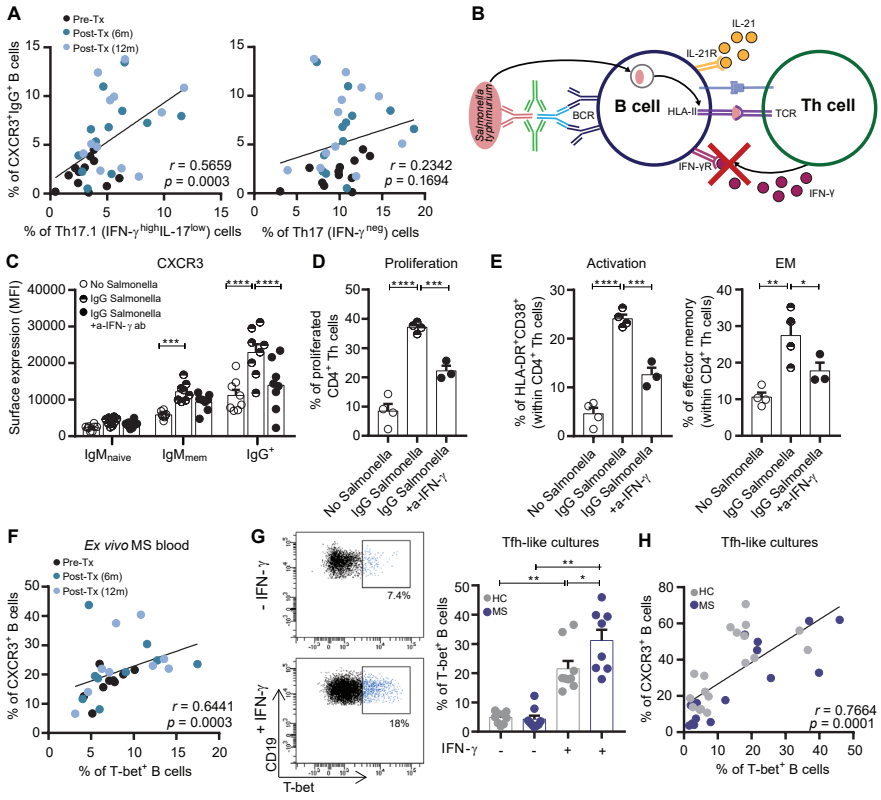


Figure 4. T helper 1 cytokine IFN-γ is a major trigger of CXCR3⁺(T-bet⁺) B-cell differentiation in MS.

(**A**) Correlation of *ex vivo* CXCR3⁺IgG⁺ B cells with Th17.1 (IFN-γ^{high}IL-17^{low}) and Th17 (IFN-γ^{neg}) cells in MS blood pre- and post-natalizumab treatment (pre-Tx and post-Tx; $n = 12$). (**B**) Experimental model of *Salmonella*-primed autologous B- and T-cell co-cultures. (**D-E**) B cells from healthy donor blood were primed with *S. typhimurium* through BCR crosslinking using a tetrameric antibody complex, as described in the methods. This allows BCR-mediated *Salmonella* uptake, processing and presentation on MHC II molecules to Th cells. IL-21 was added with and without an IFN-γ blocking antibody to analyze the effects on CXCR3 expression by B cells (**C**), and on the proliferation, activation and effector memory phenotype of Th cells (**D, E**). These experiments were performed in two independent experiments and in duplicate for (**C**) four and (**D, E**) two different blood donors. (**F**) Correlation of surface CXCR3 and intracellular T-bet expression in *ex vivo* B cells of MS patients pre- and post-natalizumab treatment (pre-Tx and post-Tx; $n = 9$). (**G, H**) Total B cells from the blood of MS patients ($n = 9$) and both age-/gender-matched healthy controls (HC; $n = 10$) were cultured *in vitro* under T_{FH}-like conditions with IL-21, 3T3-CD40L cells and with or without IFN-γ for 11 days. Representative FACS plots and quantification of *in vitro*-induced T-bet⁺ B cells (**G**) and correlation of CXCR3 and T-bet expression in these cultured B cells (**H**) are shown. Data are presented as the mean ± SEM. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$. The p values were calculated by 2-way ANOVA (**c**), 1-way ANOVA (**D, E**) and Wilcoxon matched-pairs signed rank (**G**) tests. The correlation coefficients and p values for **A, F**, **H** were calculated by Spearman rank.

by the selectivity of the BCR, surface IgG was crosslinked with Th1-associated pathogen *S. typhimurium* for efficient internalization, processing and presentation to autologous Th cells (Fig 4B) [23, 30]. After 6 days of co-culture, we found that the induced expression of CXCR3 on *Salmonella*-containing IgG^+ and not IgM_{naive} or IgM_{mem} B cells was abrogated by the addition of an IFN-γ blocking antibody ($p < 0.0001$; Fig 4C). Furthermore, blocking of IFN-γ impaired B cell-induced T-cell proliferation, activation and effector memory formation (Fig 4D and E). This implies that IFN-γ additionally stimulates the antigen-presenting function of $CXCR3^+IgG^+$ B cells, in parallel with previous findings in mice [31].

CXCR3 expression is under the direct control of IFN-γ-inducible transcription factor T-bet, a critical regulator of memory B-cell differentiation in mice [13, 14]. Consistent with this, intracellular T-bet positively correlated to surface CXCR3 expression in *ex vivo* B cells ($r = 0.644$, $p = 0.0003$) and showed a similar association with Th17.1 ($IFN\text{-}\gamma^{high}IL\text{-}17^{low}$) cells in MS patients (Fig 4F and data not shown) [20]. To further explore the susceptibility of B cells to IFN-γ in MS [7], we compared B cells from MS and matched healthy control blood for IFN-γ-mediated T-bet induction under T_{FH}-like culture conditions. After 11 days of stimulation with 3T3-CD40L cells, IL-21 and IFN-γ, T-bet was predominantly upregulated in B cells of MS patients ($p = 0.021$; Fig 4G), while conditions without IFN-γ did not show this. This *in vitro*-induced T-bet was co-expressed with surface CXCR3 ($r = 0.766$, $p = 0.0001$; Fig 4H), in line with our *ex vivo* results (Fig 4F). These findings reveal that Th cell-derived IFN-γ is a major trigger of peripheral $CXCR3(T\text{-}bet)^+$ B cells in MS.

IFN-γ stimulates plasmablast formation and synergizes with CpG-DNA to establish IgG1 switching during human T_{FH}-like B-cell cultures

Besides IFN-γ, also TLR9 ligand CpG-DNA has been reported to induce T-bet in murine B cells [14, 18] and promote pro-inflammatory cytokine responses of B cells from MS patients [7]. To assess how TLR9 signals integrate with IFN-γ to regulate human $T\text{-}bet^+$ B-cell development, we first determined whether naive or memory B cells are more prone to this type of co-activation. Naive ($CD27^-IgG^-$) and memory ($CD27^+IgG^+$) B cells were sorted from healthy donor blood and stimulated with 3T3-CD40L cells, IL-21, IFN-γ and/or CpG-DNA. After 11 days of naive B-cell cultures, both T-bet and CXCR3 expression was induced by IFN-γ, and further enhanced after addition of both IFN-γ and CpG-DNA ($p = 0.001$ and $p = 0.021$; Fig 5A). This additional effect of CpG-DNA was not found when using sorted memory B cells (Fig 5B). Both IFN-γ- and CpG-DNA-induced $T\text{-}bet(CXCR3)^+$ B cells also showed strongly reduced CD21 expression (data not shown), a typical feature seen for T-bet-expressing B cells [31].

During a germinal center response, naive B cells can either differentiate into plasmablast or memory populations, depending on the local inflammatory environment [10, 32]. We investigated the effects of IFN-γ and CpG-DNA on plasmablast formation and IgG

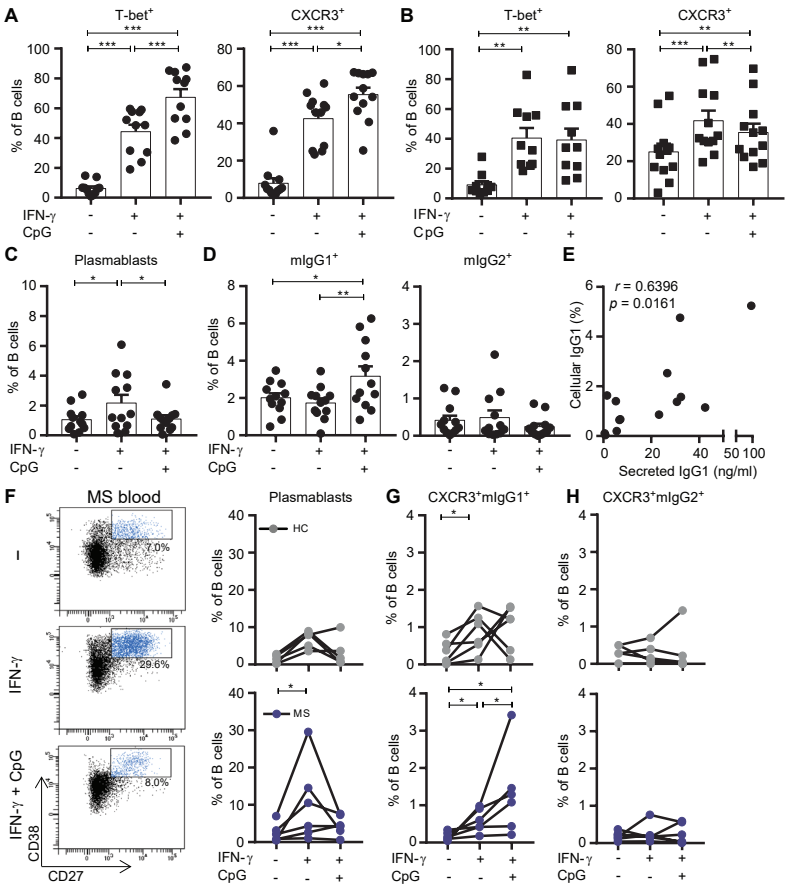


Figure 5. IFN-γ induces plasmablast differentiation, while both IFN-γ and CpG-DNA further upregulate T-bet and trigger IgG1 switching in B cells of MS patients.

(**A-D**) Naive (IgG$^-$CD27$^-$; dots) and memory (IgG$^+$CD27$^+$; squares) B cells were sorted from peripheral blood of healthy donors and were cultured under T_{FH}-like conditions with IL-21, 3T3-CD40L cells, with or without IFN-γ and/or CpG-DNA. Frequencies of T-bet$^+$ and CXCR3$^+$ B cells after 11 days of culture using (**A**) naive B cells (n = 12) and 6 days of culture using (**B**) memory B cells (n = 10-12). The frequencies of (**C**) plasmablasts (CD38^{high}CD27$^+$; n = 12) and (**D**) membrane-bound (m) mIgG1$^+$ and mIgG2$^+$ B cells were analyzed after culturing naive populations for 11 days (n = 12). (**E**) Correlation between cellular expression and secretion of IgG1, as determined by FACS and ELISA (pooled stimulation conditions for 5 donors). Naive B cells from the blood of MS patients (n = 6; dark blue dots) and healthy controls (n = 6; grey dots) were cultured under the same T_{FH}-like conditions and analyzed for (**F**) plasmablast (CD38^{high}CD27$^+$) and (**G, H**) CXCR3$^+$mIgG1$^+$ and CXCR3$^+$mIgG2$^+$ B-cell differentiation after 11 days of culture. Data are presented as the mean ± SEM. ***p < 0.001, **p < 0.01 and *p < 0.05. The p values for **A-D** and **F-H** were calculated by the Wilcoxon matched-pairs signed rank test. The correlation coefficient and p value for **E** was calculated by Spearman rank correlation.

subclass switching during IL-21-/CD40L-induced naive B-cell differentiation. After 11 days of culture, sorted naive B cells from healthy donors developed into plasmablasts under IFN-γ stimulatory conditions only ($p = 0.034$; Fig 5C). IFN-γ and CpG-DNA together did not induce plasmablast formation, but instead triggered IgG1 and not IgG2 expression on differentiating B cells (IFN-γ only versus IFN-γ + CpG-DNA: $p = 0.002$; Fig 5D). Interestingly, this *in vitro*-induced IgG1 switching was subjected to differentiation of sorted naive (Fig 5D) and not memory (data not shown) B cells. CpG-DNA alone did not upregulate CXCR3, T-bet and IgG1 in differentiating naive B cells (data not shown), indicating that both IFN-γ and TLR9 signaling are required for enhanced expression of these markers. To verify that B-cell intrinsic expression also corresponds with secretion of IgG1, we performed ELISAs on culture supernatants of these B cells. Indeed, the percentage of $IgG1^+$ B cells positively correlated to IgG1 secretion ($r = 0.640$, $p = 0.016$; Fig 5E).

Finally, to address how this is regulated in MS, we isolated naive B cells from MS patients and performed similar culture experiments. IFN-γ-mediated plasmablast formation was more induced after 11 days of culture compared to matched controls ($p = 0.031$; Fig 5F). This was not seen after stimulation with both IFN-γ and CpG-DNA. Instead, this type of triggering resulted in a robust induction of $CXCR3^+IgG1^+$, and not $CXCR3^+IgG2^+$ subsets in MS (Fig 5G and H). Collectively, these data demonstrate that TLR9 signaling potentiates IFN-γ-induced T-bet and CXCR3 expression during naive B-cell differentiation *in vitro*, and that this is important for IFN-γ-mediated formation of $IgG1^+$ memory B cells rather than plasmablasts under T_{FH}-like circumstances in MS.

DISCUSSION

Evidence has accumulated that at least in the periphery, antibody-independent roles of B cells are driving the pathology of MS [7]. However, local production of autoantibodies should not be ruled out as an underlying B-cell mechanism in this disease [33]. Even though autoreactive naive B cells are highly active in MS blood [9], the vast majority of B cells identified in the MS brain have undergone further maturation into antibody-producing cells [34, 35]. It has also been demonstrated that memory B cells of MS patients are the most potent antigen-presenting cells and likely have specific pro-inflammatory propensities, including the capacity to express enhanced levels of immune activating molecules [35]. This is of special interest considering the presence of meningeal B cell-rich follicle-like structures in MS and the adjacent subpial cortical demyelinating injury [8], which probably contributes to progressive loss of neurological function in patients with MS. Thus, identification of the particular B-cell subsets that can preferentially migrate into the CNS and clarifying how they may contribute to propagating local injury responses is of

considerable interest in such an organ-specific disease. In this study, we demonstrate that integrating IFN-γ and pathogen-associated TLR9 signals are critical for the development of human T-bet$^+$ memory B cells, probably underlying their selective recruitment to the CNS of MS patients.

Recent studies have shown that in MS identical B-cell clones are present in both the periphery and CNS [2-4]. The fact that these B-cell populations further undergo somatic hypermutation in the brain implies the presence of functional germinal centers within the CNS. Indeed, such structures have been identified in the meninges of MS patients and are obvious localizations to play a role here [8]. The enrichment that we observe of CXCR3$^+$ B cells in paired CSF, meninges and brain tissue compartments compared to blood of MS patients is in line with studies that show higher levels of CXCR3 ligand CXCL10 in the CSF of MS patients [24]. These results are also consistent with our previous findings that CXCR3$^+$ T cells, including Th17.1, are abundant within the CNS [20, 24], suggesting a common CXCR3-driven lymphocyte recruitment pathway in MS [24, 36, 37]. Other studies have also put forward CXCR5 and its ligand CXCL13 as important contributors to B-cell recruitment to the CNS [3, 38]. We did not find differences in CXCR5-expressing B cells between CNS tissues and paired blood. Hence, the CXCR5/CXCL13 axis is probably related to local organization rather than recruitment of pathogenic B (and T) cells [39, 40], in a process similar to that in secondary lymphoid organs [41]. While these studies indicate a role for germinal center B cells within the CNS, little is known about which peripheral mechanisms underlie their development and local recruitment. In mice, it has been shown that in an autoimmune setting, IFN-γ, likely produced by activated T_{FH} cells, induce germinal centers [10], which can be found in meningeal follicle-like structures [8]. In these situations, IFN-γ induced B cell-intrinsic expression of T-bet, possibly resulting in enhanced Ig class switching and CXCR3 expression [13, 14]. This points to a central role of IFN-γ-associated CXCR3$^+$ B-cell subsets in the meningeal process [10, 12].

While the inducing effects of peripheral B cells on autoreactive Th1 cells are currently being elucidated [37], far less is known about the impact of Th1 cells on peripheral B-cell differentiation and function in MS patients. Therefore, we were interested in the signals needed for B cells to differentiate into T-bet$^+$ cells and postulated that IFN-γ- and IL-21-producing $T_{FH}1$ cells in germinal centers can trigger development of such B cells. Indeed, in MS patients, B cells were found to express higher T-bet levels under $T_{FH}1$-like culture conditions. Furthermore, IL-21-based B- and T-cell co-cultures revealed that CXCR3-expressing IgG$^+$ memory B cells were less induced after blocking of IFN-γ, which corresponds to studies that show IFN-γ regulates CXCR3 expression in human B cells [29]. Th cell proliferation, activation and effector memory formation were also affected in these cultures. In line with our findings, a recent study demonstrated that memory B cells induce proliferation of CNS-infiltrating Th1 cells in MS, which was inhibited after IFN-γ abrogation [37]. Therefore, in MS

patients, peripheral interaction of CXCR3(T-bet)$^{+}$ B and IFN-γ-producing Th cells probably generates a feedforward loop, in which IFN-γ enhances the potency of B cells as antigen-presenting cells, resulting in the activation of (IFN-γ-producing) pathogenic Th cells.

Furthermore, we found that naive B cells from MS patients developed into plasmablasts rather than IgG1-switched memory B cells under IFN-γ-only conditions. Since T-bet mediates class-switching in murine B cells [13], we expected that an additional signal would be required for triggering such a mechanism in human B cells. Besides $T_{FH}1$ cells, also innate TLR signaling is critical for naive B-cell differentiation [42]. Especially pathogen-associated TLR9 and its ligand CpG-DNA have been shown to promote the development of T-bet^{+} B cells in mice [19, 31, 42]. Correspondingly, we found that the induction of MS-blood derived naive B cells with both IFN-γ and CpG-DNA resulted in the development of IgG1-switched, T-bet^{high} B cells during T_{FH}-like cultures. Likewise, CXCR3 surface expression was more enhanced under these conditions, reflecting the high CXCR3 levels on *ex vivo* IgG1^{+} B cells. This additional effect of TLR9 signaling on human T-bet^{+} B cells in MS links to the role of TLR9 in driving neuroinflammatory responses, including increased production of chemokines in the CNS [43]. Moreover, CXCR3^{+}IgG1^{+} B cells showed an enhanced transmigration potential over brain endothelial layers, and selectively accumulated in MS blood after natalizumab therapy. The importance of pathogenic immune cells in contributing to MS disease progression, such as CXCR3^{+} memory B cells, has been put forward by the recurrence of often-fatal clinical relapses in MS patients when discontinuing the use of natalizumab [27, 44, 45]. During these rebounds, EBV-infected memory B cells that have accumulated in the blood show massive influx into brain tissues of MS patients [45]. Furthermore, persistent viral infections are suggested to sustain the development of T-bet-expressing B cells [15], which further supports the enhanced differentiation and local recruitment of CXCR3(T-bet)^{high} memory B cells in an organ-specific autoimmune disease such as MS (Fig 6).

Although the exact role of (local) autoantibody production in MS is not clear, the question whether and how T-bet^{+} B cells are involved this process deserves further attention. In SLE, T-bet^{+} B cells have autoreactive BCRs and are prone to differentiate into IgG autoantibody producing plasmablasts [46]. Inappropriate T-bet expression in B cells also impaired CXCR3-mediated plasmablast differentiation within germinal centers [14] and autoantibody production [13]. In our study, IFN-γ- and CpG-DNA-induced human CXCR3(T-bet)^{high} B cells showed increased IgG1 expression and secretion. This strongly suggests that after preferential recruitment and re-activation in the CNS, CXCR3(T-bet)^{high}IgG1^{+} B cells are responsible for local production of IgG1 in MS (Fig 6) [28]. Although B cells within the CNS of MS patients show characteristics of an antigen-driven response, the specific antigens driving this response remain unknown. MS disease heterogeneity is reflected by the identification of several candidate target antigens, including non-myelin proteins such as

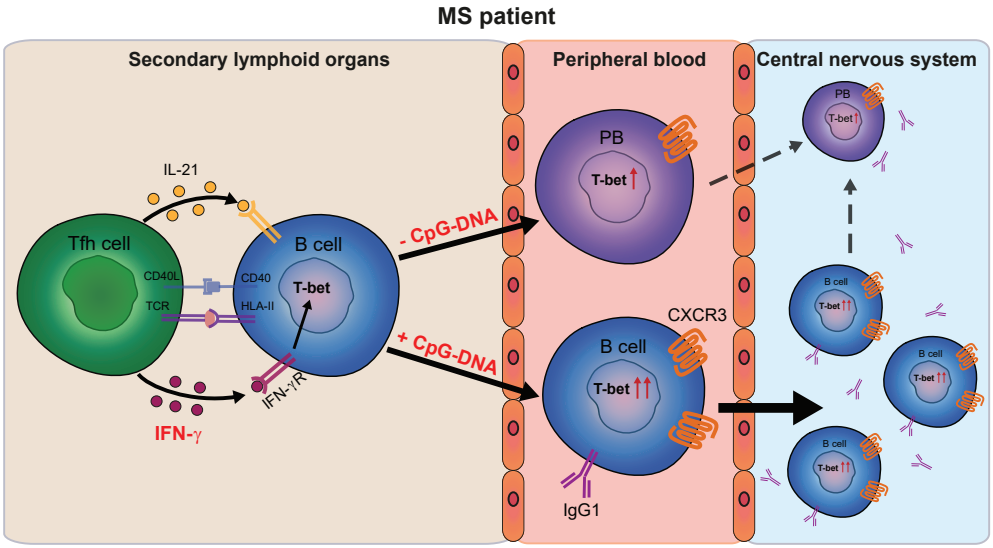


Figure 6. IFN-γ and TLR9 signalling upregulate T-bet in peripheral B cells, likely driving CXCR3-mediated recruitment and IgG1 production in the CNS of MS patients.

Our findings suggest that in the secondary lymphoid organs of MS patients, IFN-γ triggers naive B cells to differentiate into T-bet-expressing populations in a T_{FH}-dependent manner. Human T-bet$^+$ B cells either can develop into plasmablasts or undergo further differentiation into IgG1$^+$ memory B cells mediated by TLR9 ligation. The enhanced CXCR3 expression on both IFN-γ- and TLR9-induced IgG1$^+$ B cells makes these subsets highly capable of transmigrating across the blood-brain barrier and mediate local pathology in MS.

neurofilament light and RAS guanyl releasing protein 2 (RASGRP2) [33, 37]. In addition to this, increased Epstein-Barr nuclear antigen 1 (EBNA1)-specific IgG1 titers have been found in active MS, which may be explained by the interaction of B cells with pathogen-associated TLR ligands and EBNA1-specific, IFN-γ-producing T cells that cross-recognize myelin antigens [47]. Therefore, we propose that the relevant antigen specificity of B cells in MS can be found within this subset, which should be further explored in the near future.

Taken together, not only a disrupted blood-brain barrier, but also peripheral T-bet-mediated differentiation and transmigration of IgG1$^+$ memory populations could explain how B cells are eventually able to mediate CNS pathology in MS patients (Fig 6). The relevance of T- and B-cell interaction in tolerance breakthrough is stressed by the fact that antigen-specific B cells are potentially 1000 to 10,000 times better presenters of autologous peptides to T cells than non-specific B cells [48]. We here reveal that human CXCR3(T-bet)$^+$ B cells are a product of T- and B-cell interaction. Similar to SLE, such populations probably serve as potent antigen-presenting cells in CNS-specific autoimmune diseases such as MS [31]. Anti-CD20 therapy exerts immediate effects and is assumed to predominantly affect

this function of B cells in MS patients [35]. The potential role of CXCR3(T-bet)$^+$ B cells as prime targets of this therapy is further supported by their abundant CD20 expression, as shown in the current study. The development of new targeted strategies to inhibit T-bet function have the potential to become a double-edged sword in MS by suppressing pathogenic, IFN-γ-producing T (Th17.1) cells together with their counterpart CXCR3(T-bet)$^+$ B cells. Small molecule inhibitors of IFN-γ signaling (jakinhibs) [49]c and the TLR/myD88 pathway [50] are already used in the clinics for other inflammatory diseases, and are promising candidates for combined suppression of IFN-γ and TLR signals to control pathogenic T-bet$^+$ B cells in autoimmune diseases such as MS.

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REFERENCES

1 **Farh, K. K. H., Marson, A., Zhu, J., Kleinewietfeld, M., Housley, W. J., Beik, S., Shoresh, N., et al.,** Genetic and Epigenetic Fine-Mapping of Causal Autoimmune Disease Variants. *Nature* 2015. **518**: 337-343.

2 **von Büdingen, H. C., Kuo, T. C., Sirota, M., van Belle, C. J., Apeltsin, L., Glanville, J., Cree, B. A., et al.,** B cell exchange across the blood-brain barrier in multiple sclerosis. *The Journal of Clinical Investigation* 2012. **122**: 4533-4543.

3 **Eggers, E. L., Michel, B. A., Wu, H., Wang, S.-z., Bevan, C. J., Abounasr, A., Pierson, N. S., et al.,** Clonal relationships of CSF B cells in treatment-naive multiple sclerosis patients. *JCI Insight* 2017. **2**.

4 **Greenfield, A. L., Dandekar, R., Ramesh, A., Eggers, E. L., Wu, H., Laurent, S., Harkin, W., et al.,** Longitudinally persistent cerebrospinal fluid B cells can resist treatment in multiple sclerosis. *JCI Insight* 2019. **4**.

5 **Stern, J. N. H., Yaari, G., Vander Heiden, J. A., Church, G., Donahue, W. F., Hintzen, R. Q., Huttner, A. J., et al.,** B cells populating the multiple sclerosis brain mature in the draining cervical lymph nodes. *Science Translational Medicine* 2014. **6**: 107.

6 **Hauser, S. L., Waubant, E., Arnold, D. L., Vollmer, T., Antel, J., Fox, R. J., Bar-Or, A., et al.,** B-Cell Depletion with Rituximab in Relapsing–Remitting Multiple Sclerosis. *New England Journal of Medicine* 2008. **358**: 676-688.

7 **Bar-Or, A., Fawaz, L., Fan, B., Darlington, P. J., Rieger, A., Ghorayeb, C., Calabresi, P. A., et al.,** Abnormal B-cell cytokine responses a trigger of T-cell–mediated disease in MS? *Annals of Neurology* 2010. **67**: 452-461.

8 **Magliozzi, R., Howell, O., Vora, A., Serafini, B., Nicholas, R., Puopolo, M., Reynolds, R., et al.,** Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. *Brain* 2007. **130**: 1089-1104.

9 **Kinnunen, T., Chamberlain, N., Morbach, H., Cantaert, T., Lynch, M., Preston-Hurlburt, P., Herold, K. C., et al.,** Specific peripheral B cell tolerance defects in patients with multiple sclerosis. *J Clin Invest* 2013. **123**: 2737-2741.

10 **Rawlings, D. J., Metzler, G., Wray-Dutra, M. and Jackson, S. W.,** Altered B cell signalling in autoimmunity. *Nat Rev Immunol* 2017. **17**: 421-436.

11 **Michel, L., Touil, H., Pikor, N. B., Gommerman, J. L., Prat, A. and Bar-Or, A.,** B Cells in the Multiple Sclerosis Central Nervous System: Trafficking and Contribution to CNS-Compartmentalized Inflammation. *Front Immunol* 2015. **6**.

12 **Jackson, S. W., Jacobs, H. M., Arkatkar, T., Dam, E. M., Scharping, N. E., Kolhatkar, N. S., Hou, B., et al.,** B cell IFN-gamma receptor signaling promotes autoimmune germinal centers via cell-intrinsic induction of BCL-6. *J Exp Med* 2016. **213**: 733-750.

13 **Peng, S. L., Szabo, S. J. and Glimcher, L. H.,** T-bet regulates IgG class switching and pathogenic autoantibody production. *Proc Natl Acad Sci U S A* 2002. **99**: 5545-5550.

14 **Piovesan, D., Tempany, J., Di Pietro, A., Baas, I., Yiannis, C., O'Donnell, K., Chen, Y., et al.,** c-Myb Regulates the T-Bet-Dependent Differentiation Program in B Cells to Coordinate Antibody Responses. *Cell Reports*. **19**: 461-470.

15 **Barnett, B. E., Staupe, R. P., Odorizzi, P. M., Palko, O., Tomov, V. T., Mahan, A. E., Gunn, B., et al.,** Cutting Edge: B Cell-Intrinsic T-bet Expression Is Required To Control Chronic Viral Infection. *J Immunol* 2016. **197**: 1017-1022.

16 **Knox, J. J., Buggert, M., Kardava, L., Seaton, K. E., Eller, M. A., Canaday, D. H., Robb, M. L., et al.,** T-bet+ B cells are induced by human viral infections and dominate the HIV gp140 response. *JCI Insight* 2017. **2**.

17 **Buljevac, D., Flach, Z., Hop, W., Hijdra, D., Laman, J., Savelkoul, H., van der Meché, F., et al.,** Prospective study on the relationship between infections and multiple sclerosis exacerbations. *Brain: a journal of neurology* 2002.

18 **Sindhava, V. J., Oropallo, M. A., Moody, K., Naradikian, M., Higdon, L. E., Zhou, L., Myles, A., et al.,** A TLR9-dependent checkpoint governs B cell responses to DNA-containing antigens. *The Journal of Clinical Investigation* 2017. **127**: 1651-1663.

19 **Jegerlehner, A., Maurer, P., Bessa, J., Hinton, H. J., Kopf, M. and Bachmann, M. F.,** TLR9 Signaling in B Cells Determines Class Switch Recombination to IgG2a. *The Journal of Immunology* 2007. **178**: 2415-2420.

20 **van Langelaar, J., van der Vuurst de Vries, R. M., Janssen, M., Wierenga-Wolf, A. F., Spilt, I. M., Siepman, T. A., Dankers, W., et al.,** T helper 17.1 cells associate with multiple sclerosis disease activity: perspectives for early intervention. *Brain* 2018. **141**: 1334-1349.

21 **van Nierop, G. P., van Luijn, M. M., Michels, S. S., Melief, M. J., Janssen, M., Langerak, A. W., Ouwendijk, W. J. D., et al.,** Phenotypic and functional characterization of T cells in white matter lesions of multiple sclerosis patients. *Acta Neuropathol* 2017. **134**: 383-401.

22 **Weksler, B. B., Subileau, E. A., Perriere, N., Charneau, P., Holloway, K., Leveque, M., Tricoire-Leignel, H., et al.,** Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *Faseb J* 2005. **19**: 1872-1874.

23 **Souwer, Y., Griekspoor, A., Jorritsma, T., de Wit, J., Janssen, H., Neefjes, J. and van Ham, S. M.,** B Cell Receptor-Mediated Internalization of *Salmonella*: A Novel Pathway for Autonomous B Cell Activation and Antibody Production. *The Journal of Immunology* 2009. **182**: 7473-7481.

24 **Sørensen, T. L., Trebst, C., Kivisäkk, P., Klaege, K. L., Majmudar, A., Ravid, R., Lassmann, H., et al.,** Multiple sclerosis: a study of CXCL10 and CXCR3 co-localization in the inflamed central nervous system. *Journal of Neuroimmunology* 2002. **127**: 59-68.

25 **Kalinowska-Łyszczarz, A., Szczuciński, A., Pawlak, M. A. and Losy, J.,** Clinical study on CXCL13, CCL17, CCL20 and IL-17 as immune cell migration navigators in relapsing–remitting multiple sclerosis patients. *Journal of the Neurological Sciences* 2011. **300**: 81-85.

26 **Elgueta, R., Marks, E., Nowak, E., Menezes, S., Benson, M., Raman, V. S., Ortiz, C., et al.,** CCR6-Dependent Positioning of Memory B Cells Is Essential for Their Ability To Mount a Recall Response to Antigen. *The Journal of Immunology* 2015. **194**: 505-513.

27 **Saraste, M., Penttila, T. L. and Airas, L.,** Natalizumab treatment leads to an increase in circulating CXCR3-expressing B cells. *Neurol Neuroimmunol Neuroinflamm* 2016. **3**: e292.

28 **Eickhoff, K., Kaschka, W., Skvaril, F., Theilkaes, L. and Heipertz, R.,** Determination of IgG subgroups in cerebrospinal fluid of multiple sclerosis patients and others. *Acta Neurologica Scandinavica* 1979. **60**: 277-282.

29 **Muehlinghaus, G., Cigliano, L., Huehn, S., Peddinghaus, A., Leyendeckers, H., Hauser, A. E., Hiepe, F., et al.,** Regulation of CXCR3 and CXCR4 expression during terminal differentiation of memory B cells into plasma cells. *Blood* 2005. **105**: 3965-3971.

30 **O'Donnell, H. and McSorley, S.,** Salmonella as a model for non-cognate Th1 cell stimulation. *Frontiers in Immunology* 2014. **5**.

31 **Rubtsova, K., Rubtsov, A. V., Thurman, J. M., Mennona, J. M., Kappler, J. W. and Marrack, P.,** B cells expressing the transcription factor T-bet drive lupus-like autoimmunity. *The Journal of Clinical Investigation* 2017. **127**: 1392-1404.

32 **Zhang, Y., Garcia-Ibanez, L. and Toellner, K.-M.,** Regulation of germinal center B-cell differentiation. *Immunological reviews* 2016. **270**: 8-19.

33 **Willis, S. N., Stathopoulos, P., Chastre, A., Compton, S. D., Hafler, D. A. and O'Connor, K. C.,** Investigating the Antigen Specificity of Multiple Sclerosis Central Nervous System-Derived Immunoglobulins. *Frontiers in immunology* 2015. **6**: 600-600.

34 **Palanichamy, A., Apeltsin, L., Kuo, T. C., Sirota, M., Wang, S., Pitts, S. J., Sundar, P. D., et al.,** Immunoglobulin class-switched B cells form an active immune axis between CNS and periphery in multiple sclerosis. *Science translational medicine* 2014. **6**: 106.

35 **Baker, D., Pryce, G., Amor, S., Giovannoni, G. and Schmierer, K.,** Learning from other autoimmunities to understand targeting of B cells to control multiple sclerosis. *Brain* 2018. **141**: 2834-2847.

36 **Balashov, K. E., Rottman, J. B., Weiner, H. L. and Hancock, W. W.,** $CCR5^+$ and $CXCR3^+$ T cells are increased in multiple sclerosis and their ligands MIP-1α and IP-10 are expressed in demyelinating brain lesions. *Proceedings of the National Academy of Sciences* 1999. **96**: 6873-6878.

37 **Jelcic, I., Al Nimer, F., Wang, J., Lentsch, V., Planas, R., Jelcic, I., Madjovski, A., et al.,** Memory B Cells Activate Brain-Homing, Autoreactive CD4+ T Cells in Multiple Sclerosis. *Cell* 2018. **175**: 85-100.e123.

38 **Krumbholz, M., Theil, D., Cepok, S., Hemmer, B., Kivisäkk, P., Ransohoff, R. M., Hofbauer, M., et al.,** Chemokines in multiple sclerosis: CXCL12 and CXCL13 up-regulation is differentially linked to CNS immune cell recruitment. *Brain* 2006. **129**: 200-211.

39 **Magliozzi, R., Columba-Cabezas, S., Serafini, B. and Aloisi, F.,** Intracerebral expression of CXCL13 and BAFF is accompanied by formation of lymphoid follicle-like structures in the meninges of mice with relapsing experimental autoimmune encephalomyelitis. *Journal of Neuroimmunology* 2004. **148**: 11-23.

40 **Rainey-Barger, E. K., Rumble, J. M., Lalor, S. J., Esen, N., Segal, B. M. and Irani, D. N.,** The lymphoid chemokine, CXCL13, is dispensable for the initial recruitment of B cells to the acutely inflamed central nervous system. *Brain, behavior, and immunity* 2011. **25**: 922-931.

41 **Allen, C. D. C., Ansel, K. M., Low, C., Lesley, R., Tamamura, H., Fujii, N. and Cyster, J. G.,** Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nature Immunology* 2004. **5**: 943.

42 **Ruprecht, C. R. and Lanzavecchia, A.,** Toll-like receptor stimulation as a third signal required for activation of human naive B cells. *European Journal of Immunology* 2006. **36**: 810-816.

43 **Butchi, N. B., Woods, T., Du, M., Morgan, T. W. and Peterson, K. E.,** TLR7 and TLR9 Trigger Distinct Neuroinflammatory Responses in the CNS. *The American Journal of Pathology* 2011. **179**: 783-794.

44 **Sorensen, P. S., Koch-Henriksen, N., Petersen, T., Ravnborg, M., Oturai, A. and Sellebjerg, F.,** Recurrence or rebound of clinical relapses after discontinuation of natalizumab therapy in highly active MS patients. *Journal of Neurology* 2014. **261**: 1170-1177.

45 **Serafini, B., Scorsi, E., Rosicarelli, B., Rigau, V., Thouvenot, E. and Aloisi, F.,** Massive intracerebral Epstein-Barr virus reactivation in lethal multiple sclerosis relapse after natalizumab withdrawal. *Journal of Neuroimmunology* 2017. **307**: 14-17.

46 **Wang, S., Wang, J., Kumar, V., Karnell, J. L., Naiman, B., Gross, P. S., Rahman, S., et al.,** IL-21 drives expansion and plasma cell differentiation of autoreactive CD11chiT-bet+ B cells in SLE. *Nature Communications* 2018. **9**: 1758.

47 **Lünemann, J. D., Jelcić, I., Roberts, S., Lutterotti, A., Tackenberg, B., Martin, R. and Münz, C.,** EBNA1-specific T cells from patients with multiple sclerosis cross react with myelin antigens and co-produce IFN-gamma and IL-2. *The Journal of experimental medicine* 2008. **205**: 1763-1773.

48 **Lanzavecchia, A.,** Antigen-specific interaction between T and B cells. *Nature* 1985. **314**: 537-539.

49 **Schwartz, D. M., Kanno, Y., Villarino, A., Ward, M., Gadina, M. and O'Shea, J. J.,** JAK inhibition as a therapeutic strategy for immune and inflammatory diseases. *Nature Reviews Drug Discovery* 2017. **16**: 843.

50 **Capolunghi, F., Rosado, M. M., Cascioli, S., Girolami, E., Bordasco, S., Vivarelli, M., Ruggiero, B., et al.,** Pharmacological inhibition of TLR9 activation blocks autoantibody production in human B cells from SLE patients. *Rheumatology* 2010. **49**: 2281-2289.

3

The role of autoimmunity-related gene *CLEC16A* in the B cell receptor-mediated HLA class II pathway

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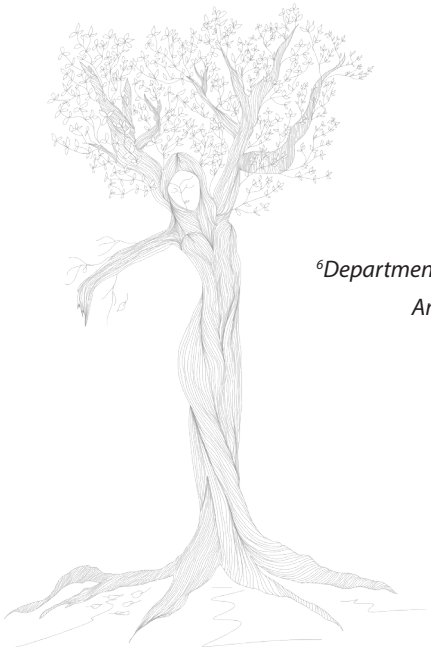

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ABSTRACT

C-type lectin *CLEC16A* is located next to *CIITA*, the master transcription factor of HLA class II (HLA-II), at a susceptibility locus for several autoimmune diseases including multiple sclerosis (MS). We previously found that *CLEC16A* promotes the biogenesis of HLA-II peptide-loading compartments (MIICs) in myeloid cells. Given the emerging role of B cells as antigen-presenting cells in these diseases, we here addressed whether and how *CLEC16A* is involved in the B cell receptor (BCR)-dependent HLA-II pathway. *CLEC16A* was co-expressed with surface class II-associated invariant chain peptide (CLIP) in human EBV-positive and not EBV-negative B-cell lines. Stable knockdown of *CLEC16A* in EBV-positive Raji B cells resulted in an upregulation of surface HLA-DR and CD74 (invariant chain), while CLIP was slightly, but significantly, reduced. In addition, IgM-mediated *Salmonella* uptake was decreased and MIICs were less clustered in *CLEC16A*-silenced Raji cells, implying that *CLEC16A* controls both HLA-DR/CD74 and BCR/antigen processing in MIICs. In primary B cells, *CLEC16A* was only induced under CLIP-stimulating conditions *in vitro* and was predominantly expressed in $CLIP^{high}$ naive populations. Finally, CLIP-loaded HLA-DR molecules were abnormally enriched and co-regulation with *CLEC16A* was abolished in blood B cells of patients who rapidly develop MS. These findings demonstrate that CLEC16A participates in the BCR-dependent HLA-II pathway in human B cells and that this regulation is impaired during MS disease onset. The abundance of CLIP already on naive B cells of MS patients may point to a chronically induced stage and a new mechanism underlying B cell-mediated autoimmune diseases such as MS.

INTRODUCTION

HLA class II (HLA-II) is a major risk factor for various autoimmune diseases, including multiple sclerosis (MS) [1]. During autoimmune pathogenesis, certain antigens are alternatively processed and/or presented by HLA-II molecules to trigger autoreactive, or maybe even suppress regulatory $CD4^+$ T cells [2, 3]. The strong clinical effects of anti-CD20 therapy put forward B cells as key players in such diseases by acting as potent antigen-presenting cells [4-6]. Currently, not much is known about which and how genes related to autoimmunity contribute to changes in the HLA-II antigen presentation pathway of B cells [2, 7, 8].

Multiple genome-wide association studies (GWAS) have demonstrated that C-type lectin gene *CLEC16A* is associated with susceptibility to type I diabetes [9], rheumatoid arthritis [10], multiple sclerosis (MS) [11] and other autoimmune diseases [12]. *CLEC16A* is located at the 16p13 region next to *CIITA,* which is the master transcription factor of HLA-II [13]. C-type lectins are known to recognize and guide antigens into the endosomal system for processing and loading onto HLA-II molecules [14]. Although it lacks a functional antigen recognition domain, *CLEC16A* has been demonstrated to induce autoimmunity in mice, probably by stimulating thymic epithelial cell antigen presentation [15]. Previously, we found that CLEC16A participates in retrograde transport of HLA-II-containing compartments (MIICs) in myeloid cells [16]. How this gene influences the B cell-intrinsic HLA-II pathway has not been studied so far.

The HLA-II pathway in B cells is a unique and tightly controlled process, in which the B-cell receptor (BCR) mediates antigen capture, uptake and processing in MIICs [17]. To reach these compartments, newly synthesized HLA-II α- and β-chains in the endoplasmic reticulum need to bind to the invariant chain (CD74), which guides their transport into the MIICs either directly or indirectly via the plasma membrane [18]. In the MIICs, the invariant chain is cleaved by specific proteases, leaving class II-associated invariant chain peptides (CLIP) bound to the HLA-II peptide-binding groove. After exchange of CLIP for antigenic peptides, HLA-II/peptide complexes are transported to the plasma membrane for presentation [19].

In this study, we addressed how *CLEC16A* is regulated and influences the HLA-II pathway in human B cells. *CLEC16A* is found to mediate BCR-mediated antigen uptake, HLA-DR/CD74 processing and MIIC biogenesis in Raji B cells. Furthermore, surface CLIP is co-regulated with *CLEC16A* in both B-cell lines and primary B cells, which is enhanced after CD40 triggering. This process seems to be disturbed during the development of MS, since CLIP is abnormally expressed and its co-regulation with *CLEC16A* is lost in B cells from early MS patients.

MATERIALS AND METHODS

Patients

Both clinically isolated syndrome (CIS) and relapsing-remitting MS (RRMS) patients, as well as healthy controls (HC) were included in the MS Center ErasMS at Erasmus MC (Rotterdam, The Netherlands). All patients and controls gave written informed consent and study protocols were approved by the medical ethics committee (MEC) of the Erasmus MC. CIS was defined as a first clinical attack of demyelination in the central nervous system [20] and diagnosed according to the 2017 revised McDonald criteria [21]. The diagnosis of clinically definite MS (CDMS) was made when a patient experienced two attacks with clinical evidence of two separate lesions according to the Poser criteria [22]. CIS patients were sampled within 4 months after their first attack. From our prospective CIS cohort (PROUD, MEC-2006-188), we selected patients who were diagnosed with CDMS within 1 year after CIS diagnosis (high-risk CIS) and patients who did not develop CDMS for at least 5 years of follow-up (low-risk CIS). These patients were matched for age and gender. RRMS patients were diagnosed according to the McDonalds criteria [23] and matched with healthy controls for both age and gender (MEC-2014-033). Patient characteristics are summarized in Table I. Buffy coats were obtained from healthy volunteers (Sanquin, Amsterdam, The Netherlands).

Table I . Clinical information of patients and healthy controls.

		Patients, no	Gender, female no (%)	Age in years, average (IQR)	Follow-up time in years, median (IQR)*	Disease duration in months before analysis, median (IQR) †	Treatment, no
HC		12	9 (75%)	43 (21-65)	NA	NA	NA
CIS	Low-risk	11	7 (64%)	38 (23-48)	8.4 (6.2 - 11.8)	2.0 (0.9 - 3.8)	2 (MP)
	High-risk	9	7 (78%)	30 (25-37)	4.2 (3.1 - 5.1)	2.1 (0.1 - 3.9)	2 (MP)
RRMS		12	9 (75%)	43 (21-63)	NA	NA	1 (Copaxone)

*, at time of sampling; †, time CIS to sampling; RRMS according to the McDonald 2010 criteria. Abbreviations: HC, healthy control; CIS, clinically isolated syndrome; IQR, interquartile range; MP, methylprednisolone; NA, not applicable or available; RRMS, relapsing-remitting MS.

Blood sampling

PBMCs and plasma were isolated from whole blood with the use of CPT™ heparin tubes, while serum was isolated using coagulation tubes (both BD Biosciences, San Jose, CA). Samples were processed according to the manufacturer's instructions. PBMCs were stored in liquid nitrogen; plasma and serum were stored in -80°C until analysis.

SNP genotyping

Whole blood samples were collected from CIS patients and DNA was isolated by a standardized method [24]. Samples were genotyped on the Immuno-Chip and were subjected to the standard quality controls [25]. Genotyping was done within the framework of the International Multiple Sclerosis Genetics Consortium (IMSGC) [26].

Human B-cell lines

Ten Epstein-Barr virus (EBV)-positive (Raji, Daudi, EB1, JVM2, JVM3, JVM13, Namalwa, DoHH2, EHEB, AKATA) and eight EBV-negative (Ramos, DG75, CA24, U698-M, MC116, ROS-50, WSU-NHL, Karpas422) human B lymphoma and leukemia cell lines (B-LCLs) were used in this study. All B-LCLs were purchased from DSMZ or ATCC.

CLEC16A antibody production

CLEC16A monoclonal antibodies (clone 7A4 [16] and clone 4F11; both IgG2b) were generated by immunizing F344 (SAS FISCH) rats with a 27-mer C-terminal human peptide (CLEC16A954-980: VIVNETEADSKPSKNVARSAAVETASL) linked to KLH and standard monoclonal antibody production services performed by ImmunoPrecise Ltd (Victoria, Canada). The fidelity of hybridoma clones was initially screened by ELISA using peptide-BSA coated wells and then by probing HEK293T cells transfected with either vector alone (pEGFP; Clontech, Palo Alto, CA) or bearing full-length human *CLEC16A* cDNA. Monoclonal CLEC16A antibodies were found to be applicable for flow cytometry by binding to paraformaldehyde-fixed protein and Western blotting using CLEC16A-transfected HEK293T cells.

Flow cytometry and cell sorting

In-depth flow cytometric analysis and cell sorting of B cells were performed using anti-human monoclonal antibodies against CD3 (SK7), CD19 (HIB16), CD38 (HIT2), CD74 (LN2), HLA-DM (MaP.DM1), HLA-DR (L243), IgM (MHM-88, all Biolegend, London, UK), CD27 (M-T271), IgD (IA6-2), IgG (G18-145, all BD Biosciences) CLIP (CerCLIP.1, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and IgA (IS11-8E10, Miltenyi-Biotec GmbH, Bergisch Gladbach, Germany). FVS700 (BD Biosciences) was used for the detection of viable cells. The used CerCLIP.1 antibody specifically recognizes CLIP-loaded HLA-II, while L243 antibody detects peptide-loaded HLA-II (called HLA-DR; [7]). For intracellular staining of CLIP and HLA-DR, cells were fixed with 2% paraformaldehyde and permeabilized with PBS pH7.4 containing 0.2% BSA and 0.5% saponin (Sigma-Aldrich). For intracellular staining of CLEC16A, the BD Cytofix/Cytoperm™ kit (BD Biosciences) was used and staining was performed according to the manufacturer's protocol. Cells were first stained with rat-anti-human CLEC16A antibody (7A4) followed by a FITC-labeled $F(ab')_2$ fragment donkey-anti-rat IgG (H+L) (Jackson Immuno Research, UK). A rat-anti-human IgG2b antibody (Biolegend)

was used as a negative control. Total ($CD19^+$) and naive mature ($CD38^{dim}CD27^-$) B cells were isolated using a high-speed cell sorter (FACSAriaIII; BD Biosciences), resulting in purities of more than 95%. All measurements were performed on an LSRFortessa flow cytometer and data were analyzed using FACSDiva 8.1 software (both BD Biosciences).

RNA isolation and quantitative PCR

Total RNA was isolated using GenElute™ Mammalian RNA Kit (Sigma-Aldrich, St Louis, MO) and mRNA was reversely transcribed into cDNA following a standard laboratory protocol with the use of SuperScript II® Reverse Transcriptase (Invitrogen, Paisley, UK). Primers and probes were selected by using the Universal Probe Library Assay Design Centre (Roche Applied Science, Penzberg, Germany). To quantify target gene expression levels, qPCR was performed using an Applied Biosystems QuantStudio 5, which was programmed for the initial step of 2 min at 50°C and 10 min 95°C, followed by 40 thermal cycles of 15s at 95°C and 1 min at 60°C. For the calculation of relative mRNA expression, CT values were related to standard curves, which were generated for each gene of interest. All data were normalized to 18S rRNA levels. *HLA-DOB* expression was determined using Power SYBR green (Invitrogen) and measured on an Applied Biosystems StepOnePlus. Primer sequences are listed in Table II.

Table II. Primers used for qPCR.

Gene	Forward primer	Reverse primer
BTK	TGTTGAAACAGTGGTTCCTGA	TGCTCCATTTCACTGGACTCT
CBLB	GTGCACCTCTTGCCTTACG	CCTTTTATTTCACAACGACAGAAA
CD74	ATGAGCAACTGCCCATGC	CAGGATGGAAAAGCCTGTGT
CIITA	AACAGGATTCACGGATCAGC	CAGCGTGGTTAGTGTCCTCA
CLEC16A	TGCCCCTCTACGTGTACTCA	GAGACACCGGCAGGCTAAT
HLA-DMB	GAACTCCCGGCATCTTTACA	CAACAGACAGGTGCTTTCCA
HLA-DOB	GGAGAAAGATGCTGAGTGGC	GCTCTTGAGACCTCATTACC
HLA-DRA	AGCCTCTTCTCAAGCACTGG	GGCACACACCACGTTCTCT
IFI30	CTACGGAAACGCACAGGAA	TCTTCTCCATGCTGGCACTT
MARCH1	TCAGGACATCTGCAGAATCTGTCAC	TCACAGCAGCGTGTATCTGAG

In vitro activation of human B cells

B cells of healthy donors were negatively sorted using the B-cell isolation kit II and MACS (Miltenyi Biotec). B cells were cultured in RPMI supplemented with 5% fetal calf serum, 1% penicillin/streptavidin, 0.1% beta-mercaptoethanol, 1% Ultraglutamine, 0.1% apotransferrin (depleted for human IgG with prot-G sapharose). B cells were activated for 24 and 48 hours using various stimulation cocktails including anti-IgM (10 µg/ml, Jackson Immuno Research), sCD40L (100 ng/ml, Enzo Life Sciences, New York, USA), CpG (10 µg/ml,

Invitrogen), IFN-γ (100 ng/ml, Peprotech, London, UK) or IL-21 (50 ng/ml, Thermo Fisher Scientific, Massachusetts, USA).

Western blotting

For western blotting, cells were lysed in radio-immunoprecipitation assay (RIPA) lysis buffer supplemented with 10% complete protease inhibitor cocktail (Roche, Mannheim, Germany) on ice for 30 minutes, centrifuged at 4°C for 10 minutes at 10,000 x *g*. Cell lysates were reduced with 10% 2-mercaptoethanol, denatured for 5min at 95°C, loaded onto a 10% precast polyacrylamide gel (Bio-Rad, Hercules, CA) followed by immunoblotting on a Immobilon-P membrane (Merck Millipore, Darmstadt, Germany) for 1 hours at 4°C. Membranes were blocked in 5% nonfat dry milk and incubated with rat-anti-human CLEC16A (clone 7A4 and 4F11; both IgG2b), rat-anti-human IgG2b isotype control (Biolegend) or mouse anti-human β-actin (AC-15; Abcam, Cambridge, UK). Horseradish peroxidase (HRP)-conjugated rabbit-anti-rat or goat-anti-mouse (Dako, Glostrup, Denmark) were used as secondary antibodies. Protein bands were visualized using Western Lightning Plus-ECL (Perkin Elmer Inc., Waltham, MA).

Generation of stable CLEC16A shRNA B-cell transfectants

For CLEC16A knockdown, human B-LCLs Raji and Ramos were transfected with CLEC16A shRNA-containing pLKO.1 constructs (MISSION® shRNA Library, Sigma-Aldrich) using Nucleofector Kit V and AMAXA nucleofector II, both from Lonza (Basel, Switzerland). Two CLEC16A shRNAs were used to exclude off-target effects: #1, CAGCTCTGTATTTGACTTCTT and #2, GCTAAGACTGAACAGGATATT. After three days, puromycin (InvivoGen, Toulouse, France) was added at 0.25 µg/ml, which was increased to 0.5 µg/ml after one week of culturing. CLEC16A levels were compared between CLEC16A and scrambled shRNA transfectants at the same day of culture.

BCR-mediated uptake and processing of Salmonella

Salmonella Typhimurium SL1344 was used as a model system to assess IgM-mediated uptake and localization in MIICs, as described previously [27-29]. In short, anti-human IgM (MH15, Sanquin, Amsterdam, The Netherlands) was mixed with anti-*Salmonella* LPS (1E6, Invitrogen) and rat anti-mouse IgG1 (RM161-1, Sanquin) to generate stable tetrameric antibody complexes. Exponentially grown bacteria were washed twice with PBS, incubated with tetrameric antibody complexes for 30 min at RT and washed again to remove unbound antibodies. Scrambled and CLEC16A shRNA-transfected Raji cells were incubated with viable anti-IgM coated GFP-*Salmonella T.* [30] at 20 bacteria/cell for 45 min at 37°C without antibiotics. Cells were washed 3 times and cultured for 1 h in RPMI supplemented with 5% fetal calf serum, 1% penicillin/streptavidin, 0.1% beta-mercaptoethanol,

1% Ultraglutamine, 0.1% apotransferrin (depleted for human IgG using prot-G sepharose) and 100 mg/ml gentamicin (Invitrogen) to eliminate non-phagocytosed bacteria. Finally, cells were added to poly-L-lysine coated diagnostic black slides for 45 min and fixed with 4% PFA for 15 min at RT.

Immunofluorescence microscopy and analysis

For immunofluorescence staining, fixed cells were permeabilized with 0.1% Triton X-100/PBS for 10 min at RT. Slides were then subsequently incubated with primary antibodies mouse-anti-human CD63 (MEM-259, Abcam) and rabbit-anti-HLA-DR (kind gift from J. Neefjes) (1 h, RT) followed by staining with goat anti-rabbit AF594 and anti-mouse AF647 (Molecular Probes) (30 min, RT) as well as DAPI (Thermo Fisher Scientific; 10 min, RT). Finally, slides were mounted in Vectashield (Vector Laboratories, Peterborough, UK) and analyzed using the confocal laser-scanning microscope Zeiss LSM 700 and ZEN software (Carl Zeiss, Oberkochen, Germany). We counted the number and calculated the percentage of B cells with clustered MIICs based on CD63 and HLA-DR expression in at least ten different images, with a minimum of 10 cells per image. The images were scored by two independent observers. We also determined the percentage of cells with *Salmonella T.* present inside MIICs. We analyzed 21-133 cells and 57-291 *Salmonella T.* bacteria in at least 13 images per condition, with an average of two bacteria per cell in each condition.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA). Data are expressed as median ± interquartile range (IQR), unless stated otherwise. Mann-Whitney-U tests and unpaired t-tests were used to compare data between two groups. Wilcoxon matched-pairs signed-rank and paired t-tests were used to analyze paired datasets. Kruskal-Wallis or Friedman including Dunn's multiple comparison tests, as well as one- or two-way ANOVA with Bonferroni's corrections were used to compare more than two groups. All correlations were tested based on Spearman rho. P values less than 0.05 were considered as statistically different.

RESULTS

EBV-positive and not -negative human B-cell lines show a correlation between surface CLIP and CLEC16A expression

Previously, we have shown that autoimmunity-related gene *CLEC16A* regulates MIIC biogenesis in human myeloid cells [16]. Since HLA-II peptide loading efficiency is determined in MIICs [18] and the presence of CLIP/HLA-II complexes on the plasma membrane

is considered a measure of inefficient antigenic peptide presentation [7], we assessed how both surface CLIP and HLA-DR levels are influenced by *CLEC16A* expression in human B cells. Since it is at least a challenge to modify genes in primary B cells, we first screened 10 EBV$^+$ and 8 EBV$^-$ human B-LCLs for CLIP, HLA-DR and *CLEC16A* expression. CLIP and not HLA-DR was expressed at a higher level on EBV$^+$ versus EBV$^-$ B-LCLs (Figure 1A). Only in EBV$^+$ B-LCLs, CLIP and CLIP-loaded HLA-DR molecules positively correlated to *CLEC16A* expression, which was not seen for HLA-DR expression alone (Figure 1B and Supplementary Figure 1A) or in EBV$^-$ B-LCLs (data not shown). These correlations were not driven by expression differences in HLA-DM and HLA-DO, two known chaperones of HLA-II peptide loading

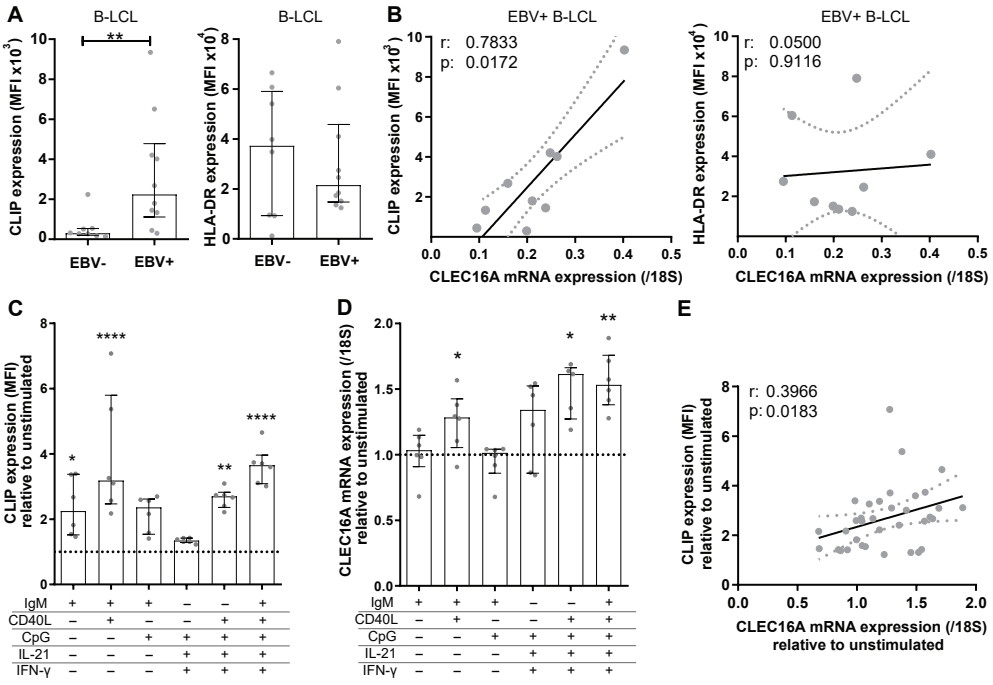

Figure 1. Association of surface CLIP and *CLEC16A* expression in unstimulated and stimulated human B-cell lines.

(A) Eight EBV$^-$ and ten EBV$^+$ B-LCLs were screened for surface CLIP and HLA-DR expression using FACS. **(B)** Correlation of *CLEC16A* with surface CLIP and HLA-DR levels in EBV$^+$ B-LCLs. **(C-E)** CLIP (48h) and *CLEC16A* (24h) induction in CLIP^{low} (EBV$^-$) B-LCL Ramos after CD40L-mediated stimulation. Expression levels are depicted as median ± IQR and were calculated as relative to unstimulated cells (dotted line). Mann-Whitney U test **(A)** or Friedman including Dunn's multiple comparison tests **(C, D)** were performed on unprocessed data (Supplementary Figure 1E). Conditions were compared to unstimulated B cells from the same donor (dotted line). For **B** and **E**, Spearman's correlation coefficients were calculated. * p<0.05, ** p<0.01, **** p<0.0001.

[31] (Supplementary Figure 1B), or other MS- and B cell-associated genes, including *CBLB*, *IFI30* and *MARCH1* (Supplementary Figure 1C). *In vitro* activation of CLIP^{low} (EBV^{-}) Ramos B cells (Supplementary Figure 1D) using CD40L-containing cocktails triggered both surface CLIP and *CLEC16A* expression (Figure 1C-E and Supplementary Figure 1E). These findings suggest that *CLEC16A* is associated with processing and presentation of CLIP-loaded HLA-II molecules by B cells.

CLEC16A knockdown in EBV-positive Raji B cells causes cytoplasmic scattering of MIIC and interferes with CLIP/HLA-DR and CD74 surface expression

In line with previous work on HLA-II biology in human B cell lines [32], we used CLIP^{high} Raji (EBV^{+}) B cells (Supplementary Figure 1D) to generate stable CLEC16A knockdowns using two different shRNA constructs. In both CLEC16A shRNA transfectants, we found an approximately 50% reduction in *CLEC16A* expression, while levels of *HLA-DRA* and its master regulator *CIITA* remained unaffected (Figure 2A) compared to scrambled controls (dotted line). This resulted in significantly elevated HLA-DR (mean increase: 37%) and CD74 (the precursor of CLIP; mean increase: 70%) and reduced CLIP (mean decrease: 15%) surface expression levels (Figure 2B and Supplementary Figure 2A). Stable *CLEC16A* knockdown in CLIP^{low} Ramos B cells did not affect surface HLA-DR, CD74 and CLIP (Supplementary Figure 2B). Based on our earlier study [16], these discrepancies in HLA-II-associated CD74 and CLIP surface expression in Raji B cells after *CLEC16A* knockdown are probably related to disrupted processing in MIICs. To test whether the localization of MIICs was affected in *CLEC16A*-silenced Raji B cells, we analyzed how and where late endosomal markers HLA-DR and CD63 were co-expressed using confocal microscopy. MIICs in *CLEC16A* shRNA transfectants were more scattered than clustered (Supplementary Figure 2C) compared to scrambled shRNA transfectants from the same experiments (Figure 2C and D; n=5). Consistent with our FACS results, HLA-DR seemed to be more expressed at the plasma membrane of CLEC16A shRNA transfectants (Figure 2C). These results imply that the recruitment of HLA-DR/CD74 to MIICs is controlled by *CLEC16A* in Raji B cells.

BCR-mediated antigen uptake and transport into MIICs is impaired in CLEC16A-silenced Raji B cells

In contrast to other types of antigen-presenting cells, antigen uptake and processing by human B cells critically depends on interaction with highly specific BCRs. This interaction not only results in internalization into the late endosomal system, but also triggers the biogenesis of MIICs [17, 33]. To determine whether *CLEC16A* participates in this tightly regulated process and overcome the highly diverse antigen specificity of BCRs, we used a model in which GFP^{+} *Salmonella Typhimurium* was coated with tetrameric antibody complexes for

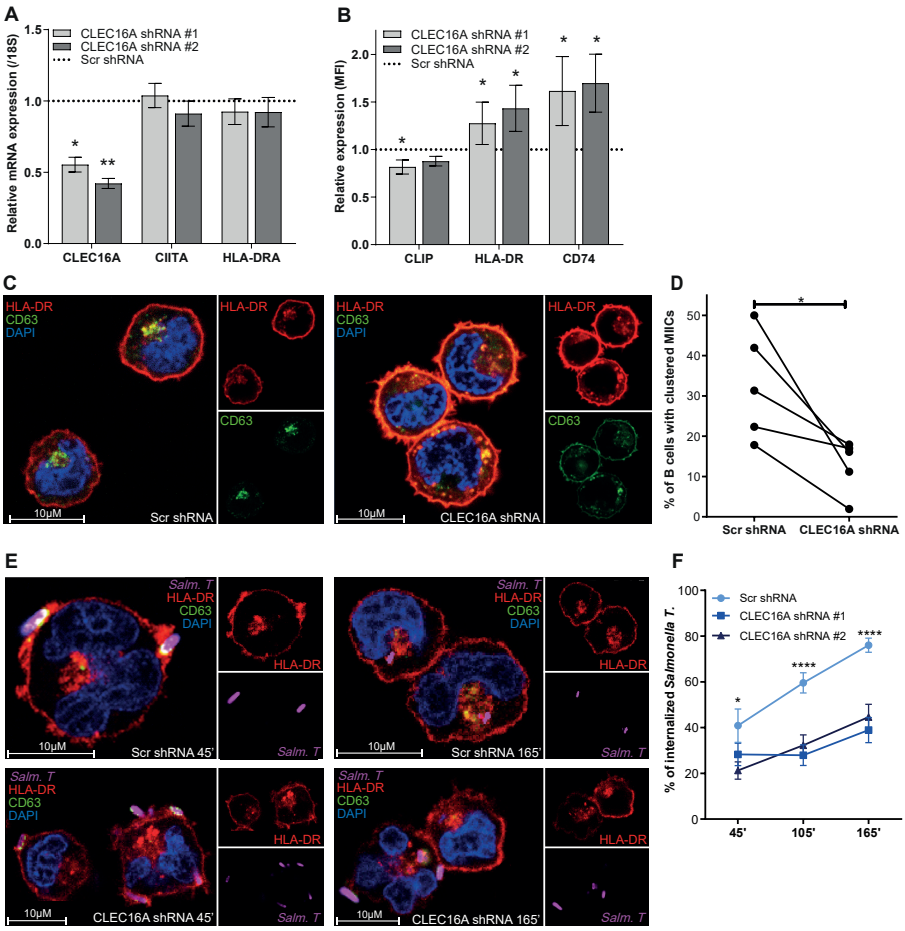

Figure 2. Effects of *CLEC16A* knockdown on surface expression of CLIP, HLA-DR and CD74 and cytosolic distribution of MIICs in CLIP^{high} Raji.

Relative *CLEC16A, CIITA* and *HLA-DRA* **(A)** and surface CLIP, HLA-DR and CD74 **(B)** levels in two different CLEC16A shRNA Raji transfectants after 25 days of puromycin selection. Data were compared to paired scrambled (scr) transfectants at the same day (dotted line, n=7-9). Expression levels are depicted as mean ± standard error of the mean, and are normalized to scrambled transfectants (dotted line). Paired t-tests were performed on unprocessed data (Supplementary Figure 2A). **(C)** Representative confocal images for HLA-DR and CD63 expression in CLEC16A and scrambled shRNA Raji transfectants (more examples in Supplementary Figure 2C). **(D)** Percentages of CLEC16A and scrambled shRNA transfectants with clustered MIICs (n=5). Representative images **(E)** and quantification of IgM-mediated *Salmonella* uptake after various incubation times **(F)** for two different CLEC16A and scrambled shRNA Raji transfectants. Data are representative for two independent experiments, with 21-133 cells and 57-291 bacteria in at least 13 images per condition analyzed. Paired t-test **(D)** or two-way ANOVA **(F)** was performed. In **F**, CLEC16A shRNA #1 and #2 were compared to the scrambled shRNA transfectants from the same experiment. * p<0.05, ** p<0.01, **** p<0.0001.

BCR-mediated transport into MIICs [27]. Both scrambled and CLEC16A shRNA Raji transfectants were incubated with viable anti-IgM-coated GFP-*Salmonella* and analyzed for internalization after different time points. In scrambled shRNA transfectants, *Salmonella* uptake and delivery to MIICs increased in time, resulting in 75-80% of *Salmonella*-containing cells after 165 min (Figure 2E and F). This was significantly lower in the two different CLEC16A shRNA transfectants, reaching a maximum uptake of approximately 40% (Figure 2F). These data indicate that *CLEC16A* regulates BCR-mediated uptake of antigens into MIICs in Raji B cells. Together with our earlier results, this implies that after antigen encounter, *CLEC16A* promotes the B cell-intrinsic HLA-II pathway by driving the retrograde trafficking of both HLA-DR/CD74 and BCR/antigen complexes for processing in the MIICs.

Surface CLIP positively correlates to CLEC16A expression in ex vivo and in vitro-activated human B cells

To translate our findings to primary B cells, we studied the correlation between CLIP-loaded HLA-DR molecules and CLEC16A expression in *ex vivo* and *in vitro*-stimulated blood B cells. Similar to EBV^+ B-cell lines, a strong correlation was found between *CLEC16A* levels and CLIP, but not HLA-DR surface expression in B cells of healthy blood donors (Figure 3A). In contrast, CLIP did not correlate to other genes that regulate the HLA-II pathway such as *CIITA, CD74, HLA-DMB* and *HLA-DOB* expression levels (Supplementary Figure 2D). To analyze this association on subset level, we separated transitional, naive mature, non-switched (IgM-only and natural effector) as well as switched (IgG^+ and IgA^+) memory B cells from buffy coats (Supplementary Figure 3A). Both surface CLIP (Figure 3B) and *CLEC16A* (Figure 3C) were more expressed in transitional, naive mature and natural effector than in germinal center-associated IgM-only, IgG^+ and IgA^+ B cells. This suggests that *CLEC16A* is particularly involved in the HLA-II pathway of naive cells, and is downregulated together with surface CLIP after maturation into potent antigen-presenting memory cells in germinal centers [34].

To further explore this co-regulation of CLIP and *CLEC16A*, B cells from healthy donors were stimulated with various combinations of T cell-dependent and -independent B-cell stimuli (anti-IgM, sCD40L, IL-21, CpG-ODN and IFN-γ) and analyzed using qPCR (24h) and FACS (48h). Under both germinal center-related and unrelated conditions (with and without IL-21), sCD40L was the strongest trigger for CLIP-loaded HLA-DR molecules and *CLEC16A* in B cells (Figure 4A-C and Supplementary Figure 3B). The positive correlation between surface CLIP and *CLEC16A* expression during *in vitro* activation (Figure 4D) is consistent with our *ex vivo* findings (see Figure 3A). We were able to validate the association of *CLEC16A* with surface CLIP on protein level; CLEC16A was induced and co-expressed with CLIP in B cells after stimulation with anti-IgM and CD40L, as determined by Western blotting and FACS (Figure 4E-H; Supplementary Figure 3C).

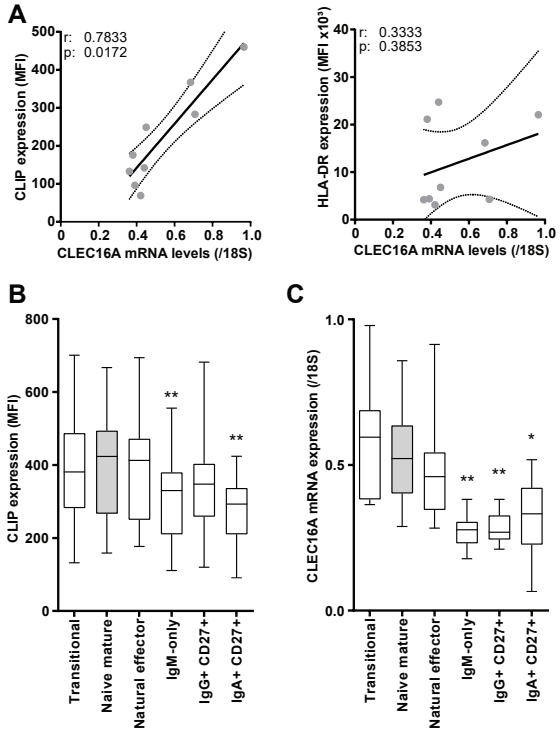

Figure 3. Association of surface CLIP and *CLEC16A* expression levels in GC-independent and -dependent B-cell subsets.

Total B cells of healthy controls (n=9, **A**) as well as individually sorted naive and memory B-cell subsets **(B-C)** from healthy blood donors (n=7-11) were analyzed for CLIP, HLA-DR **(A)** and CLEC16A expression using flow cytometry and qPCR, respectively. The following subsets were sorted by FACS: transitional ($IgM^+CD27^-CD38^{high}$), naive mature ($IgM^+CD27^-CD38^{-/dim}$), natural effector ($IgM^+IgD^+CD27^+$), IgM-only ($IgM^+IgD^-CD27^+$), IgG^+CD27^+ and IgA^+CD27^+ B cells. The used gating strategy is depicted in Supplementary Figure 3A. Data are shown as median ± IQR. Spearman's rank-order correlation **(A)** or Wilcoxon matched-pairs signed rank tests **(B, C)** were applied.* $p<0.05$, ** $p<0.01$

These data demonstrate that T cell-dependent B-cell activation by CD40L triggers both surface CLIP and *CLEC16A* expression in healthy individuals. This probably makes naive B cells ready for optimal processing of IgM-internalized antigens in MIICs before developing into professional antigen-presenting memory cells [33].

CLIP-loaded HLA-II molecules are upregulated on ex vivo blood B cells of early onset MS patients

To explore the antigen-presenting efficiency of B cells in the context of human autoimmunity, we analyzed both CLIP and HLA-DR surface expression on blood B cells from 12 clinically definite, relapsing-remitting MS (RRMS) patients and 12 age- and gender-matched healthy controls (HC; Figure 5A, Table I). In contrast to HLA-DR, CLIP expression was significantly higher on B cells in RRMS (p=0.020; Figure 5B-C), resulting in increased CLIP/HLA-DR surface ratios (p=0.011; Figure 5D). To determine whether this was already true before clinically definite diagnosis, similar analyses were carried out for B cells of patients with clinically isolated syndrome (CIS), the first clinical presentation of suspected MS [20]. In patients with very rapid onset of MS (<1 y, high-risk CIS; n=9), we found that both CLIP surface levels (p=0.002) and CLIP/HLA-DR expression ratios (p=0.007) were

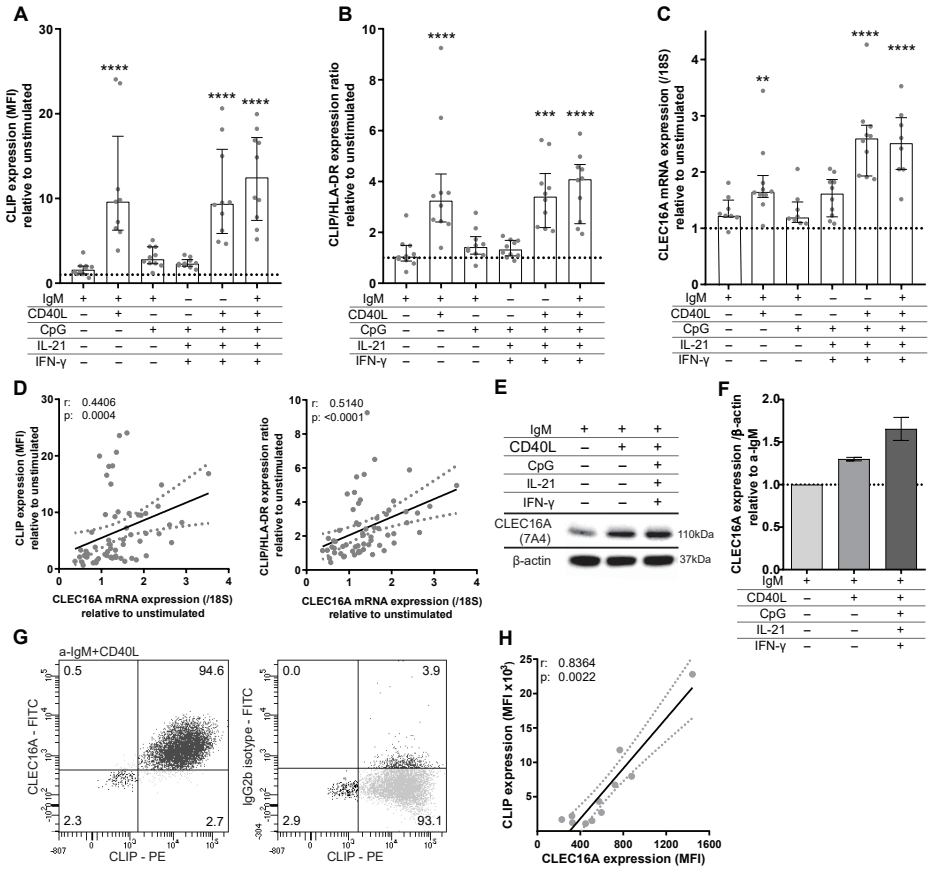

Figure 4. Surface CLIP and *CLEC16A* induction in blood B cells after *in vitro* activation with GC-dependent and -independent stimuli.

Total B cells from healthy blood donors were sorted and stimulated for 24h and 48h with combinations of anti-IgM, sCD40L, IL-21, IFN-γ and CpG-ODN. CLIP, CLIP/HLA-DR (FACS, 48h) and *CLEC16A* (qPCR, 24h) expression levels were compared between the groups (n=8-10; **A-D**). Data are depicted as the expression relative to unstimulated cells (dotted line; **A-C**). **(E-F)** Western blot analysis of CLEC16A expression in blood B cells using the 7A4 monoclonal antibody. Cells were stimulated with a-IgM, a-IgM+sCD40L and a-IgM+sCD40L+IL-21+CpG-ODN+IFN-γ for 48h. CLEC16A levels were quantified and corrected for differences in β-actin expression using B cells from two independent experiments (**F**). **(G)** CLIP and CLEC16A expression on anti-IgM and CD40L-stimulated B cells from healthy blood donors, as determined by intracellular FACS. Rat IgG2b isotype was used as a negative control for CLEC16A intracellular staining. Percentages of cell in the gates are indicated in the plots. **(H)** Correlation of CLEC16A and CLIP expression as determined by FACS before and after stimulation with a-IgM, with and without CD40L (n=4). Data are shown as median ± IQR. For **A-C**, Friedman including Dunn's multiple comparison tests were performed on unprocessed data (Supplementary Figure 3B). All conditions were compared to unstimulated cells of each donor (dotted line). Spearman's correlation coefficients were calculated for **D** and **H**. ** p<0.01, *** p<0.001, **** p<0.0001.

elevated on B cells as compared to patients with slow or no onset of MS (>5 y, low-risk CIS; n=11; Figure 5E-G). After correction for MP treatment (n=2 in each group), CLIP expression remained significantly different (Table I and data not shown). In both RRMS and high-risk CIS, CLIP was upregulated on all naive and memory subsets analyzed, including transitional ($IgM^+CD27^-CD38^{high}$), naive mature ($IgM^+CD27^-CD38^{-/dim}$), non-switched (IgM^+CD27^+) as well as switched (IgG^+CD27^+ and IgA^+CD27^+) B-cell subsets (Supplementary Figure 3A and Figure 6A). None of these subsets showed differences in surface HLA-DR levels (Figure 6B). CLIP and HLA-DR expression on B cells from CIS patients were not influenced by the presence of *HLA-DRB1*1501* (rs3135388) or previously described risk SNPs in *CLEC16A*

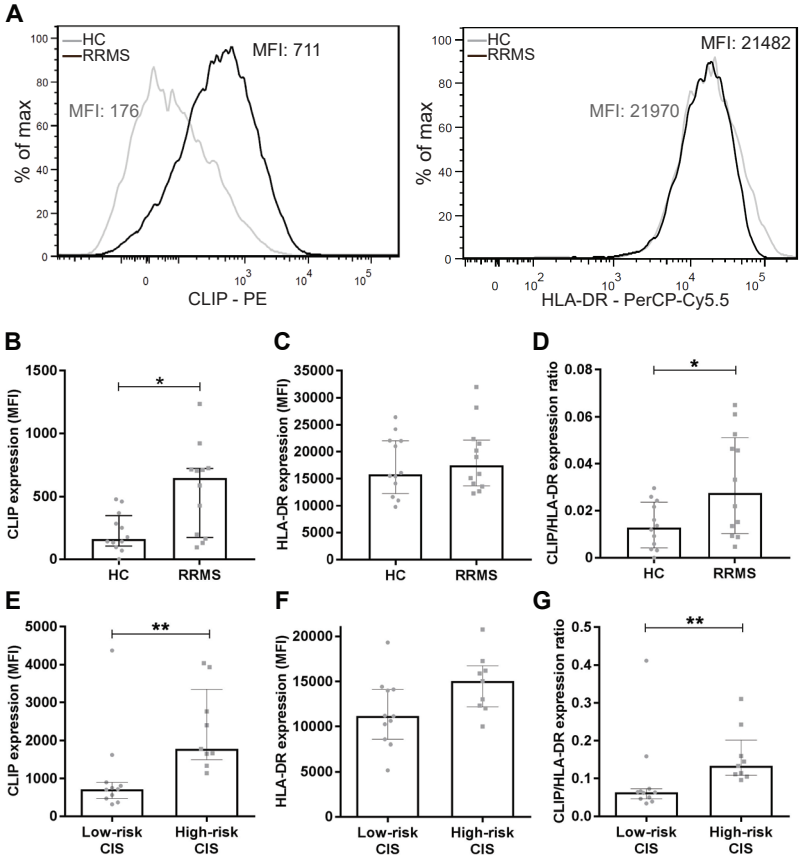

Figure 5. CLIP and HLA-DR expression on *ex vivo* B cells of different early MS patients.

(A) Representative CLIP and HLA-DR expression on viable B cells. Surface expression levels and ratios (MFI) were compared between 12 relapsing-remitting MS (RRMS) patients and 12 matched healthy controls (HC) **(B-D)**, as well as 11 low-risk and 9 high-risk CIS patients **(E-G)** using flow cytometry. Data are shown as median ± IQR. Unpaired t-tests **(E, G)** or Mann-Whitney U tests **(B-D, F)** were used to compare the groups.* $p<0.05$, ** $p<0.01$.

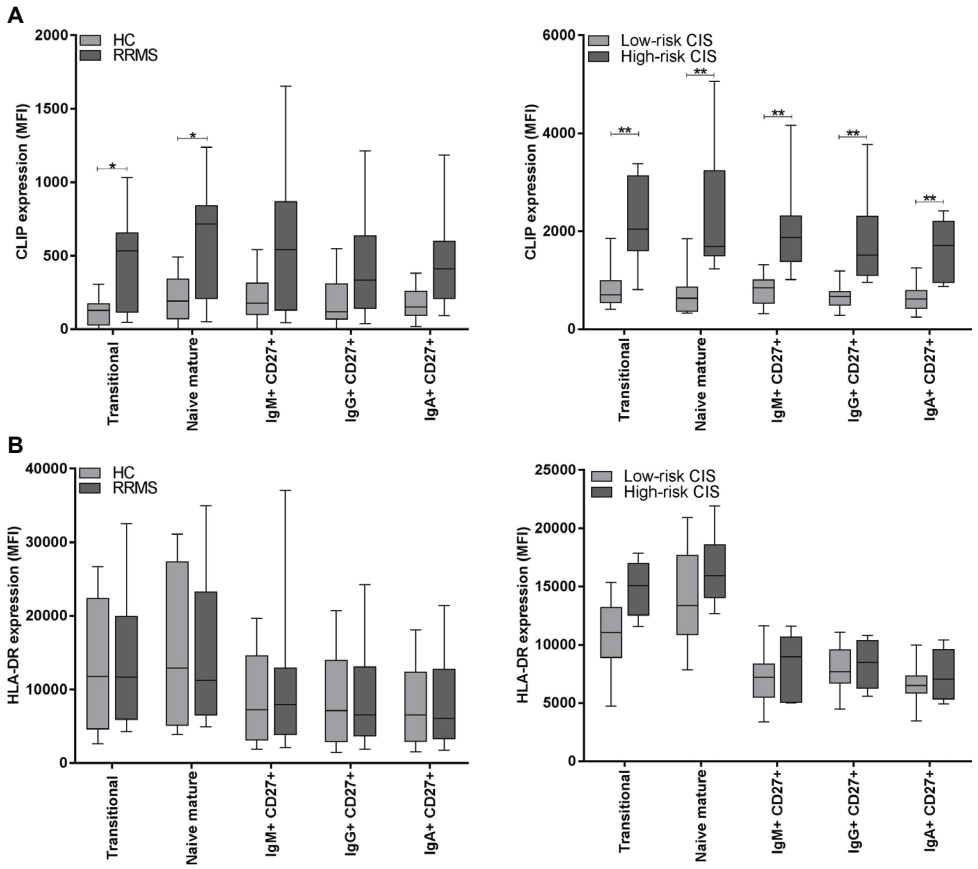

Figure 6. CLIP and HLA-DR expression on distinct naive and memory B-cell subsets of early MS patients. CLIP **(A)** and HLA-DR **(B)** expression on different B-cell subsets from early MS blood, including transitional ($IgM^+CD27^-CD38^{high}$), naive mature ($IgM^+CD27^-CD38^{-/dim}$), non-switched memory (IgM^+CD27^+), IgG^+CD27^+ and IgA^+CD27^+ B cells. The used gating strategy was similar to that in Supplementary Figure 3A. CLIP and HLA-DR MFI were compared between subsets of RRMS and matched healthy controls (n=12**)** and low-risk and high-risk CIS (n=9-11**)** groups. Data are shown as median ± IQR. Kruskal-Wallis test including Dunn's multiple comparison were used to compare groups.* $p<0.05$, ** $p<0.01$.

(rs1985372 and rs12708716) [26] (Supplementary Figure 4A), and other genes related to the HLA-II pathway (*IFI30*, rs11554159; *MARCH1*, rs72989863; *CBLB*, rs2289746) [35-39] (Supplementary Figure 4B).

Notably, in contrast to healthy donors (Figure 3A), CLIP (and HLA-DR) did not correlate with *CLEC16A* levels in *ex vivo* B cells from RRMS patients (Figure 7A). Furthermore, B cells of patients already showed an upregulation of CLIP and *CLEC16A* after stimulation with

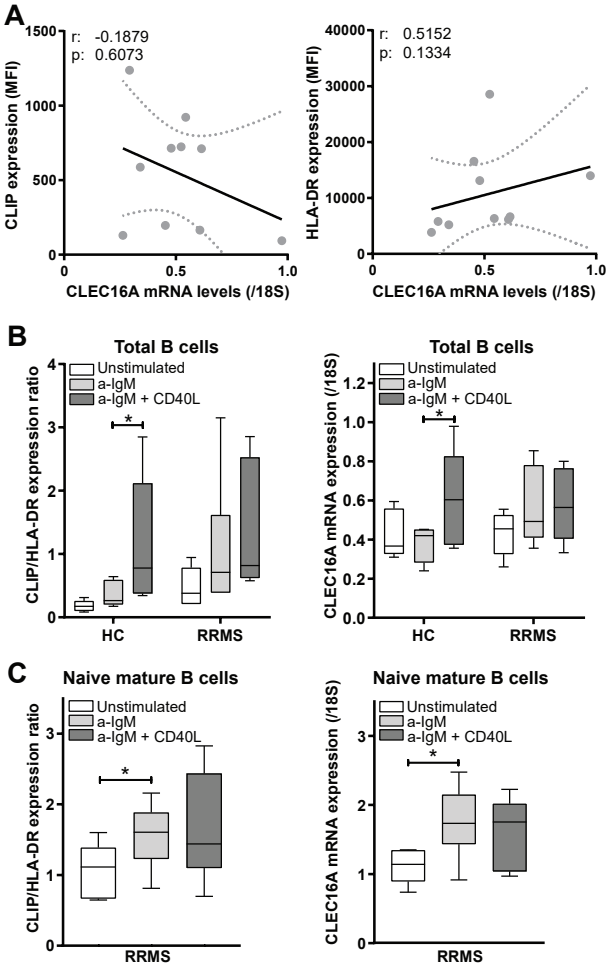

Figure 7. Surface CLIP and *CLEC16A* expression levels in *ex vivo* and *in vitro*-activated B cells from early MS patients.

(A) Total B cells of 10 RRMS patients were analyzed for CLIP, HLA-DR and *CLEC16A* expression using flow cytometry and qPCR. Spearman's correlation coefficients were calculated. **(B)** Surface CLIP/HLA-DR and *CLEC16A* levels for B cells from RRMS patients and age- and gender matched healthy controls before and after stimulation with anti-IgM and sCD40L for 24h (*CLEC16A*) and 48h (CLIP/HLA-DR; n=5-6). **(C)** Surface CLIP/HLA-DR and *CLEC16A* levels in sorted naive mature B cells from RRMS patients before and after stimulation with anti-IgM and sCD40L for 24h (*CLEC16A*) and 48h (CLIP/HLA-DR; n=7). Data are shown as median ± IQR. Friedman including Dunn's multiple comparison tests were performed. * $p<0.05$.

IgM. In contrast to HC, this was not further upregulated after CD40L stimulation (Figure 7B). Moreover, there was no correlation between surface CLIP and *CLEC16A* expression after *in vitro* activation in these RRMS patients (Supplementary Figure 3D), in contrast to healthy donors (Figure 4D). We did not find differences in *ex vivo* naive and memory subset distribution (Supplementary Figure 3E) and similar results were obtained *in vitro* using sorted naive mature B cells of these patients (Figure 7C). These data demonstrate that CLIP is abnormally increased in B cells of MS patients, and within co-regulation between CLIP and *CLEC16A* expression is lost.

DISCUSSION

This study reveals that CLEC16A contributes to the tightly coordinated antigen presentation pathway in B cells by influencing both BCR-mediated antigen uptake and MIIC biogenesis (Figure 8). *CLEC16A* expression associated with the presentation of CLIP-loaded HLA-II molecules by human B-cell lines and primary B cells. This association was not found in B cells from the blood of early MS patients, showing abnormal expression of surface CLIP and impaired co-regulation with *CLEC16A*.

Our *ex vivo* and *in vitro* results reveal that *CLEC16A* at least plays a role in the HLA-II pathway of IgM^+ B cells. This is supported by an earlier study in mice [40]. Normally, CLIP remains bound to the peptide-binding groove of HLA-II molecules until the BCR is engaged with antigen. In the absence of antigen, surface CLIP and *CLEC16A* were upregulated after CD40L triggering, in line with previous studies [41, 42]. The co-regulation with *CLEC16A* may be explained by the fact that this gene is located next to *CIITA*, which is induced in CD40L-stimulated B cells [7]. This co-regulation was not present or further enhanced by CD40 triggering for naive B cells of MS patients. It has been shown that antigen-independent, polyclonal activation of B cells triggers surface CLIP expression [43]. The increased expression of CLIP found on B cells of these patients may thus be promoted by chronic inflammation [44]. In our recent work, CD74 was downregulated particularly on naive B cells [45]. This possibly represents increased uptake and processing into CLIP, which has been shown to impede Treg activation in mice [46].

Both surface HLA-DR and CD74 were upregulated in *CLEC16A*-silenced Raji B cells. Since CLEC16A promotes dynein-mediated retrograde transport of late endosomes [16], CLEC16A is likely involved in the transport of HLA-DR/CD74 via the plasma membrane to MIICs [18]. After CLEC16A silencing, MIICs were found to be more dispersed throughout Raji B cells, which agrees with our previous observations in human myeloid cells [16]. The relatively small difference in CLIP expression in silenced Raji B cells can be explained by the fact that CLIP-loaded HLA-II is already highly abundant at the plasma membrane of wild-type cells. In addition to HLA-DR/CD74 complexes, we found evidence that BCR/antigen complexes are trapped at the plasma membrane of *CLEC16A*-silenced Raji cells, suggesting that also antigens do not properly reach the MIICs for exchange with CLIP bound to HLA-II. Like CLEC16A, non-classical HLA-II chaperone HLA-DO is highly expressed in naive B cell populations [47], which inhibits such exchange via HLA-DM [31]. The antigen-presenting capacity of human B cells can also be influenced by other mechanisms such as EBV infection, BCR-driven actin reorganization and MIIC biogenesis, and even by BCR/antigen complexes that interact with intracellular HLA-II proteins [17, 48, 49].

The discrepancy in effect of CLEC16A knockdown on HLA-DR surface expression between DCs [16] and B cells (this study) is likely the result of the different mechanisms

contributing to HLA-II expression in these antigen presenting cell types [50, 51]. In our previous study, *CLEC16A* was silenced in primary monocytes differentiating into immature DCs. In immature DCs, the majority of HLA-II molecules is present in MIICs [52]. This is in contrast to resting B cells, in which a large amount of HLA-II proteins is expressed at the plasma membrane [52]. DCs also differentially regulate antigen processing and presentation upon maturation, and internalize surface HLA-II more efficiently than B cells [52].

Previously, it was reported that *CLEC16A* silencing in human B leukemia cell line K562 did not affect allogeneic T-cell activation [53]. However, in this study, transiently silenced cells were analyzed in a BCR/antigen-independent manner. For our work, we aimed to address the more long-term effects of *CLEC16A* on the BCR-mediated HLA-II pathway in human B cells and preferred the use of vector-based shRNAs because this also prevents the dilution of silencing RNAs through cell replication. Although the use of human B-LCLs is a limitation in this study, it does provide an indication that EBV infection (a strong but poorly understood risk factor for MS) has an impact on surface CLIP expression in B cells.

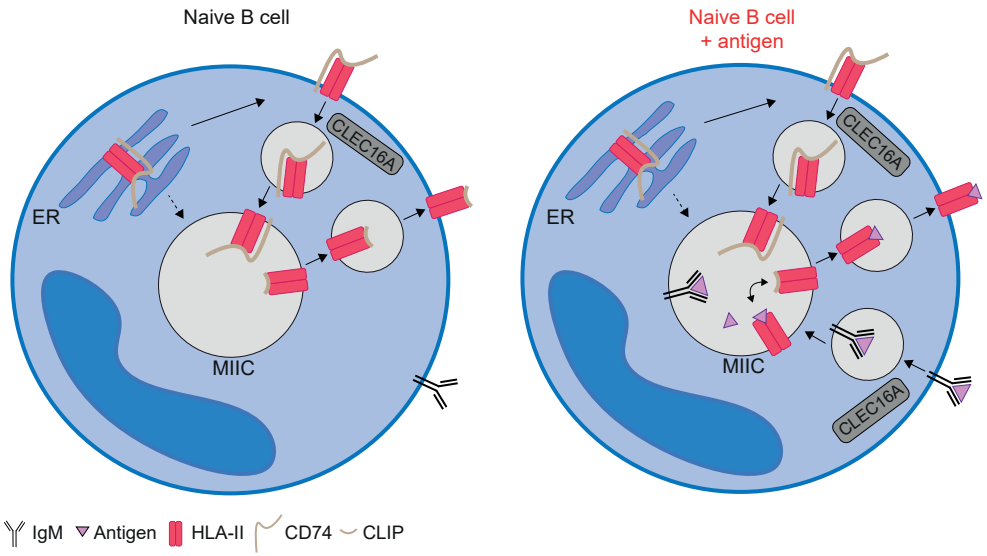

Figure 8. Model for *CLEC16A* as a regulator of BCR/antigen and HLA-II/CD74 processing in MIICs of human B cells.

Before naive B cells encounter an antigen, CLEC16A likely mediates the retrograde trafficking of HLA-II/CD74 complexes to MIICs for cleavage into CLIP. After BCR-mediated antigen uptake and processing in these compartments, CLIP is exchanged for antigenic peptides within the HLA-II peptide-binding groove, which is followed by the transport of HLA-II/peptide complexes to the plasma membrane for presentation to $CD4^+$ T cells.

After uptake of antigen within follicles, B cells process and present (self-)antigens to receive signals from cognate T follicular helper cells [54] and differentiate into potent antigen-presenting memory cells. Memory B cells are key triggers of pathogenic T cells infiltrating the brain in MS patients carrying the major HLA-DRB1*1501 risk allele [55]. Previous in silico analyses revealed that genetic variation in HLA-II risk loci influences the peptide-binding groove [56-58], and thus possibly the binding of CLIP. Although we focused on the role of *CLEC16A* here, there are several other minor risk loci that link to the (BCR-mediated) HLA-II pathway, including *CIITA*, *IFI30*, *MARCH1* and *CBLB* [8, 57, 59, 60]. We did not find an association between B cell-intrinsic CLIP expression and the presence of these risk loci in CIS, including *CLEC16A*. However, this study lacks sufficient power to draw definite conclusions on this matter. In addition, many SNPs in *CLEC16A* have been reported to associate with MS risk [11, 26], which makes it a challenge to pinpoint the actual causal risk SNP, let alone whether *CLEC16A* is affected at transcriptional or post-transcriptional level.

This work offers new insights into the regulation of the HLA-II pathway in human B cells. The co-regulation of CLIP and *CLEC16A* in B cells indicates that this gene contributes to the regulation of HLA-II peptide loading efficiency. We find indications that this goes via the internalization of both BCR/antigen and HLA-DR/CD74 complexes into MIICs. The abundance of CLIP on (naive) B cells and the loss of its co-regulation with *CLEC16A* in MS implies that antigen processing and presentation to T cells is dysregulated. Further research into this phenomenon will be required to better understand the role of B cells as potent antigen presenting cells in patients with autoimmune diseases such as MS.

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REFERENCES

1 **Fernando, M. M., Stevens, C. R., Walsh, E. C., De Jager, P. L., Goyette, P., Plenge, R. M., Vyse, T. J., et al.,** Defining the role of the MHC in autoimmunity: a review and pooled analysis. *PLoS Genet* 2008. **4**: e1000024.

2 **Unanue, E. R., Turk, V. and Neefjes, J.,** Variations in MHC Class II Antigen Processing and Presentation in Health and Disease. *Annu Rev Immunol* 2016. **34**: 265-297.

3 **Vanderlugt, C. L. and Miller, S. D.,** Epitope spreading in immune-mediated diseases: implications for immunotherapy. *Nat Rev Immunol* 2002. **2**: 85-95.

4 **Hauser, S. L., Bar-Or, A., Comi, G., Giovannoni, G., Hartung, H. P., Hemmer, B., Lublin, F., et al.,** Ocrelizumab versus Interferon Beta-1a in Relapsing Multiple Sclerosis. *N Engl J Med* 2017. **376**: 221-234.

5 **Montalban, X., Hauser, S. L., Kappos, L., Arnold, D. L., Bar-Or, A., Comi, G., de Seze, J., et al.,** Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis. *N Engl J Med* 2017. **376**: 209-220.

6 **Du, F. H., Mills, E. A. and Mao-Draayer, Y.,** Next-generation anti-CD20 monoclonal antibodies in autoimmune disease treatment. *Auto Immun Highlights* 2017. **8**: 12.

7 **Paul, P., van den Hoorn, T., Jongsma, M. L., Bakker, M. J., Hengeveld, R., Janssen, L., Cresswell, P., et al.,** A Genome-wide multidimensional RNAi screen reveals pathways controlling MHC class II antigen presentation. *Cell* 2011. **145**: 268-283.

8 **Farh, K. K., Marson, A., Zhu, J., Kleinewietfeld, M., Housley, W. J., Beik, S., Shoresh, N., et al.,** Genetic and epigenetic fine mapping of causal autoimmune disease variants. *Nature* 2015. **518**: 337-343.

9 **Hakonarson, H. and Grant, S. F.,** Genome-wide association studies in type 1 diabetes, inflammatory bowel disease and other immune-mediated disorders. *Semin Immunol* 2009. **21**: 355-362.

10 **Skinningsrud, B., Lie, B. A., Husebye, E. S., Kvien, T. K., Forre, O., Flato, B., Stormyr, A., et al.,** A CLEC16A variant confers risk for juvenile idiopathic arthritis and anti-cyclic citrullinated peptide antibody negative rheumatoid arthritis. *Ann Rheum Dis* 2010. **69**: 1471-1474.

11 **Berge, T., Leikfoss, I. S. and Harbo, H. F.,** From Identification to Characterization of the Multiple Sclerosis Susceptibility Gene CLEC16A. *Int J Mol Sci* 2013. **14**: 4476-4497.

12 **Wellcome Trust Case Control, C.,** Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 2007. **447**: 661-678.

13 **Choi, N. M., Majumder, P. and Boss, J. M.,** Regulation of major histocompatibility complex class II genes. *Curr Opin Immunol* 2011. **23**: 81-87.

14 **Geijtenbeek, T. B., van Vliet, S. J., Engering, A., t Hart, B. A. and van Kooyk, Y.,** Self- and nonself-recognition by C-type lectins on dendritic cells. *Annu Rev Immunol* 2004. **22**: 33-54.

15 **Schuster, C., Gerold, K. D., Schober, K., Probst, L., Boerner, K., Kim, M. J., Ruckdeschel, A., et al.,** The Autoimmunity-Associated Gene CLEC16A Modulates Thymic Epithelial Cell Autophagy and Alters T Cell Selection. *Immunity* 2015. **42**: 942-952.

16 **van Luijn, M. M., Kreft, K. L., Jongsma, M. L., Mes, S. W., Wierenga-Wolf, A. F., van Meurs, M., Melief, M. J., et al.,** Multiple sclerosis-associated CLEC16A controls HLA class II expression via late endosome biogenesis. *Brain* 2015. **138**: 1531-1547.

17 **Yuseff, M. I., Pierobon, P., Reversat, A. and Lennon-Dumenil, A. M.,** How B cells capture, process and present antigens: a crucial role for cell polarity. *Nat Rev Immunol* 2013. **13**: 475-486.

18 **Rocha, N. and Neefjes, J.,** MHC class II molecules on the move for successful antigen presentation. *EMBO J* 2008. **27**: 1-5.

19 **Neefjes, J., Jongsma, M. L., Paul, P. and Bakke, O.,** Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol* 2011. **11**: 823-836.

20 **Miller, D. H., Chard, D. T. and Ciccarelli, O.,** Clinically isolated syndromes. *Lancet Neurol* 2012. **11**: 157-169.

21 **van der Vuurst de Vries, R. M., Mescheriakova, J. Y., Wong, Y. Y. M., Runia, T. F., Jafari, N., Samijn, J. P., de Beukelaar, J. W. K., et al.,** Application of the 2017 Revised McDonald Criteria for Multiple Sclerosis to Patients With a Typical Clinically Isolated Syndrome. *JAMA Neurol* 2018. **75**: 1392-1398.

22 **Poser, C. M., Paty, D. W., Scheinberg, L., McDonald, W. I., Davis, F. A., Ebers, G. C., Johnson, K. P., et al.,** New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann Neurol* 1983. **13**: 227-231.

23 **Polman, C. H., Reingold, S. C., Banwell, B., Clanet, M., Cohen, J. A., Filippi, M., Fujihara, K., et al.,** Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol* 2011. **69**: 292-302.

24 **Miller, S. A., Dykes, D. D. and Polesky, H. F.,** A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988. **16**: 1215.

25 **Anderson, C. A., Pettersson, F. H., Clarke, G. M., Cardon, L. R., Morris, A. P. and Zondervan, K. T.,** Data quality control in genetic case-control association studies. *Nat Protoc* 2010. **5**: 1564-1573.

26 **International Multiple Sclerosis Genetics, C.,** Multiple sclerosis genomic map implicates peripheral immune cells and microglia in susceptibility. *Science* 2019. **365**.

27 **Souwer, Y., Griekspoor, A., de Wit, J., Martinoli, C., Zagato, E., Janssen, H., Jorritsma, T., et al.,** Selective infection of antigen-specific B lymphocytes by Salmonella mediates bacterial survival and systemic spreading of infection. *PLoS One* 2012. **7**: e50667.

28 **Souwer, Y., Griekspoor, A., Jorritsma, T., de Wit, J., Janssen, H., Neefjes, J. and van Ham, S. M.,** B cell receptor-mediated internalization of salmonella: a novel pathway for autonomous B cell activation and antibody production. *J Immunol* 2009. **182**: 7473-7481.

29 **Verstegen, N. J. M., Unger, P. A., Walker, J. Z., Nicolet, B. P., Jorritsma, T., van Rijssel, J., Spaapen, R. M., et al.,** Human B Cells Engage the NCK/PI3K/RAC1 Axis to Internalize Large Particles via the IgM-BCR. *Front Immunol* 2019. **10**: 415.

30 **Marsman, M., Jordens, I., Kuijl, C., Janssen, L. and Neefjes, J.,** Dynein-mediated vesicle transport controls intracellular Salmonella replication. *Mol Biol Cell* 2004. **15**: 2954-2964.

31 **van Ham, S. M., Tjin, E. P., Lillemeier, B. F., Gruneberg, U., van Meijgaarden, K. E., Pastoors, L., Verwoerd, D., et al.,** HLA-DO is a negative modulator of HLA-DM-mediated MHC class II peptide loading. *Curr Biol* 1997. **7**: 950-957.

32 **Roucard, C., Thomas, C., Pasquier, M. A., Trowsdale, J., Sotto, J. J., Neefjes, J. and van Ham, M.,** In vivo and in vitro modulation of HLA-DM and HLA-DO is induced by B lymphocyte activation. *J Immunol* 2001. **167**: 6849-6858.

33 **Adler, L. N., Jiang, W., Bhamidipati, K., Millican, M., Macaubas, C., Hung, S. C. and Mellins, E. D.,** The Other Function: Class II-Restricted Antigen Presentation by B Cells. *Front Immunol* 2017. **8**: 319.

34 **Berkowska, M. A., Driessen, G. J., Bikos, V., Grosserichter-Wagener, C., Stamatopoulos, K., Cerutti, A., He, B., et al.,** Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. *Blood* 2011. **118**: 2150-2158.

35 **Patsopoulos, N. A., Baranzini, S. E., Santaniello, A., Shoostari, P., Cotsapas, C., Wong, G., Beecham, A. H., et al.,** The Multiple Sclerosis Genomic Map: Role of peripheral immune cells and resident microglia in susceptibility. *bioRxiv* 2017: 143933.

36 **Hastings, K. T.,** GILT: Shaping the MHC Class II-Restricted Peptidome and CD4(+) T Cell-Mediated Immunity. *Front Immunol* 2013. **4**: 429.

37 **Reith, W., LeibundGut-Landmann, S. and Waldburger, J. M.,** Regulation of MHC class II gene expression by the class II transactivator. *Nat Rev Immunol* 2005. **5**: 793-806.

38 **Steimle, V., Siegrist, C. A., Mottet, A., Lisowska-Grospierre, B. and Mach, B.,** Regulation of MHC class II expression by interferon-gamma mediated by the transactivator gene CIITA. *Science* 1994. **265**: 106-109.

39 **Veselits, M., Tanaka, A., Lipkowitz, S., O'Neill, S., Sciammas, R., Finnegan, A., Zhang, J., et al.,** Recruitment of Cbl-b to B cell antigen receptor couples antigen recognition to Toll-like receptor 9 activation in late endosomes. *PLoS One* 2014. **9**: e89792.

40 **Li, J., Jorgensen, S. F., Maggadottir, S. M., Bakay, M., Warnatz, K., Glessner, J., Pandey, R., et al.,** Association of CLEC16A with human common variable immunodeficiency disorder and role in murine B cells. *Nat Commun* 2015. **6**: 6804.

41 **Clatza, A., Bonifaz, L. C., Vignali, D. A. and Moreno, J.,** CD40-induced aggregation of MHC class II and CD80 on the cell surface leads to an early enhancement in antigen presentation. *J Immunol* 2003. **171**: 6478-6487.

42 **Faassen, A. E., Dalke, D. P., Berton, M. T., Warren, W. D. and Pierce, S. K.,** CD40-CD40 ligand interactions stimulate B cell antigen processing. *Eur J Immunol* 1995. **25**: 3249-3255.

43 **Newell, M. K., Tobin, R. P., Cabrera, J. H., Sorensen, M. B., Huckstep, A., Villalobos-Menuey, E. M., Burnett, M., et al.,** TLR-mediated B cell activation results in ectopic CLIP expression that promotes B cell-dependent inflammation. *J Leukoc Biol* 2010. **88**: 779-789.

44 **Tan, C., Mueller, J. L., Noviski, M., Huizar, J., Lau, D., Dubinin, A., Molofsky, A., et al.,** Nur77 Links Chronic Antigen Stimulation to B Cell Tolerance by Restricting the Survival of Self-Reactive B Cells in the Periphery. *J Immunol* 2019. **202**: 2907-2923.

45 **Rijvers, L., Melief, M. J., van der Vuurst de Vries, R. M., Stephant, M., van Langelaar, J., Wierenga-Wolf, A. F., Hogervorst, J. M., et al.,** The macrophage migration inhibitory factor pathway in human B cells is tightly controlled and dysregulated in multiple sclerosis. *Eur J Immunol* 2018. **48**: 1861-1871.

46 **Zou, T., Satake, A., Corbo-Rodgers, E., Schmidt, A. M., Farrar, M. A., Maltzman, J. S. and Kambayashi, T.,** Cutting edge: IL-2 signals determine the degree of TCR signaling necessary to support regulatory T cell proliferation in vivo. *J Immunol* 2012. **189**: 28-32.

47 **Chen, X., Laur, O., Kambayashi, T., Li, S., Bray, R. A., Weber, D. A., Karlsson, L., et al.,** Regulated expression of human histocompatibility leukocyte antigen (HLA)-DO during antigen-dependent and antigen-independent phases of B cell development. *J Exp Med* 2002. **195**: 1053-1062.

48 **Morandi, E., Jagessar, S. A., t Hart, B. A. and Gran, B.,** EBV Infection Empowers Human B Cells for Autoimmunity: Role of Autophagy and Relevance to Multiple Sclerosis. *J Immunol* 2017. **199**: 435-448.

49 **Barroso, M., Tucker, H., Drake, L., Nichol, K. and Drake, J. R.,** Antigen-B Cell Receptor Complexes Associate with Intracellular major histocompatibility complex (MHC) Class II Molecules. *J Biol Chem* 2015. **290**: 27101-27112.

50 **Blum, J. S., Wearsch, P. A. and Cresswell, P.,** Pathways of antigen processing. *Annu Rev Immunol* 2013. **31**: 443-473.

51 **Roche, P. A. and Furuta, K.,** The ins and outs of MHC class II-mediated antigen processing and presentation. *Nat Rev Immunol* 2015. **15**: 203-216.

52 **Ma, J. K., Platt, M. Y., Eastham-Anderson, J., Shin, J. S. and Mellman, I.,** MHC class II distribution in dendritic cells and B cells is determined by ubiquitin chain length. *Proc Natl Acad Sci U S A* 2012. **109**: 8820-8827.

53 **Zouk, H., D'Hennezel, E., Du, X., Ounissi-Benkalha, H., Piccirillo, C. A. and Polychronakos, C.,** Functional evaluation of the role of C-type lectin domain family 16A at the chromosome 16p13 locus. *Clin Exp Immunol* 2014. **175**: 485-497.

54 **Rawlings, D. J., Metzler, G., Wray-Dutra, M. and Jackson, S. W.,** Altered B cell signalling in autoimmunity. *Nat Rev Immunol* 2017. **17**: 421-436.

55 **Jelcic, I., Al Nimer, F., Wang, J., Lentsch, V., Planas, R., Jelcic, I., Madjovski, A., et al.,** Memory B Cells Activate Brain-Homing, Autoreactive CD4(+) T Cells in Multiple Sclerosis. *Cell* 2018. **175**: 85-100 e123.

56 **Ito, Y., Ashenberg, O., Pyrdol, J., Luoma, A. M., Rozenblatt-Rosen, O., Hofree, M., Christian, E., et al.,** Rapid CLIP dissociation from MHC II promotes an unusual antigen presentation pathway in autoimmunity. *J Exp Med* 2018. **215**: 2617-2635.

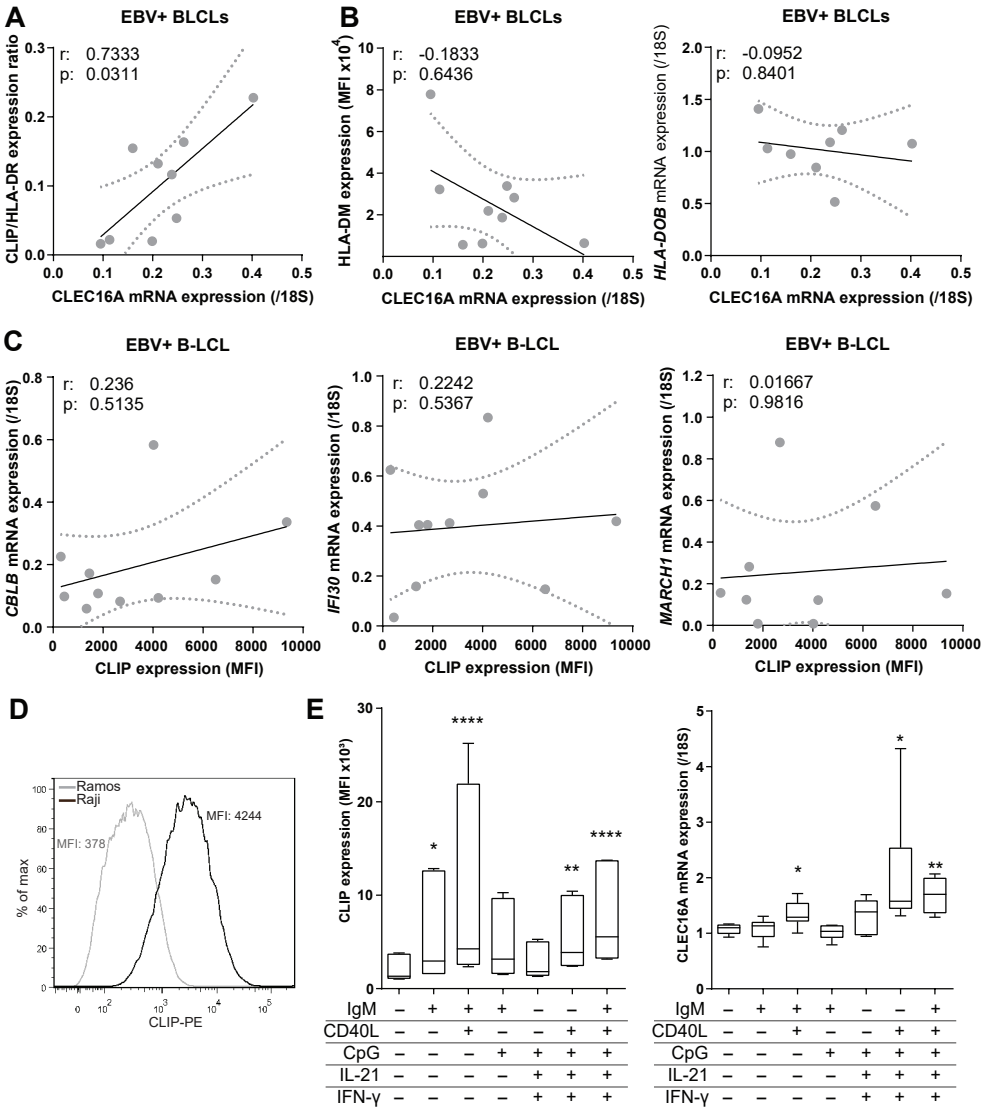
57 **Patsopoulos, N. A., Barcellos, L. F., Hintzen, R. Q., Schaefer, C., van Duijn, C. M., Noble, J. A., Raj, T., et al.,** Fine-mapping the genetic association of the major histocompatibility complex in multiple sclerosis: HLA and non-HLA effects. *PLoS Genet* 2013. **9**: e1003926.

58 **Raychaudhuri, S., Sandor, C., Stahl, E. A., Freudenberg, J., Lee, H. S., Jia, X., Alfredsson, L., et al.,** Five amino acids in three HLA proteins explain most of the association between MHC and seropositive rheumatoid arthritis. *Nat Genet* 2012. **44**: 291-296.

59 **International Multiple Sclerosis Genetics, C., Beecham, A. H., Patsopoulos, N. A., Xifara, D. K., Davis, M. F., Kemppinen, A., Cotsapas, C., et al.,** Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis. *Nat Genet* 2013. **45**: 1353-1360.

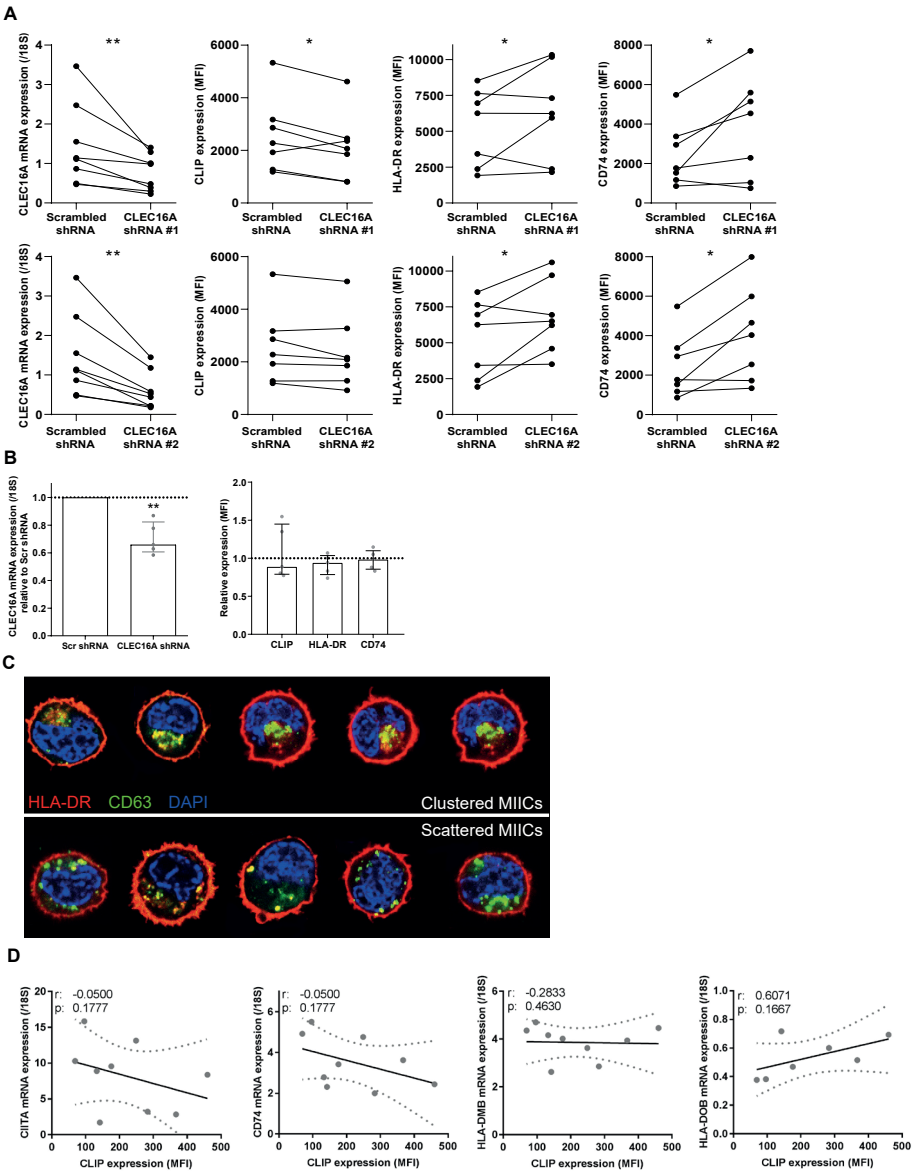
60 **International Multiple Sclerosis Genetics, C., Wellcome Trust Case Control, C., Sawcer, S., Hellenthal, G., Pirinen, M., Spencer, C. C., Patsopoulos, N. A., et al.,** Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* 2011. **476**: 214-219.

SUPPLEMENTARY FILES



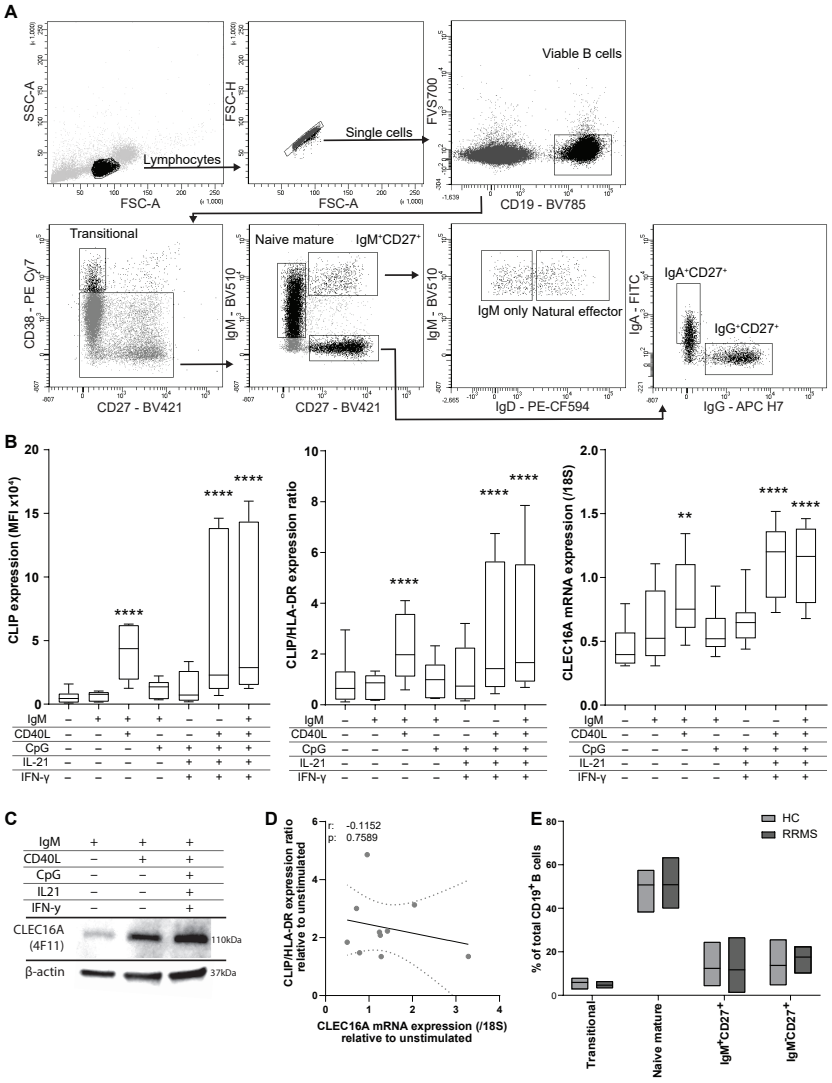
Supplementary Figure 1.

(A) Correlation of *CLEC16A* with surface CLIP/HLA-DR expression ratio in EBV⁺ B-LCLs. **(B)** The association of CLEC16A with HLA-DM expression as well as *HLA-DOB* levels in EBV⁺ B-LCLs (n=9). **(C)** The association of surface CLIP levels with *CBLB, IFI30* and *MARCH1* expression in human EBV⁺ B-LCLs (n=9-10). Spearman's correlation coefficients were calculated. **(D)** CLIP surface expression on Raji and Ramos wild type cells. **(E)** CLIP (48h) and *CLEC16A* (24h) induction in CLIP^{low} (EBV⁻) B-LCL Ramos after CD40L-mediated stimulations. Expression levels are depicted as median ± range and Friedman including Dunn's multiple comparison tests were performed. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.



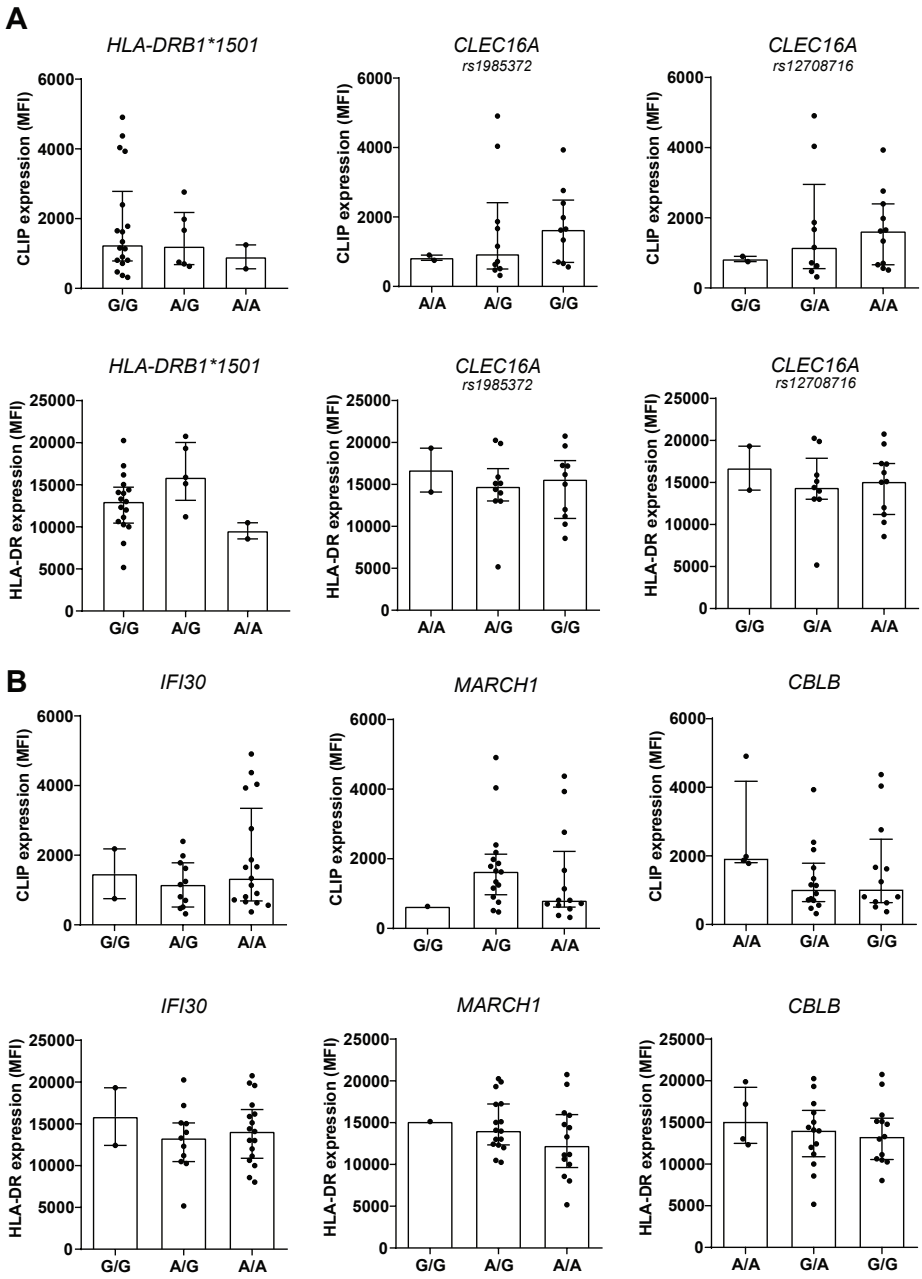
Supplementary Figure 2.

(A) *CLEC16A* and surface CLIP, HLA-DR and CD74 levels in two different CLEC16A shRNA Raji transfectants. Paired t-tests were performed. **(B)** Relative expression levels of *CLEC16A* **(**qPCR**)** as well as surface CLIP, HLA-DR and CD74 **(**FACS**)** were compared between CLEC16A and scrambled shRNA transfectants of Ramos cells after 25 days of puromycin selection (n=5). Data were normalized and compared to the scrambled control per experiment (dotted line). Data are depicted as median ± IQR and paired t-tests were performed on unprocessed data. ** $p<0.01$ **(C)** Representative confocal images of clustered and scattered MIICs in Raji cells used for the quantification in Figure 2D. **(D)** The association of surface CLIP levels with *CIITA, CD74, HLA-DMB* and *HLA-DOB* expression in B cells from healthy blood (n=7-9). Spearman's correlation coefficients were calculated.



Supplementary Figure 3.

(A) Used gating strategy to define transitional ($IgM^+CD27^-CD38^{high}$), naive mature ($IgM^+CD27^-CD38^{-/dim}$), natural effector ($IgM^+IgD^+CD27^+$), IgM-only ($IgM^+IgD^-CD27^+$), IgG^+CD27^+ and IgA^+CD27^+ B cells. **(B)** Total B cells from healthy blood donors were sorted and stimulated with combinations of anti-IgM, sCD40L, IL-21, IFN-γ and CpG-ODN. CLIP, CLIP/HLA-DR (FACS, 48h) and *CLEC16A* (qPCR, 24h) expression levels were compared between the groups (n=8-10). Data are depicted as median ± range and Friedman including Dunn's multiple comparison tests were used. ** $p<0.01$, **** $p<0.0001$ **(C)** Western blot analysis of CLEC16A in B cells from healthy blood using the 4F11 monoclonal antibody. Cells were stimulated with a-IgM, a-IgM+sCD40L and a-IgM+sCD40L+IL-21+CpG-ODN+IFN-γ for 48h. **(D)** Correlation of *CLEC16A* and CLIP/HLA-DR expression ratios on total B cells from RRMS patients (n=5) after stimulation with a-IgM and both a-IgM and sCD40L. Spearman's correlation coefficients were calculated. **(E)** Distribution of B-cell subsets within the blood of RRMS patients (n=6) and HC (n=3) used for stimulation experiments, of which the results are shown in Figure 7B. Data are shown as percentage of total $CD19^+$ B cells.



Supplementary Figure 4.

CLIP and HLA-DR expression on B cells of CIS patients (n=24) were stratified based on the presence of risk alleles **(A)** *HLA-DRB1*1501* **(**rs3135388, A=risk allele), *CLEC16A* (rs1985372, G=risk allele; rs12708716, A=risk allele) and **(B)** *IFI30* (rs11554159; A=risk allele) *MARCH1* (rs72989863; A=risk allele) and *CBLB* (rs2289746; G=risk allele). Data are shown as median ± IQR.

4

The macrophage migration inhibitory factor pathway in human B cells is tightly controlled and dysregulated in multiple sclerosis

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ABSTRACT

In multiple sclerosis (MS), B cells survive peripheral tolerance checkpoints to mediate local inflammation, but the underlying molecular mechanisms are relatively underexplored. In mice, the macrophage migration inhibitory factor (MIF) pathway controls B-cell development and the induction of experimental autoimmune encephalomyelitis. Here, we found that MIF and MIF receptor CD74 are downregulated, while MIF receptor CXCR4 is upregulated in B cells from early onset MS patients. B cells were identified as the main immune subset in blood expressing MIF. Blocking of MIF and CD74 signaling in B cells triggered CXCR4 expression, and vice versa, with separate effects on their pro-inflammatory activity, proliferation and sensitivity to Fas-mediated apoptosis. This study reveals a new reciprocal negative regulation loop between CD74 and CXCR4 in human B cells. The disturbance of this loop during MS onset provides further insights into how pathogenic B cells survive peripheral tolerance checkpoints to mediate disease activity in MS.

INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system, in which infiltrating pro-inflammatory immune cells mediate local pathology [1]. The strong effects of anti-CD20 monoclonal antibody therapy in MS patients demonstrate a key role for peripheral B cells during the pathogenesis [2]. Immature B cells survive peripheral tolerance checkpoints in MS [3], but underlying mechanisms are poorly understood.

An important factor associated with chronic inflammation and cell survival is the macrophage migration inhibitory factor (MIF) [4]. In the animal model of MS, i.e. experimental autoimmune encephalomyelitis (EAE), MIF levels are increased in the central nervous system [5]. Furthermore, anti-MIF treatment prevents disease onset and improves the course of the disease by decreasing the expression of VCAM-1, which impairs the homing of neuroantigen-specific T cells to the central nervous system [6]. Which and how immune subsets are regulated by MIF to promote disease activity in MS patients remains to be determined.

In murine B cells, triggering of the cognate receptor of MIF, CD74 (invariant chain), results in enhanced proliferation and pro-inflammatory cytokine production via NF-κB [7, 8]. Besides functioning as an MHC class II chaperone protein, CD74 also has an MHC class II-independent role in B-cell maturation [9]. Interestingly, MIF is also a non-cognate ligand for chemokine receptor CXCR4 [10], which probably cooperate with CD74 to regulate B-cell development and function through MIF [11-13].

This study explores whether MIF, CD74 and CXCR4 expression in B cells is associated with early MS disease activity, and how the regulation and downstream effects of MIF receptors CXCR4 and CD74 affect human B-cell function. We show that MIF and CD74 are downregulated and CXCR4 is upregulated in blood B cells from early MS patients. This is dependent on a B cell-intrinsic negative regulation loop between MIF, CXCR4 and CD74, which mediates their pro-inflammatory activity, proliferation and sensitivity to Fas-mediated apoptosis.

MATERIAL AND METHODS

Patients

Patient characteristics are summarized in Supplementary Table 1. All clinically isolated syndrome (CIS) and relapsing-remitting MS (RRMS) patients as well as healthy controls (HC) were included in MS Center ErasMS at Erasmus MC (Rotterdam, The Netherlands). CIS was defined as a first clinical attack of demyelination in the central nervous system [14]. Clinically definite MS (CDMS) was diagnosed when a patient experienced two attacks with

clinical evidence of two separate lesions according to the Poser criteria [15]. CIS patients were sampled within 4 months after their first attack. From our prospective cohort, we selected CIS patients who did not develop CDMS for at least 5 years of follow-up (low-risk CIS) and CIS patients who were diagnosed with CDMS within 1 year after CIS diagnosis (high-risk CIS). Fatigue severity was assessed using Krupp's Fatigue Severity Scale (FSS) [16]. RRMS patients were diagnosed according to the McDonalds criteria [17] and were age- and gender-matched with healthy control subjects. For functional studies, buffy coats (Sanquin, Amsterdam, The Netherlands) were obtained from healthy volunteers. All patients and controls gave written informed consent and study protocols were approved by the medical ethics committee of the Erasmus MC.

Peripheral blood sampling

PBMCs and plasma were isolated from whole blood with the use of CPT™ heparin tubes, while serum was isolated using coagulation tubes (both BD Biosciences, San Jose, CA). Samples were processed according to the manufacturer's instructions. PBMCs were stored in liquid nitrogen; plasma and serum were stored in -80^{0}C until analysis.

Human MIF ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to measure human MIF levels in serum and plasma using the four-span approach, as previously described [18]. In brief, anti-human MIF polyclonal antibodies raised in chicken and rabbit were used as capture and trapping antibodies. Microtiter plates were coated with a duck anti-chicken antibody. A horseradish peroxidase-labeled goat anti-rabbit antibody was used for the detection. Distinct concentrations of rhMIF (R&D Systems, Minneapolis, MN) were used to generate a standard curve. The analytic sensitivity of the human MIF ELISA was 39 pg/mL and the coefficients of variation were 6% for intrarun and 12% for interrun.

Flow cytometry and cell sorting

In-depth flow cytometric analysis of B cells were performed using anti-human monoclonal antibodies against CD3, CD19, CD24, CD27, CD38, CD69, CD74, CD95, CXCR4, HLA-DR, IgA, IgD, IgG and IgM. Details of these antibodies are indicated in Supplementary Table 2. All measurements were performed on an LSRFortessa™ flow cytometer and data were analyzed using FACSDiva 8.1 software (both BD Biosciences). Guidelines for the use of flow cytometry in immunological studies have been followed [19].

Intracellular cytokine staining

Cells were stimulated for 5h using phorbol 12-myristate 13-acetate (PMA; 20ng/ml) and ionomycin (500ng/ml, both Sigma-Aldrich), in the presence of BD GolgiStop™. Stimulated

cells were stained with BD Horizon™ Fixable Viability Stain 700, fixed and permeabilized using BD Cytofix/Cytoperm™ according to the provided protocol and stained for IL-6 and TNF-α (Supplementary Table 2, BD Biosciences).

RNA isolation and quantitative PCR

$CD19^+$ B cells, $CD3^+$ T cells, $CD14^+$ monocytes and $CD56^-HLA\text{-}DR^+$ dendritic cells were sorted using a high-speed cell sorter (FACSAria III™; BD Biosciences), resulting in a purity of more than 95%. Subsequently, mRNA was isolated using GenElute™ Mammalian RNA Kit (Sigma-Aldrich, St Louis, MO) and reversely transcribed into cDNA following a standard laboratory protocol with the use of SuperScript II® Reverse Transcriptase (Invitrogen, Paisley, UK). Primers and probes were selected by using the Universal Probe Library Assay Design Centre (Roche Applied Science, Penzberg, Germany). To determine target gene mRNA expression levels, qPCR was performed using an Applied Biosystems 7900 Sequence Detector, which was programmed for the initial step of 2 min at 50^0C and 10 min 95^0C, followed by 40 thermal cycles of 15s at 95^0C and 1min at 60^0C. For the calculation of relative mRNA levels, CT values per gene were related to standard curves, which were generated for each gene of interest. 18S levels were measured as a control to normalize for RNA input. Primer sequences are listed in Supplementary Table 3.

In vitro activation and modulation of B cells

$CD19^+$ B cells of healthy donors were isolated untouched by depleting all other cell types from peripheral mononuclear blood cells (PBMCs) using the B-cell isolation kit II and MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD19^+$ B cells of patients and healthy controls were sorted using FACS and were cultured in RPMI supplemented with 10% fetal calf serum and 1% penicillin/streptavidin for 24 hours or 3 days with various stimuli; anti-IgM ($F(ab')_2$, 10µg/ml, Jackson ImmunoResearch Inc., West Grove, PA) with or without MIF inhibitor ISO-1 (100 µM, R&D Systems), a neutralizing anti-human CD74 antibody (LN2; 10 µg/ml, BD Biosciences) or AMD3100 (10 µg/ml, Sigma-Aldrich). Proliferation rates were addressed by labelling B cells with CFSE (eBioscience, San Diego, CA) before *in vitro* activation. For MIF knockdown, the human B-cell line Raji was transfected with MIF shRNA-containing pLKO.1 constructs (MISSION® shRNA Library, Sigma-Aldrich) using Nucleofector Kit V from Lonza (Basel, Switzerland). Three different MIF shRNA constructs were used: #1 GACAGGGTCTACATCAACTAT, #2 CTACATCAACTATTACGACAT, and #3 CCTGCACAGCATCGGCAAGAT. MIF mRNA and CD74 surface expression was analyzed and compared to scrambled shRNA controls at day 3.

Western blotting

Cells were lysed in radio-immunoprecipitation assay (RIPA) lysis buffer supplemented with 10% complete protease inhibitor cocktail (Roche, Mannheim, Germany) on ice for 30 minutes, centrifuged at 4°C for 10 minutes at 10,000 x *g*. Cell lysates were reduced with 10% 2-mercaptoethanol, denatured for 5min at 95°C, loaded onto a 10% precast polyacrylamide gel (Bio-Rad, Hercules, CA) followed by immunoblotting on a Immobilon-P membrane (Merck Millipore, Darmstadt, Germany) for 1 hours at 4°C. Membranes were blocked in 5% nonfat dry milk and incubated with rabbit anti-human NF-κB1 p105/p50 (D7H5M, Cell signaling technology, Danvers, MA) or mouse anti-human β-actin (AC-15; Abcam, Cambridge, UK) and horseradish peroxidase (HRP)-conjugated swine-anti-rabbit Ig or goat-anti-mouse Ig (Dako, Glostrup, Denmark). Protein bands were visualized using Western Lightning Plus-ECL (Perkin Elmer Inc., Waltham, MA).

Statistical analyses

Statistical analyses were performed using Graphpad Prism 7 (GraphPad Software Inc., San Diego, CA). Data are expressed as mean ± SEM. Data sets were tested for normal distribution. Two-tailed t tests or analysis of variance were used to compare groups. Correlations between two parameters were tested by using Pearson's or Spearman correlation coefficients. P-values <0.05 were considered as statistically different.

RESULTS

The expression ratio of MIF receptors CXCR4 and CD74 on B cells is increased during rapid MS onset

To determine whether the B cell-intrinsic MIF pathway is differentially regulated in early MS, we assessed the expression levels of MIF receptors CXCR4 and CD74 on blood B cells of relapsing-remitting MS (RRMS) patients and healthy controls (HC). CXCR4 was 1.4-fold increased on B cells from 15 RRMS patients compared to 15 age- and gender-matched HC (p=0.002, Fig. 1A, 1B and 1D), which was reproduced and validated in additional cohorts (Supplementary Fig. 1A and 1B). In contrast, CD74 expression was 1.4-fold reduced on B cells in RRMS versus HC (p=0.038, Fig. 1A, 1C and 1E). The ratio of CXCR4 and CD74 expression levels on B cells was even further enhanced in RRMS (2.1-fold, p=0.004; Fig. 1F; Supplementary Fig. 1C and 1D), suggesting that both MIF receptors are dysregulated on a per-patient basis.

Next to RRMS patients, patients with clinically isolated syndrome (CIS), a first manifestation of suspected MS [14], were analyzed. Similar to RRMS, B cells from CIS patients with a very rapid onset of clinically definite MS (CDMS) ('high-risk CIS', n=16) showed 1.5-fold

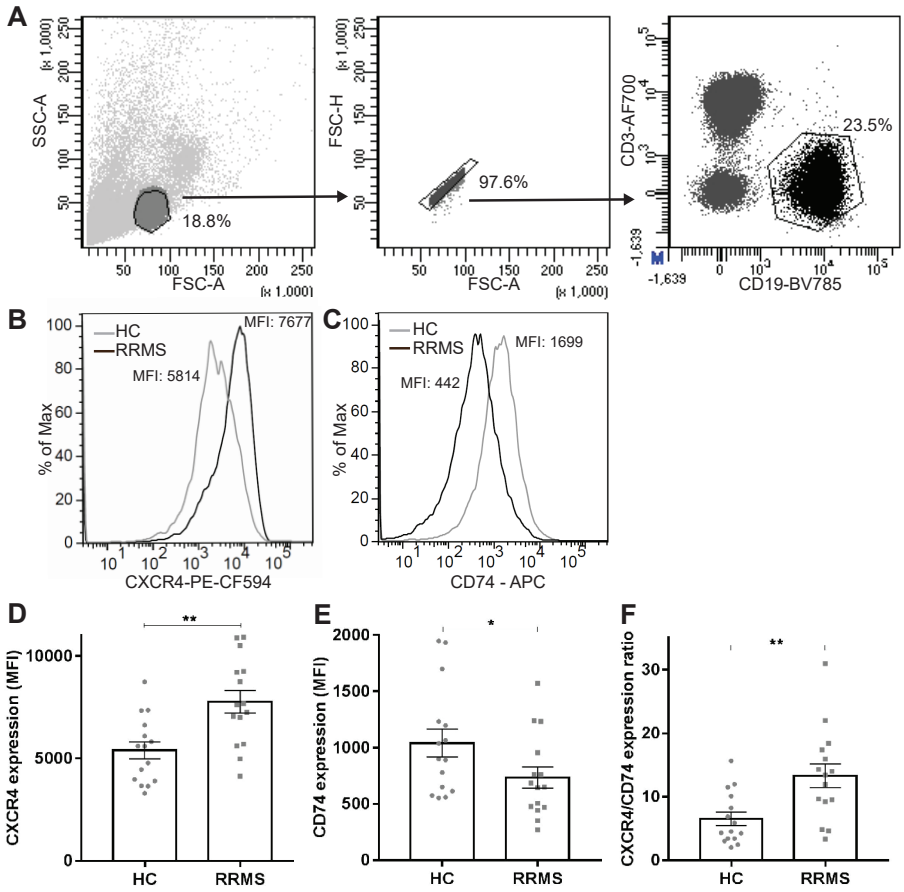

Figure 1. CXCR4 upregulation and CD74 downregulation on B cells of clinically definite MS patients.
(A) Gating strategy for CD19+ B cells. (B-C) representative histograms of CXCR4 **(B)** and CD74 **(C)** expression levels in HC and RRMS. **(D-F)** Expression of MIF receptors CXCR4 (D) and CD74 **(E)** and their ratios **(F)** on blood B cells from 15 RRMS patients and 15 age- and gender-matched healthy controls (HC) as determined by FACS. Data was measured in 3 individual experiments, with 5 HC and 5 RRMS patients per experiment. Data are shown as mean ± SEM. Unpaired t-tests were used to compare groups. * $p<0.05$, ** $p<0.01$.

increased CXCR4 (p=0.014, Fig. 2A) and 1.3-fold reduced CD74 surface levels (p=0.004, Fig. 2B) compared to CIS patients with slow or no onset of CDMS ('low-risk CIS', n=17). This resulted in strongly elevated CXCR4/CD74 expression ratios per patient in the high-risk CIS group (2.5-fold; Fig. 2C). In CIS, a negative correlation was found between CXCR4 and CD74 levels on B cells (r=-0.44, p=0.01; Fig. 2D), and CXCR4/CD74 expression ratios positively associated with fatigue (r=0.53, p=0.003; Fig. 2E), an independent predictor of rapid CIS to CDMS transition [20].

In RRMS and high-risk CIS blood, transitional ($IgM^+CD27^-CD38^{hi}CD24^{hi}$) as well as naive mature ($IgM^+CD27^-CD38^{-/dim}$) B-cell subsets displayed the highest CXCR4/CD74 expression ratios as compared to class-switched ($CD27^+/CD27^-$ IgG^+ and IgA^+) and non–class-switched (IgM^+CD27^+) memory subsets (Fig. 3; Supplementary Fig. 2), implying that the $CXCR4^{hi}CD74^{lo}$ phenotype of B cells in early MS reflects a more immature state. These data demonstrate that MIF receptors CXCR4 and CD74 are inversely expressed on B cells, which is dysregulated during early disease onset in MS.

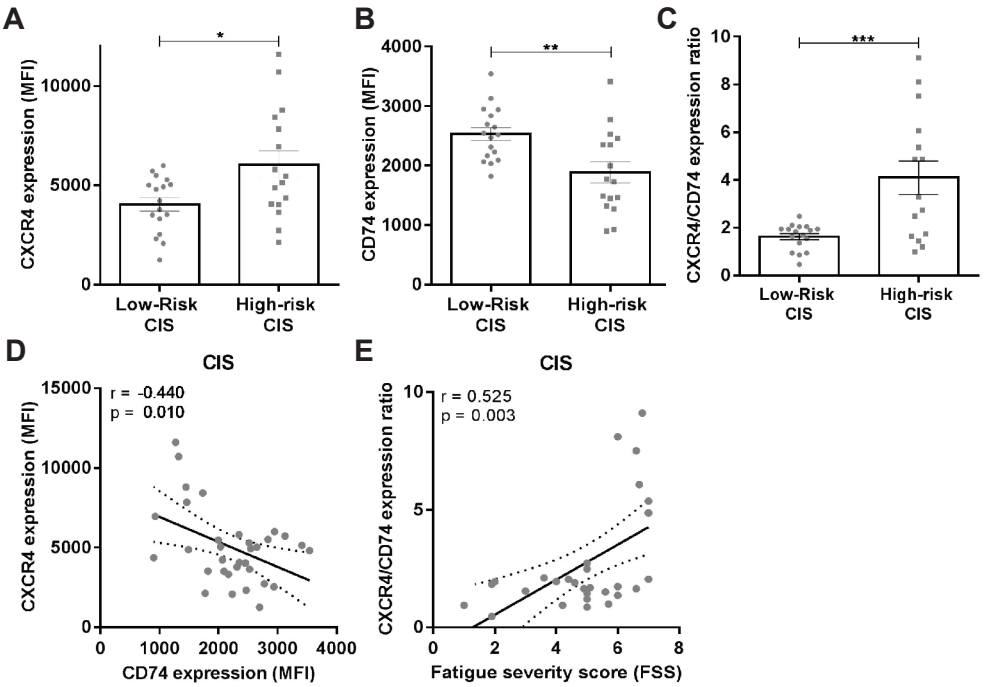

Figure 2. High CXCR4/CD74 expression ratios on B cells of CIS patients associate with rapid MS diagnosis. Expression of MIF receptors CXCR4 and CD74 and their ratios on blood B cells 17 low-risk CIS and 16 high-risk CIS patients **(A-C)**, as determined by FACS. Gating on CD19+ B cells is depicted in Fig. 1. Data was measured in 9 individual experiments, with 1-2 low-risk CIS and 1-2 high-risk CIS patients were measured per experiment. Data are shown as mean ± SEM. Unpaired t-tests were used to compare groups. **(D)** Correlation between CXCR4 and CD74 expression on B cells in CIS patients (n=33). **(E)** Correlation between CXCR4/CD74 surface expression ratios on B cells and fatigue severity scores (FSS) for CIS patients (n=30). r = Pearson's correlation coefficient **(D)** or Spearman correlation **(E)**. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

MIF is predominantly expressed by B cells in healthy blood and downregulated in early MS patients

MIF levels in both serum and plasma were not different between CIS patients and healthy controls (Fig. 4A and Supplementary Fig. 3A) or high-risk and low-risk CIS subgroups (Fig. 4B and Supplementary Fig. 3B), and did not correlate with CXCR4 and CD74 expression levels on B cells from the same individuals (Supplementary Fig. 3C and 3D).

However, among blood immune subsets, MIF mRNA was predominantly expressed by B cells compared to paired T cells, monocytes and dendritic cells (Fig. 4C), but downregulated in B cells from RRMS patients versus HC ($p<0.01$) and even further in B cells of CIS patients (CIS vs HC, $p<0.0001$; CIS vs RRMS, $p<0.01$; Fig. 4D). We found no differences in MIF mRNA levels between B cells from low-risk and high-risk CIS subgroups (Fig. 4E). Low MIF mRNA levels corresponded to high CXCR4/CD74 surface expression ratios in B cells of patients and controls ($p=0.005$; Fig. 4F). This points to the existence of a disturbed regulation loop between MIF and MIF receptors in B cells of early MS patients.

MIF, CD74 and CXCR4 are part of a reciprocal negative regulation loop in human B cells

In vitro, B cells showed enhanced MIF expression and secretion after activation with anti-IgM, which was comparable between patients and controls (Fig. 5A and 5B). To determine potential crosstalk between MIF, CXCR4 and CD74, we used the specific MIF inhibitor ISO-1 [21] to block MIF-mediated signaling in *in vitro*-activated B cells. ISO-1 treatment of these B cells resulted in a CXCR4 upregulation and CD74 downregulation (Fig. 5C), reflecting the inverse correlation between MIF and CXCR4/CD74 expression levels in B cells *ex vivo* (Fig. 4F). In parallel to this, in the human B-cell line Raji, which abundantly expresses CD74 [22], the percentage of $CD74^{hi}$ cells decreased after MIF knockdown using three distinct shRNA constructs (Fig. 5D and 5E). This indicates that CXCR4 and CD74 surface expression is inversely regulated by endogenous MIF in B cells. After treatment with anti-CD74 antibody (LN2), CXCR4 surface expression was increased, whereas MIF expression was reduced in *in vitro*-activated B cells (Fig. 5F and 5H). Vice versa, activated B cells treated with CXCR4 antagonist AMD3100 [23] showed increased CD74 and MIF levels (Fig. 5G and 5H). These data demonstrate that MIF, CD74 and CXCR4 expression in human B cells is tightly and mutually controlled.

CXCR4/CD74 controls the inflammatory, proliferative and Fas-mediated apoptotic potential of B cells

To determine how the reciprocal negative regulation of CD74 and CXCR4 is associated with the function of B cells, we compared the pro-inflammatory, proliferative and survival capacity of *in vitro*-activated B cells before and after blocking of these MIF receptors.

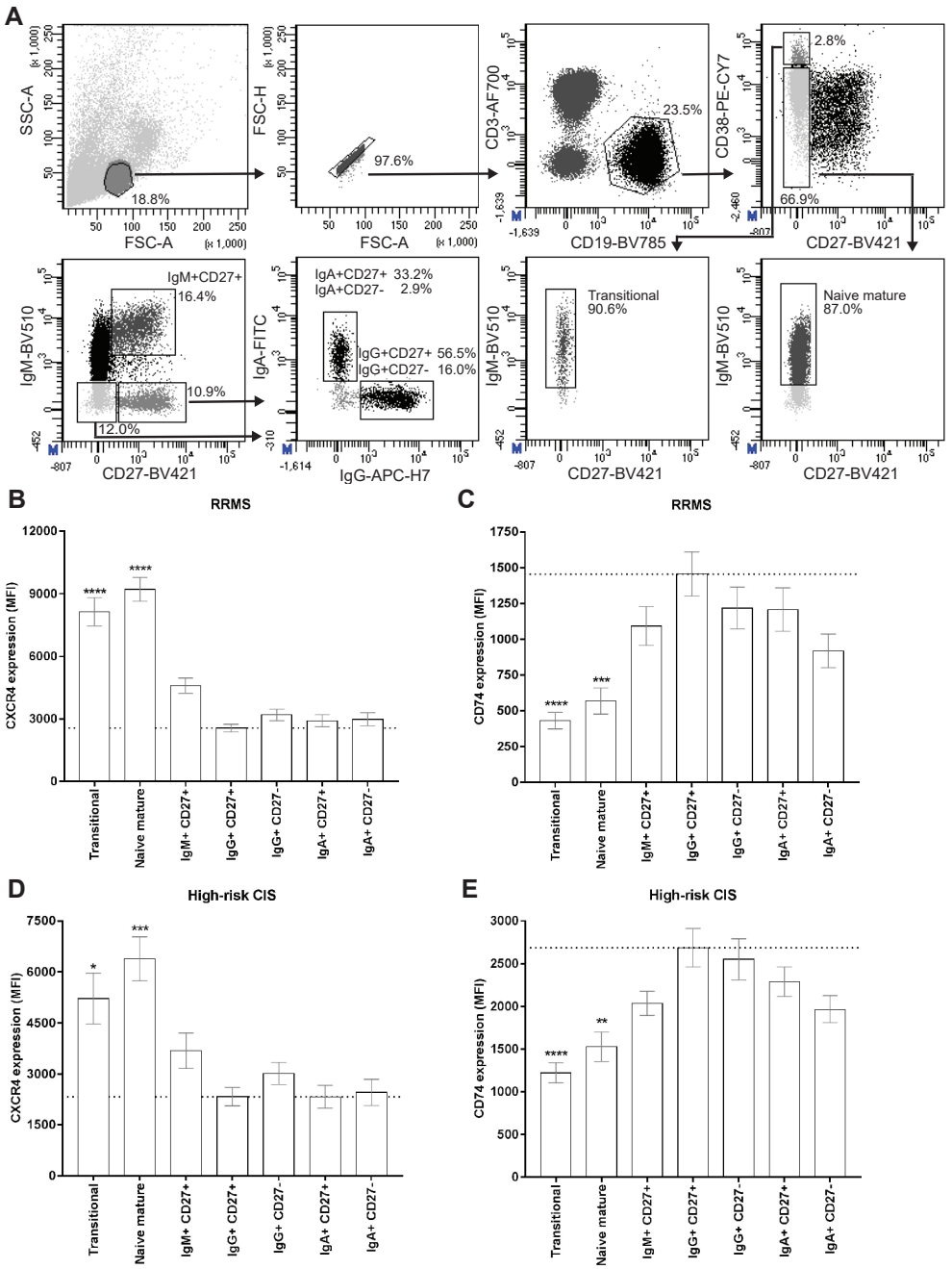
A
SSC-A
FSC-A
18.8%
FSC-H
97.6%
CD3-AF700
23.5%
CD19-BV785
CD38-PE-CY7
2.8%
66.9%
CD27-BV421
IgM-BV510
IgM+CD27+
16.4%
10.9%
12.0%
IgA-FITC
IgA+CD27+ 33.2%
IgA+CD27- 2.9%
IgG+CD27+ 56.5%
IgG+CD27- 16.0%
IgG-APC-H7
Transitional
90.6%
Naive mature
87.0%
B
RRMS
CXCR4 expression (MFI)
C
RRMS
CD74 expression (MFI)
D
High-risk CIS
CXCR4 expression (MFI)
E
High-risk CIS
CD74 expression (MFI)
Transitional
Naive mature
IgM+ CD27+
IgG+ CD27+
IgG+ CD27-
IgA+ CD27+
IgA+ CD27-

Figure 3 (see left page). The CXCR4^{hi}CD74^{lo} phenotype of B cells in early MS blood is linked to transitional and naive mature populations.

(A) Used gating strategy for the different blood B-cell subsets analyzed in **B-E**: transitional (IgM$^+$CD27$^-$ CD38^{hi}CD24^{hi}), naive mature (IgM$^+$CD27$^-$CD38$^{-/dim}$), IgM$^+$CD27$^+$ (non–class-switched memory), IgG$^+$CD27$^+$, IgG$^+$CD27$^-$, IgA$^+$CD27$^+$ and IgA$^+$CD27$^-$ B cells. Blood from RRMS (**B, C**; n=15) and high-risk CIS (**D, E**; n=16) patients was used to assess CXCR4 **(B, D)** and CD74 **(C, E)** expression on these subsets. For RRMS patients, the data was measured in 3 individual experiments with 5 RRMS patients per experiment. For high-risk CIS patients, data was measured in 9 individual experiments with 1-2 patients per experiment. The expression level on each subset was compared to that of IgG$^+$CD27$^+$ memory B cells. Data are shown as mean ± SEM. Kruskal-Wallis test with Dunn's correction for multiple comparison was used to compare groups. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Treatment of these B cells with anti-CD74 antibody LN2 (24h) suppressed the induction of pro-inflammatory genes *NFKB1, IL6* and *TNF* (Fig. 6A). This was not found in *in vitro*-activated B cells treated with CXCR4 antagonist AMD3100 (Fig. 6A). These differences were verified on protein level (Supplementary Fig. 4). Also B-cell proliferation was inhibited after treatment for 3 days with LN2 antibody and not with AMD3100, as determined by CFSE labeling (Fig. 6B and 6C). Finally, surface expression of the death receptor Fas (CD95) was triggered in AMD3100- and not in LN2-treated B cells after 3 days of activation (Fig. 6D). These results imply that in humans, CD74 primarily boosts the pro-inflammatory and proliferative capacity of B cells, while CXCR4 makes B cells less sensitive for Fas-mediated apoptosis [24].

DISCUSSION

The aim of this study was to elucidate the impact of the B cell-intrinsic MIF pathway on early disease onset in MS patients. We demonstrate that decreased CD74 and increased CXCR4 expression on B cells in blood are associated with early MS diagnosis. This was shown for CIS patients who will rapidly develop MS as well as for clinically definite MS patients. *In vitro* experiments supported the inverse regulation of MIF/CD74 and CXCR4 expression in B cells *ex vivo*, which differentially controlled their pro-inflammatory capacity, proliferation and sensitivity to Fas-mediated apoptosis (Supplementary Fig. 5). The observed CXCR4^{hi}CD74^{lo} B-cell phenotype in early MS blood points to the presence of more immature B cell populations with senescent features that survived peripheral tolerance checkpoints in MS [3].

There are several lines of evidence implying that CD74 downregulation and CXCR4 upregulation disrupt the selection of immature B cells. During B-cell development, 'new emigrant' transitional subsets in blood are negatively selected in secondary lymphoid

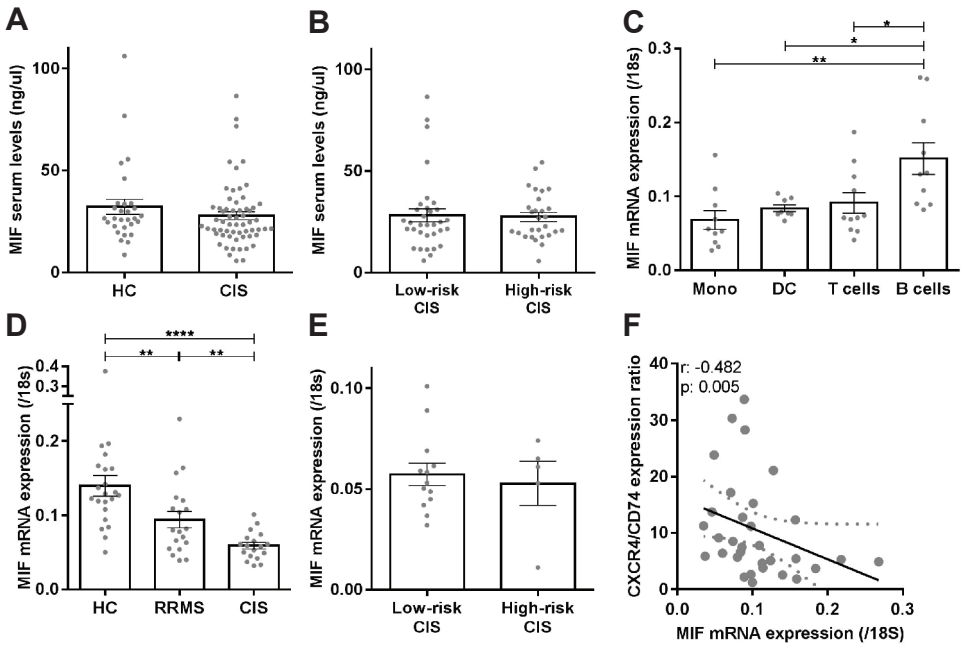

Figure 4. Serum MIF levels are not different, while the predominant MIF expression in B cells is reduced in CIS and RRMS patients.

MIF serum levels (ng/µl) were compared between CIS patients (n=61) and HC (n=29; **A**), as well as between low-risk CIS (n=33) and high-risk CIS (n=28) subgroups **(B)** using ELISA. Data is measured in one single experiment. Each dot represents the mean value of one individual, measured in duplicates. **(C)** Monocytes (n=10), dendritic cells (n=8), T cells (n=11) and B cells (n=10) were sorted from healthy blood and assessed for MIF mRNA expression relative to those of 18S mRNA using qPCR. Data was collected in 4 independent experiments with 2-4 donors per experiment. Similar analyses were performed for blood B cells from CIS patients (n=18), RRMS patients (n=19) and HC (**D**; n=22), as well as low-risk CIS (n=13) and high-risk CIS (n=5) groups **(E)**. B cells of CIS patients were sorted in 9 independent experiments with 2-4 patients per experiment. B cells of HC and RRMS patients were sorted in 17 independent experiments, with 1-2 HC and 1-2 RRMS patients per experiment. MIF and 18S mRNA expression was measured in 6 individual experiments, with 2 independent experiments per patient group. Data are shown as mean ± SEM. Each dot represents the mean value of one individual, measured in duplicates. Student's t-tests were used to compare groups. **(F)** Correlation between MIF mRNA levels and CXCR4/CD74 surface expression ratios in B cells from patients and healthy controls (n=30). B cells were sorted, mRNA was measured in duplicates, and surface expression was analyzed in 6 independent experiments with 2 HC and 2-4 patients per experiment. r = Spearman's correlation; * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.

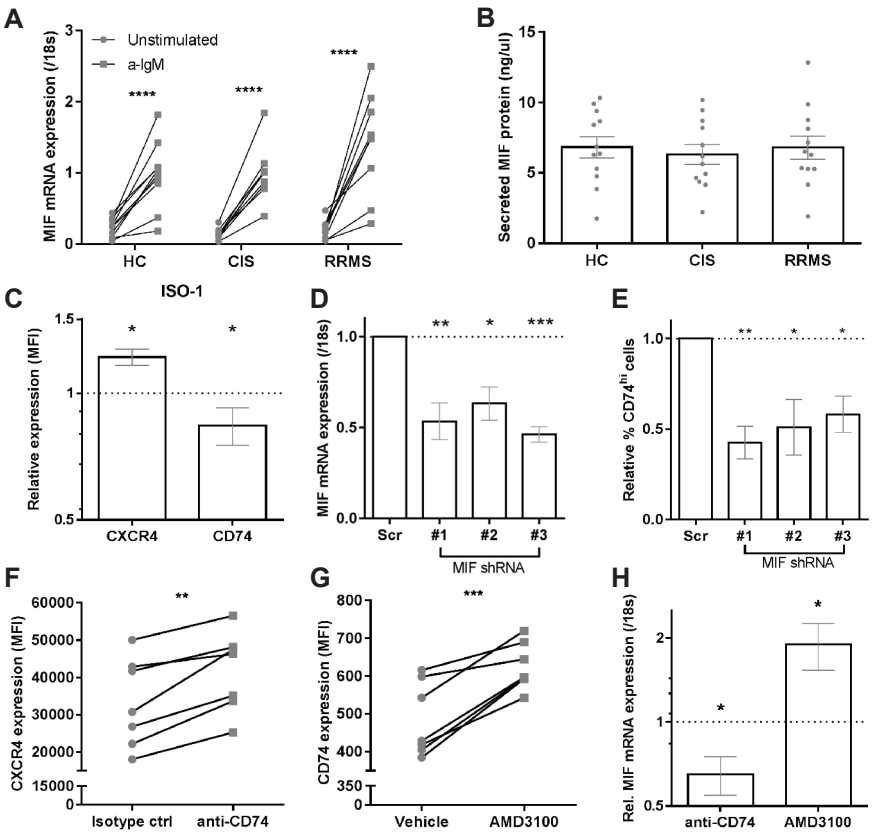

Figure 5. Interference with MIF signaling pathways reveals mutual regulation of CD74, CXCR4 and MIF expression in in vitro-activated B cells.

B cells sorted from CIS, RRMS and HC blood (n=9-12 per group) were activated *in vitro* for 24h using anti-IgM and analyzed for MIF expression (**A**; qPCR) and secretion into culture media (**B**; ELISA). Stimulations have been done in 3 individual experiments with 3-4 patients per group per experiment. Each dot represents the mean value of one individual, measured in duplicates. **(C)** *In vitro*-activated B cells from healthy blood were treated with and without MIF inhibitor ISO-1 for 24h and assessed for CXCR4 and CD74 surface expression using FACS (n=6). Stimulations have been done in 3 individual experiments with 2 controls per group per experiment. **(D, E)** The human B-cell line Raji ($CD74^{hi}$) was transfected with three distinct MIF shRNA constructs and compared with scrambled controls for MIF mRNA levels **(D)** and the percentage of $CD74^{hi}$ cells **(E)** at day 3 (n=5). Data was measured in 5 individual experiments with one set of scrambled and 3 different shRNA's per experiment. *In vitro*-activated B cells were also evaluated for CXCR4 (**F**, n=7), CD74 (**G**, n=7) and MIF mRNA (**H**, n=5-6, measured in duplicates) expression after 24h treatment with anti-CD74 (LN2) or isotype antibody **(F, H)** and CXCR4 antagonist AMD3100 **(G, H)**. Stimulations have been done in 3 individual experiments with 2-3 controls per group per experiment. All used controls were set at 1 (dotted line). Data are shown as mean ± SEM. Paired t-tests were performed to compare groups. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

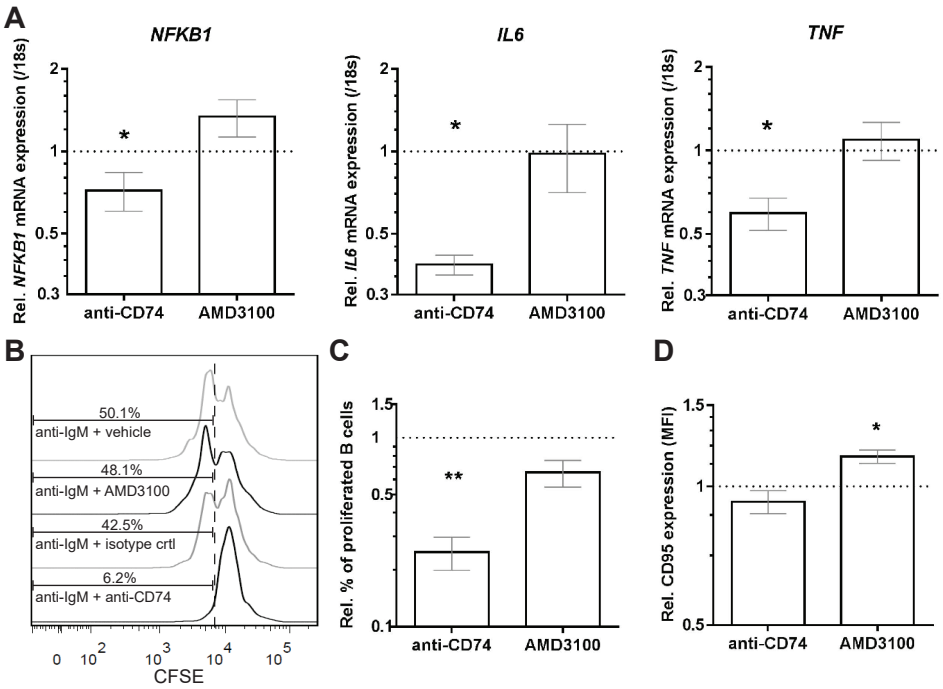

Figure 6. CD74 and CXCR4 on human B cells differentially control pro-inflammatory gene expression, proliferation and sensitivity to Fas-mediated apoptosis.

B cells from healthy blood were *in vitro*-activated with a-IgM and subsequently treated with anti-CD74 antibody (LN2) or AMD3100. Data was compared to their respective controls for relative NF-κB, IL-6 and TNF-α mRNA expression after 24h using qPCR (**A**, n=4-6, measured in duplicates), as well as CFSE-based proliferation (**B** and **C**, n=5) and Fas (CD95) surface levels (**D**, n=5) after 3 days using FACS. Stimulations have been done in 3 individual experiments with 2 controls per group per experiment. All used controls were set at 1 (dotted line). Data are shown as mean ± SEM. Paired t-tests were performed to compare groups. * $p<0.05$, ** $p<0.01$.

organs for autoreactive clones before developing into naive mature subsets. B cells in CD74-deficient mice revealed an arrest at the transitional stage [25] and a reduced lifespan [26]. Consistently, decreased CD74 expression impaired B-cell maturation in patients with X-linked lymphoproliferative disease [27]. Both cell autonomous and non-autonomous roles of CD74 could explain these defects in B-cell development. As a cell surface receptor, CD74 triggers B-cell proliferation in mice [8] and humans (current study), supporting the lowered proliferative capacity of naive ($CD74^{lo}$) versus memory ($CD74^{hi}$) populations [28]. The reduced expression of CD74 on B cells in early MS blood thus might reflect a functional state of anergy, contributing to the persistence of pathogenic immature B cells in the periphery [29]. This is underlined by the down-modulation of pro-inflammatory

cytokines TNF-α and IL-6, as well as MIF after blockade of CD74 on B cells. Alternatively, defective processing of CD74 results in the accumulation of an N-terminal fragment, which interferes with B-cell receptor signaling to suppress B-cell maturation in an MHC class II-independent manner [9, 30, 31]. In addition to these cell-intrinsic effects, a loss of CD74 can also influence T helper cell-mediated selection of naive mature B cells via altered MHC class II antigen presentation [32].

In contrast to CD74, CXCR4 was found to be the most abundant on naive B cells, which controls their development in germinal centers. $CXCR4^{hi}$ B cells are localized in the dark zone to undergo somatic hypermutation, whereas antigen- and T helper cell-based selection of $CXCR4^{lo}$ B cells occurs in the light zone [33, 34]. Our data show that blocking of CXCR4 signaling in B cells increases CD95 (Fas) expression, which is essential for the elimination of autoreactive clones by T helper cells [35]. Hence, it may be speculated that overexpression of CXCR4 on B cells as observed in early MS results in the escape of naive populations from T helper cell-based selection in the light zone, via reduced Fas expression and enhanced migration to the dark zone. Central tolerance checkpoints were not defective in MS patients [3], making it unlikely that the abundance of CXCR4 affects precursor B-cell selection in the bone marrow [36]. *In vivo* studies need to be performed in the future to confirm these roles of CD74 and CXCR4 in peripheral B-cell tolerance in MS.

To our knowledge, the co-regulation of MIF, CD74 and CXCR4 in human B cells and MS patients has never been studied before. Although abundantly expressed by B cells compared to other immune populations, MIF is downregulated in blood B cells from early MS patients. This downregulation links to the survival of autoreactive naive B cells, as seen in atherosclerotic mice [13] and MS [3], and coincides with decreased CD74 and increased CXCR4 surface expression, as part of a tightly controlled regulation loop in B cells. The reciprocal expression of CD74 and CXCR4 was supported by the increased migration capacity of B cells towards CXCL12 after inhibition of CD74 [12, 37]. Since MIF has a higher affinity for CD74 than for CXCR4 [7, 10], a possible underlying mechanism of this reciprocal expression is that MIF-mediated endocytosis of CD74 results in interaction with the adaptor molecule β-arrestin, thereby preventing binding to and internalization of CXCR4 [38-40] (Supplementary Fig. 5).

Extracellular MIF levels were not different in the blood of early MS patients. However, previous studies showed increased levels of MIF in MS CSF [41] as well as in MS lesions and not normal-appearing white matter [42]. This suggests that local MIF production predominantly attracts $CXCR4^{hi}$ B cells from the blood [10, 12] to mediate early MS disease activity. Another CXCR4 ligand, CXCL12, was also found to be abundant in MS CSF, but did not correlate to local B-cell infiltration and activation [43]. After recruitment to the central nervous system, $CXCR4^{hi}CD74^{lo}$ B cells will probably be activated, resulting in increased CD74 expression and MIF production. CD74 triggering by autocrine MIF may then enhance

their ability to proliferate, as shown for tumor cells [44], and produce pro-inflammatory cytokines to mediate central nervous system pathology in MS.

This study shows that MIF and MIF receptors CD74 and CXCR4 are coordinately expressed in B cells to control their inflammatory, proliferative and apoptotic potential in humans. The dysregulation of this B cell-intrinsic loop in early MS pleads for future studies on the processing and cooperation of CD74 and CXCR4 during autoreactive B-cell development. Also more insights into the effects of autocrine and T helper cell-derived MIF on their development will lead to better understanding of the role of B cells as central players in MS and other autoimmune diseases.

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REFERENCES

1 **Nylander, A. and Hafler, D. A.,** Multiple sclerosis. *J Clin Invest* 2012. **122**: 1180-1188.

2 **Hauser, S. L., Waubant, E., Arnold, D. L., Vollmer, T., Antel, J., Fox, R. J., Bar-Or, A., et al.,** B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med* 2008. **358**: 676-688.

3 **Kinnunen, T., Chamberlain, N., Morbach, H., Cantaert, T., Lynch, M., Preston-Hurlburt, P., Herold, K. C., et al.,** Specific peripheral B cell tolerance defects in patients with multiple sclerosis. *J Clin Invest* 2013. **123**: 2737-2741.

4 **Calandra, T. and Roger, T.,** Macrophage migration inhibitory factor: a regulator of innate immunity. *Nat Rev Immunol* 2003. **3**: 791-800.

5 **Baker, D., O'Neill, J. K. and Turk, J. L.,** Cytokines in the central nervous system of mice during chronic relapsing experimental allergic encephalomyelitis. *Cell Immunol* 1991. **134**: 505-510.

6 **Denkinger, C. M., Denkinger, M., Kort, J. J., Metz, C. and Forsthuber, T. G.,** In vivo blockade of macrophage migration inhibitory factor ameliorates acute experimental autoimmune encephalomyelitis by impairing the homing of encephalitogenic T cells to the central nervous system. *J Immunol* 2003. **170**: 1274-1282.

7 **Leng, L., Metz, C. N., Fang, Y., Xu, J., Donnelly, S., Baugh, J., Delohery, T., et al.,** MIF signal transduction initiated by binding to CD74. *J Exp Med* 2003. **197**: 1467-1476.

8 **Starlets, D., Gore, Y., Binsky, I., Haran, M., Harpaz, N., Shvidel, L., Becker-Herman, S., et al.,** Cell-surface CD74 initiates a signaling cascade leading to cell proliferation and survival. *Blood* 2006. **107**: 4807-4816.

9 **Matza, D., Lantner, F., Bogoch, Y., Flaishon, L., Hershkoviz, R. and Shachar, I.,** Invariant chain induces B cell maturation in a process that is independent of its chaperonic activity. *Proc Natl Acad Sci U S A* 2002. **99**: 3018-3023.

10 **Bernhagen, J., Krohn, R., Lue, H., Gregory, J. L., Zernecke, A., Koenen, R. R., Dewor, M., et al.,** MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. *Nat Med* 2007. **13**: 587-596.

11 **Schwartz, V., Lue, H., Kraemer, S., Korbiel, J., Krohn, R., Ohl, K., Bucala, R., et al.,** A functional heteromeric MIF receptor formed by CD74 and CXCR4. *FEBS Lett* 2009. **583**: 2749-2757.

12 **Klasen, C., Ohl, K., Sternkopf, M., Shachar, I., Schmitz, C., Heussen, N., Hobeika, E., et al.,** MIF promotes B cell chemotaxis through the receptors CXCR4 and CD74 and ZAP-70 signaling. *J Immunol* 2014. **192**: 5273-5284.

13 **Schmitz, C., Noels, H., El Bounkari, O., Straussfeld, E., Megens, R. T. A., Sternkopf, M., Alampour-Rajabi, S., et al.,** Mif-deficiency favors an atheroprotective autoantibody phenotype in atherosclerosis. *FASEB J* 2018: fj201800058R.

14 **Miller, D. H., Chard, D. T. and Ciccarelli, O.,** Clinically isolated syndromes. *Lancet Neurol* 2012. **11**: 157-169.

15 **Poser, C. M., Paty, D. W., Scheinberg, L., McDonald, W. I., Davis, F. A., Ebers, G. C., Johnson, K. P., et al.,** New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann Neurol* 1983. **13**: 227-231.

16 **Krupp, L. B., LaRocca, N. G., Muir-Nash, J. and Steinberg, A. D.,** The fatigue severity scale. Application to patients with multiple sclerosis and systemic lupus erythematosus. *Arch Neurol* 1989. **46**: 1121-1123.

17 **Polman, C. H., Reingold, S. C., Banwell, B., Clanet, M., Cohen, J. A., Filippi, M., Fujihara, K., et al.,** Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol* 2011. **69**: 292-302.

18 **Radstake, T. R., Sweep, F. C., Welsing, P., Franke, B., Vermeulen, S. H., Geurts-Moespot, A., Calandra, T., et al.,** Correlation of rheumatoid arthritis severity with the genetic functional variants and circulating levels of macrophage migration inhibitory factor. *Arthritis Rheum* 2005. **52**: 3020-3029.

19 **Cossarizza, A., Chang, H. D., Radbruch, A., Akdis, M., Andra, I., Annunziato, F., Bacher, P., et al.,** Guidelines for the use of flow cytometry and cell sorting in immunological studies. *Eur J Immunol* 2017. **47**: 1584-1797.

20 **Runia, T. F., Jafari, N., Siepman, D. A. and Hintzen, R. Q.,** Fatigue at time of CIS is an independent predictor of a subsequent diagnosis of multiple sclerosis. *J Neurol Neurosurg Psychiatry* 2015. **86**: 543-546.

21 **Al-Abed, Y., Dabideen, D., Aljabari, B., Valster, A., Messmer, D., Ochani, M., Tanovic, M., et al.,** ISO-1 binding to the tautomerase active site of MIF inhibits its pro-inflammatory activity and increases survival in severe sepsis. *J Biol Chem* 2005. **280**: 36541-36544.

22 **Wraight, C. J., van Endert, P., Moller, P., Lipp, J., Ling, N. R., MacLennan, I. C., Koch, N., et al.,** Human major histocompatibility complex class II invariant chain is expressed on the cell surface. *J Biol Chem* 1990. **265**: 5787-5792.

23 **Hatse, S., Princen, K., Bridger, G., De Clercq, E. and Schols, D.,** Chemokine receptor inhibition by AMD3100 is strictly confined to CXCR4. *FEBS Lett* 2002. **527**: 255-262.

24 **Koncz, G. and Hueber, A. O.,** The Fas/CD95 Receptor Regulates the Death of Autoreactive B Cells and the Selection of Antigen-Specific B Cells. *Front Immunol* 2012. **3**: 207.

25 **Shachar, I. and Flavell, R. A.,** Requirement for invariant chain in B cell maturation and function. *Science* 1996. **274**: 106-108.

26 **Benlagha, K., Park, S. H., Guinamard, R., Forestier, C., Karlsson, L., Chang, C. H. and Bendelac, A.,** Mechanisms governing B cell developmental defects in invariant chain-deficient mice. *J Immunol* 2004. **172**: 2076-2083.

27 **Radomir, L., Cohen, S., Kramer, M. P., Bakos, E., Lewinsky, H., Barak, A., Porat, Z., et al.,** T Cells Regulate Peripheral Naive Mature B Cell Survival by Cell-Cell Contact Mediated through SLAMF6 and SAP. *J Immunol* 2017. **199**: 2745-2757.

28 **Allman, D., Lindsley, R. C., DeMuth, W., Rudd, K., Shinton, S. A. and Hardy, R. R.,** Resolution of three nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation. *J Immunol* 2001. **167**: 6834-6840.

29 **Yarkoni, Y., Getahun, A. and Cambier, J. C.,** Molecular underpinning of B-cell anergy. *Immunol Rev* 2010. **237**: 249-263.

30 **Schneppenheim, J., Loock, A. C., Huttl, S., Schweizer, M., Lullmann-Rauch, R., Oberg, H. H., Arnold, P., et al.,** The Influence of MHC Class II on B Cell Defects Induced by Invariant Chain/CD74 N-Terminal Fragments. *J Immunol* 2017. **199**: 172-185.

31 **Huttl, S., Klasener, K., Schweizer, M., Schneppenheim, J., Oberg, H. H., Kabelitz, D., Reth, M., et al.,** Processing of CD74 by the Intramembrane Protease SPPL2a Is Critical for B Cell Receptor Signaling in Transitional B Cells. *J Immunol* 2015. **195**: 1548-1563.

32 **Yeh, C.-H., Nojima, T., Kuraoka, M. and Kelsoe, G. H.,** The Limit of MHC Class II-driven Selection in Germinal Centers. *The Journal of Immunology* 2017. **198**: 152.114-152.114.

33 **Allen, C. D., Ansel, K. M., Low, C., Lesley, R., Tamamura, H., Fujii, N. and Cyster, J. G.,** Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol* 2004. **5**: 943-952.

34 **Victora, G. D., Dominguez-Sola, D., Holmes, A. B., Deroubaix, S., Dalla-Favera, R. and Nussenzweig, M. C.,** Identification of human germinal center light and dark zone cells and their relationship to human B-cell lymphomas. *Blood* 2012. **120**: 2240-2248.

35 **Rathmell, J. C., Cooke, M. P., Ho, W. Y., Grein, J., Townsend, S. E., Davis, M. M. and Goodnow, C. C.,** CD95 (Fas)-dependent elimination of self-reactive B cells upon interaction with CD4+ T cells. *Nature* 1995. **376**: 181-184.

36 **Nie, Y., Waite, J., Brewer, F., Sunshine, M. J., Littman, D. R. and Zou, Y. R.,** The role of CXCR4 in maintaining peripheral B cell compartments and humoral immunity. *J Exp Med* 2004. **200**: 1145-1156.

37 **Frolich, D., Blassfeld, D., Reiter, K., Giesecke, C., Daridon, C., Mei, H. E., Burmester, G. R., et al.,** The anti-CD74 humanized monoclonal antibody, milatuzumab, which targets the invariant chain of MHC II complexes, alters B-cell proliferation, migration, and adhesion molecule expression. *Arthritis Res Ther* 2012. **14**: R54.

38 **Orsini, M. J., Parent, J. L., Mundell, S. J., Marchese, A. and Benovic, J. L.,** Trafficking of the HIV coreceptor CXCR4. Role of arrestins and identification of residues in the c-terminal tail that mediate receptor internalization. *J Biol Chem* 1999. **274**: 31076-31086.

39 **Xie, L., Qiao, X., Wu, Y. and Tang, J.,** beta-Arrestin1 mediates the endocytosis and functions of macrophage migration inhibitory factor. *PLoS One* 2011. **6**: e16428.

40 **van der Vorst, E. P., Doring, Y. and Weber, C.,** Chemokines and their receptors in Atherosclerosis. *J Mol Med (Berl)* 2015. **93**: 963-971.

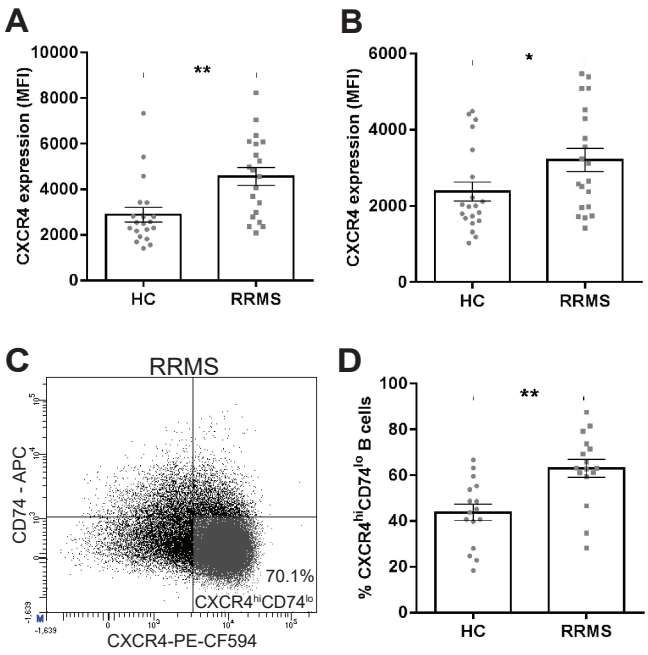
41 **Niino, M., Ogata, A., Kikuchi, S., Tashiro, K. and Nishihira, J.,** Macrophage migration inhibitory factor in the cerebrospinal fluid of patients with conventional and optic-spinal forms of multiple sclerosis and neuro-Behcet's disease. *J Neurol Sci* 2000. **179**: 127-131.

42 **Cox, G. M., Kithcart, A. P., Pitt, D., Guan, Z., Alexander, J., Williams, J. L., Shawler, T., et al.,** Macrophage migration inhibitory factor potentiates autoimmune-mediated neuroinflammation. *J Immunol* 2013. **191**: 1043-1054.

43 **Krumbholz, M., Theil, D., Cepok, S., Hemmer, B., Kivisakk, P., Ransohoff, R. M., Hofbauer, M., et al.,** Chemokines in multiple sclerosis: CXCL12 and CXCL13 up-regulation is differentially linked to CNS immune cell recruitment. *Brain* 2006. **129**: 200-211.

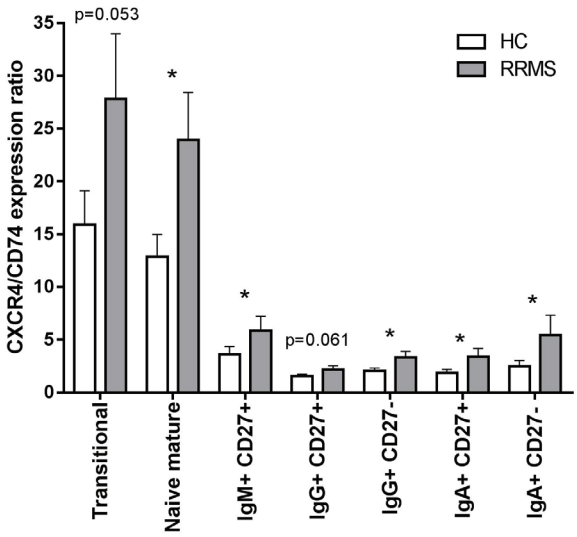
44 **Verjans, E., Noetzel, E., Bektas, N., Schutz, A. K., Lue, H., Lennartz, B., Hartmann, A., et al.,** Dual role of macrophage migration inhibitory factor (MIF) in human breast cancer. *BMC Cancer* 2009. **9**: 230.

SUPPLEMENTARY FILES



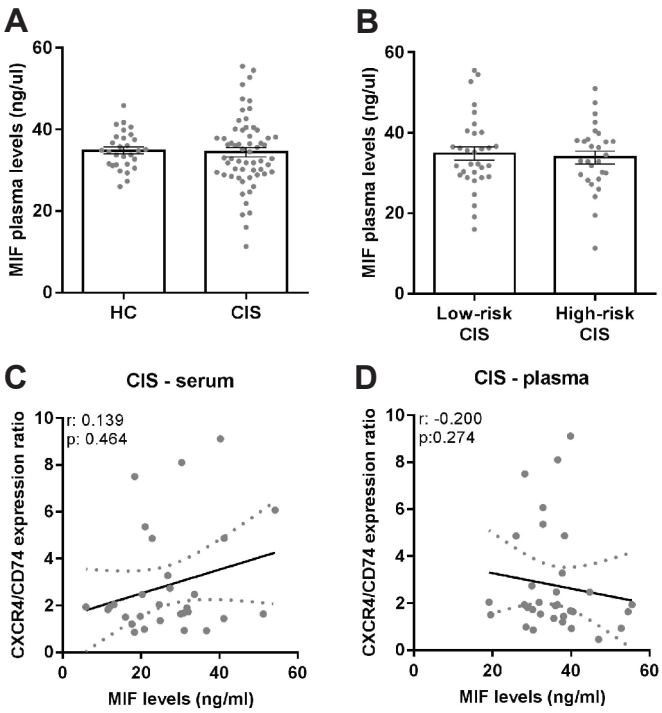
Supplementary Figure 1. Reproducibility and validation of the CXCR4^{hi}CD74^{lo} B-cell phenotype in early MS patients.

(A) Blood B cells of RRMS patients (n=20) and HC (n=20) from the screening cohort (see Supplementary Table 1) were reassessed for CXCR4 expression levels using the 12G5 antibody labeled with APC instead of PE-CF594. The latter antibody was used for the FACS analysis in Fig. 1B and 1D. Similar experiments were performed for B cells from an additional cohort of RRMS patients (n=20) and matched HC (n=20, see Supplementary Table 1; **B**). Data were obtained from 10 individual experiments, with B cells from 2 HC and 2 RRMS patients analyzed per experiment. **(C-D)** Representative dotplot and quantification of CXCR4^{hi}CD74^{lo} frequencies of B cells from RRMS patients and HC (screening cohort, see Supplementary Table 1). Data are shown as mean ± SEM. Student's t-tests were used to compare the groups. * $p<0.05$, ** $p<0.01$.



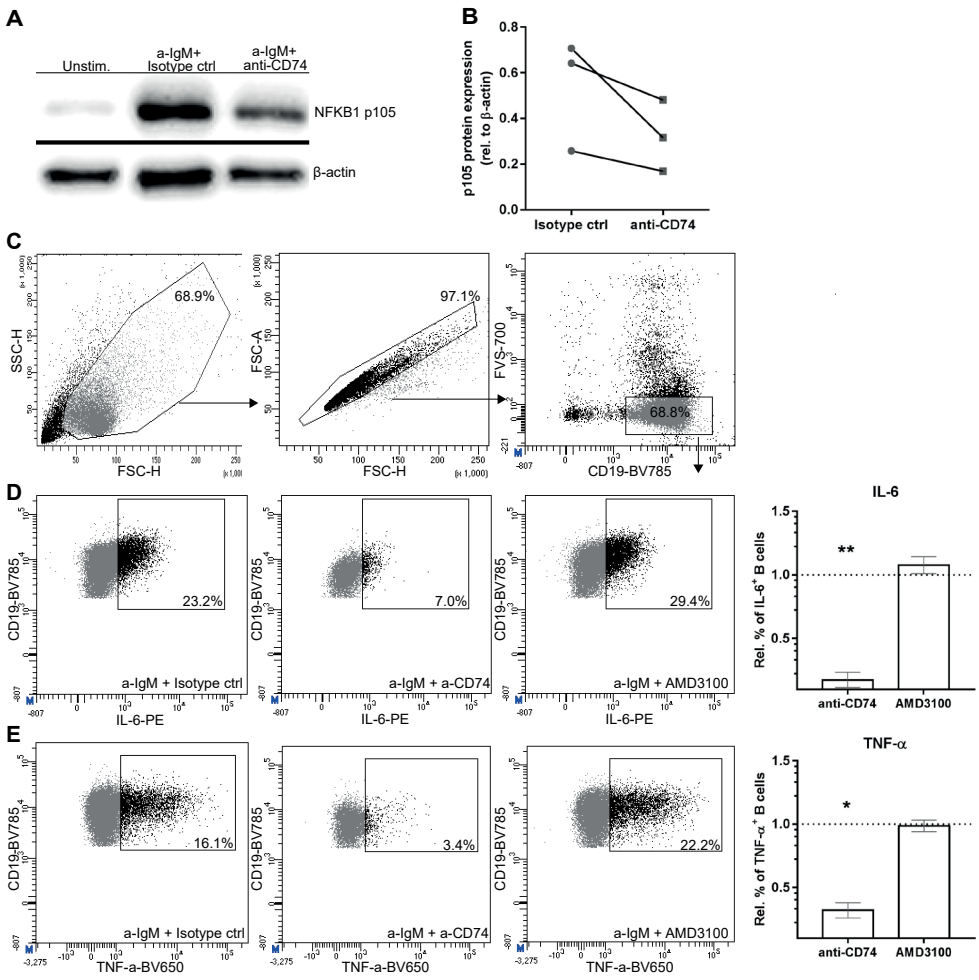
Supplementary Figure 2. CXCR4/CD74 expression ratios on distinct B-cell subsets in RRMS versus HC blood.

Gating of the subsets is shown in Fig. 3. Data were obtained from 3 individual FACS experiments, with B cells from 5 HC and 5 RRMS patients analyzed per experiment. Data are shown as mean ± SEM. Mann-Whitney U tests were used to compare subsets between the RRMS and HC groups. * $p<0.05$.



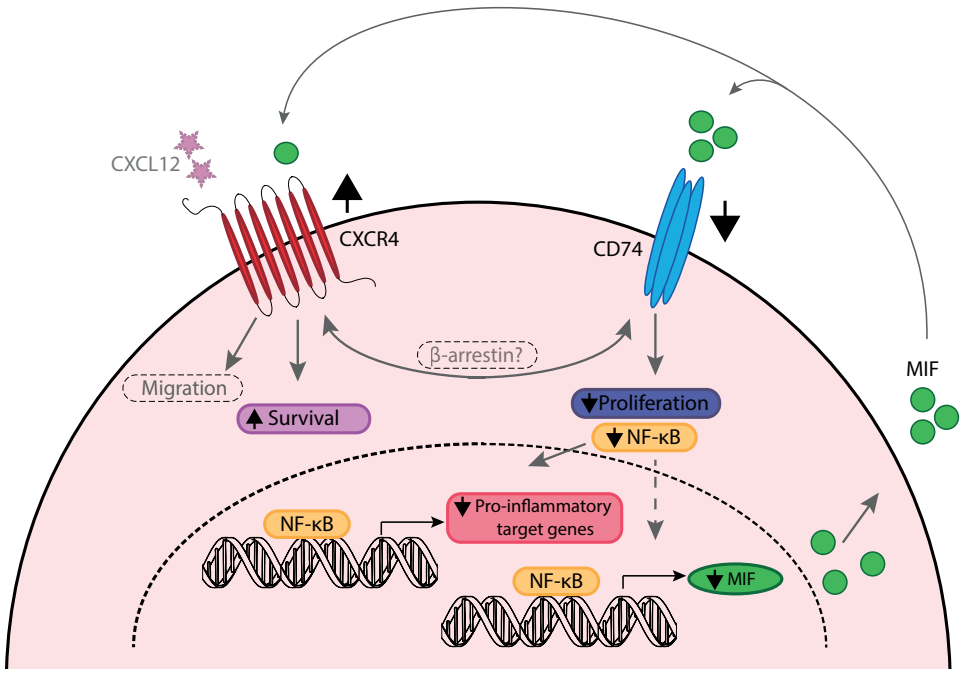
Supplementary Figure 3. Circulating MIF levels and their association with CXCR4/CD74 expression ratios on blood B cells in CIS.

MIF plasma levels (ng/µl) were compared between CIS patients (n=60) and HC (n=29; **A**), as well as low-risk CIS (n=32) and high-risk CIS (n=28; **B**) subgroups using ELISA. Data is measured in one single experiment. Each dot represents the mean value of one individual, measured in duplicates. Data are shown as mean ± SEM. The presence of MIF in serum **(C)** and plasma **(D)** was correlated to CXCR4/CD74 expression ratios on B cells in CIS blood (Fig. 2C). No significant differences and correlations were found.



Supplementary Figure 4. Effects of CD74 and CXCR4 blocking on NF-κB1, IL-6 and TNF-α protein expression in primary B cells.

(A-B) Representative image **(A)** and quantification **(B)** of NF-κB1 protein p105 expression in untreated as well anti-CD74 (LN2) or isotype control treated B cells from healthy blood after 24h of a-IgM stimulation (n=3, measured in one experiment). NF-κB1 p105 protein levels were normalized based on β-actin expression using the same blot. **(C-E)** IL-6 And TNF-α protein expression in anti-CD74 antibody (LN2) or AMD3100 treated healthy blood after *in vitro*-activation with a-IgM for 72h. Data was compared to their respective controls (n=3, measured in one experiment). **(C)** Gating of viable CD19+ B cells. **(D-E)** Gating and quantification of IL-6$^+$ **(D)** and TNF-α$^+$ fractions of treated B cells **(E)**. All used controls were set at 1 (dotted line). Data are shown as mean ± SEM. Paired t-tests were performed to compare groups. * p<0.05, ** p<0.01.



Supplementary Figure 5. Potential counter-regulatory mechanism and downstream effects of CD74 and CXCR4 signaling in peripheral B cells of early MS patients.

Our data demonstrate that CXCR4 is upregulated and CD74 is downregulated on peripheral B cells from early MS patients. Mechanistically, this counter-regulation of CXCR4 and CD74 could be explained by the higher affinity of MIF for CD74 than for CXCR4. After MIF-mediated internalization, CD74 potentially interacts with the adaptor molecule β-arrestin, preventing binding to and endocytosis of surface CXCR4. Functionally, peripheral B cells are less able to proliferate and express pro-inflammatory cytokines (MIF, IL-6, TNF-α) via NF-κB (mediated by CD74), but more capable of surviving peripheral tolerance checkpoints and infiltrating the central nervous system via local MIF production (mediated by CXCR4) in early MS. We postulate that within the central nervous system, $CXCR4^{hi}CD74^{lo}MIF^{lo}$ B cells are activated, proliferate and shift from a quiescent to a more pro-inflammatory state ($CXCR4^{lo}CD74^{hi}MIF^{hi}$).

Supplementary Table 1. Clinical information of patients and healthy controls.

Cohorts		Patients, no	Gender, female no (%)	Age in years, median (IQR)*	Follow-up time in years, median (IQR)*	Disease duration in months, median (IQR) †
FACS (B cells, *ex vivo*)						
HC	Screening	15	12 (80%)	46 (32-60)	NA	NA
	Replication	20	16 (80%)	45 (32-60)	NA	NA
	Validation	20	13 (65%)	46 (24-60)	NA	NA
CIS	Total	33	24 (73%)	34 (22-48)	6.04 (2.17 - 9.81)	2.0 (0.1 - 4.0)
	Low-risk	17	12 (71%)	36 (22-48)	7.08 (5.21 - 9.81)	2.25 (1.0 - 4.0)
	High-risk	16	12 (81%)	32 (24-39)	4.08 (2.17 - 8.69)	2.0 (0.1 - 3.75)
RRMS	Screening	15	12 (80%)	46 (31-61)	NA	NA
	Replication	20	16 (80%)	46 (31-61)	NA	NA
	Validation	20	13 (65%)	46.5 (24-61)	NA	NA
RT-PCR (B cells, *ex vivo*)						
HC		22	14 (64%)	43.5 (24-66)	NA	NA
CIS	Total	18	13 (72%)	35.5 (24-48)	6.65 (2.17-9.81)	2.25 (1.0-4.0)
	Low-risk	13	10 (77%)	36 (25-48)	7.07 (5.21-9.81)	2.25 (1.0-4.0)
	High-risk	5	3 (60%)	32 (24-39)	3.36 (2.17-8.69)	3.0 (2.0-3.75)
RRMS		19	14 (74%)	42 (32-65)	NA	NA
RT-PCR (B cells, *in vitro*)						
HC		10	7 (70%)	30 (25-58)	NA	NA
CIS		9	7 (78%)	33 (23-39)	4.31 (1.34-10.99)	3 (1.5-5.25)
RRMS		9	8 (89%)	39 (21-54)	NA	NA
ELISA (serum and plasma)						
HC		29	19 (66%)	46 (24-60)	NA	NA
CIS	Total	61	43 (70%)	34,5 (19-51)	6.07 (2.17-9.81)	2.0 (0.0-4.0)
	Low-risk	33	24 (73%)	36 (22-51)	6.45 (3.78-9.81)	2.25 (0.5-4.0)
	High-risk	28	19 (68%)	33,5 (19-46)	5.51 (2.17-9.49)	2.0 (0.0-3.75)
RRMS		NA	NA	NA	NA	NA
ELISA (culture medium)						
HC		12	9 (75%)	40.8 (25-58)	NA	NA
CIS		12	8 (67%)	36 (23-47)	4.6 (0.55-8.53)	4.0 (0.0-6.0)
RRMS		12	9 (75%)	40.5 (21-66)	NA	NA

*, at time of sampling; †, time CIS to sampling; RRMS according to the McDonald 2010 criteria. Abbreviations: HC, healthy control; CIS, clinically isolated syndrome; IQR, interquartile range; NA, not applicable or available; RRMS, relapsing-remitting MS.

Supplementary Table 2. Monoclonal antibodies used for FACS.

Marker	Fluorochrome	Clone	Company
CD3	AF700	SK7	Biolegend
CD14	APC	MOP9	BD Biosciences
CD19	BV785	HIB19	Biolegend
CD24	BV605	ML5	BD Biosciences
CD27	BV421	M-T271	BD Biosciences
CD38	PE-Cy7	HIT2	Biolegend
CD56	PE-CF594	B159	BD Biosciences
CD69	BV421	FN50	Biolegend
CD74	APC	LN2	Biolegend
CD95	BV605	DX2	Biolegend
CXCR4	PE-CF594	12G5	BD Biosciences
CXCR4	APC	12G5	BD Biosciences
HLA-DR	PerCP-Cy5.5	L243	Biolegend
IgA	FITC	IS11-8E10	Miltenyi-Biotec GmbH
IgD	PE-CF594	IA6-2	BD Biosciences
IgG	APC-H7	G18-145	BD Biosciences
IgM	BV510	MHM-88	Biolegend
IL-6	PE	MQ2-6A3	BD Biosciences
TNF	BV650	Mab11	BD Biosciences

Supplementary Table 3. Primer sequences used for real-time PCR.

Gene	Forward primer	Reverse primer
MIF	ACCGCTCCTACAGCAAGC	CGCGTTCATGTCGTAATAGTTG
IL6	ATGAGTACAAAAGTCCTGATCCA	CTGCAGCCACTGGTTCTGT
TNF	GCCCAGGCAGTCAGATCATC	GGGTTTGCTACAACATGGGCT
NFKB1	CTGGCAGCTCTTCTCAAAGC	TCCAGGTCATAGAGAGGCTCA

5

Pregnancy-induced effects on memory B-cell development in multiple sclerosis

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ABSTRACT

Background: In MS, pathogenic memory B cells infiltrate the brain and develop into antibody-secreting cells. Chemokine receptors not only define their brain-infiltrating capacity, but also assist in their maturation in germinal centers. How this corresponds to pregnancy, as a naturally occurring modifier of MS, is underexplored.

Objective: To study the impact of pregnancy on both *ex vivo* and *in vitro* B-cell differentiation in MS.

Methods: The composition and outgrowth of peripheral B cells were compared between 19 MS pregnant patients and 12 healthy controls during the third trimester of pregnancy (low-relapse risk) and postpartum (high-relapse risk).

Results: Transitional, and not naive mature, B-cell frequencies dropped in the third trimester, which was most prominent in patients that experienced a pre-pregnancy relapse. Postpartum, these frequencies raised again, while memory B cell frequencies modestly declined. CXCR4 was downregulated and CXCR5, CXCR3 and CCR6 were upregulated on postpartum memory B cells, implying enhanced recruitment into germinal center light zones for interaction with T follicular helper (T_{FH}) cells. Postpartum memory B cells of MS patients expressed higher levels of CCR6 and preferentially developed into plasma cells during T_{FH}-like cultures.

Conclusions: These findings imply that memory B cell differentiation contributes to postpartum relapse risk in MS.

INTRODUCTION

MS is a chronic inflammatory and demyelinating disease of the CNS, for which a key role for B cells in the pathogenesis has been shown in recent years [1]. In the relapsing phase of MS, naive B cells escape peripheral tolerance checkpoints [2] and develop into memory populations that activate CNS-infiltrating, IFN-γ-producing $CD4^+$ T cells [3]. This is further supported by the reduction in T-cell activation in MS patients after receiving anti-CD20 therapy [4]. Normally, memory B cells interact with T cells within germinal centers (GCs) of peripheral secondary lymphoid organs. Our group recently demonstrated that B cells utilize IFN-γ to drive their CXCR3-expression, boosting their ability to recruit to MS brain tissue as well [5]. Hence, in MS, CNS-infiltrating memory B cells are probably reactivated by T cells in perivascular spaces[6] and ectopic lymphoid structures in the cerebral meninges [7] to further mature into plasmablasts/plasma cells and contribute to focal inflammation [6].

IFN-γ-induced CXCR3 expression on B cells is not only associated with increased homing to inflamed tissue and the formation of ectopic lymphoid structures, but also with aberrant plasma cell differentiation within GCs [8, 9]. This is further controlled by the expression of other chemokine receptors, such as CXCR4, CXCR5 and CCR6. CXCR4 and CXCR5 orchestrate the GC response by guiding B cells into dark and light zones respectively. Centroblasts undergo several rounds of proliferation and somatic hypermutation in the dark zone, while centrocytes interact with follicular dendritic cells and T follicular helper (T_{FH}) cells to undergo antigen-specific selection in the light zone [10]. CCR6 has been reported to promote their development and antigen responsiveness in the light zone [11, 12]. Therefore, specific aberrancies in chemokine receptor profiles may affect the outgrowth of B cells into memory and plasmablasts/plasma cells [13]. Currently, it remains to be determined whether this is related to relapse risk in MS.

Notably, in pregnant MS patients, relapse risk is reduced by approximately 70% in the third trimester. This increases in the first three months after delivery, with almost 30% of patients having a postpartum relapse [14]. Although current diagnostic and treatment strategies probably contribute to an attenuation of this fluctuation [15], these specific phases of pregnancy can still be considered periods of relatively low and high relapse risk in MS. Pregnancy is known to cause a shift from T_H1 to T_H2 responses [16] and promote the expansion of circulating T_{FH} cells [17], therefore likely affecting the maturation of B cells in GCs.

In this experimental study, we assessed the frequencies and GC-related chemokine receptor profiles on *ex vivo* B-cell subsets in paired first trimester, third trimester and early postpartum blood samples from MS patients and healthy controls. In addition, we used a T_{FH}-like cell culture system to investigate how *in vitro* memory B-cell differentiation into

plasmablasts or plasma cells differs between periods of relatively low (third trimester) and high (early postpartum) relapse risk.

METHODS

Participants

Nineteen pregnant women with RRMS were included at the MS Center ErasMS as part of our previous study [18] and retrospectively validated to match the most recent McDonald 2017 criteria [19]. Patients did not use any immune modulatory medication before, during and early after pregnancy. Two patients gave birth via a cesarean section and all others had vaginal deliveries. No relapses were observed during pregnancy. During the early postpartum period, 6 patients developed a clinically defined relapse [20]. These patients did not differ from non-relapsing patients in their third trimester expanded disability status scale (EDSS) scores. No MRI evaluations for disease activity were performed. Additionally, 12 age-matched healthy pregnant women were included, who did not have central nervous system or inflammatory disease and were seen at the outpatient obstetric clinic at Erasmus MC. None of the healthy pregnant women used immunomodulatory medication before or during the study, were hypertensive, experienced recurrent abortions or were diagnosed with diabetes mellitus. Clinical characteristics of patients and controls are depicted in Table 1. All participants gave written informed consent and this study was approved by the medical ethics committee of Erasmus MC.

PBMC isolation, flow cytometry and antibodies

PBMCs from patients and controls were collected in the first and third trimester of pregnancy as well as 4-8 weeks after delivery (postpartum). For sample collection, we used Vacutainer CPT® tubes containing sodium heparin according to manufacturer's instructions (BD Biosciences, Erembodegem, Belgium). After isolation, cells were taken up in RPMI 1640 (Lonza, Basel, Switzerland) containing 20% fetal calf serum (Lonza) and 10% dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO) and stored in liquid nitrogen until further use. Cells were pre-incubated with Fixable Viability Stain 700 (BD Biosciences) for 15 min at 4°C. The following monoclonal antibodies were used stained for 30 min at 4°C: CD27 (BV421, M-T271), CD138 (BV605 and PE-CF594, MI15), CXCR4 (PE-CF594, 12G5), CXCR5 (PercCP, RF8B2), IgD (PE-CF594, IA6), IgG (APC-H7, G18-145; all BD Biosciences), CCR6 (PE, G034E3), CD19 (BV785, HIB19), CD38 (BV605 and PE-Cy7, HIT2), CXCR3 (APC and PE-Cy7, G025H7), CXCR4 (APC-Cy7, 12G5) and IgM (BV510, MHM-88; all Biolegend, London, UK). Stained cells were measured using an LSRII-Fortessa flow cytometer. For *in vitro* culture experiments, memory ($CD19^+CD3^-CD27^+$) B cells were purified using a FACSAria III sorter.

Table 1. Clinical information of pregnant MS patients and healthy controls.

	Ex vivo		
	RRMS, no PP relapse	RRMS, PP relapse	HC
Number of individuals	13	6	12
Median maternal age (range)	33.3 (26.8-33.8)	32.1 (29.8-33.7)	33.3 (27.7-34.1)
Median EDSS third trimester (range)	1.5 (0.0-2.0)	1.0 (0.0-2.0)	N.A.
Median EDSS postpartum (range)	1.0 (0.0-2.0)	1.8 (1.0-4.5)	N.A.
Nullipara	4	1	9
Caesarean section	2	0	1
(Pre)eclampsia	0	0	0
Median gestation (weeks, range)	40 (38.0-42.0)	38.0 (38.0-40.0)	39.0 (32.0-42.0)
	In vitro		
	RRMS, no PP relapse	RRMS, PP relapse	HC
Number of individuals	8	3	5
Median maternal age	31.9 (26.8-37.3)	31.5 (N.A)	27.9 (27.2-27.9)
Median EDSS third trimester (range)	1.5 (0.0-2.0)	2.0 (0.0-2.0)	N.A.
Median EDSS postpartum (range)	1.0 (0.0-2.0)	2.0 (1.5-3)	N.A.
Nullipara	5	2	1
Caesarean section	2	0	0
(Pre)eclampsia	0	0	0
Median gestation (weeks, range)	39.5 (38.0-41.0)	39 (38.0-40.0)	38.6 (34.0-42.0)

Information on cesarean section, nullipara, pre(eclampsia) and gestational period was missing for 1 RRMS patient with a postpartum (PP) relapse.

Data were analyzed using FACS Diva software, version 8.0.1 (all BD Biosciences). Since *ex vivo* B-cell phenotyping of the relapsing MS group was performed in a separate study, it was not possible to compare the MFI of functional markers with the non-relapsing MS or control groups.

Germinal center-like B-cell differentiation assay

In vitro B-cell differentiation assays were performed as recently described [5, 21]. In short, irradiated murine 3T3 fibroblasts expressing human CD40L were co-cultured with sorted memory B cells in the presence of IL-21 (50 ng/ml; Thermo Fisher Scientific, Landsmeer, The Netherlands). After 6 days of culturing, viable $CD19^+$ cells were analyzed using flow cytometry and supernatants were collected and stored at -80°C until further use.

IgM and IgG ELISA

IgM and IgG levels were determined in supernatants of memory B cells cultured for 6 days using ELISA. After overnight coating with goat anti-human Ig (1 mg/ml; Southern Biotech, Birmingham, USA) at 4°C, flat-bottom 96-well plates (Corning, Tewksbury, USA) were washed with PBS/0.05%Tween-20 and subsequently blocked with PBS/5%FCS for 2 h at RT. Samples were added for 1.5 h at room temperature. After washing, peroxidase-conjugated

goat anti-human IgG (Thermo Fisher Scientific) or rabbit anti-human IgM (Jackson, Uden, The Netherlands) were used to detect bound antibody. 3,3',5,5'-Tetramethylbenzidine substrate (Thermo Fisher Scientific) was used to reveal peroxidase activity. Reactions were stopped with sulfuric acid and optical densities were measured at 450 nm using a BioTek Synergy 2 reader (Winooski, USA). Concentrations were calculated using standard curves for IgM and IgG.

Statistical analysis

Graphpad Prism software (version 8) was used for statistical analyses. Both percentages and mean fluorescent intensity (MFI) are shown as individual data points together with the corresponding mean. We compared paired data using Wilcoxon signed-rank tests and data between clinical groups using 2-way analysis of variance (ANOVA) with Bonferroni's multiple comparison tests, unless stated otherwise. *P* values < 0.05 were considered statistically significant.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

RESULTS

Pregnancy alters the peripheral B-cell compartment resulting in memory populations with increased Ig expression in the early postpartum period

To explore whether *ex vivo* B-cell differentiation profiles are associated with pregnancy-induced relapse risk in MS, we analyzed the proportions of different naive and memory populations in paired first trimester (T1), third trimester (T3) and early postpartum (PP) blood. For this, we thawed PBMCs of 19 MS patients and 12 healthy controls (Table 1) [18] and distinguished transitional ($CD38^{high}CD27^{-}$), naive mature ($CD38^{-/dim}CD27^{-}IgM^{+}$) as well as IgM^{+} and IgG^{+} memory ($CD38^{-/dim}CD27^{+}$) B cells using flow cytometry (Figure 1A).

In MS patients without a postpartum relapse (n = 13) and healthy controls, the proportion of transitional B cells declined from first to third trimester and recuperated after delivery (Figure 1B). Naive mature B-cell frequencies were not different between periods, resulting in elevated naive mature/transitional B-cell ratios per individual in the third trimester (Supplementary Figure 1A). These ratios were further increased in patients with a relapse one year before pregnancy (Supplementary Figure 1B) and were lower in MS patients with an early postpartum relapse (n = 6; Table 1 and Supplementary Figure 1C). IgM and not IgD expression was significantly increased on postpartum transitional B cells,

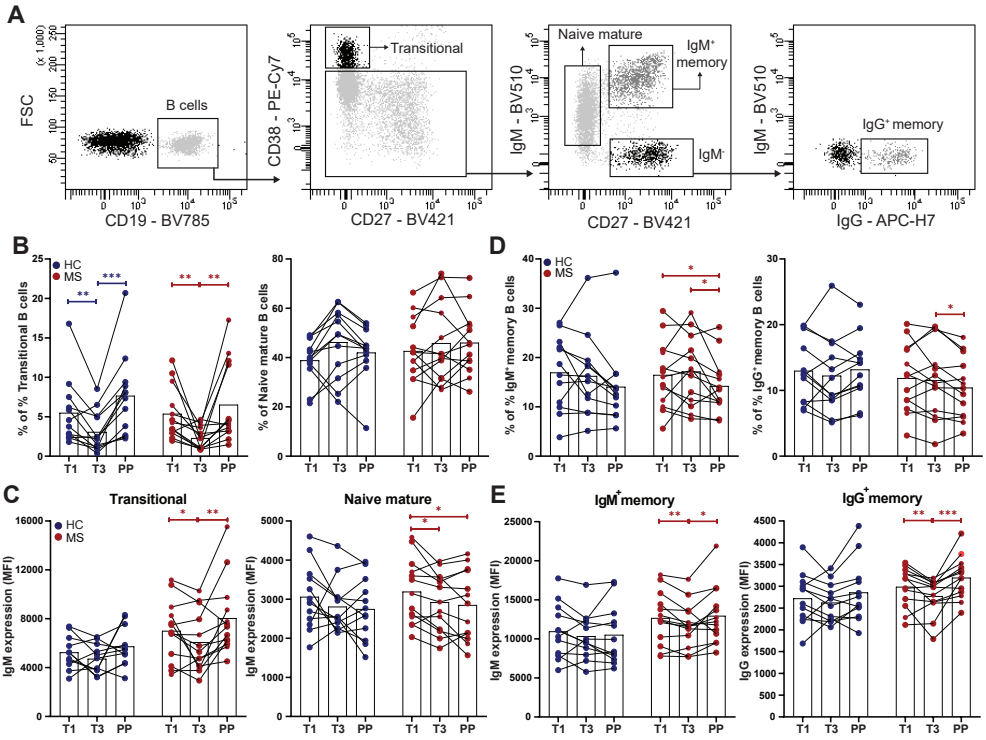

Figure 1. Frequencies of circulating naive and memory B-cell subsets during and early after pregnancy in MS patients and healthy controls.

(**A**) Representative gating of viable CD19^{+} B-cell subsets: transitional (CD38^{high}CD27^{-}), naive mature (CD38$^{-/dim}$CD27^{-}IgM^{+}), IgM memory (IgM^{+}CD27^{+}) and IgG memory (IgG^{+}CD27^{+}) B cells. The percentages of transitional and naive mature B cells (**B**) as well as IgM and IgD expression (MFI) on these subsets (**C**) were compared between paired first trimester (T1), third trimester (T3) and postpartum (PP) blood samples of 13 MS patients who did not experience a postpartum relapse (red) and 12 HC (blue). Similar analyses were performed for IgM^{+} and IgG^{+} memory B cells (**D** and **E**). Wilcoxon signed-rank test was performed to compared the different gestational periods. * p<0.05, ** p<0.01, *** p<0.001.

which was not seen for naive mature B cells and was the most pronounced in MS patients (p = 0.0056, Figure 1C and Supplementary Figure 1D). Both IgM^{+} and IgG^{+} memory B cells showed a moderate decline in frequencies, but a significant increase in Ig surface expression in the postpartum period (p = 0.0473 for IgM^{+} and p = 0.0010 for IgG^{+}; Figure 1D-E). These differences in expression level and memory fractions were not influenced by an early pre-pregnancy and postpartum relapse (Supplementary Figure 2). The pregnancy-induced disturbances in naive to memory B-cell development *ex vivo* may imply favored differentiation in GCs in the early postpartum period [22].

Memory B cells reveal a more GC light zone-related chemokine receptor expression profile in postpartum versus third trimester samples

Chemokine receptors selectively regulate GC organization and maturation of B cells (Figure 2A). First trimester, third trimester and postpartum B cells were analyzed for the expression of CXCR4 and CXCR5, which mediate dark and light zone localization, respectively [10], as well as CCR6 and CXCR3, which contribute to memory recall and antibody responses [8, 23], respectively.

CXCR4 was downregulated, while CXCR5 was upregulated on postpartum versus third trimester B cells. This was seen for both memory (IgM^+ and IgG^+; Figure 2B and C) and naive (Supplementary Figure 3A) B cells. The postpartum rise in CXCR5 was significant in the

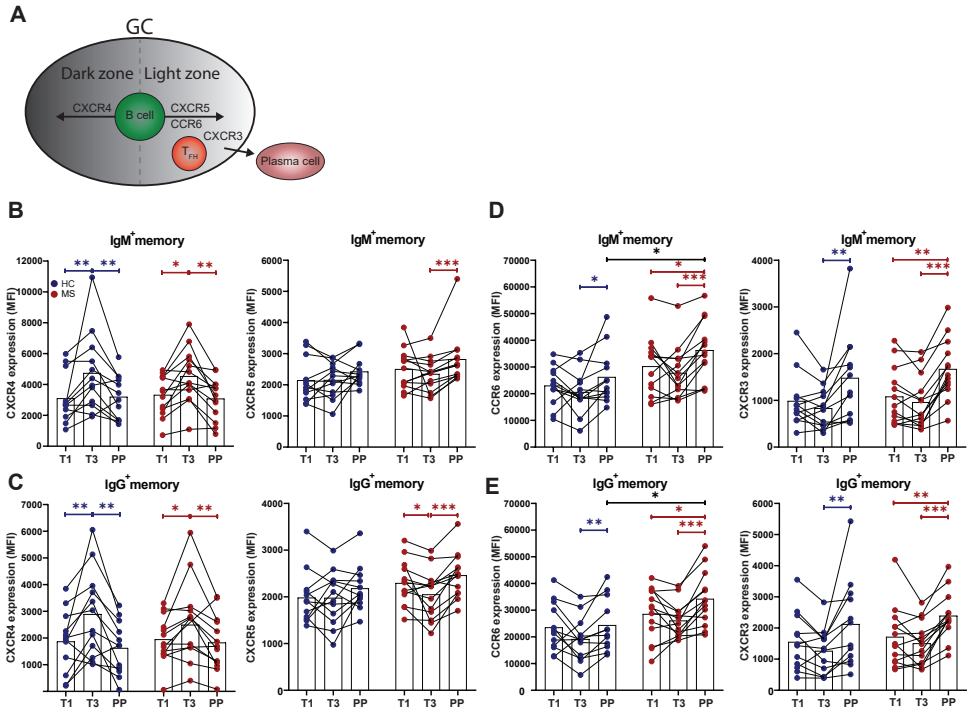

Figure 2. Chemokine receptor expression on circulating memory B cells during and early after pregnancy in MS patients and healthy controls.

(**A**) Schematic display of chemokine receptors involved in GC-dependent organization and maturation of B cells. Expression of dark zone-associated CXCR4 and light zone-associated CXCR5, CCR6 and CXCR3 was compared between IgM^+ (**B, D**) and IgG^+ (**C, E**) memory B cells from first trimester (T1), third trimester (T3) and postpartum (PP) samples from 13 MS patients without a postpartum relapse (red) and 12 HC (blue). Wilcoxon signed-rank test was performed to compare the different gestational periods. Two-way ANOVA with Bonferroni's multiple comparison test was performed to compare HC with MS patients. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

MS but not in the healthy control group. CXCR4 and CXCR5 levels were higher on naive than memory B cells (Supplementary Figure 4A). CCR6 and CXCR3 were upregulated on postpartum B cells in both groups (Figure 2D and E; Supplementary Figure 3B). In the MS group, postpartum CCR6 levels were higher compared to healthy controls as well as first trimester B cells (Figure 2D and E). For all the groups and periods, CCR6 was predominantly expressed on naive B cells, while CXCR3 expression was most pronounced on memory B cells (Supplementary Figure 4B). In contrast to CXCR3, CCR6 was not upregulated on postpartum B cells from 4 of 6 MS patients experiencing a postpartum relapse (Supplementary Figure 5). We found no significant impact of a pre-pregnancy relapse on chemokine receptor expression (data not shown).

Together with the increased Ig surface levels (Figure 1E), the distinct chemokine receptor expression profile for *ex vivo* postpartum memory B cells particularly from MS patients ($CXCR3^{high}CXCR4^{low}CXCR5^{high}CCR6^{high}$) implies an increased potential of these cells to recruit and further develop in the GC light zone.

Postpartum memory B cells of MS patients preferentially develop into Ig-secreting plasma cells under TFH-like conditions in vitro

B-cell memory formation is generated through the help of T_{FH} cells within the GC light zone. After reaching the CNS, memory B cells are likely reactivated to develop into potent antibody-secreting cells in MS patients. To assess whether B cells are prone for such recall responses in the high-risk postpartum period in MS, memory ($CD27^{+}$) B cells were purified from paired third trimester and postpartum samples of 11 MS patients and compared for their outgrowth into plasmablasts/plasma cells *in vitro*. Under IL-21- and CD40L-inducing conditions, which mimic a T_{FH} cell response, more antibody-secreting cells ($CD38^{high}CD27^{high}$) were formed in cultures with postpartum versus third trimester memory B cells ($p = 0.023$, Figure 3A and B). A similar trend was seen for memory B cells derived from paired samples of healthy controls (Figure 3B). When we analyzed plasma cell ($CD138^{+}CD38^{high}CD27^{high}$) frequencies, these were mainly increased for postpartum memory B cells from MS patients, which were significantly higher than those from healthy controls ($p = 0.001$, Figure 3C). For the MS group, IgM^{+} and not IgG^{+} plasmablasts ($CD38^{high}CD27^{high}$) were more induced in both third trimester and postpartum memory B-cell cultures (Figure 3D). Especially IgM levels were elevated in the supernatants of postpartum memory B-cell cultures, but were not different between patients and healthy controls (Figure 3E).

These results show that memory B cells are highly capable of differentiating into antibody-secreting cells early after parturition, which may contribute to the high postpartum relapse risk in MS.

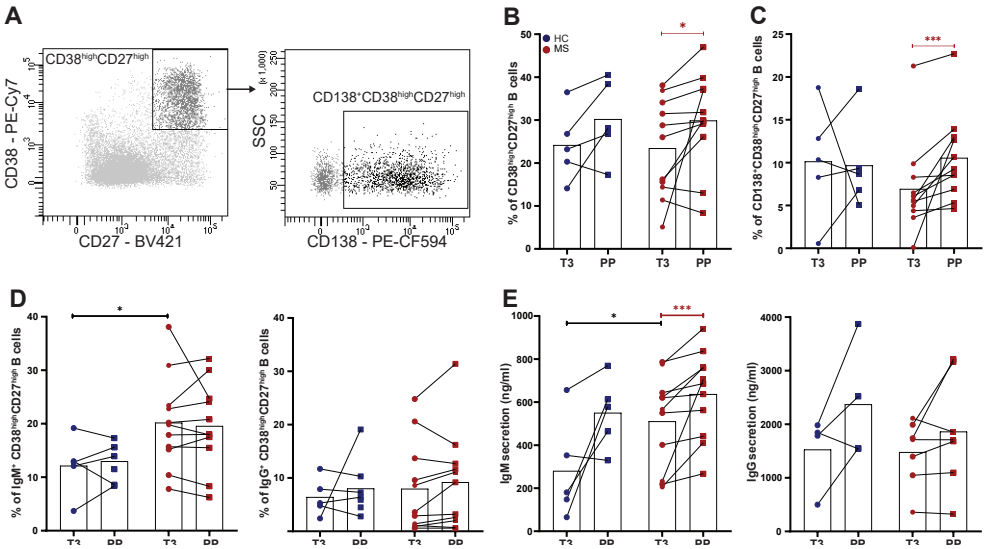

Figure 3. *In vitro* outgrowth of antibody-secreting cells using memory B cells from paired third trimester and postpartum blood samples.

(**A**) Representative gating of CD138-expressing antibody-secreting cells ($CD38^{high}CD27^{high}$) after culturing of memory B cells under IL-21/CD40L-stimulating (T_{FH}-like) conditions for 6 days. Fractions of $CD38^{high}CD27^{high}$ plasmablasts/plasma cells (**B**), $CD138^{+}CD38^{high}CD27^{high}$ plasma cells (**C**) and IgM^{+} and IgG^{+} $CD38^{high}CD27^{high}$ plasmablasts (**D**; FACS), as well as IgM and IgG secretion (**E**; ELISA) were compared between cultures with third trimester (T3) and postpartum (PP) memory B cells from 11 MS patients (red) and 5 HC (blue). Wilcoxon signed-rank test was performed to compare third trimester and postpartum samples. Two-way ANOVA was performed to compare MS and HC groups. * $p<0.05$, *** $p<0.001$.

DISCUSSION

Pregnancy causes a transient period of immune suppression, which is lost early after delivery in MS. Here, we studied how peripheral B-cell differentiation is regulated in pregnancy-associated low and high relapse risk periods in MS patients. The differential chemokine receptor expression pattern ($CXCR3^{high}CXCR4^{low}CXCR5^{high}CCR6^{high}$) found for *ex vivo* postpartum B cells implied a marked propensity to recruit to the T_{FH} cell-containing GC light zone [10, 11]. The elevated expression of CCR6 on *ex vivo* postpartum memory B cells of MS patients compared to healthy controls pointed towards increased, T cell-dependent recall responsiveness [11, 23]. This was supported by their ability to differentiate into plasma cells under T_{FH}-like conditions *in vitro*.

Regarding naive B cells, the proportion of circulating transitional B cells was found to be decreased in third trimester compared to postpartum samples. A similar observation was made in a previous study [24]. We additionally show that this decline occurs in both MS patients and healthy controls and is also seen when comparing to samples from the first trimester, a phase in which relapse rates are less reduced [14]. These pregnancy-induced alterations in B lymphopoiesis are probably the result of increased hormone levels in the third trimester, keeping transitional B cells in check due to a lack of multidrug resistance receptor 1 (MDR1) [25], a glycoprotein which pumps steroids out of cells. We recently found a similar impact of steroid treatment in patients with MS, AQP4-IgG^{+} neuromyelitis optica spectrum disorder and MOG-IgG-associated disease [26]. Because of the rise in IgM^{high} transitional B cells early after delivery, one could speculate that this results in increased entrance of potentially autoreactive naive mature B cells to GCs, resulting in the development of pathogenic memory subsets that are destined to enter the CNS and contribute to an MS relapse. Although we did not touch upon their CNS-infiltrating ability in this study, we can at least assume that the postpartum increase in CXCR3 expression mediates local B-cell enrichment in MS [5]. This may be further induced by the observed abundance of CCR6 on postpartum B cells of MS patients.

The postpartum upregulation of CXCR5 especially seen in MS patients is likely involved in B-cell organization rather than recruitment in the CNS [10, 27]. Our group previously reported an upregulation of CXCR4 during MS onset in non-pregnant patients [28]. The observed reduction in CXCR4 expression during the high relapse risk postpartum period is therefore counterintuitive. A possible explanation for this discrepancy may be that during a primary response, CXCR4^{high} naive mature B cells escape from T_{FH}-mediated selection in the GC light zones of peripheral secondary lymphoid organs [13], while CXCR4^{low} memory B cells are more prone to interact with T_{FH} cells and develop into long-lived plasma cells during a recall response in the CNS. CCR6 could facilitate such recall responses [23] and, together with CXCR3, has also been associated with the production of high affinity antibodies [29].

Thus far, the potential of functionally distinct memory B cells to develop into plasma cells has been relatively understudied in MS. Despite the current focus on antibody-independent B-cell functions, this is of high relevance as long-lived plasma cells reside within the chronically inflamed CNS of MS patients [30]. The clinical relevance of local Ig production has become apparent from the increased risk of CIS to MS conversion in patients with CSF oligoclonal bands [31], which are present in more than 95% of MS patients and indicates ongoing IgG production in the CNS. Consistently, the absence of B cells in brain lesions of MS patients is associated with a lack of CSF oligoclonal bands, a lower intrathecal IgG production, and a more favorable outcome [32]. The observation that anti-CD20 treatment reduces CSF B cell numbers while oligoclonal bands persist [33] suggests that

intrathecal IgG are mainly produced by ($CD20^-$) long-lived plasma cells in the CNS. Recently, it has been shown that MS myelin is bound by IgG and that IgG immune complexes trigger human microglia, resulting in enhanced production of pro-inflammatory cytokines [34].

Our study has some limitations. The relatively low numbers of included subjects hampered the analysis of MS risk groups based on disease activity before and after pregnancy. Sequential data collection of such patients is difficult, but the accumulating evidence for the safe continuation during pregnancy of various disease modifying therapies may increase options [35]. Furthermore, we did not perform *in vitro* B-cell cultures with pregnancy-related sera or hormones to verify whether the observed GC light zone phenotype is controlled by extrinsic factors. Finally, although beyond the scope of this study, intrinsic factors such as memory B-cell EBV load could contribute to the increased CXCR3 expression and plasma cell formation [21] as seen early after delivery.

Together, this work provides new insights into how B-cell development is affected during high and low risk periods associated with pregnancy in MS. We demonstrate a first link between chemokine receptor expression profiles and the capacity of (potentially brain-infiltrating) memory B cells to differentiate into plasma cells, which should be further studied in the near future. This may not only help to decipher underlying mechanisms of local B-cell accumulation and antibody production, but also offer new tools to better predict disease activity in patients with MS.

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REFERENCES

1 **Thompson, A. J., Baranzini, S. E., Geurts, J., Hemmer, B. and Ciccarelli, O.,** Multiple sclerosis. *The Lancet* 2018. **391**: 1622-1636.

2 **Kinnunen, T., Chamberlain, N., Morbach, H., Cantaert, T., Lynch, M., Preston-Hurlburt, P., Herold, K. C., et al.,** Specific peripheral B cell tolerance defects in patients with multiple sclerosis. *J Clin Invest* 2013. **123**: 2737-2741.

3 **Jelcic, I., Al Nimer, F., Wang, J., Lentsch, V., Planas, R., Jelcic, I., Madjovski, A., et al.,** Memory B Cells Activate Brain-Homing, Autoreactive CD4(+) T Cells in Multiple Sclerosis. *Cell* 2018. **175**: 85-100 e123.

4 **Bar-Or, A., Fawaz, L., Fan, B., Darlington, P. J., Rieger, A., Ghorayeb, C., Calabresi, P. A., et al.,** Abnormal B-cell cytokine responses a trigger of T-cell-mediated disease in MS? *Ann Neurol* 2010. **67**: 452-461.

5 **van Langelaar, J., Rijvers, L., Janssen, M., Wierenga-Wolf, A. F., Melief, M. J., Siepman, T. A., de Vries, H. E., et al.,** Induction of brain-infiltrating T-bet-expressing B cells in multiple sclerosis. *Ann Neurol* 2019. **86**: 264-278.

6 **Machado-Santos, J., Saji, E., Troscher, A. R., Paunovic, M., Liblau, R., Gabriely, G., Bien, C. G., et al.,** The compartmentalized inflammatory response in the multiple sclerosis brain is composed of tissue-resident CD8+ T lymphocytes and B cells. *Brain* 2018. **141**: 2066-2082.

7 **Serafini, B., Rosicarelli, B., Magliozzi, R., Stigliano, E. and Aloisi, F.,** Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. *Brain Pathol* 2004. **14**: 164-174.

8 **Piovesan, D., Tempany, J., Di Pietro, A., Baas, I., Yiannis, C., O'Donnell, K., Chen, Y., et al.,** c-Myb Regulates the T-Bet-Dependent Differentiation Program in B Cells to Coordinate Antibody Responses. *Cell Rep* 2017. **19**: 461-470.

9 **Rawlings, D. J., Metzler, G., Wray-Dutra, M. and Jackson, S. W.,** Altered B cell signalling in autoimmunity. *Nat Rev Immunol* 2017. **17**: 421-436.

10 **Allen, C. D., Ansel, K. M., Low, C., Lesley, R., Tamamura, H., Fujii, N. and Cyster, J. G.,** Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol* 2004. **5**: 943-952.

11 **Suan, D., Krautler, N. J., Maag, J. L. V., Butt, D., Bourne, K., Hermes, J. R., Avery, D. T., et al.,** CCR6 Defines Memory B Cell Precursors in Mouse and Human Germinal Centers, Revealing Light-Zone Location and Predominant Low Antigen Affinity. *Immunity* 2017. **47**: 1142-1153 e1144.

12 **Reimer, D., Lee, A. Y. S., Bannan, J., Fromm, P., Kara, E. E., Comerford, I., McColl, S., et al.,** Early CCR6 expression on B cells modulates germinal centre kinetics and efficient antibody responses. *Immunol Cell Biol* 2017. **95**: 33-41.

13 **Wu, B., Zhao, L. and Zhang, X.,** CXCR4 and CXCR5 orchestrate dynamic germinal center reactions and may contribute to the pathogenesis of systemic lupus erythematosus. *Cell Mol Immunol* 2019. **16**: 724-726.

14 **Confavreux, C., Hutchinson, M., Hours, M. M., Cortinovis-Tourniaire, P. and Moreau, T.,** Rate of Pregnancy-Related Relapse in Multiple Sclerosis. *N Engl J Med* 1998. **339**: 285-291.

15 **Dobson, R., Jokubaitis, V. G. and Giovannoni, G.,** Change in pregnancy-associated multiple sclerosis relapse rates over time: a meta-analysis. *Mult Scler Relat Disord* 2020. **44**: 102241.

16 **Wang, W., Sung, N., Gilman-Sachs, A. and Kwak-Kim, J.,** T Helper (Th) Cell Profiles in Pregnancy and Recurrent Pregnancy Losses: Th1/Th2/Th9/Th17/Th22/Tfh Cells. *Front Immunol* 2020. **11**.

17 **Monteiro, C., Kasahara, T. M., Castro, J. R., Sacramento, P. M., Hygino, J., Centurião, N., Cassano, T., et al.,** Pregnancy favors the expansion of circulating functional follicular helper T Cells. *J Reprod Immunol* 2017. **121**: 1-10.

18 **Neuteboom, R. F., Verbraak, E., Wierenga-Wolf, A. F., van Meurs, M., Steegers, E. A. P., de Groot, C. J. M., Laman, J. D., et al.,** Pregnancy-induced fluctuations in functional T-cell subsets in multiple sclerosis patients. *Multiple Sclerosis Journal* 2010. **16**: 1073-1078.

19 **Thompson, A. J., Banwell, B. L., Barkhof, F., Carroll, W. M., Coetzee, T., Comi, G., Correale, J., et al.,** Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *Lancet Neurol* 2018. **17**: 162-173.

20 **Neuteboom, R. F., Verbraak, E., Voerman, J. S. A., van Meurs, M., Steegers, E. A. P., de Groot, C. J. M., Laman, J. D., et al.,** Serum leptin levels during pregnancy in multiple sclerosis. *Multiple Sclerosis Journal* 2009. **15**: 907-912.

21 **van Langelaar, J., Wierenga-Wolf, A. F., Samijn, J. P. A., Luijks, C. J. M., Siepman, T. A., van Doorn, P. A., Bell, A., et al.,** The association of Epstein-Barr virus infection with CXCR3(+) B-cell development in multiple sclerosis impact of immunotherapies. *Eur J Immunol* 2020.

22 **Bautista, D., Vásquez, C., Ayala-Ramírez, P., Téllez-Sosa, J., Godoy-Lozano, E., Martínez-Barnetche, J., Franco, M., et al.,** Differential Expression of IgM and IgD Discriminates Two Subpopulations of Human Circulating IgM(+)IgD(+)CD27(+) B Cells That Differ Phenotypically, Functionally, and Genetically. *Front Immunol* 2020. **11**: 736.

23 **Elgueta, R., Marks, E., Nowak, E., Menezes, S., Benson, M., Raman, V. S., Ortiz, C., et al.,** CCR6-Dependent Positioning of Memory B Cells Is Essential for Their Ability To Mount a Recall Response to Antigen. *J Immunol* 2015. **194**: 505-513.

24 **Lima, J., Martins, C., Leandro, M. J., Nunes, G., Sousa, M.-J., Branco, J. C. and Borrego, L.-M.,** Characterization of B cells in healthy pregnant women from late pregnancy to post-partum: a prospective observational study. *BMC Pregnancy and Childbirth* 2016. **16**: 139.

25 **Wirths, S. and Lanzavecchia, A.,** ABCB1 transporter discriminates human resting naive B cells from cycling transitional and memory B cells. *Eur J Immunol* 2005. **35**: 3433-3441.

26 **Janssen, M., Bruijstens, A. L., van Langelaar, J., Wong, Y., Wierenga-Wolf, A. F., Melief, M.-J., Rijvers, L., et al.,** Naive B cells in neuromyelitis optica spectrum disorders: impact of steroid use and relapses. *Brain Communications* 2020. **2**.

27 **Rainey-Barger, E. K., Rumble, J. M., Lalor, S. J., Esen, N., Segal, B. M. and Irani, D. N.,** The lymphoid chemokine, CXCL13, is dispensable for the initial recruitment of B cells to the acutely inflamed central nervous system. *Brain Behav Immun* 2011. **25**: 922-931.

28 **Rijvers, L., Melief, M.-J., van der Vuurst de Vries, R. M., Stéphant, M., van Langelaar, J., Wierenga-Wolf, A. F., Hogervorst, J. M., et al.,** The macrophage migration inhibitory factor pathway in human B cells is tightly controlled and dysregulated in multiple sclerosis. *Eur J Immunol* 2018. **48**: 1861-1871.

29 **Wiede, F., Fromm, P. D., Comerford, I., Kara, E., Bannan, J., Schuh, W., Ranasinghe, C., et al.,** CCR6 is transiently upregulated on B cells after activation and modulates the germinal center reaction in the mouse. *Immunol Cell Biol* 2013. **91**: 335-339.

30 **Pollok, K., Mothes, R., Ulbricht, C., Liebheit, A., Gerken, J. D., Uhlmann, S., Paul, F., et al.,** The chronically inflamed central nervous system provides niches for long-lived plasma cells. *Acta Neuropathol Commun* 2017. **5**: 88.

31 **Dobson, R., Ramagopalan, S., Davis, A. and Giovannoni, G.,** Cerebrospinal fluid oligoclonal bands in multiple sclerosis and clinically isolated syndromes: a meta-analysis of prevalence, prognosis and effect of latitude. *J Neurol Neurosurg Psychiatry* 2013. **84**: 909-914.

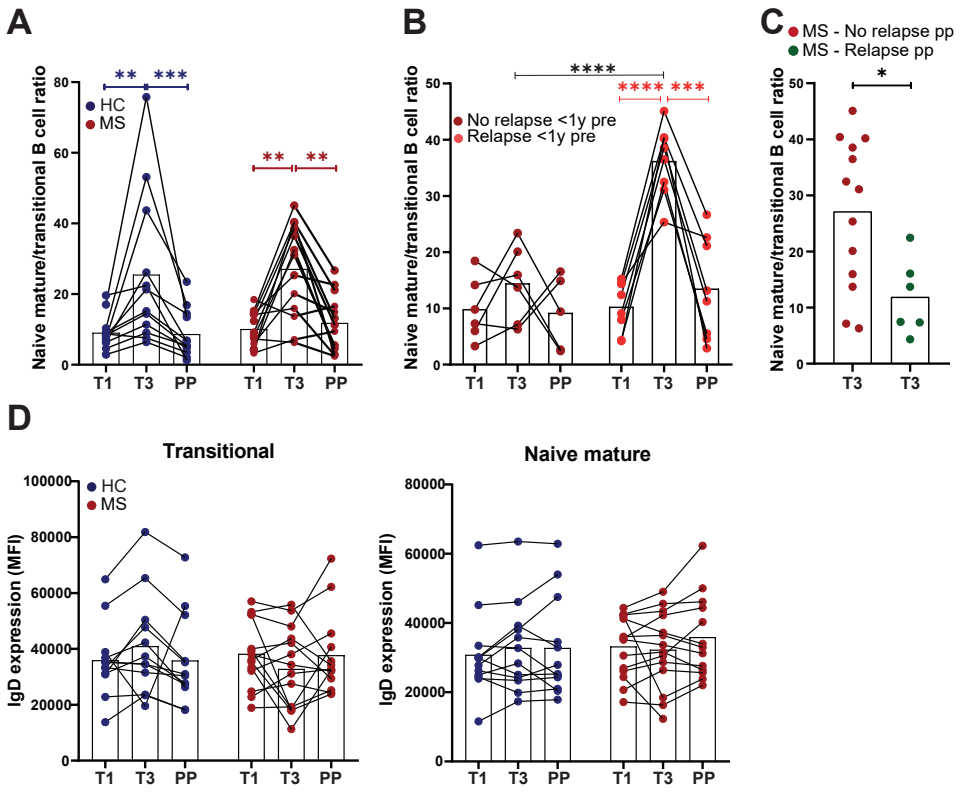
32 **Fransen, N. L., de Jong, B. A., Heß, K., Kuhlmann, T., Vincenten, M. C. J., Hamann, J., Huitinga, I., et al.,** Absence of B Cells in Brainstem and White Matter Lesions Associates With Less Severe Disease and Absence of Oligoclonal Bands in MS. *Neurol Neuroimmunol Neuroinflamm* 2021. **8**.

33 **Hauser, S. L., Waubant, E., Arnold, D. L., Vollmer, T., Antel, J., Fox, R. J., Bar-Or, A., et al.,** B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med* 2008. **358**: 676-688.

34 **van der Poel, M., Hoepel, W., Hamann, J., Huitinga, I. and Dunnen, J. d.,** IgG Immune Complexes Break Immune Tolerance of Human Microglia. *J Immunol* 2020: ji2000130.

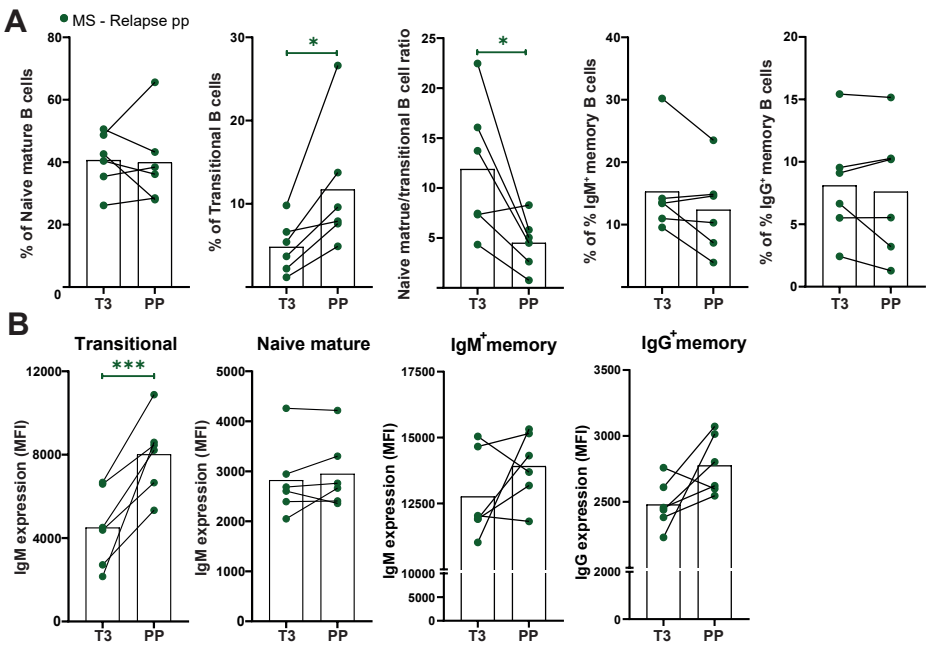
35 **Alroughani, R., Inshasi, J., Al-Asmi, A., Alkhabouri, J., Alsaadi, T., Alsalti, A., Boshra, A., et al.,** Disease-Modifying Drugs and Family Planning in People with Multiple Sclerosis: A Consensus Narrative Review from the Gulf Region. *Neurology and Therapy* 2020. **9**: 265-280.

SUPPLEMENTARY FILES



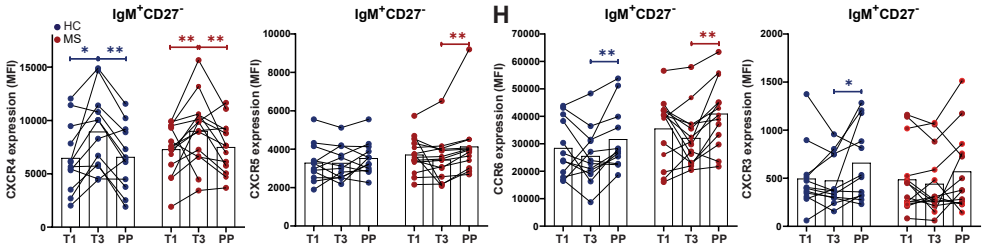
Supplementary Figure 1. The naive mature/transitional B cell ratio and the IgD surface expression on these subsets.

(**A**) The naive mature/transitional B cell ratio in the different gestational periods of 12 HC (blue) and 13 MS patients (red), which in **(B)** were separated into MS patients with (n = 8) or without (n = 6) a relapse within 1 year before pregnancy. **(C)** The naive mature/transitional B cell ratio in the 3rd trimester of MS patients with (n=6) or without (n=13) a postpartum relapse. **(D)** IgD expression on transitional and naive mature B cells was compared between paired first trimester (T1), third trimester (T3) and early postpartum (PP) samples of 12 HC (blue) and 13 MS patients without a postpartum relapse (red). Wilcoxon signed-rank test was performed to compare the different gestational periods. Mann-Whitney U test was performed in **C**. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.



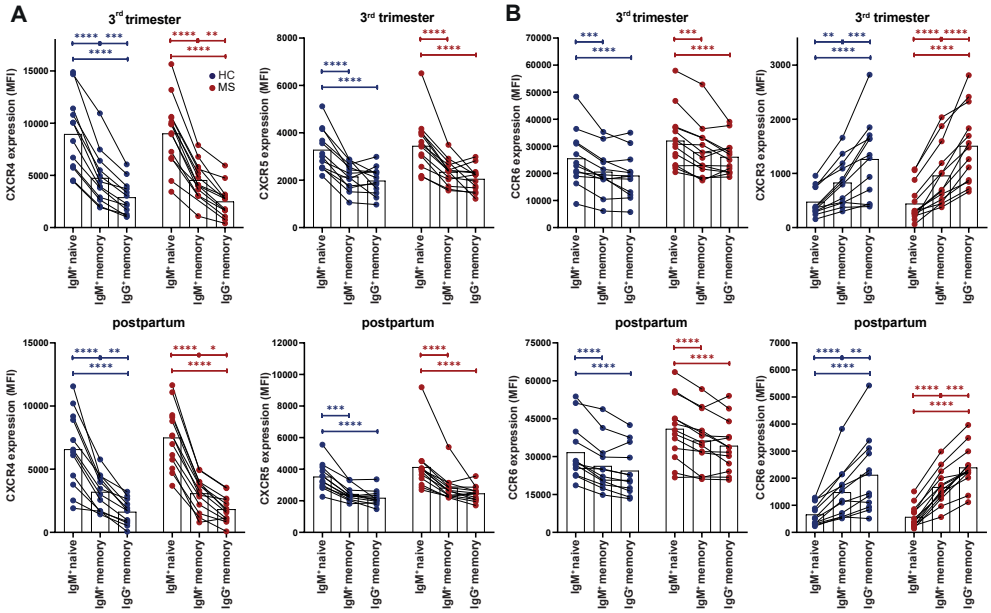
Supplementary Figure 2. Frequencies of B cell subsets and immunoglobulin expression in MS patients with a postpartum relapse.

(A) The percentage of transitional and naive mature B cells of third trimester (T3) and early postpartum (PP) periods in MS patients with a post-pregnancy relapse (n = 6), as well as their ratio and the frequency of IgM^+ and IgG^+ memory B cells. (**B**) Immunoglobulin expression on memory B cells of MS patients with a postpartum relapse (n = 6). Wilcoxon signed-rank test was performed to compare the different gestational periods. * $p<0.05$, *** $p<0.001$.



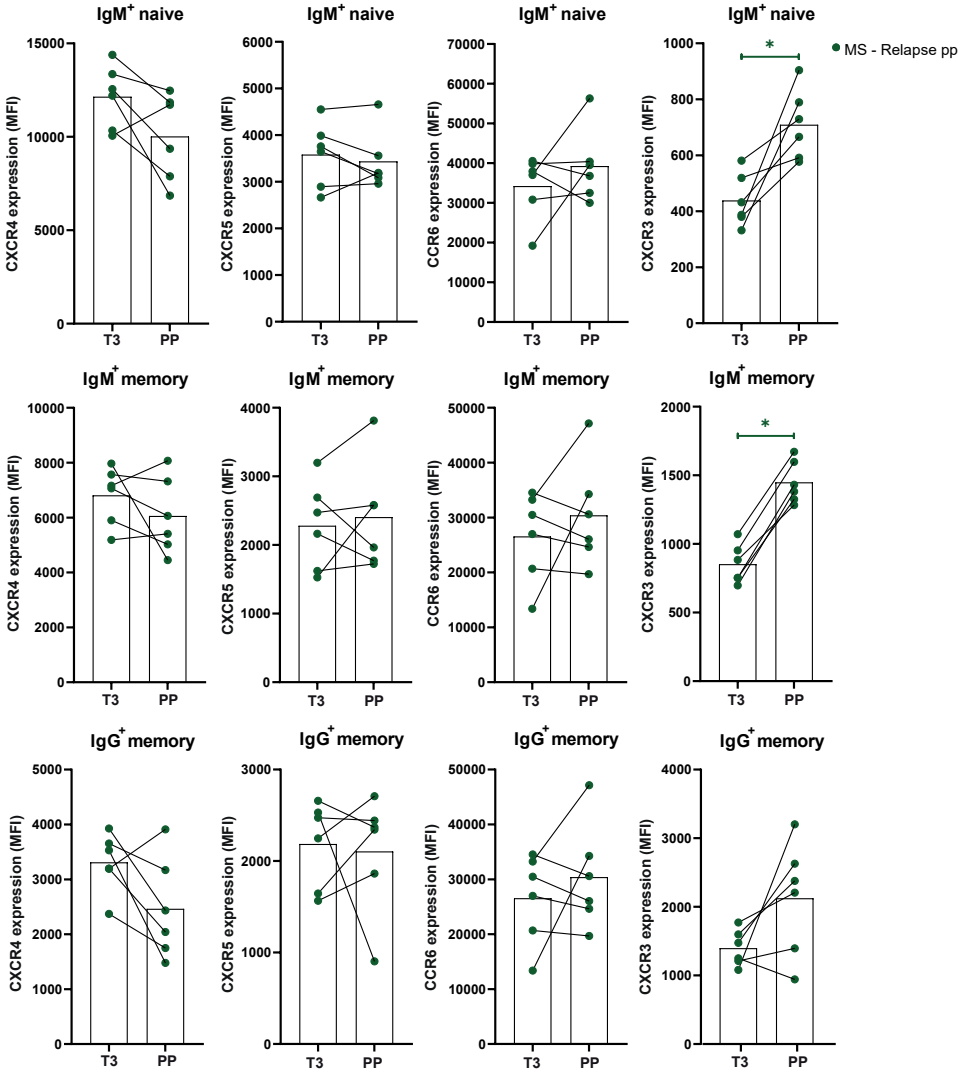
Supplementary Figure 3. Chemokine expression levels on naive mature B cells of HC and MS patients.

Surface expression of dark zone-associated CXCR4 and light zone-associated CXCR5, CCR6 and CXCR3 on IgM$^+$CD27$^-$ naive B cells in the first trimester (T1), third trimester (T3) and postpartum period (PP) for 12 HC (blue) and 13 MS patients without a postpartum relapse (red). Wilcoxon signed-rank test was performed to compare the different gestational periods. p<0.05, ** p<0.01.



Supplementary Figure 4. Chemokine expression levels on naive mature, IgM$^+$ and IgG$^+$ memory B cells in the third trimester and early after delivery.

Surface expression levels of dark zone-associated CXCR4 and light zone-associated CXCR5, CCR6 and CXCR3 were compared between IgM$^+$ naive, IgM$^+$ memory and IgG$^+$ memory B cells from third trimester and early postpartum samples from 12 HC (blue) and 13 MS patients without a postpartum relapse (red). Two-way ANOVA was performed to compare the different B-cell subsets within a group. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.



Supplementary Figure 5. Chemokine expression levels on naive mature, IgM+ and IgG+ memory B cells in the third trimester and early after delivery of MS patients experiencing a postpartum relapse.

Surface expression levels of dark zone-associated CXCR4 and light zone-associated CXCR5, CCR6 and CXCR3 on IgM+ naive, IgM+ memory and IgG+ memory B cells in the first trimester, third trimester (T3) and postpartum period (PP) for MS patients with a postpartum relapse (n = 6). Wilcoxon signed-rank test was performed to compare the groups. * $p<0.05$.

6

B and T cells driving multiple sclerosis: Identity, mechanisms and potential triggers

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ABSTRACT

Historically, multiple sclerosis (MS) has been viewed as being primarily driven by T cells. However, the effective use of anti-CD20 treatment now also reveals an important role for B cells in MS patients. The results from this treatment put forward T-cell activation rather than antibody production by B cells as a driving force behind MS. The main question of how their interaction provokes both B and T cells to infiltrate the CNS and cause local pathology remains to be answered. In this review, we highlight key pathogenic events involving B and T cells that most likely contribute to the pathogenesis of MS. These include 1) peripheral escape of B cells from T cell-mediated control, 2) interaction of pathogenic B and T cells in secondary lymph nodes, and 3) reactivation of B and T cells accumulating in the CNS. We will focus on the functional programs of CNS-infiltrating lymphocyte subsets in MS patients and discuss how these are defined by mechanisms such as antigen presentation, co-stimulation and cytokine production in the periphery. Furthermore, the potential impact of genetic variants and viral triggers on candidate subsets will be debated in the context of MS.

INTRODUCTION

In multiple sclerosis (MS) patients, pathogenic lymphocytes are triggered in the periphery to infiltrate the central nervous system (CNS) and cause local inflammation and demyelination. Anti-CD20 therapy has recently been approved as a novel treatment modality for MS [1-3]. Although this underscores the fact that B cells play a key role in MS, the exact triggers, subsets and effector mechanisms contributing to the disease course are incompletely understood. The impact of this therapy on the antigen-presenting rather than the antibody-producing function of B cells in MS indicates that their interaction with T cells is an important driver of the pathogenesis [1, 4]. Alterations in cytokine production, co-stimulation and antigen presentation most likely contribute to the development of pathogenic B and T cells that are prone to enter the CNS [4, 6]. Such mechanisms might be influenced by the interplay between genetic and environmental risk factors [7]. The major *HLA-DRB1*1501* locus accounts for 30% of the overall risk [7] and has been shown to promote B cell-mediated induction of brain-infiltrating T helper (Th) cells in MS patients [4]. Besides for *HLA-DRB1*1501*, other genetic risk variants that have been identified in the past decades also appear to potentiate B and Th cell activation, a feature that is shared amongst several autoimmune disorders [5]. Furthermore, infectious triggers such as the Epstein-Barr virus (EBV) alter their function and reactivity in MS [6-9]. The current view is that transmigration of lymphocyte subsets into the CNS signifies relapsing disease, while compartmentalized CNS inflammation, as seen during disease progression, seems to be driven by tissue-resident populations [10, 11]. Since there is a clear association of relapse occurrence and radiological disease activity early in MS with the severity of disability progression later in MS [12], it is crucial to understand what motivates these cells to invade the CNS and why these cells instigate local pathology in MS patients.

In this review, we will discuss which and how brain-infiltrating lymphocyte subsets can contribute to MS pathogenesis. These pathogenic events are characterized by: 1) peripheral escape of pathogenic B cells from T cell-mediated control, 2) mutual activation of pathogenic B and T cells within peripheral germinal centers, and 3) re-activation of infiltrating B and T cells within the CNS. We will use current knowledge to consider the extent to which genetic and viral triggers may drive these pathogenic events in MS.

IMPAIRED T CELL-MEDIATED CONTROL OF PATHOGENIC B CELLS IN MS

B and T cells closely interact in secondary lymphoid organs to generate an optimal immune response against invading pathogens. Within follicles, B cells recognize antigens via the highly specific B-cell receptor (BCR), resulting in internalization, processing and presentation to T cells. This mechanism is unique and tightly coordinated involving five consecutive and interdependent steps: 1) B-cell receptor signaling, 2) actin remodeling, 3) endosomal formation and transport, 4) HLA class II synthesis and trafficking to specialized late endosomes (i.e. MIICs), and 5) antigen processing and loading onto HLA class II molecules for presentation to $CD4^+$ Th cells [13, 14]. Through their interaction with Th cells, germinal center (GC)-dependent and -independent memory B cells are formed, a process that is governed by the strength of the HLA/peptide signal [15]. GC B cells respond to interleukin (IL)-21-producing follicular Th (Tfh) cells to develop into class-switched (IgG^+) subsets or antibody-producing plasmablasts/plasma cells [15, 16]. Memory B cells, in return, specifically trigger Th effector subsets that help $CD8^+$ cytotoxic T cells (CTLs) to kill the infected cell [17]. In MS, this crosstalk between B and T cells is likely disturbed, eventually causing pathogenic instead of protective immunity. This may already start during selection of naive autoreactive B cells in the periphery.

Normally, after removal of the majority of B-cell clones expressing polyreactive antibodies in the bone marrow (central tolerance), surviving autoreactive B cells are kept in check by peripheral tolerance checkpoints [18]. In contrast to most other autoimmune diseases, only peripheral and not central B-cell tolerance checkpoints are defective in MS, which coincides with increased frequencies of naive polyreactive populations in the blood [18-21]. Although the exact cause is currently unknown, the escape of pathogenic B cells from peripheral control may be related to 1) chronic T-cell stimulation and 2) T cell-intrinsic defects (see Figure 1).

EBV is one of the most thoroughly investigated pathogens regarding T-cell responses in MS. Many theories have been proposed how EBV can influence MS pathogenesis [9]. One hypothesis is that, due to the chronic nature of this infection, continuous antigen presentation by B cells leads to functionally impaired, so-called 'exhausted' T cells [8, 22]. This, together with the impact of HLA and other risk alleles [23], may result in inappropriate T cell-mediated control of EBV-infected (pathogenic) B cells. Consistent with this, peripheral $CD8^+$ CTLs show decreased responses to EBV and not to cytomegalovirus antigens during the MS course [8]. EBV antigens can also induce IL-10-producing $CD4^+$ T regulatory cells (Tregs) capable of suppressing effector T-cell responses to recall antigens [24], as seen for other persistent viral infections such as lymphocytic choriomeningitis virus [25, 26]. However, forkhead box P3 ($FOXP3^+$) Tregs have also been described to control infections

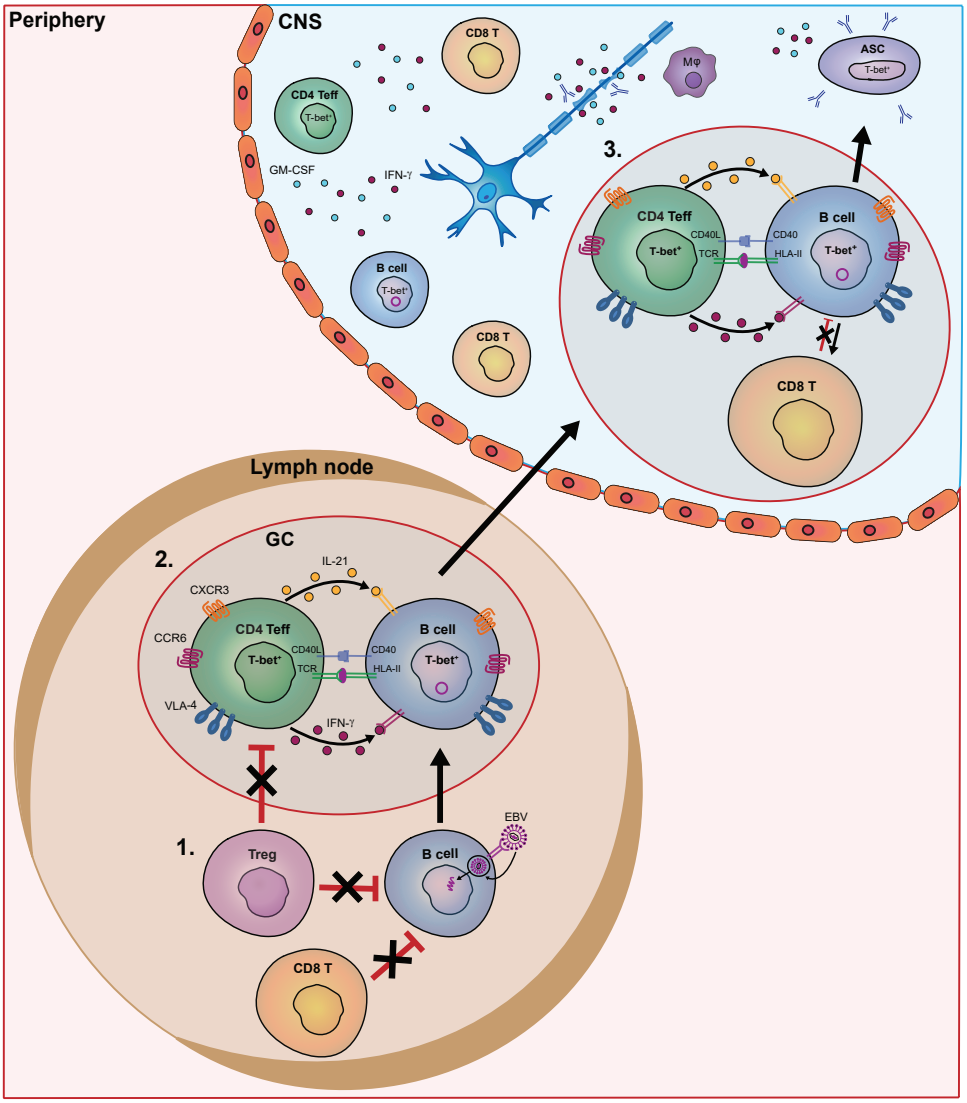

Figure 1. Model of the key pathogenic events involving human B- and T-cell subsets driving MS disease activity.

In MS patients, B- and T-cells interact in the periphery and central nervous system (CNS) to contribute to disease pathogenesis. In this model, we put forward three important meeting points of pathogenic B and T cells that drive the disease course of MS. In secondary lymphoid organs, B-cell tolerance defects in MS patients allow EBV-infected B cells to escape from suppression by $CD8^+$ and T regulatory (Treg) cells (**1**). Subsequently, these activated B cells enter germinal centers (GCs) and interact with follicular Th cells to further differentiate into pathogenic memory B cells. Under the influence of IFN-γ and IL-21, B cells develop into T-bet-expressing memory cells, which in turn activate Th effector cells such as Th17.1 (**2**). These subsets are prone for infiltrating the CNS of MS patients by distinct expression of chemokine receptors (CXCR3, CCR6), adhesion molecules (VLA-4) as well as pro-inflammatory cytokines. (**3**) Within the CNS, IFN-γ- and GM-CSF-producing T cells and $T\text{-bet}^+$ memory B cells probably come into contact in follicle-like structures, resulting in clonal expansion inflammation and demyelination. $T\text{-bet}^+$ memory B cells further differentiate into plasmablasts/plasma cells to secrete high numbers of potentially harmful antibodies (oligoclonal bands).

[27] T-Lymphocyte, suggesting that additional T cell-intrinsic defects are involved. For example, Treg populations that are enriched in MS patients produce increased levels of interferon gamma (IFN-γ), express reduced levels of FOXP3 and have defective suppressive activity *in vitro* [28]. This is not only accompanied with less suppression of effector T cells [29, 30], but possibly also with impaired removal of pathogenic B cells, as described for other autoimmune diseases [18, 31, 32]. The direct impact of Tregs on B cells in MS patients is still unknown. Treg function may be altered by variation in *IL2RA* and *IL7RA*, two known MS risk loci [33, 34]. FOXP3 correlates with IL-2 receptor (IL-2R) as well as IL-7 receptor (IL-7R) expression in Tregs [35]. It can thus be expected that *IL2RA* and *IL7RA* [33, 34], but also *BACH2* [36] variants impair Treg development in MS. This may even influence FOXP3- and IL-2R-expressing $CD8^+$ T cells, which can suppress pro-inflammatory $CD4^+$ Th cells [37] and are reduced in the blood during MS relapses [38-40].

THE GERMINAL CENTER AS A POWERHOUSE OF PATHOGENIC B- AND TH-CELL INTERACTION IN MS

Th cells as inducers of pathogenic memory B cells

After their escape from peripheral tolerance checkpoints, naive B cells likely interact with Th cells in GCs to eventually develop into memory populations potentially capable of infiltrating the MS brain (Figure 1). Little is known about how peripheral effector Th cells mediate the development of such pathogenic B cells in MS patients. In GCs of autoimmune mice, autoreactive B cells are triggered by Tfh cells producing high levels of IFN-γ [16]. IFN-γ induces the expression of the T-box transcription factor T-bet, which upregulates CXC chemokine receptor 3 (CXCR3), elicits IgG class switching and enhanced antiviral responsiveness of murine B cells [41-43] . Recently, we found that B cells from MS patients preferentially develop into $CXCR3^+$ populations that transmigrate into the CNS [44]. The IFN-γ receptor (IFNGR) and downstream molecule signal transducer and activator of transcription (STAT)1 in B cells are major determinants of autoimmune GC formation in mice [45, 46]. After ligation of the IFNGR, STAT1 is phosphorylated, dimerizes and translocates into the nucleus to induce genes involved in GC responses, such as T-bet and B-cell lymphoma 6 (BCL-6) [16, 47]. Although IFN-γ-stimulated B cells of MS patients show enhanced pro-inflammatory capacity [44, 48], it is unclear whether alterations in the IFN-γ signaling pathway contribute to the development of $T\text{-}bet^+$ B cells infiltrating the CNS. Interestingly, a missense SNP in *IFNGR2* has been found in MS, which may alter their development [49, 50]. Another target gene of the IFN-γ pathway is *IFI30*, which encodes for the IFN-γ-inducible lysosomal thiol reductase (GILT) and is considered one of the causal risk variants in MS [5]. GILT is a critical regulator of antigen processing for presentation by HLA class

II molecules [51-53]. Together, these findings point to T-bet-expressing B cells as potent antigen-presenting cells that are highly susceptible to triggering by IFN-γ-producing Th effector subsets in MS [44, 54] (Figure 2).

EBV may be an additional player in the formation of T-bet-expressing B cells. In mice, persistent viral infections sustain the development of these types of B cells, in which T-bet enhances their ability to recognize viral and self-antigens [41, 55]. EBV is hypothesized to persist latently in pathogenic B cells and mimic T-cell help for further differentiation in GCs [6, 22, 56, 57]. During acute infection, EBV uses a series of latency programs that drive B cells towards a GC response in an antigen-independent manner. Latent membrane protein (LMP)2A and LMP1 resemble signals coming from the BCR and CD40 receptor [56, 57]. In addition to their regulation of GC responses independently of T-cell help [58], recent evidence implicates that LMP2A and LMP1 can synergize with BCR and CD40 signaling as well [59]. Interestingly, downstream molecules of the BCR (e.g. Syk, CBL-B) and CD40 receptor (e.g. TRAF3) are genetic risk factors for MS [23, 60], therefore potentially cooperating with these latent proteins to enhance pathogenic B-cell development (Figure 2). This is supported by the binding of LMP2A to Syk in B cells and their escape from deletion in GCs of transgenic mice [61]. Alternatively, pathogenic B cells can be induced via pathogen-associated TLR9, which binds to unmethylated CpG DNA and further integrate with BCR, CD40 and cytokine signals [62-65]. Moreover, pathogenic B-cell responses in systemic autoimmune diseases such as systemic lupus erythematosus are enhanced after IFN-γ and virus-mediated induction of the T-bet [45, 55, 64, 65]. In MS patients, TLR9 ligation is also a major trigger of pro-inflammatory B cells [48] and crucial for the differentiation of T-bet-expressing IgG1$^+$ B cells during IFN-γ- and CD40-dependent GC-like cultures *in vitro*. Thus, under influence of specific genetic factors, EBV might join forces with IFN-γ-producing Th cells to stimulate pathogenic (T-bet$^+$) GC B cells both in a direct (via infection and persistence in pathogenic subsets) and indirect (via TLR7/9) fashion in MS (Figure 2).

B cells as inducers of pathogenic memory Th cells

Synchronously, within peripheral GCs, T-bet-expressing memory B cells are ideal candidates to trigger IFN-γ-producing, CNS-infiltrating Th cells in MS (Figure 1). In both mice and humans, T-bet promotes the antigen-presenting cell function of B cells. This may be related to the impact of EBV infection on B cell-intrinsic processing and presentation of antigens such as myelin oligodendrocyte glycoprotein (MOG)[6]. The potent antigen-presenting cell function of B cells in MS patients is further reflected by the effective use of anti-CD20 therapy. This therapy does not affect antibody serum levels, but significantly reduces pro-inflammatory Th-cell responses in MS, both *ex vivo* and *in vivo* [1]. CD20 was found to be enriched on IFN-g-inducible T-bet-expressing IgG$^+$ B cells in MS blood [44], pointing to this pathogenic subset as an important therapeutic target. Furthermore, genetic changes

in HLA class II molecules, as well as costimulatory molecules (e.g. CD80 [66, 67] and CD86 [68]), may additionally enhance Th cell activation by such memory B cells (Figure 2). HLA class II expression on murine B cells was reported to be indispensable for EAE disease onset [69, 70]. The *in silico* evidence that autoimmunity-associated HLA class II molecules have an altered peptide-binding groove [71, 72], together with the potential role of several minor risk variants in the HLA class II pathway (e.g. *CIITA, CLEC16A, IFI30* [Figure 2]), insinuates that antigens are differently processed and presented by B cells [4, 6]. This is supported by the increased ability of memory B cells to trigger CNS-infiltrating Th cells in MS patients carrying *HLA-DRB1*1501* [4]. These CNS-infiltrating T cells induced by B cells showed features of both Th1 and Th17, therefore representing highly pathogenic subsets. Such subsets are characterized by master transcription factors T-bet and RORγt [73, 74], of which the latter

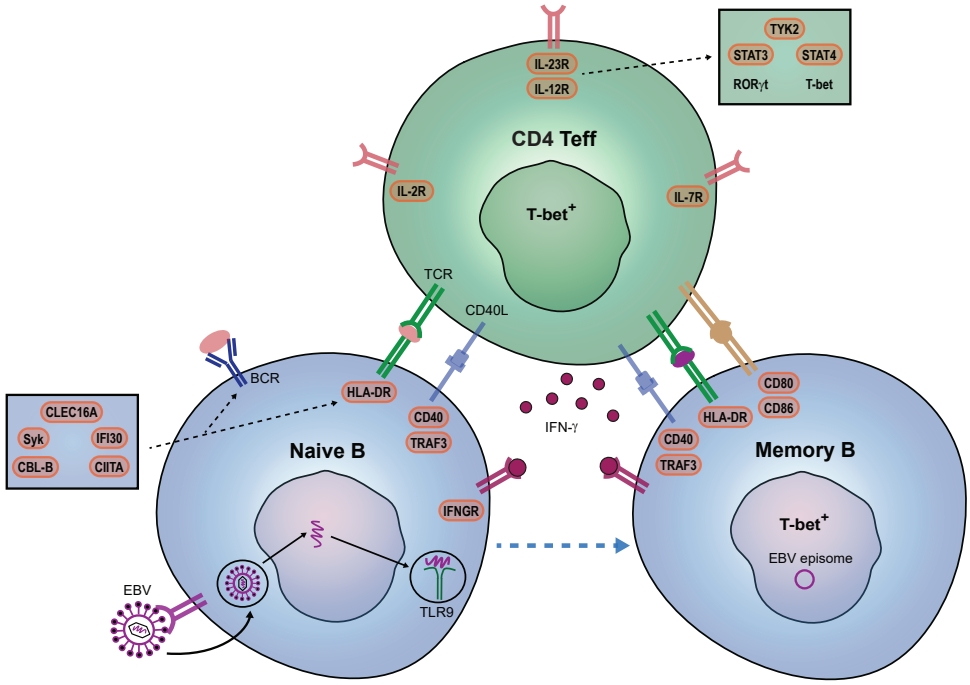

Figure 2. Potential contribution of EBV and genetic risk factors to pathogenic B- and Th-cell development in MS patients. IFN-γ is a key player in autoreactive B- and Th-cell interaction and autoimmune germinal center (GC) formation in mice. In MS, we propose that EBV infection together with specific genetic risk variants promote the IFN-γ-mediated interplay between B and T cells within GCs. EBV directly infects naive B cells and mimic GC responses. EBV DNA can also bind to TLR7/9, and together with IFN-γ, induces T-bet$^+$ memory B cells. Their interplay may be additionally stimulated by both B cell-intrinsic (IFN-γ sensitivity: *IFNGR2*; B cell receptor-antigen uptake: *CBLB*, *SYK*, *CLEC16A*; HLA class II pathway: *CLEC16A*, *CIITA*, *IFI30*; co-stimulation: *CD80, CD86*) and Th cell-intrinsic (surface receptors: *IL2RA*, *IL7RA*, *IL12RB1*; downstream molecules: *TYK2*, *STAT3*, *STAT4*) genetic risk variants. IL12R/IL-23R complexes trigger JAK2/STAT3-dependent RORγt and TYK2/STAT4-dependent T-bet expression in Th effector cells.

is involved in the co-expression of IL-17 and GM-CSF in mice but not in humans [75, 76]. GM-CSF is an emerging pro-inflammatory cytokine produced by Th cells in MS [33, 75, 77]. Our group recently revealed that a Th subset producing high levels of IFN-γ and GM-CSF, but low levels of IL-17, termed Th17.1, plays a key role in driving early disease activity in MS patients [78]. Proportions of Th17.1 cells were reduced in the blood and highly enriched in the CSF of rapid-onset MS patients. In addition, Th17.1 cells and not classical Th1 and Th17 cells accumulated in the blood of MS patients who clinically responded to natalizumab (anti-VLA-4 mAb). The increased pathogenicity of Th17.1 is further exemplified by their high levels of multidrug resistance, anti-apoptotic and cytotoxicity-associated genes *ABCB1* (MDR1), *FCMR* (TOSO) and *GZMB* (granzyme B), respectively [78-81]. Th17.1 cells also show pronounced expression of the IL-23 receptor (IL-23R) [78], which is essential for maintaining the pathogenicity of Th17 cells during CNS autoimmunity [82]. IL-23 signals through the IL-23R and IL-12 receptor beta chain (IL-12Rβ1), resulting in JAK2-mediated STAT3 and TYK2-mediated STAT4 phosphorylation, and thereby inducing RORγt and T-bet, respectively [83]. *IL-12RB1*, *TYK2*, *STAT3* and *STAT4* are known genetic risk variants and thus may directly induce Th effector cells in MS (Figure 2). In addition to its potential effect on Tregs (see above), MS-associated risk variant *IL-2RA* enhances GM-CSF production by human Th effector cells [33]. To confirm the influence of these and other risk loci [84] on the induction of pathogenic Th cells such as Th17.1 in MS, functional studies need to be performed in the near future.

The increased pathogenicity of Th effector cells may additionally be skewed by IL-6-producing B cells [85, 86], which have been shown to trigger autoimmune GC formation and EAE in mice [87, 88]. Blocking of IL-6 prevents the development of myelin-specific Th1 and Th17 cells in EAE [89]. The IL-6-mediated resistance of pathogenic Th cells to Treg mediated suppression in MS [90, 91] further links to the abundant expression of anti-apoptotic gene *FCMR* in Th17.1 [78, 92]. Intriguingly, B cell-derived GM-CSF can be an additional cytokine driving pathogenic Th cells in MS patients by inducing pro-inflammatory myeloid cells [93]. Although the causal MS autoantigen is still unknown, previous work implies that B cell-mediated presentation of EBV antigens at least contributes to pathogenic Th-cell induction [6, 94]. As mentioned above, antiviral $CD8^+$ CTLs can become exhausted during persistent viral infections. Normally, this mechanism is compensated by the presence of cytotoxic $CD4^+$ Th cells, which keep these types of infections under control [95]. Such Th populations have been associated with MS progression [96] and are also formed after EBV infection, producing high levels of IFN-γ, IL-2, granzyme B and perforin [97, 98]. Similarly, EBV- and myelin-reactive Th cells from MS patients produce high levels of IFN-γ and IL-2 [7] and strongly respond to memory B cells presenting myelin peptides [99]. These studies indicate that the involvement of EBV-infected B cells, especially those expressing T-bet (see

3.1), in activating Th effector cells with cytotoxic potential [78, 100, 101] deserves further attention in MS.

REACTIVATION OF CNS-INFILTRATING B AND T CELLS IN MS

Mechanisms of infiltration

Under normal physiological conditions, the CNS has been considered an immune privileged environment and consists of a limited number of lymphocytes that cross the blood brain barrier (BBB) [102]. However, the revelation of meningeal lymphatic structures emphasized the cross-talk between CNS and peripheral lymphocytes in secondary lymphoid organs [103]. The choroid plexus has been identified as the main entry of memory cells into the CNS, which is in the case of T cells mostly mediated by CCR6 [104, 105]. The normal human CSF, as is acquired from the arachnoid space by lumbar spinal taps, contains more $CD4^+$ Th cells compared to $CD8^+$ T cells with central memory characteristics [106-108]. The arachnoid space is a continuum with the perivascular space surrounding penetrating arterial and venous structures into the parenchyma [109]. Within the brain parenchyma, more $CD8^+$ T cells than $CD4^+$ Th cells are found, however their numbers remain low and can be found virtually restricted to the perivascular space [11, 110]. These T cells display a phenotype mostly associated with non-circulating tissue resident memory T cells. The perivenular perivascular space has been argued to be the common drainage site of antigens mobilized with the glymphatics flow [111]. The exact relationship between memory T cells in the subarachnoid and perivascular space has been poorly identified in terms of replenishment and clonal association.

The BBB is dysfunctional during the early phase of MS, resulting in or is due to local recruitment of pathogenic T and B cells [112]. Differential expression of pro-inflammatory cytokines, chemokine receptors and integrins by infiltrating lymphocytes have been argued to mediate disruption of the BBB in MS [104, 113]. Myelin-reactive $CCR6^+$ and not $CCR6^-$ memory Th cells from MS patients not only produce high levels of IL-17, but also IFN-γ and GM-CSF [80]. Previous studies mainly focused on the migration of IL-17-producing $CCR6^+$ Th cells through the choroid plexus in EAE and *in vitro* human brain endothelial cell layers in MS brain tissues [104, 114]. In our recent study, we subdivided these $CCR6^+$ memory Th cells into distinct Th17 subsets and found that especially IFN-γ producing Th17.1 ($CCR6^+CXCR3^+CCR4^-$) cells were capable of infiltrating the CNS, both in *ex vivo* autopsied brain tissues and in *in vitro* transmigration assays [78]. The fact that Th17.1 cells have cytotoxic potential and strongly co-express IFN-γ with GM-CSF [78] suggests that these cells are involved in disrupting the permeability of the BBB in MS [115, 116]. The impact of CXCR3 on their transmigration capacity is likely the result of binding to the

chemokine ligand CXCL10, which is produced by brain endothelial cells and is abundant in the CSF of MS patients [117, 118]. Similar observations were made for CXCR3(T-bet)$^+$ B cells [44]. CCR6 is also highly expressed on memory B-cell precursors within the Th cell-containing light zone of GCs [119], and on IFN-γ-producing CD8$^+$ T cells infiltrating the MS brain [120]. This implies that both populations are susceptible to enter the CNS of MS patients. In addition to chemokine receptors and pro-inflammatory cytokines, adhesion molecules such as activated leukocyte cell adhesion molecule (ALCAM) enhance transmigration of pathogenic B and T cell subsets [115, 121, 122]. Furthermore, CXCR3 is co-expressed with integrin α4β1 (VLA-4), which allows both B- and T-cell populations to bind to vascular cell adhesion protein 1 (VCAM-1) on brain endothelial cells [123]. This is supported by the reducing effects of VLA-4 inhibition on B- and Th17-cell infiltration into the CNS and disease susceptibility in EAE [124]. Natalizumab, a monoclonal antibody against VLA-4, is used as an effective second-line treatment for MS [125]. Discontinuation of this treatment often results in severe MS rebound effects [126]. Hence, the peripheral entrapment of populations like Th17.1 and T-bet$^+$ B cells in natalizumab-treated patients [44, 78] probably underlies the massive influx of blood cells causing these effects. The same is true for EBV-reactivated B cells, which are enriched in lesions from MS patients after natalizumab withdrawal [127]. A previous gene network approach using several GWAS datasets further highlights the relevance of adhesion molecules on the BBB endothelium for the crossing of T and B cells [128], especially those affected by IFN-γ [115].

Local organization and impact

Both B and T cells accumulate in active white matter lesions of the MS brain [10, 129]. In diagnostic biopsy studies, T cell-dominated inflammation is a characteristic of all lesion-types observed [130]. Also in post-mortem MS lesions, white matter MS lesions with active demyelination associate with an increase in T cell numbers [10, 129]. Although CD4$^+$ Th cells are in general outnumbered by CD8$^+$ CTLs in brain lesions as investigated in autopsy studies [10], their role as triggers of local pathology should not be overlooked in MS. This is consistent with the enrichment of CD4$^+$ Th cells in white matter lesions with active demyelination [10]. An abundant number of CD4$^+$ Th cells were also visible in pre-active lesion sites, suggesting an involvement of these cells in the early stages of lesion formation [131]. Additionally, it was demonstrated that in contrast to CD8$^+$ CTLs, brain-associated CD4$^+$ Th-cell clonotypes are reduced in MS blood, indicating specific recruitment (as described above) or, alternatively, clonal expansion in the CNS [132]. Furthermore, dominant Th-cell clones were undetectable following reconstitution after autologous hematopoietic stem cell transplantation in MS patients, which was not seen for CD8$^+$ T cells [133]. Interestingly, T-cell clones are shared between CNS compartments within a patient, including CSF and

anatomically separated brain lesions [134-137]. This suggests that brain-infiltrating T cells bear similar reactivity against local (auto)antigens.

In subsets of MS autopsy cases with acute and relapsing remitting MS, B cells can also be found predominantly in the perivascular space in association with active white matter lesions [10]. The role of these perivascular B cells, including T-bet$^+$ B cells [44], could be to re-activate (infiltrating) pro-inflammatory CD4$^+$ and CD8$^+$ T cells to cause MS pathology (Figure 1). Identical B-cell clones have been found in different CNS compartments of MS patients, including the meninges [138, 139]. Within the meninges, B- and T cell-rich follicle-like structures have been found that localize next to cortical lesions, presumably mediating progressive loss of neurological function in MS [140, 141]. Interestingly, MS brain-infiltrating lymphocytes express and respond to IL-21 [142], the cytokine that drives follicular T- and B-cell responses. Additionally, IFN-γ triggering of B cells promotes ectopic follicle formation in autoimmune mice [16, 45], suggesting that the structures observed in the MS CNS are induced by B cells interacting with IFN-γ-producing T cells. However, the role of IL-17 in this process should not be ruled out, as shown in EAE [143].

Besides mediating migration and organization of pathogenic lymphocytes in the MS brain, cytokines are likely relevant effector molecules. IFN-γ production by Th cells also associates with the presence of demyelinating lesions in the CNS [144-146]. IFN-γ, and possibly also GM-CSF, can activate microglia or infiltrated macrophages to cause damage to oligodendrocytes [93, 147, 148]. As for B cells, increased production of TNF-α, IL-6 and GM-CSF has been found [48, 87] and we have recently shown that during Tfh-like cultures, IFN-γ drives IgG-producing plasmablasts in MS [44]. One could speculate that after their re-activation by IFN-γ-producing Th cells within the meningeal follicles, T-bet$^+$ memory B cells rapidly develop into antibody-producing plasmablasts/plasma cells (Figure 1). IFN-γ-induced GC formation promotes the generation of autoantibodies in lupus mice [16, 45]. The targeting of B cells and not plasmablasts/plasma cells by clinically effective anti-CD20 therapies in MS, as well as the abundance of oligoclonal bands in MS CSF, at least support the local differentiation of B cells into antibody-secreting cells [48, 149]. We argue that IgG secreted by local T-bet-expressing plasmablasts/plasma cells are highly reactive in the MS brain [43, 44, 55], although the (auto)antigen specificity and pathogenicity of such antibodies remain unclear in MS, as well as their contribution as effector molecules to MS pathology.

Several antigenic targets have been proposed to contribute to MS pathology. Next to myelin, which is one of the most intensively studied antigens [150], also EBV antigens are considered as major candidates. EBNA-1 specific IgG antibodies are predictive for early disease activity [151] and are present in CSF from MS patients [152, 153]. Some studies imply that reactivated B cells in ectopic meningeal follicles [154, 155] cross-present EBV peptides to activate myelin- and EBNA-1 specific Th cells [7, 156, 157]. Whether EBV is detected in

the brain or solely recognized in the periphery and how this contributes to local pathology is still a matter of intense debate in the field [127, 158-162]. In addition to myelin [150] and EBV [7], other antigenic targets of locally produced IgG and infiltrating T cells have been suggested, such as sperm-associated antigen 16 (SPAG16 [163]), neurofilament light, RAS guanyl-releasing protein 2 (RASGRP2 [4]), αB-crystallin and GDP-l-fucose synthase [135].

CONCLUDING REMARKS

In this review, we have discussed potential triggers and mechanisms through which interacting B and T cells drive the pathogenesis of MS. In our presented model, peripheral B cells escape from tolerance checkpoints as the result of impaired control by chronically exhausted or genetically altered regulatory T cells. Subsequently, B cells interact with IFN-γ-producing effector Th cells in germinal centers of lymphoid organs to create a feed-forward loop, after which highly pathogenic subsets break through blood-CNS barriers and, together with infiltrating CD8+ CTLs are locally reactivated to cause MS pathology. Although definite proof is still lacking, these pathogenic events are likely mediated by an interplay between persistent infections such as EBV and genetic risk variants. Together, these factors may alter the selection, differentiation and pathogenic features of B- and T-cell subsets. In our view, more in-depth insights into how infections and genetic burden define the CNS-infiltrating potential and antigen specificity of such subsets should be the next step to take in the near future. The development of small molecule therapeutics against subsets driving the disease course would be an effective way of generating clinically relevant benefits without harmful effects in MS patients.

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REFERENCES

1 **Hauser, S. L., Waubant, E., Arnold, D. L., Vollmer, T., Antel, J., Fox, R. J., Bar-Or, A., et al.,** B-Cell Depletion with Rituximab in Relapsing–Remitting Multiple Sclerosis. *New England Journal of Medicine* 2008. **358**: 676-688.

2 **Bar-Or, A., Calabresi, P. A., Arnold, D., Markowitz, C., Shafer, S., Kasper, L. H., Waubant, E., et al.,** Rituximab in relapsing-remitting multiple sclerosis: a 72-week, open-label, phase I trial. *Ann Neurol* 2008. **63**: 395-400.

3 **Hauser, S. L., Bar-Or, A., Comi, G., Giovannoni, G., Hartung, H.-P., Hemmer, B., Lublin, F., et al.,** Ocrelizumab versus Interferon Beta-1a in Relapsing Multiple Sclerosis. *New England Journal of Medicine* 2016. **376**: 221-234.

4 **Jelcic, I., Al Nimer, F., Wang, J., Lentsch, V., Planas, R., Jelcic, I., Madjovski, A., et al.,** Memory B Cells Activate Brain-Homing, Autoreactive CD4+ T Cells in Multiple Sclerosis. *Cell* 2018. **175**: 85-100.e123.

5 **Farh, K. K., Marson, A., Zhu, J., Kleinewietfeld, M., Housley, W. J., Beik, S., Shoresh, N., et al.,** Genetic and epigenetic fine mapping of causal autoimmune disease variants. *Nature* 2015. **518**: 337-343.

6 **Morandi, E., Jagessar, S. A., 't Hart, B. A. and Gran, B.,** EBV Infection Empowers Human B Cells for Autoimmunity: Role of Autophagy and Relevance to Multiple Sclerosis. *The Journal of Immunology* 2017: ji1700178.

7 **Lunemann, J. D., Jelcic, I., Roberts, S., Lutterotti, A., Tackenberg, B., Martin, R. and Munz, C.,** EBNA1-specific T cells from patients with multiple sclerosis cross react with myelin antigens and co-produce IFN-gamma and IL-2. *J Exp Med* 2008. **205**: 1763-1773.

8 **Pender, M. P., Csurhes, P. A., Burrows, J. M. and Burrows, S. R.,** Defective T-cell control of Epstein-Barr virus infection in multiple sclerosis. *Clin Transl Immunology* 2017. **6**: e126.

9 **Bar-Or, A., Pender, M. P., Khanna, R., Steinman, L., Hartung, H.-P., Maniar, T., Croze, E., et al.,** Epstein–Barr Virus in Multiple Sclerosis: Theory and Emerging Immunotherapies. *Trends in Molecular Medicine* 2019.

10 **Machado-Santos, J., Saji, E., Troscher, A. R., Paunovic, M., Liblau, R., Gabriely, G., Bien, C. G., et al.,** The compartmentalized inflammatory response in the multiple sclerosis brain is composed of tissue-resident CD8+ T lymphocytes and B cells. *Brain* 2018. **141**: 2066-2082.

11 **Smolders, J., Heutinck, K. M., Fransen, N. L., Remmerswaal, E. B. M., Hombrink, P., ten Berge, I. J. M., van Lier, R. A. W., et al.,** Tissue-resident memory T cells populate the human brain. *Nature Communications* 2018. **9**: 4593.

12 **Rotstein, D. and Montalban, X.,** Reaching an evidence-based prognosis for personalized treatment of multiple sclerosis. *Nature Reviews Neurology* 2019. **15**: 287-300.

13 **Yuseff, M.-I. and Lennon-Duménil, A. M.,** B Cells use Conserved Polarity Cues to Regulate Their Antigen Processing and Presentation Functions. *Frontiers in immunology* 2015. **6**: 251-251.

14 **Flora, C. and Ronald, N. G.,** Cooperation between $CD4^+$ and $CD8^+$ T cells: When, Where, and How. *Annual Review of Immunology* 2006. **24**: 519-540.

15 **Kurosaki, T., Kometani, K. and Ise, W.,** Memory B cells. *Nature Reviews Immunology* 2015. **15**: 149.

16 **Rawlings, D. J., Metzler, G., Wray-Dutra, M. and Jackson, S. W.,** Altered B cell signalling in autoimmunity. *Nat Rev Immunol* 2017. **17**: 421-436.

17 **Swain, S. L., McKinstry, K. K. and Strutt, T. M.,** Expanding roles for CD4○T cells in immunity to viruses. *Nature reviews Immunology* 2012. **12**: 136-148.

18 **Kinnunen, T., Chamberlain, N., Morbach, H., Cantaert, T., Lynch, M., Preston-Hurlburt, P., Herold, K. C., et al.,** Specific peripheral B cell tolerance defects in patients with multiple sclerosis. *J Clin Invest* 2013. **123**: 2737-2741.

19 **Samuels, J., Ng, Y. S., Coupillaud, C., Paget, D. and Meffre, E.,** Impaired early B cell tolerance in patients with rheumatoid arthritis. *J Exp Med* 2005. **201**: 1659-1667.

20 **Menard, L., Saadoun, D., Isnardi, I., Ng, Y. S., Meyers, G., Massad, C., Price, C., et al.,** The PTPN22 allele encoding an R620W variant interferes with the removal of developing autoreactive B cells in humans. *J Clin Invest* 2011. **121**: 3635-3644.

21 **Cotzomi, E., Stathopoulos, P., Lee, C. S., Ritchie, A. M., Soltys, J. N., Delmotte, F. R., Oe, T., et al.,** Early B cell tolerance defects in neuromyelitis optica favour anti-AQP4 autoantibody production. *Brain* 2019. **142**: 1598-1615.

22 **Pender, M. P.,** Infection of autoreactive B lymphocytes with EBV, causing chronic autoimmune diseases. *Trends in Immunology* 2003. **24**: 584-588.

23 **International Multiple Sclerosis Genetics, C., Wellcome Trust Case Control, C., Sawcer, S., Hellenthal, G., Pirinen, M., Spencer, C. C., Patsopoulos, N. A., et al.,** Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* 2011. **476**: 214-219.

24 **Marshall, N. A., Vickers, M. A. and Barker, R. N.,** Regulatory T Cells Secreting IL-10 Dominate the Immune Response to EBV Latent Membrane Protein 1. *The Journal of Immunology* 2003. **170**: 6183-6189.

25 **Brooks, D. G., Trifilo, M. J., Edelmann, K. H., Teyton, L., McGavern, D. B. and Oldstone, M. B.,** Interleukin-10 determines viral clearance or persistence in vivo. *Nat Med* 2006. **12**: 1301-1309.

26 **Blackburn, S. D. and Wherry, E. J.,** IL-10, T cell exhaustion and viral persistence. *Trends Microbiol* 2007. **15**: 143-146.

27 **Voo, K. S., Peng, G., Guo, Z., Fu, T., Li, Y., Frappier, L. and Wang, R. F.,** Functional characterization of EBV-encoded nuclear antigen 1-specific CD4+ helper and regulatory T cells elicited by in vitro peptide stimulation. *Cancer Res* 2005. **65**: 1577-1586.

28 **Dominguez-Villar, M., Baecher-Allan, C. M. and Hafler, D. A.,** Identification of T helper type 1-like, Foxp3+ regulatory T cells in human autoimmune disease. *Nat Med* 2011. **17**: 673-675.

29 **Viglietta, V., Baecher-Allan, C., Weiner, H. L. and Hafler, D. A.,** Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med* 2004. **199**: 971-979.

30 **Kumar, M., Putzki, N., Limmroth, V., Remus, R., Lindemann, M., Knop, D., Mueller, N., et al.,** CD4+CD25+FoxP3+ T lymphocytes fail to suppress myelin basic protein-induced proliferation in patients with multiple sclerosis. *J Neuroimmunol* 2006. **180**: 178-184.

31 **Venken, K., Hellings, N., Broekmans, T., Hensen, K., Rummens, J. L. and Stinissen, P.,** Natural naive CD4+CD25+CD127low regulatory T cell (Treg) development and function are disturbed in multiple sclerosis patients: recovery of memory Treg homeostasis during disease progression. *J Immunol* 2008. **180**: 6411-6420.

32 **Kinnunen, T., Chamberlain, N., Morbach, H., Choi, J., Kim, S., Craft, J., Mayer, L., et al.,** Accumulation of peripheral autoreactive B cells in the absence of functional human regulatory T cells. *Blood* 2013. **121**: 1595-1603.

33 **Hartmann, F. J., Khademi, M., Aram, J., Ammann, S., Kockum, I., Constantinescu, C., Gran, B., et al.,** Multiple sclerosis-associated IL2RA polymorphism controls GM-CSF production in human TH cells. *Nature Communications* 2014. **5**: 5056.

34 **Kreft, K. L., Verbraak, E., Wierenga-Wolf, A. F., van Meurs, M., Oostra, B. A., Laman, J. D. and Hintzen, R. Q.,** Decreased systemic IL-7 and soluble IL-7Rα in multiple sclerosis patients. *Genes And Immunity* 2012. **13**: 587.

35 **Liu, W., Putnam, A. L., Xu-yu, Z., Szot, G. L., Lee, M. R., Zhu, S., Gottlieb, P. A., et al.,** CD127 expression inversely correlates with FoxP3 and suppressive function of human $CD4^+$ T reg cells. *The Journal of Experimental Medicine* 2006. **203**: 1701-1711.

36 **Roychoudhuri, R., Hirahara, K., Mousavi, K., Clever, D., Klebanoff, C. A., Bonelli, M., Sciume, G., et al.,** BACH2 represses effector programs to stabilize T(reg)-mediated immune homeostasis. *Nature* 2013. **498**: 506-510.

37 **Correale, J. and Villa, A.,** Role of CD8+ CD25+ Foxp3+ regulatory T cells in multiple sclerosis. *Annals of Neurology* 2010. **67**: 625-638.

38 **Baughman, E. J., Mendoza, J. P., Ortega, S. B., Ayers, C. L., Greenberg, B. M., Frohman, E. M. and Karandikar, N. J.,** Neuroantigen-specific CD8+ regulatory T-cell function is deficient during acute exacerbation of multiple sclerosis. *J Autoimmun* 2011. **36**: 115-124.

39 **Correale, J. and Villa, A.,** Isolation and characterization of CD8+ regulatory T cells in multiple sclerosis. *Journal of Neuroimmunology* 2008. **195**: 121-134.

40 **Frisullo, G., Nociti, V., Iorio, R., Plantone, D., Patanella, A. K., Tonali, P. A. and Batocchi, A. P.,** CD8(+) Foxp3(+) T cells in peripheral blood of relapsing-remitting multiple sclerosis patients. *Hum Immunol* 2010. **71**: 437-441.

41 **Barnett, B. E., Staupe, R. P., Odorizzi, P. M., Palko, O., Tomov, V. T., Mahan, A. E., Gunn, B., et al.,** Cutting Edge: B Cell–Intrinsic T-bet Expression Is Required To Control Chronic Viral Infection. *The Journal of Immunology* 2016: 1500368.

42 **Rubtsova, K., Rubtsov, A. V., van Dyk, L. F., Kappler, J. W. and Marrack, P.,** T-box transcription factor T-bet, a key player in a unique type of B-cell activation essential for effective viral clearance. *Proc Natl Acad Sci U S A* 2013. **110**: E3216-3224.

43 **Peng, S. L., Szabo, S. J. and Glimcher, L. H.,** T-bet regulates IgG class switching and pathogenic autoantibody production. *Proc Natl Acad Sci U S A* 2002. **99**: 5545-5550.

44 **van Langelaar, J., Rijvers, L., Janssen, M., Wierenga-Wolf, A. F., Melief, M.-J., Siepman, T. A., de Vries, H. E., et al.,** Induction of brain-infiltrating T-bet–expressing B cells in multiple sclerosis. *Annals of Neurology* 2019. **86**: 264-278.

45 **Jackson, S. W., Jacobs, H. M., Arkatkar, T., Dam, E. M., Scharping, N. E., Kolhatkar, N. S., Hou, B., et al.,** B cell IFN-gamma receptor signaling promotes autoimmune germinal centers via cell-intrinsic induction of BCL-6. *J Exp Med* 2016. **213**: 733-750.

46 **Domeier, P. P., Chodisetti, S. B., Soni, C., Schell, S. L., Elias, M. J., Wong, E. B., Cooper, T. K., et al.,** IFN-gamma receptor and STAT1 signaling in B cells are central to spontaneous germinal center formation and autoimmunity. *J Exp Med* 2016. **213**: 715-732.

47 **Dalpke, A. H., Eckerle, S., Frey, M. and Heeg, K.,** Triggering of Toll-like receptors modulates IFN-γ signaling: involvement of serine 727 STAT1 phosphorylation and suppressors of cytokine signaling. *European Journal of Immunology* 2003. **33**: 1776-1787.

48 **Bar-Or, A., Fawaz, L., Fan, B., Darlington, P. J., Rieger, A., Ghorayeb, C., Calabresi, P. A., et al.,** Abnormal B-cell cytokine responses a trigger of T-cell–mediated disease in MS? *Annals of Neurology* 2010. **67**: 452-461.

49 **Lill, C. M., Luessi, F., Alcina, A., Sokolova, E. A., Ugidos, N., de la Hera, B., Guillot-Noël, L., et al.,** Genome-wide significant association with seven novel multiple sclerosis risk loci. *Journal of Medical Genetics* 2015. **52**: 848-855.

50 **Patsopoulos, N. A., Baranzini, S. E., Santaniello, A., Shoostari, P., Cotsapas, C., Wong, G., Beecham, A. H., et al.,** The Multiple Sclerosis Genomic Map: Role of peripheral immune cells and resident microglia in susceptibility. *bioRxiv* 2017: 143933.

51 **Phipps-Yonas, H., Semik, V. and Hastings, K. T.,** GILT expression in B cells diminishes cathepsin S steady-state protein expression and activity. *Eur J Immunol* 2013. **43**: 65-74.

52 **Hastings, K. T.,** GILT: Shaping the MHC Class II-Restricted Peptidome and CD4(+) T Cell-Mediated Immunity. *Front Immunol* 2013. **4**: 429.

53 **Hastings, K. T., Lackman, R. L. and Cresswell, P.,** Functional requirements for the lysosomal thiol reductase GILT in MHC class II-restricted antigen processing. *J Immunol* 2006. **177**: 8569-8577.

54 **Rubtsov, A. V., Rubtsova, K., Kappler, J. W., Jacobelli, J., Friedman, R. S. and Marrack, P.,** CD11c-Expressing B Cells Are Located at the T Cell/B Cell Border in Spleen and Are Potent APCs. *J Immunol* 2015. **195**: 71-79.

55 **Piovesan, D., Tempany, J., Di Pietro, A., Baas, I., Yiannis, C., O'Donnell, K., Chen, Y., et al.,** c-Myb Regulates the T-Bet-Dependent Differentiation Program in B Cells to Coordinate Antibody Responses. *Cell Reports*. **19**: 461-470.

56 **Pender, M. P.,** The essential role of Epstein-Barr virus in the pathogenesis of multiple sclerosis. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry* 2011. **17**: 351-367.

57 **Tracy, S. I., Kakalacheva, K., Lünemann, J. D., Luzuriaga, K., Middeldorp, J. and Thorley-Lawson, D. A.,** Persistence of Epstein-Barr Virus in Self-Reactive Memory B Cells. *Journal of Virology* 2012. **86**: 12330-12340.

58 **Thorley-Lawson, D. A. and Gross, A.,** Persistence of the Epstein-Barr virus and the origins of associated lymphomas. *N Engl J Med* 2004. **350**: 1328-1337.

59 **Roughan, J. E. and Thorley-Lawson, D. A.,** The Intersection of Epstein-Barr Virus with the Germinal Center. *Journal of Virology* 2009. **83**: 3968-3976.

60 **James, T., Linden, M., Morikawa, H., Fernandes, S. J., Ruhrmann, S., Huss, M., Brandi, M., et al.,** Impact of genetic risk loci for multiple sclerosis on expression of proximal genes in patients. *Hum Mol Genet* 2018. **27**: 912-928.

61 **Caldwell, R. G., Wilson, J. B., Anderson, S. J. and Longnecker, R.,** Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. *Immunity* 1998. **9**: 405-411.

62 **Sindhava, V. J., Oropallo, M. A., Moody, K., Naradikian, M., Higdon, L. E., Zhou, L., Myles, A., et al.,** A TLR9-dependent checkpoint governs B cell responses to DNA-containing antigens. *The Journal of Clinical Investigation* 2017. **127**: 1651-1663.

63 **Jegerlehner, A., Maurer, P., Bessa, J., Hinton, H. J., Kopf, M. and Bachmann, M. F.,** TLR9 Signaling in B Cells Determines Class Switch Recombination to IgG2a. *The Journal of Immunology* 2007. **178**: 2415-2420.

64 **Knox, J. J., Buggert, M., Kardava, L., Seaton, K. E., Eller, M. A., Canaday, D. H., Robb, M. L., et al.,** T-bet+ B cells are induced by human viral infections and dominate the HIV gp140 response. *JCI Insight* 2017. **2**.

65 **Rubtsova, K., Rubtsov, A. V., Thurman, J. M., Mennona, J. M., Kappler, J. W. and Marrack, P.,** B cells expressing the transcription factor T-bet drive lupus-like autoimmunity. *The Journal of Clinical Investigation* 2017. **127**: 1392-1404.

66 **Sawcer, S., Ban, M., Maranian, M., Yeo, T. W., Compston, A., Kirby, A., Daly, M. J., et al.,** A high-density screen for linkage in multiple sclerosis. *Am J Hum Genet* 2005. **77**: 454-467.

67 **Good-Jacobson, K. L., Song, E., Anderson, S., Sharpe, A. H. and Shlomchik, M. J.,** CD80 expression on B cells regulates murine T follicular helper development, germinal center B cell survival, and plasma cell generation. *Journal of immunology (Baltimore, Md : 1950)* 2012. **188**: 4217-4225.

68 **Smets, I., Fiddes, B., Garcia-Perez, J. E., He, D., Mallants, K., Liao, W., Dooley, J., et al.,** Multiple sclerosis risk variants alter expression of co-stimulatory genes in B cells. *Brain* 2018. **141**: 786-796.

69 **Molnarfi, N., Schulze-Topphoff, U., Weber, M. S., Patarroyo, J. C., Prod'homme, T., Varrin-Doyer, M., Shetty, A., et al.,** MHC class II–dependent B cell APC function is required for induction of CNS autoimmunity independent of myelin-specific antibodies. *The Journal of Experimental Medicine* 2013. **210**: 2921-2937.

70 **Parker Harp, C. R., Archambault, A. S., Sim, J., Ferris, S. T., Mikesell, R. J., Koni, P. A., Shimoda, M., et al.,** B cell antigen presentation is sufficient to drive neuroinflammation in an animal model of multiple sclerosis. *J Immunol* 2015. **194**: 5077-5084.

71 **Raychaudhuri, S., Sandor, C., Stahl, E. A., Freudenberg, J., Lee, H. S., Jia, X., Alfredsson, L., et al.,** Five amino acids in three HLA proteins explain most of the association between MHC and seropositive rheumatoid arthritis. *Nat Genet* 2012. **44**: 291-296.

72 **Patsopoulos, N. A., Barcellos, L. F., Hintzen, R. Q., Schaefer, C., van Duijn, C. M., Noble, J. A., Raj, T., et al.,** Fine-mapping the genetic association of the major histocompatibility complex in multiple sclerosis: HLA and non-HLA effects. *PLoS Genet* 2013. **9**: e1003926.

73 **Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G. and Glimcher, L. H.,** A Novel Transcription Factor, T-bet, Directs Th1 Lineage Commitment. *Cell* 2000. **100**: 655-669.

74 **Ivanov, I. I., McKenzie, B. S., Zhou, L., Tadokoro, C. E., Lepelley, A., Lafaille, J. J., Cua, D. J., et al.,** The Orphan Nuclear Receptor RORγt Directs the Differentiation Program of Proinflammatory IL-17+ T Helper Cells. *Cell* 2006. **126**: 1121-1133.

75 **Noster, R., Riedel, R., Mashreghi, M. F., Radbruch, H., Harms, L., Haftmann, C., Chang, H. D., et al.,** IL-17 and GM-CSF expression are antagonistically regulated by human T helper cells. *Sci Transl Med* 2014. **6**: 241ra280.

76 **El-Behi, M., Ciric, B., Dai, H., Yan, Y., Cullimore, M., Safavi, F., Zhang, G. X., et al.,** The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat Immunol* 2011. **12**: 568-575.

77 **Galli, E., Hartmann, F. J., Schreiner, B., Ingelfinger, F., Arvaniti, E., Diebold, M., Mrdjen, D., et al.,** GM-CSF and CXCR4 define a T helper cell signature in multiple sclerosis. *Nat Med* 2019. **25**: 1290-1300.

78 **van Langelaar, J., van der Vuurst de Vries, R. M., Janssen, M., Wierenga-Wolf, A. F., Spilt, I. M., Siepman, T. A., Dankers, W., et al.,** T helper 17.1 cells associate with multiple sclerosis disease activity: perspectives for early intervention. *Brain* 2018. **141**: 1334-1349.

79 **Ramesh, R., Kozhaya, L., McKevitt, K., Djuretic, I. M., Carlson, T. J., Quintero, M. A., McCauley, J. L., et al.,** Pro-inflammatory human Th17 cells selectively express P-glycoprotein and are refractory to glucocorticoids. *The Journal of Experimental Medicine* 2014. **211**: 89-104.

80 **Cao, Y., Goods, B. A., Raddassi, K., Nepom, G. T., Kwok, W. W., Love, J. C. and Hafler, D. A.,** Functional inflammatory profiles distinguish myelin-reactive T cells from patients with multiple sclerosis. *Sci Transl Med* 2015. **7**: 287ra274.

81 **Acosta-Rodriguez, E. V., Rivino, L., Geginat, J., Jarrossay, D., Gattorno, M., Lanzavecchia, A., Sallusto, F., et al.,** Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* 2007. **8**: 639-646.

82 **Meyer Zu Horste, G., Wu, C., Wang, C., Cong, L., Pawlak, M., Lee, Y., Elyaman, W., et al.,** RBPJ Controls Development of Pathogenic Th17 Cells by Regulating IL-23 Receptor Expression. *Cell Rep* 2016. **16**: 392-404.

83 **Teng, M. W., Bowman, E. P., McElwee, J. J., Smyth, M. J., Casanova, J. L., Cooper, A. M. and Cua, D. J.,** IL-12 and IL-23 cytokines: from discovery to targeted therapies for immune-mediated inflammatory diseases. *Nat Med* 2015. **21**: 719-729.

84 **Hussman, J. P., Beecham, A. H., Schmidt, M., Martin, E. R., McCauley, J. L., Vance, J. M., Haines, J. L., et al.,** GWAS analysis implicates NF-kappaB-mediated induction of inflammatory T cells in multiple sclerosis. *Genes Immun* 2016. **17**: 305-312.

85 **Kimura, A. and Kishimoto, T.,** IL-6: regulator of Treg/Th17 balance. *Eur J Immunol* 2010. **40**: 1830-1835.

86 **Zhou, L., Ivanov, II, Spolski, R., Min, R., Shenderov, K., Egawa, T., Levy, D. E., et al.,** IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol* 2007. **8**: 967-974.

87 **Barr, T. A., Shen, P., Brown, S., Lampropoulou, V., Roch, T., Lawrie, S., Fan, B., et al.,** B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. *J Exp Med* 2012. **209**: 1001-1010.

88 **Arkatkar, T., Du, S. W., Jacobs, H. M., Dam, E. M., Hou, B., Buckner, J. H., Rawlings, D. J., et al.,** B cell–derived IL-6 initiates spontaneous germinal center formation during systemic autoimmunity. *The Journal of Experimental Medicine* 2017. **214**: 3207-3217.

89 **Serada, S., Fujimoto, M., Mihara, M., Koike, N., Ohsugi, Y., Nomura, S., Yoshida, H., et al.,** IL-6 blockade inhibits the induction of myelin antigen-specific Th17 cells and Th1 cells in experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* 2008. **105**: 9041-9046.

90 **Schneider, A., Long, S. A., Cerosaletti, K., Ni, C. T., Samuels, P., Kita, M. and Buckner, J. H.,** In active relapsing-remitting multiple sclerosis, effector T cell resistance to adaptive T(regs) involves IL-6-mediated signaling. *Sci Transl Med* 2013. **5**: 170ra115.

91 **Neurath, M. F. and Finotto, S.,** IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. *Cytokine Growth Factor Rev* 2011. **22**: 83-89.

92 **Gaublomme, J. T., Yosef, N., Lee, Y., Gertner, R. S., Yang, L. V., Wu, C., Pandolfi, P. P., et al.,** Single-Cell Genomics Unveils Critical Regulators of Th17 Cell Pathogenicity. *Cell* 2015. **163**: 1400-1412.

93 **Li, R., Rezk, A., Miyazaki, Y., Hilgenberg, E., Touil, H., Shen, P., Moore, C. S., et al.,** Proinflammatory GM-CSF–producing B cells in multiple sclerosis and B cell depletion therapy. *Science Translational Medicine* 2015. **7**: 310ra166-310ra166.

94 **Lünemann, J. D., Kamradt, T., Martin, R. and Münz, C.,** Epstein-Barr Virus: Environmental Trigger of Multiple Sclerosis? *Journal of Virology* 2007. **81**: 6777-6784.

95 **Takeuchi, A. and Saito, T.,** CD4 CTL, a Cytotoxic Subset of CD4+ T Cells, Their Differentiation and Function. *Frontiers in Immunology* 2017. **8**.

96 **Peeters, L. M., Vanheusden, M., Somers, V., Van Wijmeersch, B., Stinissen, P., Broux, B. and Hellings, N.,** Cytotoxic CD4+ T Cells Drive Multiple Sclerosis Progression. *Frontiers in Immunology* 2017. **8**.

97 **Meckiff, B. J., Ladell, K., McLaren, J. E., Ryan, G. B., Leese, A. M., James, E. A., Price, D. A., et al.,** Primary EBV Infection Induces an Acute Wave of Activated Antigen-Specific Cytotoxic $CD4^+$ T Cells. *The Journal of Immunology* 2019: ji1900377.

98 **Lam, J. K. P., Hui, K. F., Ning, R. J., Xu, X. Q., Chan, K. H. and Chiang, A. K. S.,** Emergence of CD4+ and CD8+ Polyfunctional T Cell Responses Against Immunodominant Lytic and Latent EBV Antigens in Children With Primary EBV Infection. *Front Microbiol* 2018. **9**: 416.

99 **Harp, C. T., Ireland, S., Davis, L. S., Remington, G., Cassidy, B., Cravens, P. D., Stuve, O., et al.,** Memory B cells from a subset of treatment-naïve relapsing-remitting multiple sclerosis patients elicit CD4(+) T-cell proliferation and IFN-γ production in response to myelin basic protein and myelin oligodendrocyte glycoprotein. *Eur J Immunol* 2010. **40**: 2942-2956.

100 **Broux, B., Markovic-Plese, S., Stinissen, P. and Hellings, N.,** Pathogenic features of CD4+CD28- T cells in immune disorders. *Trends Mol Med* 2012. **18**: 446-453.

101 **Herich, S., Schneider-Hohendorf, T., Rohlmann, A., Khaleghi Ghadiri, M., Schulte-Mecklenbeck, A., Zondler, L., Janoschka, C., et al.,** Human CCR5high effector memory cells perform CNS parenchymal immune surveillance via GZMK-mediated transendothelial diapedesis. *Brain* 2019. **142**: 3411-3427.

102 **Hickey, W. F.,** Leukocyte traffic in the central nervous system: the participants and their roles. *Semin Immunol* 1999. **11**: 125-137.

103 **Louveau, A., Smirnov, I., Keyes, T. J., Eccles, J. D., Rouhani, S. J., Peske, J. D., Derecki, N. C., et al.,** Structural and functional features of central nervous system lymphatic vessels. *Nature* 2015. **523**: 337-341.

104 **Reboldi, A., Coisne, C., Baumjohann, D., Benvenuto, F., Bottinelli, D., Lira, S., Uccelli, A., et al.,** C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat Immunol* 2009. **10**: 514-523.

105 **Ransohoff, R. M., Kivisakk, P. and Kidd, G.,** Three or more routes for leukocyte migration into the central nervous system. *Nat Rev Immunol* 2003. **3**: 569-581.

106 **de Graaf, M. T., de Jongste, A. H. C., Kraan, J., Boonstra, J. G., Smitt, P. A. E. S. and Gratama, J. W.,** Flow cytometric characterization of cerebrospinal fluid cells. *Cytometry Part B: Clinical Cytometry* 2011. **80B**: 271-281.

107 **Giunti, D., Borsellino, G., Benelli, R., Marchese, M., Capello, E., Valle, M. T., Pedemonte, E., et al.,** Phenotypic and functional analysis of T cells homing into the CSF of subjects with inflammatory diseases of the CNS. *J Leukoc Biol* 2003. **73**: 584-590.

108 **Kivisakk, P., Trebst, C., Liu, Z., Tucky, B. H., Sorensen, T. L., Rudick, R. A., Mack, M., et al.,** T-cells in the cerebrospinal fluid express a similar repertoire of inflammatory chemokine receptors in the absence or presence of CNS inflammation: implications for CNS trafficking. *Clin Exp Immunol* 2002. **129**: 510-518.

109 **Sorokin, L.,** The impact of the extracellular matrix on inflammation. *Nature Reviews Immunology* 2010. **10**: 712-723.

110 **Smolders, J., Remmerswaal, E. B., Schuurman, K. G., Melief, J., van Eden, C. G., van Lier, R. A., Huitinga, I., et al.,** Characteristics of differentiated CD8(+) and CD4 (+) T cells present in the human brain. *Acta Neuropathol* 2013. **126**: 525-535.

111 **Plog, B. A. and Nedergaard, M.,** The Glymphatic System in Central Nervous System Health and Disease: Past, Present, and Future. *Annu Rev Pathol* 2018. **13**: 379-394.

112 **Dendrou, C. A., Fugger, L. and Friese, M. A.,** Immunopathology of multiple sclerosis. *Nature Reviews Immunology* 2015. **15**: 545.

113 **Filippi, M., Bar-Or, A., Piehl, F., Preziosa, P., Solari, A., Vukusic, S. and Rocca, M. A.,** Multiple sclerosis. *Nature Reviews Disease Primers* 2018. **4**: 43.

114 **Brucklacher-Waldert, V., Stuerner, K., Kolster, M., Wolthausen, J. and Tolosa, E.,** Phenotypical and functional characterization of T helper 17 cells in multiple sclerosis. *Brain* 2009. **132**: 3329-3341.

115 **Cayrol, R., Wosik, K., Berard, J. L., Dodelet-Devillers, A., Ifergan, I., Kebir, H., Haqqani, A. S., et al.,** Activated leukocyte cell adhesion molecule promotes leukocyte trafficking into the central nervous system. *Nat Immunol* 2008. **9**: 137-145.

116 **Rahman, M. T., Ghosh, C., Hossain, M., Linfield, D., Rezaee, F., Janigro, D., Marchi, N., et al.,** IFN-γ, IL-17A, or zonulin rapidly increase the permeability of the blood-brain and small intestinal epithelial barriers: Relevance for neuro-inflammatory diseases. *Biochemical and Biophysical Research Communications* 2018. **507**: 274-279.

117 **Subileau, E. A., Rezaie, P., Davies, H. A., Colyer, F. M., Greenwood, J., Male, D. K. and Romero, I. A.,** Expression of chemokines and their receptors by human brain endothelium: implications for multiple sclerosis. *J Neuropathol Exp Neurol* 2009. **68**: 227-240.

118 **Sørensen, T. L., Trebst, C., Kivisäkk, P., Klaege, K. L., Majmudar, A., Ravid, R., Lassmann, H., et al.,** Multiple sclerosis: a study of CXCL10 and CXCR3 co-localization in the inflamed central nervous system. *Journal of Neuroimmunology* 2002. **127**: 59-68.

119 **Suan, D., Krautler, N. J., Maag, J. L. V., Butt, D., Bourne, K., Hermes, J. R., Avery, D. T., et al.,** CCR6 Defines Memory B Cell Precursors in Mouse and Human Germinal Centers, Revealing Light-Zone Location and Predominant Low Antigen Affinity. *Immunity* 2017. **47**: 1142-1153 e1144.

120 **Annibali, V., Ristori, G., Angelini, D. F., Serafini, B., Mechelli, R., Cannoni, S., Romano, S., et al.,** CD161(high)CD8+T cells bear pathogenetic potential in multiple sclerosis. *Brain* 2011. **134**: 542-554.

121 **Michel, L., Grasmuck, C., Charabati, M., Lécuyer, M.-A., Zandee, S., Dhaeze, T., Alvarez, J. I., et al.,** Activated leukocyte cell adhesion molecule regulates B lymphocyte migration across central nervous system barriers. *Science Translational Medicine* 2019. **11**: eaaw0475.

122 **Lecuyer, M. A., Saint-Laurent, O., Bourbonniere, L., Larouche, S., Larochelle, C., Michel, L., Charabati, M., et al.,** Dual role of ALCAM in neuroinflammation and blood-brain barrier homeostasis. *Proc Natl Acad Sci U S A* 2017. **114**: E524-E533.

123 **Elices, M. J., Osborn, L., Takada, Y., Crouse, C., Luhowskyj, S., Hemler, M. E. and Lobb, R. R.,** VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/Fibronectin binding site. *Cell* 1990. **60**: 577-584.

124 **Lehmann-Horn, K., Sagan, S. A., Bernard, C. C., Sobel, R. A. and Zamvil, S. S.,** B-cell very late antigen-4 deficiency reduces leukocyte recruitment and susceptibility to central nervous system autoimmunity. *Ann Neurol* 2015. **77**: 902-908.

125 **Polman, C. H., O'Connor, P. W., Havrdova, E., Hutchinson, M., Kappos, L., Miller, D. H., Phillips, J. T., et al.,** A Randomized, Placebo-Controlled Trial of Natalizumab for Relapsing Multiple Sclerosis. *New England Journal of Medicine* 2006. **354**: 899-910.

126 **Sorensen, P. S., Koch-Henriksen, N., Petersen, T., Ravnborg, M., Oturai, A. and Sellebjerg, F.,** Recurrence or rebound of clinical relapses after discontinuation of natalizumab therapy in highly active MS patients. *Journal of Neurology* 2014. **261**: 1170-1177.

127 **Serafini, B., Scorsi, E., Rosicarelli, B., Rigau, V., Thouvenot, E. and Aloisi, F.,** Massive intracerebral Epstein-Barr virus reactivation in lethal multiple sclerosis relapse after natalizumab withdrawal. *Journal of Neuroimmunology* 2017. **307**: 14-17.

128 **Damotte, V., Guillot-Noel, L., Patsopoulos, N. A., Madireddy, L., El Behi, M., International Multiple Sclerosis Genetics, C., Wellcome Trust Case Control, C., et al.,** A gene pathway analysis highlights the role of cellular adhesion molecules in multiple sclerosis susceptibility. *Genes Immun* 2014. **15**: 126-132.

129 **Frischer, J. M., Bramow, S., Dal-Bianco, A., Lucchinetti, C. F., Rauschka, H., Schmidbauer, M., Laursen, H., et al.,** The relation between inflammation and neurodegeneration in multiple sclerosis brains. *Brain* 2009. **132**: 1175-1189.

130 **Lucchinetti, C., Bruck, W., Parisi, J., Scheithauer, B., Rodriguez, M. and Lassmann, H.,** Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol* 2000. **47**: 707-717.

131 **Ramaglia, V., Sheikh-Mohamed, S., Legg, K., Rojas, O. L., Zandee, S., Fu, F., Ornatsky, O., et al.,** Multiplexed imaging of immune cells in staged multiple sclerosis lesions by mass cytometry. *bioRxiv* 2019: 638015.

132 **Planas, R., Metz, I., Martin, R. and Sospedra, M.,** Detailed Characterization of T Cell Receptor Repertoires in Multiple Sclerosis Brain Lesions. *Frontiers in Immunology* 2018. **9**.

133 **Muraro, P. A., Robins, H., Malhotra, S., Howell, M., Phippard, D., Desmarais, C., de Paula Alves Sousa, A., et al.,** T cell repertoire following autologous stem cell transplantation for multiple sclerosis. *J Clin Invest* 2014. **124**: 1168-1172.

134 **Junker, A., Ivanidze, J., Malotka, J., Eiglmeier, I., Lassmann, H., Wekerle, H., Meinl, E., et al.,** Multiple sclerosis: T-cell receptor expression in distinct brain regions. *Brain* 2007. **130**: 2789-2799.

135 **Planas, R., Santos, R., Tomas-Ojer, P., Cruciani, C., Lutterotti, A., Faigle, W., Schaeren-Wiemers, N., et al.,** GDP-l-fucose synthase is a $CD4^+$ T cell–specific autoantigen in DRB3*02:02 patients with multiple sclerosis. *Science Translational Medicine* 2018. **10**: eaat4301.

136 **Skulina, C., Schmidt, S., Dornmair, K., Babbe, H., Roers, A., Rajewsky, K., Wekerle, H., et al.,** Multiple sclerosis: Brain-infiltrating $CD8^+$ T cells persist as clonal expansions in the cerebrospinal fluid and blood. *Proceedings of the National Academy of Sciences of the United States of America* 2004. **101**: 2428-2433.

137 **van Nierop, G. P., van Luijn, M. M., Michels, S. S., Melief, M. J., Janssen, M., Langerak, A. W., Ouwendijk, W. J. D., et al.,** Phenotypic and functional characterization of T cells in white matter lesions of multiple sclerosis patients. *Acta Neuropathol* 2017. **134**: 383-401.

138 **Stern, J. N. H., Yaari, G., Vander Heiden, J. A., Church, G., Donahue, W. F., Hintzen, R. Q., Huttner, A. J., et al.,** B cells populating the multiple sclerosis brain mature in the draining cervical lymph nodes. *Science Translational Medicine* 2014. **6**: 107.

139 **Lovato, L., Willis, S. N., Rodig, S. J., Caron, T., Almendinger, S. E., Howell, O. W., Reynolds, R., et al.,** Related B cell clones populate the meninges and parenchyma of patients with multiple sclerosis. *Brain : a journal of neurology* 2011. **134**: 534-541.

140 **Howell, O. W., Reeves, C. A., Nicholas, R., Carassiti, D., Radotra, B., Gentleman, S. M., Serafini, B., et al.,** Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis. *Brain* 2011. **134**: 2755-2771.

141 **Magliozzi, R., Howell, O., Vora, A., Serafini, B., Nicholas, R., Puopolo, M., Reynolds, R., et al.,** Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. *Brain* 2007. **130**: 1089-1104.

142 **Tzartos, J. S., Craner, M. J., Friese, M. A., Jakobsen, K. B., Newcombe, J., Esiri, M. M. and Fugger, L.,** IL-21 and IL-21 receptor expression in lymphocytes and neurons in multiple sclerosis brain. *Am J Pathol* 2011. **178**: 794-802.

143 **Peters, A., Pitcher, L. A., Sullivan, J. M., Mitsdoerffer, M., Acton, S. E., Franz, B., Wucherpfennig, K., et al.,** Th17 cells induce ectopic lymphoid follicles in central nervous system tissue inflammation. *Immunity* 2011. **35**: 986-996.

144 **Olsson, T.,** Cytokines in neuroinflammatory disease: role of myelin autoreactive T cell production of interferon-gamma. *Journal of Neuroimmunology* 1992. **40**: 211-218.

145 **Renno, T., Lin, J.-Y., Piccirillo, C., Antel, J. and Owens, T.,** Cytokine production by cells in cerebrospinal fluid during experimental allergic encephalomyelitis in SJL/J mice. *Journal of Neuroimmunology* 1994. **49**: 1-7.

146 **Brosnan, C. F., Cannella, B., Battistini, L. and Raine, C. S.,** Cytokine localization in multiple sclerosis lesions. *Correlation with adhesion molecule expression and reactive nitrogen species* 1995. **45**: S16-S21.

147 **Bsibsi, M., Peferoen, L. A. N., Holtman, I. R., Nacken, P. J., Gerritsen, W. H., Witte, M. E., van Horssen, J., et al.,** Demyelination during multiple sclerosis is associated with combined activation of microglia/macrophages by IFN-γ and alpha B-crystallin. *Acta Neuropathologica* 2014. **128**: 215-229.

148 **Perry, V. H. and Holmes, C.,** Microglial priming in neurodegenerative disease. *Nature Reviews Neurology* 2014. **10**: 217.

149 **Li, R., Patterson, K. R. and Bar-Or, A.,** Reassessing B cell contributions in multiple sclerosis. *Nature Immunology* 2018. **19**: 696-707.

150 **Genain, C. P., Cannella, B., Hauser, S. L. and Raine, C. S.,** Identification of autoantibodies associated with myelin damage in multiple sclerosis. *Nat Med* 1999. **5**: 170-175.

151 **Lunemann, J. D., Tintore, M., Messmer, B., Strowig, T., Rovira, A., Perkal, H., Caballero, E., et al.,** Elevated Epstein-Barr virus-encoded nuclear antigen-1 immune responses predict conversion to multiple sclerosis. *Ann Neurol* 2010. **67**: 159-169.

152 **Rand, K. H., Houck, H., Denslow, N. D. and Heilman, K. M.,** Epstein-Barr virus nuclear antigen-1 (EBNA-1) associated oligoclonal bands in patients with multiple sclerosis. *J Neurol Sci* 2000. **173**: 32-39.

153 **Castellazzi, M., Contini, C., Tamborino, C., Fasolo, F., Roversi, G., Seraceni, S., Rizzo, R., et al.,** Epstein-Barr virus-specific intrathecal oligoclonal IgG production in relapsing-remitting multiple sclerosis is limited to a subset of patients and is composed of low-affinity antibodies. *Journal of Neuroinflammation* 2014. **11**: 188.

154 **Serafini, B., Rosicarelli, B., Magliozzi, R., Stigliano, E. and Aloisi, F.,** Detection of Ectopic B-cell Follicles with Germinal Centers in the Meninges of Patients with Secondary Progressive Multiple Sclerosis. *Brain Pathology* 2004. **14**: 164-174.

155 **Serafini, B., Rosicarelli, B., Franciotta, D., Magliozzi, R., Reynolds, R., Cinque, P., Andreoni, L., et al.,** Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain. *The Journal of experimental medicine* 2007. **204**: 2899-2912.

156 **Sundström, P., Juto, P., Wadell, G., Hallmans, G., Svenningsson, A., Nyström, L., Dillner, J., et al.,** An altered immune response to Epstein-Barr virus in multiple sclerosis. *A prospective study* 2004. **62**: 2277-2282.

157 **Lang, H. L. E., Jacobsen, H., Ikemizu, S., Andersson, C., Harlos, K., Madsen, L., Hjorth, P., et al.,** A functional and structural basis for TCR cross-reactivity in multiple sclerosis. *Nature Immunology* 2002. **3**: 940-943.

158 **Owens, G. P. and Bennett, J. L.,** Trigger, pathogen, or bystander: the complex nexus linking Epstein- Barr virus and multiple sclerosis. *Mult Scler* 2012. **18**: 1204-1208.

159 **Willis, S. N., Stadelmann, C., Rodig, S. J., Caron, T., Gattenloehner, S., Mallozzi, S. S., Roughan, J. E., et al.,** Epstein-Barr virus infection is not a characteristic feature of multiple sclerosis brain. *Brain* 2009. **132**: 3318-3328.

160 **Sargsyan, S. A., Shearer, A. J., Ritchie, A. M., Burgoon, M. P., Anderson, S., Hemmer, B., Stadelmann, C., et al.,** Absence of Epstein-Barr virus in the brain and CSF of patients with multiple sclerosis. *Neurology* 2010. **74**: 1127-1135.

161 **Peferoen, L. A., Lamers, F., Lodder, L. N., Gerritsen, W. H., Huitinga, I., Melief, J., Giovannoni, G., et al.,** Epstein Barr virus is not a characteristic feature in the central nervous system in established multiple sclerosis. *Brain* 2010. **133**: e137.

162 **Aloisi, F., Serafini, B., Magliozzi, R., Howell, O. W. and Reynolds, R.,** Detection of Epstein-Barr virus and B-cell follicles in the multiple sclerosis brain: what you find depends on how and where you look. *Brain* 2010. **133**: e157.

163 **de Bock, L., Fraussen, J., Villar, L. M., Álvarez-Cermeño, J. C., Van Wijmeersch, B., van Pesch, V., Stinissen, P., et al.,** Anti-SPAG16 antibodies in primary progressive multiple sclerosis are associated with an elevated progression index. *European Journal of Neurology* 2016. **23**: 722-728.

7

General discussion

GENERAL DISCUSSION

The contribution of B cells to the pathogenesis of multiple sclerosis (MS) is indisputable. However, the exact B-cell subset and mechanisms that mediate the disease are still unclear. Previously, MS was mainly considered a T cell-driven chronic inflammatory disorder of the central nervous system (CNS), although the presence of oligoclonal bands in the cerebrospinal fluid (CSF) already indicated a pathophysiological role of B cells. This was largely strengthened by the strong clinical benefits of anti-CD20 mediated B-cell depletion in MS. Since long-lived antibody-producing plasma cells do not express CD20 and thus are not removed with this therapy, B cells also promote MS pathogenesis in an antibody-independent manner. It has become evident that B cells can drive autoimmune diseases such as MS through the presentation of autoantigens to T cells, secretion of cytokines and the formation of ectopic lymphoid structures in chronically inflamed tissue (**chapter 1**). In this thesis, we aimed to uncover genes and pathways that are functionally altered in human B cells and could explain their contribution to MS pathogenesis. To study this, we made use of gene silencing and functional assays related to B- and T_H-cell interaction and took advantage of recent genome-wide association study (GWAS) data, different patient cohorts and the effects of immunosuppressive conditions and treatment on disease activity.

In **chapter 6**, we discuss our hypothesis that multiple events involving B-T cell interaction play a key role in MS. During B cell development, naive B cells likely escape from peripheral selection to enter secondary lymphoid organs and encounter T-follicular helper (T_{FH}) cells. After mutual activation, B cells further develop into pathogenic memory subsets within germinal centers (GCs), which migrate into the CNS and meet again with T cells to cause local pathology. Within this general discussion, we elaborate on alterations in functional B-cell pathways that possibly drive these events in MS. This involves altered B-cell antigen processing and presentation via HLA-II molecules, T cell-derived interferon gamma (IFN-γ) as inducer of pathogenic memory B cells, local organization within GCs to facilitate pathogenic B-cell differentiation as well as their capacity to infiltrate the CNS.

PART I. DYSREGULATION OF THE B-CELL ANTIGEN PROCESSING AND PRESENTATION PATHWAY IN MS

Expression and impact of class II–associated invariant chain peptides (CLIP) on B cells

Naive B cells circulate within the bloodstream and recruit to secondary lymphoid follicles. When a naive B cell recognizes and processes an antigen, it will migrate towards the follicular border. Here, the B cell presents the processed antigen to a T_{FH} cell, which together with cytokine and costimulatory signals results in T_{FH}-cell activation. During this interaction, the B cell also receives survival and proliferating signals from the T cell. In **chapter 3,** we analyzed both CLIP and HLA-DR surface expression as indirect measure of B-cell antigen presentation efficiency [1] in both relapsing-remitting MS (RRMS) patients and clinically isolated syndrome (CIS) patients. The used cerCLIP.1 antibody specifically recognizes CLIP-loaded HLA-II, while L243 antibody detects peptide-loaded HLA-II (HLA-DR; [1]). CLIP was upregulated on B cells of RRMS as well as high-risk CIS patients, while surface HLA-DR expression was not increased, resulting in an increased CLIP/HLA-DR ratio. This increased CLIP/HLA-DR ratio was seen for all subsets, including transitional ($IgM^+CD27^-CD38^{high}$), naive mature ($IgM^+CD27^-CD38^{-/dim}$), non-switched (IgM^+CD27^+) as well as switched (IgG^+CD27^+ and IgA^+CD27^+) B-cell subsets.

There could be several reasons for the increased expression of CLIP on B cells of early MS patients. In naive B cells, CLIP remains bound to the peptide-binding groove of HLA-II molecules until the B-cell receptor (BCR) is engaged with an antigen. It has been proposed that before receiving an antigen-specific signal, polyclonal activation of B cells results in increased CLIP expression and improper control of T cell-induced cell death, thereby contributing to chronic inflammation in mice [2]. This polyclonal activation is mediated via toll-like receptor (TLR) activation during infections. TLR-mediated activation of B cells not only results in ectopic CLIP expression, but also increased expression of HLA-II itself. After polyclonal activation, recognition of an antigen triggers an adaptive immune response by supporting survival of antigen-specific and promoting MHC-dependent death of non-specific B cells. The increased CLIP expression on naïve B cells in the blood of MS patients may thus be the result of improper control of such polyclonal, potentially autoreactive B cells [3].

Another possible reason for the abundance of CLIP on B cells in MS might be that chronic exposure to antigens in the absence of T_H cell stimulation can eventually result in anergy [4]. This makes B cells less able to recognize and internalize antigens for exchange with CLIP on HLA-II within HLA-II peptide-loading compartments (MIICs). Furthermore, not only HLA-II, but also CD40L expressed by T cells plays a crucial role in the survival of

autoreactive naive B cells [5, 6]. In **chapter 3,** we demonstrate that CLIP upregulation on B cells from healthy subjects is mainly induced by CD40-CD40L interaction. The fact that CLIP was already highly expressed on naive B cells of MS patients, and not further enhanced by CD40 triggering, provides further insights into how $CLIP^{high}$ B cells may be able to circumvent T cell-mediated selection in MS [3]. Defective T_{REG} responses are often considered as the main cause of autoreactive B-cell survival in the periphery [7-9]. Notably, in mice, CLIP upregulation impedes T_{REG} activation [10, 11], which may represent a second mechanism by which $CLIP^{high}$ naive B cells escape from peripheral tolerance in MS.

Dual role for the invariant chain in B cells

CLIP remains in the HLA-II peptide-binding groove after cleavage of the invariant chain (CD74). In **chapter 4**, we show that surface expression of CD74 on B cells from RRMS and high-risk CIS patients is decreased compared to healthy controls and low-risk CIS patients, respectively. The downregulation of CD74 and upregulation of CLIP especially on naive B cells supports our hypothesis that invariant chain processing into CLIP is enhanced during polyclonal activation (see above). Furthermore, we demonstrate that a CD74 blocking antibody suppressed the proliferation, and induction of pro-inflammatory genes *NFKB1, IL6* and *TNF,* after α-IgM stimulation of B cells *in vitro*. The reduced expression of CD74 on B cells in early MS blood might therefore reflect a functional state of anergy, contributing to the persistence of pathogenic immature B cells in the periphery [12]. Next to its role as a chaperone of the HLA-II molecule (**chapter 1**), 2-5% of the invariant chain or CD74 is expressed at the surface independently of HLA-II [13, 14]. When present on the cell surface, CD74 can serve as a high-affinity receptor for the macrophage migration inhibitory factor (MIF) [15]. MIF is a pro-inflammatory and immune-regulatory cytokine, which is ubiquitously expressed in mammalians. Extracellular MIF binds to CD74 and triggers B-cell proliferation, migration and inhibition of apoptosis [15, 16]. Although binding of MIF to CD74 is essential for the signaling, it was not sufficient to trigger downstream transduction. It depends on the presence of co-receptors CD44, CXCR2 or CXCR4, which recruit signaling components with their cytoplasmic domains [17-19].

Very little is known about how the expression of the invariant chain is distributed between functioning as a MHC-II chaperone or as a MIF signaling receptor. There are several isoforms of CD74; p33, p35, p41 and p43 [20]. The latter two have an additional exon in the luminal domain, while p35 and p43 have an extra N-terminal extension which is an endoplasmic reticulum (ER) retention motif. The p35 and p43 therefore cannot leave the ER without assembling MHC-II, since this will mask the ER retention motif, which allows the exit of the complex to the cell surface [21]. However, p33 and p41 do not have this ER retention motif and therefore can exit the ER without MHC-II [22], potentially function as MIF receptor. However, these isoforms can also still assemble MHC-II molecules and be a

chaperone. More research is needed to separate these functions of the invariant chain and to understand the full potential and biology.

Minor risk alleles and the HLA-II pathway

After their escape from selection and BCR-mediated uptake of an antigen, B cells process and present (auto)antigens to receive signals from cognate T_{FH} cells [23] and differentiate into memory subsets or plasmablasts/plasma cells. Within GC, memory B cells serve as potent antigen-presenting cells to trigger pathogenic T_H subsets, as recently described for MS [24]. These subsets express high levels of IFN-γ and have a similar phenotype as the MS brain-homing T_H cells found by our team [25, 26]. In **chapter 2,** we found that blocking of IFN-γ suppressed human T_H cell activation, proliferation and effector memory skewing during autologous co-cultures. Although the exact antigen in MS is still unknown, the disease pathology likely involves interaction of B cells with IFN-γ-producing T_H cells within lymphoid organs, which creates a feedforward loop resulting in the development of highly pathogenic B- and T cell subsets.

Several lines of evidence indicate that the exchange of CLIP for antigenic peptides on HLA-II is at least partially controlled by genetic autoimmune traits. Firstly, the strong effects of certain HLA-II loci in autoimmune diseases such as type I diabetes, rheumatoid arthritis and MS are associated with specific amino acid changes in the peptide-binding groove [27-29]. However, in CIS, we did not find an association between B cell-intrinsic CLIP expression and the presence of *HLA-DRB*1501*. In addition, HLA-DM polymorphisms, which may affect HLA-II peptide binding [30], did not link to disease susceptibility or myelin-specific T-cell responses in MS [31]. Secondly, non-HLA risk loci are being identified that regulate the BCR-mediated HLA-II pathway [1, 23, 32].

Multiple GWAS have demonstrated that C-type lectin gene *CLEC16A* genetically links to several autoimmune diseases including MS [33]. Our group previously found that it has a non-classical C-type lectin function in myeloid cells by participating in dynein-mediated transport of MIICs [34]. In **chapter 3**, we show that *CLEC16A* is co-expressed and -regulated with surface CLIP in human B cells. Based on the expression patterns amongst distinct *ex vivo* B-cell subsets and gene silencing experiments, *CLEC16A* seems to be especially involved in the HLA-II pathway of IgM^+ B cells residing outside the GC (i.e. naive and natural effector memory subsets) [35, 36]. This is supported by an earlier study in mice [37]. The more dispersed localization of MIICs in $CLIP^{high}$ Raji B cells after CLEC16A silencing agrees with our previous findings in other antigen presenting cells (APCs) [34]. Additionally, HLA-DR and CD74 as well as IgM/antigen complexes were trapped at the plasma membrane of *CLEC16A*-silenced Raji cells. This not only confirms the role of *CLEC16A* in retrograde transport of MIICs [34, 38], but also points to a function in BCR-mediated internalization of antigens into MIICs. Although yet unknown in B cells, MIICs are known to undergo

acidification in perinuclear regions that is required for optimal antigen processing and loading onto HLA-II molecules in DCs. In the absence of *CLEC16A*, both HLA-II/invariant chain and BCR/antigen complexes probably do not reach these MIICs for proper cleavage into CLIP and exchange with antigenic peptides. Like *CLEC16A*, non-classical HLA-II chaperone HLA-DO is highly expressed in naive B cell populations [39], which inhibits this exchange via HLA-DM [40]. Considering the proposed HLA-DM-independent nature of CLIP release from autoimmunity-related HLA-II molecules [27], it will be interesting to further analyze patient B cells for *CLEC16A* and CLIP co-regulation in the context of both classical and non-classical HLA-II proteins.

Next to *CLEC16A,* GWAS studies have identified multiple other single nucleotide polymorphisms (SNPs) that can be linked to the regulation of the HLA-II pathway. Two of those SNPs are located in *CBLB* and *MARCH1* [41-43]. CBL-B is an E3 ubiquitin ligase that tags proteins with ubiquitin for degradation. CBL-B acts as a scaffolding molecule to coordinate the delivery of BCR-captured antigens to TLR9 in subcellular compartments [44]. Furthermore, CBL-B negatively regulates CD40-mediated B-cell responses [45]. MARCH1 is another E3 ubiquitin ligase that serves as the master regulator of MHC-II expression at the post-transcriptional level [46]. Ubiquitination of MHC-II in both murine and human cells represents a critical molecular switch that regulates its transport to and from the cell surface [47-50]. HLA-DM and costimulatory molecule CD86 are regulated by MARCH1 ubiquitination as well [51, 52] [53]. Intriguingly, one of the very few SNPs in a protein-coding region is located in *IFI30* (rs11554159) [41]. *IFI30* is a gene that encodes for GILT and mainly resides in late endosomes. Here, it reduces protein disulfide bonds and cleaves the invariant chain into CLIP, thus facilitating efficient peptide loading and presentation by HLA-II molecules [54-56]. This missense SNP is located next to the active site of GILT and is predicted to be the causal MS risk variant in *IFI30* [32]. *IFI30* as well as the master regulator of HLA-II pathway termed *CIITA* are under tight control of IFN-γ (unpublished data and [57]). Another missense risk SNP is found in *IFNGR2* (rs9808753; [43, 58]), which encodes for the IFN-γ receptor β chain and has been linked to disease progression in MS [58, 59]. The exact mechanisms by which this risk SNP in *IFNGR2* contributes to MS is still unknown. Since these two, out of the very few, exonic SNPs are located in IFN-γ-associated genes, it is highly tempting to speculate that the IFN-γ signaling pathway in B cells is alternatively regulated and contributes to the development of pathogenic subsets in MS.

PART II. INCREASED IFN-γ SENSITIVITY AND DEVELOPMENT OF BRAIN-INFILTRATING B CELLS IN MS

Function of coding MS risk variant IFNGR2

The IFN-γ receptor (IFNGR) consist of a ligand binding chain (IFNGR1) and a non-ligand binding chain (IFNGR2) [60, 61]. Once IFN-γ binds to the IFNGR, Janus kinase (Jak)1 and Jak2 are activated and signal transducer and activator of transcription 1 (STAT1) gets phosphorylated [62]. Phosphorylated STAT1 (pSTAT1) dimerizes and translocates to the nucleus where it initiates transcription of genes such as T-bet, IRF1 and BCL-6 [23, 62] (Figure 1A).

Preliminary work by our group suggests that STAT1 is highly phosphorylated and stimulates T-bet under IFN-γ-stimulating conditions in human B-cell lines carrying MS risk allele *IFNGR2*. For this, we first genotyped the IFNGR2 risk SNP (rs9808753; [43, 58]) in 19 human B-LCLs. We selected one B-LCL that carried no risk alleles (Karpas422) and one B-LCL that

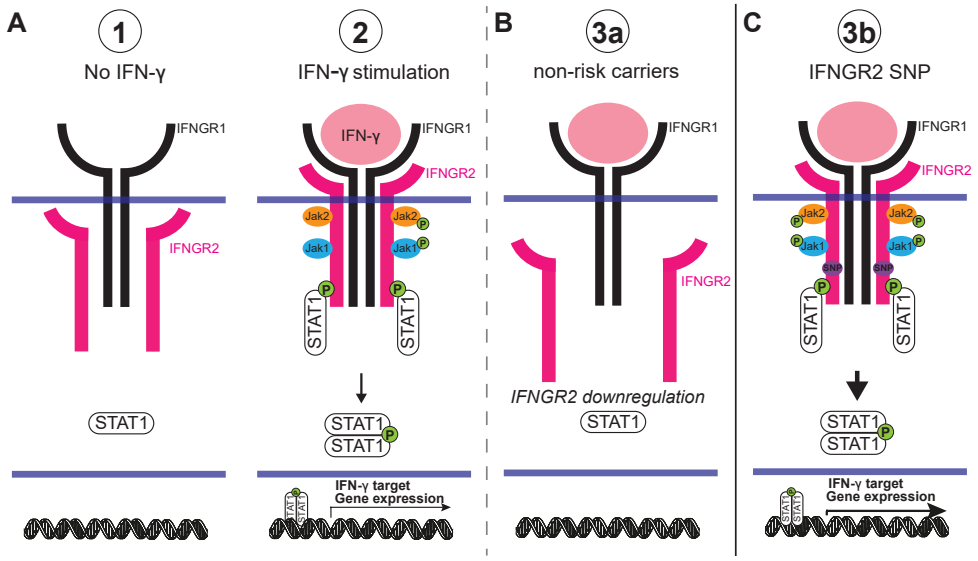

Figure 1. Model showing the potential impact of a missense risk SNP (rs9808753) in IFNGR2 on the IFN-γ signaling pathway .

A: The IFNGR is expressed at the surface of B cells and consists of an α (IFNGR1) and β (IFNGR2) chain. Once IFN-γ binds to the receptor, signaling is initiated and results in the expression of IFN-γ target genes. **B:** After the IFN-γ binding and IFNGR signaling, IFNGR2 is removed from the surface to prevent further stimulation of the B cell. **C**: In IFNGR2 SNP carriers, the IFNGR2 is possibly not removed from the surface, resulting in a prolonged signaling of the IFNGR pathway and subsequent activation of target genes.

carried both risk alleles (MC116). After stimulation with IFN-γ for 10 min, we analyzed STAT1 and pSTAT1 protein expression by western blotting (Figure 2A). We found that the B-LCL carrying both risk alleles had an enhanced phosphorylation of STAT1 compared to the B-LCL carrying no risk alleles (Figure 2B). This association between STAT1 phosphorylation and the presence of the IFNGR2 risk allele was confirmed using two other B-LCLs (data not shown). Next, we screened 90 healthy controls and 130 CIS patients for the presence of the IFNGR2 risk SNP (Figure 2C). We were able to select and purify B cells from the blood of 4 CIS patients who were homozygous risk carriers. After stimulation with IFN-γ for various time points between 0 and 30 min, observed differences in pSTAT1 expression in B cells between these patients and 4 heterozygous risk and 4 non-risk carriers could not be reproduced using FACS. Alternatively, we stimulated B cells from these same patients for 24h with IFN-γ and analyzed the expression of IFN-γ-associated genes using qPCR. Interestingly, T-bet, IRF1 and IFNGR2 levels turned out to be higher in the homozygous risk

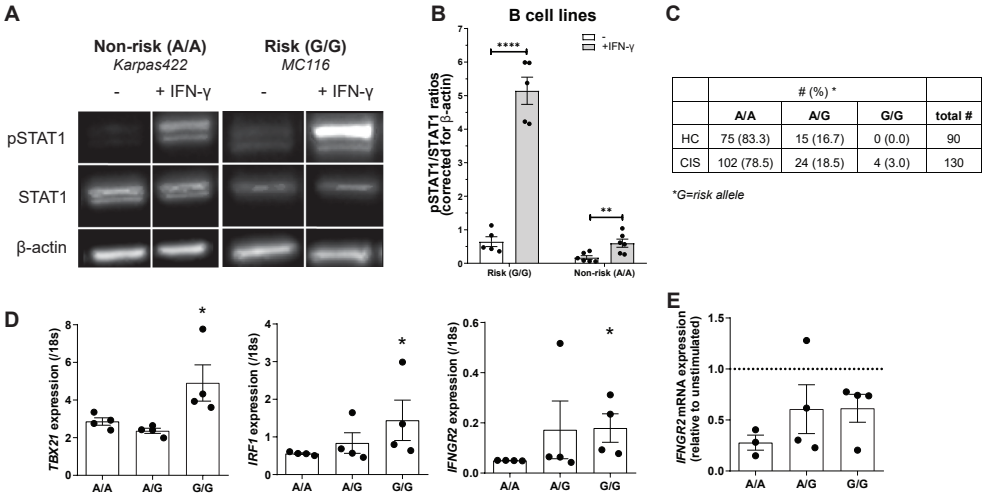

Figure 2. The impact of coding MS risk variant IFNGR2 on the IFN-γ pathway in human B cells.

A: A human EBV-negative B-LCL carrying no IFNGR2 risk alleles (Karpas422; A/A) and a human EBV-negative B-LCL carrying both IFNGR2 risk alleles (MC116; G/G) were analyzed for pSTAT1 and STAT1 protein expression before and after 10 min stimulation with IFN-γ using western blotting. This was repeated 6 times in two independent experiments, corrected for β-actin expression and depicted as pSTAT1/STAT1 ratios (**B**). (**C**) T-bet (TBX21), IRF-1 (IRF1) and IFNGR2 gene expression in 24h-stimulated B cells was compared between CIS patients who were non-risk (A/A), heterozygous (A/G) and homozygous risk (G/G) carriers (n=4 per subgroup) using qPCR. In **D,** IFNGR2 gene expression was compared to the unstimulated condition per patient (dotted line). For statistical analysis, paired t-tests (B) and Mann-Whitney U tests (C) were carried out. *p<0.05, ** p<0.01, ****p<0.0001

group (Figure 2D), suggesting that the IFN-γ signaling pathway is affected by the *IFNGR2* risk allele in human primary B cells as well. These results need to be confirmed in larger numbers of individuals.

It also remains to be determined whether and how this SNP affects the expression of IFNGR. In T cells, IFNGR2 trafficking tunes IFN-γ signaling, by altering its surface expression [63]. Under normal conditions, IFNGR2 is removed from the cell surface after ligation to prevent further stimulation by IFN-γ (Figure 1B). Although IFNGR1 expression is ubiquitous, IFNGR2 expression is fine-tuned by many factors, including ligand-dependent engulfment of IFNGR2 and downregulation of *IFNGR2* transcription [63-66]. In our analyses, we found that for the homozygous risk group, IFNGR2 expression is less decreased in B cells after IFN-γ triggering (Figure 2E). This implies that the risk SNP interferes with the removal of surface IFNGR2, resulting in prolonged IFN-γ signaling (Figure 1C). This should to be validated and further investigated in the near future.

Synergy between IFN-γ and TLR9 ligand

It has been shown previously that B cells of MS patients are more sensitive to IFN-γ signaling [67]. More general, in an autoimmune setting, it has been shown that additional production of IFN-γ by T_{FH} cells elicits autoimmune GC formation. This first became evident from studies in SLE [68], where it has been shown that deletion of the IFN-γ receptor in B-cells inhibits the formation of autoimmune GCs and production of class-switched autoantibodies. In B cells, IFN-γ has been shown to induce the expression of T-bet, which triggers class switching under inflammatory conditions in both mice and humans [69-71]. T-bet induces CXCR3 expression, which is involved in the localization of B cells and differentiation into plasma cells within GCs [72, 73]. Furthermore, class-switching and autoantibody production can also be mediated by TLR9 signaling [74]. In **chapter 2**, we show that naive B cells from MS patients are differentially influenced by IFN-γ and TLR9 ligand CpG-ODN during T_{FH}-like cultures. IFN-γ stimulated the development of plasmablasts, whereas IFN-γ together with CpG-ODN triggered the differentiation of $IgG1^+$ memory B cells. Furthermore, we demonstrate that TLR9 triggering enhanced T-bet and CXCR3 surface expression during IFN-γ-mediated GC-like cultures with naive B cells. Interestingly, these synergistic effects on T-bet/CXCR3 induction and B-cell differentiation were not seen when culturing purified memory B cells, indicating that IFN-γ and TLR9 ligand are important for the fate of naive B cells in autoimmune diseases such as MS [68, 70, 75].

GC organization versus CNS recruitment

Increased levels of CXCL10, CXCL12 and CXCL13 in the CNS of MS patients probably facilitate the recruitment and organization of memory B cells [76-78]. In **chapter 2,** we demonstrate that CXCR3 was strongly upregulated on blood B cells from MS patients

after treatment with natalizumab, an effective MS drug that blocks the entry of pro-inflammatory lymphocytes into the CNS. Additionally, $CXCR3^+$ B cells were enriched in fresh single-cell suspensions from CSF and brain tissue versus blood of progressive MS patients. Further analysis of these cells in the blood of natalizumab-treated MS patients revealed that particularly $IgG(1)^+$ memory B cells express high levels of CXCR3 and accumulate within 1 year after treatment. $CXCR3^+IgG1^+$ memory B cells also preferentially migrated towards CXCL10 in human brain endothelial *in vitro* transmigration assays. It is therefore highly likely that $CXCR3^{high}CD27^+IgG1^+$ B-cell populations in the blood preferentially recruit to the CNS during MS pathogenesis, as discussed in more detail in **chapter 6**.

Once in the CNS, B cells are possibly re-activated within ectopic lymphoid follicle-like structures found in the meninges, which could mimic a GC response (**chapter 1**). Here, the aberrant CXCR4 and CXCR5 expression might play an important role in the local organization and differentiation of memory B cells and antibody-producing plasma cells. Within the periphery, the local organization within a GC is crucial for efficient selection and development of B cells, which is mediated through various chemokines and their receptors. After interaction with pre-T_{FH} cells at the follicular border, B cells are attracted to CXCL12 within the GC dark zone through the expression of CXCR4 [79, 80] and undergo extensive proliferation and SHM (**chapter 1**). Subsequently, CXCR4 is downregulated and both CXCR5 and CCR6 direct the B cell towards the light zone [79, 81]. Here, B cells interact with follicular DCs and T_{FH} cells to undergo selection and eventually differentiate into class-switched memory or long-lived plasma cells [82]. The number of peptide-MHC complexes presented on the cell surface dictates the quantity of T cell help and thereby drives the selection of light zone B cells [83]. If required, GC B cells can be directed back into the dark zone for further rounds of somatic hypermutation and maturation before they exit the GC as memory B cells or plasma cells. In **chapter 4,** we demonstrate that CXCR4 expression is increased on B cells of CIS patients who rapidly developed MS as well as in clinically definite MS patients. CXCR4 was found to be the highest on naive B cells, which probably controls their development within GCs, as shown in SLE [84]. Our data shows that blocking of CXCR4 signaling in B cells increases CD95 (Fas) expression. Fas is essential for the elimination of autoreactive B-cell clones by T_H cells [85]. Hence, the overexpression of CXCR4 on naïve B cells, as observed in early MS patients, may promote their escape from T_H cell-based selection in the light zone via reduced Fas expression and enhanced migration into the dark zone. Next to the initial GC-response, memory B cell can re-enter the GC for a secondary immune response after antigen re-challenge [86]. Here, memory B cells can differentiate into long-lived plasma cells or undergo additional rounds of expansion, SHM and affinity maturation. Especially this reactivation of memory B cells and formation of long-lived (potentially tissue-resident) plasma cells is speculated to happen within ectopic lymphoid follicles [87].

Whether the local organization and subsequent reactivation within the CNS correspond to MS relapse risk has not been elucidated. Pregnancy results in a natural and transient period of immune suppression [88], enabling the analysis of different B-cell subsets during periods with low relapse risk (3rd trimester) and high relapse risk (early postpartum) [89, 90]. In **chapter 5,** we show that CXCR4 expression is decreased while CXCR5, CCR6 and CXCR3 expression are increased on B cells in the early postpartum period as compared to 3rd trimester. These differences were most pronounced in MS patients and reflected a GC light zone phenotype. Not only surface expression of IgM and IgG, but also *in vitro* differentiation of IgM- and IgG-secreting plasma cells was increased for memory B cells from postpartum blood samples. This indicates that the increased postpartum relapse risk may be partially explained by the increased potential of B cells to migrate into the GC light zone, interact with IFN-γ-producing T_{FH} cells and differentiate into $CXCR3^+$ memory B cells that are prone to enter the CNS and locally develop in potentially tissue-resident plasma cells in MS [91, 92].

FUTURE PERSPECTIVES

Functional B-cell genetics

GWAS identified now more than 200 SNPs that are associated with MS, however only limited research has been done on the function of such a SNP. In this thesis, we took a first step by linking MS risk variant *CLEC16A* to the HLA-II pathway and *IFNGR2* to the IFN-γ signaling pathway in B cells. However, to do real functional studies on the effect of one single nucleotide change in human lymphocytes, different techniques should be used. CRISPR/Cas9 is one of those techniques that is potentially very useful to introduce a SNP in a specific cell type. However, doing CRISPR/Cas9 SNP editing in human B cells *in vitro* is a challenge and needs further development before we can implicate this for future research.

While HLA-DRB1*15:01 is associated with an increased risk of MS development, it is still unknown why. Many studies have been performed on the functional consideration on the effect of this HLA-II allele and suggest that it has probably to do with the peptide binding motif which is unique for this allele [93]. However, the exact peptide that is presented by HLA-II molecules to T cells in MS is currently still unknown. Some studies suggest that it is a specific peptide from the MBP protein, but this has not been validated *in vivo* [94].

Another interesting strategy is to study genetic risk scores by selecting the risk genes within these GWAS analysis that are important for the function of B cells. Based on those risk SNPs calculate a genetic risk score and compare this to the actual function or phenotype of the cells. This could give more relevant insight and could possibly predict disease progression of therapeutic outcome. However, this is also a challenge since a large part

of the SNPs are located intergenic or intragenic, which makes it difficult to determine on which gene the SNP would have an effect. Hopefully, more insight is gained in the function of these SNPs in the future.

Selective B-cell therapeutics

Targeting B cells with a-CD20 B-cell depletion therapies such as rituximab and ocrelizumab is highly efficient, and is now even the first therapy to be approved for progressive MS patients [95]. However, deleting all B-cell can have severe side effects. Moreover, the sooner we could identify the pathogenic B cell subtype, the better we can target these cells more specific. Therefore, more specific targeted therapy needs to be developed that targets only selected B cell subsets, or specific B cell functions. Within this thesis we have shown that the CXCR3$^+$ (T-bet$^+$) IgG1$^+$ memory B cells is of high interest to be targeted by therapy, since this subset is most prone to migrate to the CNS to mediate the disease locally (**chapter 2**). This subset is generated after stimulation of both IFN-γ and TLR-9 pathways. Small molecule inhibitors of the IFN-γ signaling (jakinibs) [96, 97] and TLR pathway [98] are currently being used for treating other inflammatory diseases. Such targeted treatments may be applied for suppression of IFN-γ and/or TLR signals in B cells, specifically targeting the potential disease causing B cell subsets (Figure 3).

Another potential therapeutic target in B cells is Bruton's tyrosine kinase (BTK). BTK is a member of the Tec family of kinases and transmit signals through multiple receptors on the B cell, including the BCR and TLR. Furthermore, BTK also controls CXCR4 signaling as well as the APC function of the B cell (Figure 3). BTK inhibitors are currently under investigation

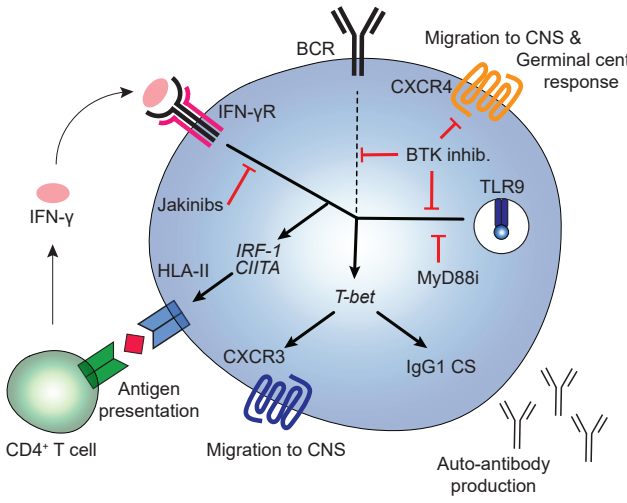

Figure 3. The different B-cell pathways addressed in this thesis and possible targets to interfere with the development of pathogenic B cells in MS.

in several autoimmune diseases, including SLE, RA and MS. A recently published placebo-controlled phase II trial of an oral BTK inhibitor (evobrutinib) showed promising results and underlines further investigation for the use as therapy in MS [99].

Currently within our group, we are studying the effect of evobrutinib on human B cell differentiation and migration *in vitro*. Furthermore, we are investigating the effect of BTK inhibition after IFN-γ and TLR9 stimulation and subsequent development of $CXCR3^+$($T\text{-}bet^+$) B cells. Although still preliminary, these result show that evobrutinib inhibits the differentiation of naive B cells towards $CXCR3^+$($T\text{-}bet^+$) memory B cells, as well as the differentiation of memory B cells towards antibody-producing plasma cells. In addition, specific migration of IFN-γ- and TLR9-induced $CXCR3^+$ B cells towards CXCL10 across the blood-brain barrier seems to be partially inhibited by evobrutinib (data not shown). These results suggest that inhibiting BTK could be a good strategy to inhibit the differentiation and CNS-homing capacity of potentially pathogenic $CXCR3^+$($T\text{-}bet^+$) B cells.

REFERENCES

1 **Paul, P., van den Hoorn, T., Jongsma, M. L., Bakker, M. J., Hengeveld, R., Janssen, L., Cresswell, P., et al.,** A Genome-wide multidimensional RNAi screen reveals pathways controlling MHC class II antigen presentation. *Cell* 2011. **145**: 268-283.

2 **Newell, M. K., Tobin, R. P., Cabrera, J. H., Sorensen, M. B., Huckstep, A., Villalobos-Menuey, E. M., Burnett, M., et al.,** TLR-mediated B cell activation results in ectopic CLIP expression that promotes B cell-dependent inflammation. *J Leukoc Biol* 2010. **88**: 779-789.

3 **Kinnunen, T., Chamberlain, N., Morbach, H., Cantaert, T., Lynch, M., Preston-Hurlburt, P., Herold, K. C., et al.,** Specific peripheral B cell tolerance defects in patients with multiple sclerosis. *J Clin Invest* 2013. **123**: 2737-2741.

4 **Andrews, S. F. and Wilson, P. C.,** The anergic B cell. *Blood* 2010. **115**: 4976-4978.

5 **Herve, M., Isnardi, I., Ng, Y. S., Bussel, J. B., Ochs, H. D., Cunningham-Rundles, C. and Meffre, E.,** CD40 ligand and MHC class II expression are essential for human peripheral B cell tolerance. *J Exp Med* 2007. **204**: 1583-1593.

6 **Lesley, R., Kelly, L. M., Xu, Y. and Cyster, J. G.,** Naive CD4 T cells constitutively express CD40L and augment autoreactive B cell survival. *Proc Natl Acad Sci U S A* 2006. **103**: 10717-10722.

7 **Kinnunen, T., Chamberlain, N., Morbach, H., Choi, J., Kim, S., Craft, J., Mayer, L., et al.,** Accumulation of peripheral autoreactive B cells in the absence of functional human regulatory T cells. *Blood* 2013. **121**: 1595-1603.

8 **Menard, L., Samuels, J., Ng, Y. S. and Meffre, E.,** Inflammation-independent defective early B cell tolerance checkpoints in rheumatoid arthritis. *Arthritis Rheum* 2011. **63**: 1237-1245.

9 **Samuels, J., Ng, Y. S., Coupillaud, C., Paget, D. and Meffre, E.,** Impaired early B cell tolerance in patients with rheumatoid arthritis. *J Exp Med* 2005. **201**: 1659-1667.

10 **Morgan, M. A., Muller, P. S., Mould, A., Newland, S. A., Nichols, J., Robertson, E. J., Cooke, A., et al.,** The nonconventional MHC class II molecule DM governs diabetes susceptibility in NOD mice. *PLoS One* 2013. **8**: e56738.

11 **Zou, T., Satake, A., Corbo-Rodgers, E., Schmidt, A. M., Farrar, M. A., Maltzman, J. S. and Kambayashi, T.,** Cutting edge: IL-2 signals determine the degree of TCR signaling necessary to support regulatory T cell proliferation in vivo. *J Immunol* 2012. **189**: 28-32.

12 **Yarkoni, Y., Getahun, A. and Cambier, J. C.,** Molecular underpinning of B-cell anergy. *Immunol Rev* 2010. **237**: 249-263.

13 **Wilson, K. M., Labeta, M. O., Pawelec, G. and Fernandez, N.,** Cell-surface expression of human histocompatibility leucocyte antigen (HLA) class II-associated invariant chain (CD74) does not always correlate with cell-surface expression of HLA class II molecules. *Immunology* 1993. **79**: 331-335.

14 **Wraight, C. J., van Endert, P., Moller, P., Lipp, J., Ling, N. R., MacLennan, I. C., Koch, N., et al.,** Human major histocompatibility complex class II invariant chain is expressed on the cell surface. *J Biol Chem* 1990. **265**: 5787-5792.

15 **Leng, L., Metz, C. N., Fang, Y., Xu, J., Donnelly, S., Baugh, J., Delohery, T., et al.,** MIF signal transduction initiated by binding to CD74. *J Exp Med* 2003. **197**: 1467-1476.

16 **Mitchell, R. A., Liao, H., Chesney, J., Fingerle-Rowson, G., Baugh, J., David, J. and Bucala, R.,** Macrophage migration inhibitory factor (MIF) sustains macrophage proinflammatory function by inhibiting p53: regulatory role in the innate immune response. *Proc Natl Acad Sci U S A* 2002. **99**: 345-350.

17 **Bernhagen, J., Krohn, R., Lue, H., Gregory, J. L., Zernecke, A., Koenen, R. R., Dewor, M., et al.,** MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. *Nat Med* 2007. **13**: 587-596.

18 **Schwartz, V., Lue, H., Kraemer, S., Korbiel, J., Krohn, R., Ohl, K., Bucala, R., et al.,** A functional heteromeric MIF receptor formed by CD74 and CXCR4. *FEBS Lett* 2009. **583**: 2749-2757.

19 **Gore, Y., Starlets, D., Maharshak, N., Becker-Herman, S., Kaneyuki, U., Leng, L., Bucala, R., et al.,** Macrophage migration inhibitory factor induces B cell survival by activation of a CD74-CD44 receptor complex. *J Biol Chem* 2008. **283**: 2784-2792.

20 **Cresswell, P.,** Invariant chain structure and MHC class II function. *Cell* 1996. **84**: 505-507.

21 **Khalil, H., Brunet, A. and Thibodeau, J.,** A three-amino-acid-long HLA-DRbeta cytoplasmic tail is sufficient to overcome ER retention of invariant-chain p35. *J Cell Sci* 2005. **118**: 4679-4687.

22 **Nordeng, T. W., Gregers, T. F., Kongsvik, T. L., Meresse, S., Gorvel, J. P., Jourdan, F., Motta, A., et al.,** The cytoplasmic tail of invariant chain regulates endosome fusion and morphology. *Mol Biol Cell* 2002. **13**: 1846-1856.

23 **Rawlings, D. J., Metzler, G., Wray-Dutra, M. and Jackson, S. W.,** Altered B cell signalling in autoimmunity. *Nat Rev Immunol* 2017. **17**: 421-436.

24 **Jelcic, I., Al Nimer, F., Wang, J., Lentsch, V., Planas, R., Jelcic, I., Madjovski, A., et al.,** Memory B Cells Activate Brain-Homing, Autoreactive CD4(+) T Cells in Multiple Sclerosis. *Cell* 2018. **175**: 85-100 e123.

25 **Koetzier, S. C., van Langelaar, J., Blok, K. M., van den Bosch, T. P. P., Wierenga-Wolf, A. F., Melief, M. J., Pol, K., et al.,** Brain-homing CD4(+) T cells display glucocorticoid-resistant features in MS. *Neurol Neuroimmunol Neuroinflamm* 2020. **7**.

26 **van Langelaar, J., van der Vuurst de Vries, R. M., Janssen, M., Wierenga-Wolf, A. F., Spilt, I. M., Siepman, T. A., Dankers, W., et al.,** T helper 17.1 cells associate with multiple sclerosis disease activity: perspectives for early intervention. *Brain* 2018. **141**: 1334-1349.

27 **Ito, Y., Ashenberg, O., Pyrdol, J., Luoma, A. M., Rozenblatt-Rosen, O., Hofree, M., Christian, E., et al.,** Rapid CLIP dissociation from MHC II promotes an unusual antigen presentation pathway in autoimmunity. *J Exp Med* 2018. **215**: 2617-2635.

28 **Patsopoulos, N. A., Barcellos, L. F., Hintzen, R. Q., Schaefer, C., van Duijn, C. M., Noble, J. A., Raj, T., et al.,** Fine-mapping the genetic association of the major histocompatibility complex in multiple sclerosis: HLA and non-HLA effects. *PLoS Genet* 2013. **9**: e1003926.

29 **Raychaudhuri, S., Sandor, C., Stahl, E. A., Freudenberg, J., Lee, H. S., Jia, X., Alfredsson, L., et al.,** Five amino acids in three HLA proteins explain most of the association between MHC and seropositive rheumatoid arthritis. *Nat Genet* 2012. **44**: 291-296.

30 **Alvaro-Benito, M., Wieczorek, M., Sticht, J., Kipar, C. and Freund, C.,** HLA-DMA polymorphisms differentially affect MHC class II peptide loading. *J Immunol* 2015. **194**: 803-816.

31 **Ristori, G., Carcassi, C., Lai, S., Fiori, P., Cacciani, A., Floris, L., Montesperelli, C., et al.,** HLA-DM polymorphisms do not associate with multiple sclerosis: an association study with analysis of myelin basic protein T cell specificity. *J Neuroimmunol* 1997. **77**: 181-184.

32 **Farh, K. K., Marson, A., Zhu, J., Kleinewietfeld, M., Housley, W. J., Beik, S., Shoresh, N., et al.,** Genetic and epigenetic fine mapping of causal autoimmune disease variants. *Nature* 2015. **518**: 337-343.

33 **Berge, T., Leikfoss, I. S. and Harbo, H. F.,** From Identification to Characterization of the Multiple Sclerosis Susceptibility Gene CLEC16A. *Int J Mol Sci* 2013. **14**: 4476-4497.

34 **van Luijn, M. M., Kreft, K. L., Jongsma, M. L., Mes, S. W., Wierenga-Wolf, A. F., van Meurs, M., Melief, M. J., et al.,** Multiple sclerosis-associated CLEC16A controls HLA class II expression via late endosome biogenesis. *Brain* 2015. **138**: 1531-1547.

35 **Berkowska, M. A., Driessen, G. J., Bikos, V., Grosserichter-Wagener, C., Stamatopoulos, K., Cerutti, A., He, B., et al.,** Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. *Blood* 2011. **118**: 2150-2158.

36 **Kurosaki, T., Kometani, K. and Ise, W.,** Memory B cells. *Nat Rev Immunol* 2015. **15**: 149-159.

37 **Li, J., Jorgensen, S. F., Maggadottir, S. M., Bakay, M., Warnatz, K., Glessner, J., Pandey, R., et al.,** Association of CLEC16A with human common variable immunodeficiency disorder and role in murine B cells. *Nat Commun* 2015. **6**: 6804.

38 **Yuseff, M. I., Pierobon, P., Reversat, A. and Lennon-Dumenil, A. M.,** How B cells capture, process and present antigens: a crucial role for cell polarity. *Nat Rev Immunol* 2013. **13**: 475-486.

39 **Chen, X., Laur, O., Kambayashi, T., Li, S., Bray, R. A., Weber, D. A., Karlsson, L., et al.,** Regulated expression of human histocompatibility leukocyte antigen (HLA)-DO during antigen-dependent and antigen-independent phases of B cell development. *J Exp Med* 2002. **195**: 1053-1062.

40 **van Ham, S. M., Tjin, E. P., Lillemeier, B. F., Gruneberg, U., van Meijgaarden, K. E., Pastoors, L., Verwoerd, D., et al.,** HLA-DO is a negative modulator of HLA-DM-mediated MHC class II peptide loading. *Curr Biol* 1997. **7**: 950-957.

41 **International Multiple Sclerosis Genetics, C., Beecham, A. H., Patsopoulos, N. A., Xifara, D. K., Davis, M. F., Kemppinen, A., Cotsapas, C., et al.,** Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis. *Nat Genet* 2013. **45**: 1353-1360.

42 **International Multiple Sclerosis Genetics, C., Wellcome Trust Case Control, C., Sawcer, S., Hellenthal, G., Pirinen, M., Spencer, C. C., Patsopoulos, N. A., et al.,** Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* 2011. **476**: 214-219.

43 **International Multiple Sclerosis Genetics, C.,** Multiple sclerosis genomic map implicates peripheral immune cells and microglia in susceptibility. *Science* 2019. **365**.

44 **Veselits, M., Tanaka, A., Lipkowitz, S., O'Neill, S., Sciammas, R., Finnegan, A., Zhang, J., et al.,** Recruitment of Cbl-b to B cell antigen receptor couples antigen recognition to Toll-like receptor 9 activation in late endosomes. *PLoS One* 2014. **9**: e89792.

45 **Qiao, G., Lei, M., Li, Z., Sun, Y., Minto, A., Fu, Y. X., Ying, H., et al.,** Negative regulation of CD40-mediated B cell responses by E3 ubiquitin ligase Casitas-B-lineage lymphoma protein-B. *J Immunol* 2007. **179**: 4473-4479.

46 **Steimle, V., Siegrist, C. A., Mottet, A., Lisowska-Grospierre, B. and Mach, B.,** Regulation of MHC class II expression by interferon-gamma mediated by the transactivator gene CIITA. *Science* 1994. **265**: 106-109.

47 **De Gassart, A., Camosseto, V., Thibodeau, J., Ceppi, M., Catalan, N., Pierre, P. and Gatti, E.,** MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH I down-regulation. *Proc Natl Acad Sci U S A* 2008. **105**: 3491-3496.

48 **Matsuki, Y., Ohmura-Hoshino, M., Goto, E., Aoki, M., Mito-Yoshida, M., Uematsu, M., Hasegawa, T., et al.,** Novel regulation of MHC class II function in B cells. *EMBO J* 2007. **26**: 846-854.

49 **Walseng, E., Furuta, K., Bosch, B., Weih, K. A., Matsuki, Y., Bakke, O., Ishido, S., et al.,** Ubiquitination regulates MHC class II-peptide complex retention and degradation in dendritic cells. *Proc Natl Acad Sci U S A* 2010. **107**: 20465-20470.

50 **Cho, K. J., Walseng, E., Ishido, S. and Roche, P. A.,** Ubiquitination by March-I prevents MHC class II recycling and promotes MHC class II turnover in antigen-presenting cells. *Proc Natl Acad Sci U S A* 2015. **112**: 10449-10454.

51 **Baravalle, G., Park, H., McSweeney, M., Ohmura-Hoshino, M., Matsuki, Y., Ishido, S. and Shin, J. S.,** Ubiquitination of CD86 is a key mechanism in regulating antigen presentation by dendritic cells. *J Immunol* 2011. **187**: 2966-2973.

52 **Corcoran, K., Jabbour, M., Bhagwandin, C., Deymier, M. J., Theisen, D. L. and Lybarger, L.,** Ubiquitin-mediated regulation of CD86 protein expression by the ubiquitin ligase membrane-associated RING-CH-1 (MARCH1). *J Biol Chem* 2011. **286**: 37168-37180.

53 **Jahnke, M., Trowsdale, J. and Kelly, A. P.,** Ubiquitination of human leukocyte antigen (HLA)-DM by different membrane-associated RING-CH (MARCH) protein family E3 ligases targets different endocytic pathways. *J Biol Chem* 2012. **287**: 7256-7264.

54 **Arunachalam, B., Phan, U. T., Geuze, H. J. and Cresswell, P.,** Enzymatic reduction of disulfide bonds in lysosomes: characterization of a gamma-interferon-inducible lysosomal thiol reductase (GILT). *Proc Natl Acad Sci U S A* 2000. **97**: 745-750.

55 **Hastings, K. T.,** GILT: Shaping the MHC Class II-Restricted Peptidome and CD4(+) T Cell-Mediated Immunity. *Front Immunol* 2013. **4**: 429.

56 **Hastings, K. T., Lackman, R. L. and Cresswell, P.,** Functional requirements for the lysosomal thiol reductase GILT in MHC class II-restricted antigen processing. *J Immunol* 2006. **177**: 8569-8577.

57 **Reith, W., LeibundGut-Landmann, S. and Waldburger, J. M.,** Regulation of MHC class II gene expression by the class II transactivator. *Nat Rev Immunol* 2005. **5**: 793-806.

58 **Lill, C. M., Luessi, F., Alcina, A., Sokolova, E. A., Ugidos, N., de la Hera, B., Guillot-Noel, L., et al.,** Genome-wide significant association with seven novel multiple sclerosis risk loci. *J Med Genet* 2015. **52**: 848-855.

59 **Schrijver, H. M., Hooper-van Veen, T., van Belzen, M. J., Crusius, J. B., Pena, A. S., Barkhof, F., Polman, C. H., et al.,** Polymorphisms in the genes encoding interferon-gamma and interferon-gamma receptors in multiple sclerosis. *Eur J Immunogenet* 2004. **31**: 133-140.

60 **Arellano, G., Ottum, P. A., Reyes, L. I., Burgos, P. I. and Naves, R.,** Stage-Specific Role of Interferon-Gamma in Experimental Autoimmune Encephalomyelitis and Multiple Sclerosis. *Frontiers in immunology* 2015. **6**: 492-492.

61 **Billiau, A.,** Interferon-γ: Biology and Role in Pathogenesis. In **Dixon, F. J.** (Ed.) *Adv Immunol*. Academic Press 1996, pp 61-130.

62 **Dalpke, A. H., Eckerle, S., Frey, M. and Heeg, K.,** Triggering of Toll-like receptors modulates IFN-γ signaling: involvement of serine 727 STAT1 phosphorylation and suppressors of cytokine signaling. *Eur J Immunol* 2003. **33**: 1776-1787.

63 **Regis, G., Conti, L., Boselli, D. and Novelli, F.,** IFNgammaR2 trafficking tunes IFNgamma-STAT1 signaling in T lymphocytes. *Trends Immunol* 2006. **27**: 96-101.

64 **Bach, E. A., Szabo, S. J., Dighe, A. S., Ashkenazi, A., Aguet, M., Murphy, K. M. and Schreiber, R. D.,** Ligand-induced autoregulation of IFN-gamma receptor beta chain expression in T helper cell subsets. *Science* 1995. **270**: 1215-1218.

65 **Bernabei, P., Coccia, E. M., Rigamonti, L., Bosticardo, M., Forni, G., Pestka, S., Krause, C. D., et al.,** Interferon-gamma receptor 2 expression as the deciding factor in human T, B, and myeloid cell proliferation or death. *J Leukoc Biol* 2001. **70**: 950-960.

66 **Rigamonti, L., Ariotti, S., Losana, G., Gradini, R., Russo, M. A., Jouanguy, E., Casanova, J. L., et al.,** Surface expression of the IFN-gamma R2 chain is regulated by intracellular trafficking in human T lymphocytes. *J Immunol* 2000. **164**: 201-207.

67 **Bar-Or, A., Fawaz, L., Fan, B., Darlington, P. J., Rieger, A., Ghorayeb, C., Calabresi, P. A., et al.,** Abnormal B-cell cytokine responses a trigger of T-cell-mediated disease in MS? *Ann Neurol* 2010. **67**: 452-461.

68 **Jackson, S. W., Jacobs, H. M., Arkatkar, T., Dam, E. M., Scharping, N. E., Kolhatkar, N. S., Hou, B., et al.,** B cell IFN-gamma receptor signaling promotes autoimmune germinal centers via cell-intrinsic induction of BCL-6. *J Exp Med* 2016. **213**: 733-750.

69 **Piovesan, D., Tempany, J., Di Pietro, A., Baas, I., Yiannis, C., O'Donnell, K., Chen, Y., et al.,** c-Myb Regulates the T-Bet-Dependent Differentiation Program in B Cells to Coordinate Antibody Responses. *Cell Reports*. **19**: 461-470.

70 **Peng, S. L., Szabo, S. J. and Glimcher, L. H.,** T-bet regulates IgG class switching and pathogenic autoantibody production. *Proc Natl Acad Sci U S A* 2002. **99**: 5545-5550.

71 **van Langelaar, J., Rijvers, L., Janssen, M., Wierenga-Wolf, A. F., Melief, M.-J., Siepman, T. A., de Vries, H. E., et al.,** Induction of brain-infiltrating T-bet–expressing B cells in multiple sclerosis. *Ann Neurol* 2019. **86**: 264-278.

72 **Bleul, C. C., Schultze, J. L. and Springer, T. A.,** B lymphocyte chemotaxis regulated in association with microanatomic localization, differentiation state, and B cell receptor engagement. *J Exp Med* 1998. **187**: 753-762.

73 **Hargreaves, D. C., Hyman, P. L., Lu, T. T., Ngo, V. N., Bidgol, A., Suzuki, G., Zou, Y. R., et al.,** A coordinated change in chemokine responsiveness guides plasma cell movements. *J Exp Med* 2001. **194**: 45-56.

74 **Jegerlehner, A., Maurer, P., Bessa, J., Hinton, H. J., Kopf, M. and Bachmann, M. F.,** TLR9 Signaling in B Cells Determines Class Switch Recombination to IgG2a. *J Immunol* 2007. **178**: 2415-2420.

75 **Rubtsova, K., Rubtsov, A. V., Thurman, J. M., Mennona, J. M., Kappler, J. W. and Marrack, P.,** B cells expressing the transcription factor T-bet drive lupus-like autoimmunity. *J Clin Invest* 2017. **127**: 1392-1404.

76 **Krumbholz, M., Theil, D., Cepok, S., Hemmer, B., Kivisakk, P., Ransohoff, R. M., Hofbauer, M., et al.,** Chemokines in multiple sclerosis: CXCL12 and CXCL13 up-regulation is differentially linked to CNS immune cell recruitment. *Brain* 2006. **129**: 200-211.

77 **Mahad, D. J., Howell, S. J. and Woodroofe, M. N.,** Expression of chemokines in the CSF and correlation with clinical disease activity in patients with multiple sclerosis. *J Neurol Neurosurg Psychiatry* 2002. **72**: 498-502.

78 **Sørensen, T. L., Trebst, C., Kivisäkk, P., Klaege, K. L., Majmudar, A., Ravid, R., Lassmann, H., et al.,** Multiple sclerosis: a study of CXCL10 and CXCR3 co-localization in the inflamed central nervous system. *J Neuroimmunol* 2002. **127**: 59-68.

79 **Allen, C. D., Ansel, K. M., Low, C., Lesley, R., Tamamura, H., Fujii, N. and Cyster, J. G.,** Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol* 2004. **5**: 943-952.

80 **Victora, G. D., Dominguez-Sola, D., Holmes, A. B., Deroubaix, S., Dalla-Favera, R. and Nussenzweig, M. C.,** Identification of human germinal center light and dark zone cells and their relationship to human B-cell lymphomas. *Blood* 2012. **120**: 2240-2248.

81 **Reimer, D., Lee, A. Y. S., Bannan, J., Fromm, P., Kara, E. E., Comerford, I., McColl, S., et al.,** Early CCR6 expression on B cells modulates germinal centre kinetics and efficient antibody responses. *Immunol Cell Biol* 2017. **95**: 33-41.

82 **Konforte, D., Simard, N. and Paige, C. J.,** IL-21: An Executor of B Cell Fate. *J Immunol* 2009. **182**: 1781-1787.

83 **Bannard, O., McGowan, S. J., Ersching, J., Ishido, S., Victora, G. D., Shin, J. S. and Cyster, J. G.,** Ubiquitin-mediated fluctuations in MHC class II facilitate efficient germinal center B cell responses. *J Exp Med* 2016. **213**: 993-1009.

84 **Wu, B. X., Zhao, L. D. and Zhang, X.,** CXCR4 and CXCR5 orchestrate dynamic germinal center reactions and may contribute to the pathogenesis of systemic lupus erythematosus. *Cell Mol Immunol* 2019. **16**: 724-726.

85 **Rathmell, J. C., Cooke, M. P., Ho, W. Y., Grein, J., Townsend, S. E., Davis, M. M. and Goodnow, C. C.,** CD95 (Fas)-dependent elimination of self-reactive B cells upon interaction with CD4+ T cells. *Nature* 1995. **376**: 181-184.

86 **Akkaya, M., Kwak, K. and Pierce, S. K.,** B cell memory: building two walls of protection against pathogens. *Nat Rev Immunol* 2020. **20**: 229-238.

87 **Serafini, B., Rosicarelli, B., Magliozzi, R., Stigliano, E. and Aloisi, F.,** Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. *Brain Pathol* 2004. **14**: 164-174.

88 **Jared, H. R., James, M. E., Lijun, X. and Sing Sing, W.,** Regulatory T cells and the immune pathogenesis of prenatal infection. *Reproduction* 2013. **146**: R191-R203.

89 **Confavreux, C., Hutchinson, M., Hours, M. M., Cortinovis-Tourniaire, P. and Moreau, T.,** Rate of Pregnancy-Related Relapse in Multiple Sclerosis. *N Engl J Med* 1998. **339**: 285-291.

90 **Airas, L., Saraste, M., Rinta, S., Elovaara, I., Huang, Y. H., Wiendl, H., Finnish Multiple, S., et al.,** Immunoregulatory factors in multiple sclerosis patients during and after pregnancy: relevance of natural killer cells. *Clin Exp Immunol* 2008. **151**: 235-243.

91 **van Langelaar, J., Wierenga-Wolf, A. F., Samijn, J. P. A., Luijks, C. J. M., Siepman, T. A., van Doorn, P. A., Bell, A., et al.,** The association of Epstein-Barr virus infection with CXCR3(+) B-cell development in multiple sclerosis impact of immunotherapies. *Eur J Immunol* 2020.

92 **Pollok, K., Mothes, R., Ulbricht, C., Liebheit, A., Gerken, J. D., Uhlmann, S., Paul, F., et al.,** The chronically inflamed central nervous system provides niches for long-lived plasma cells. *Acta Neuropathol Commun* 2017. **5**: 88.

93 **Hollenbach, J. A. and Oksenberg, J. R.,** The immunogenetics of multiple sclerosis: A comprehensive review. *J Autoimmun* 2015. **64**: 13-25.

94 **Smith, K. J., Pyrdol, J., Gauthier, L., Wiley, D. C. and Wucherpfennig, K. W.,** Crystal structure of HLA-DR2 (DRA*0101, DRB1*1501) complexed with a peptide from human myelin basic protein. *J Exp Med* 1998. **188**: 1511-1520.

95 **Montalban, X., Hauser, S. L., Kappos, L., Arnold, D. L., Bar-Or, A., Comi, G., de Seze, J., et al.,** Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis. *N Engl J Med* 2017. **376**: 209-220.

96 **Schwartz, D. M., Bonelli, M., Gadina, M. and O'Shea, J. J.,** Type I/II cytokines, JAKs, and new strategies for treating autoimmune diseases. *Nat Rev Rheumatol* 2016. **12**: 25-36.

97 **Schwartz, D. M., Kanno, Y., Villarino, A., Ward, M., Gadina, M. and O'Shea, J. J.,** JAK inhibition as a therapeutic strategy for immune and inflammatory diseases. *Nat Rev Drug Discov* 2017. **17**: 78.

98 **Capolunghi, F., Rosado, M. M., Cascioli, S., Girolami, E., Bordasco, S., Vivarelli, M., Ruggiero, B., et al.,** Pharmacological inhibition of TLR9 activation blocks autoantibody production in human B cells from SLE patients. *Rheumatology (Oxford)* 2010. **49**: 2281-2289.

99 **Montalban, X., Arnold, D. L., Weber, M. S., Staikov, I., Piasecka-Stryczynska, K., Willmer, J., Martin, E. C., et al.,** Placebo-Controlled Trial of an Oral BTK Inhibitor in Multiple Sclerosis. *N Engl J Med* 2019. **380**: 2406-2417.

Addendum

LIST OF ABBREVIATIONS

Ag	Antigen
ALCAM	Activated leukocyte cell adhesion molecule
AP	Activator protein
APC	Antigen-presenting cell
BBB	Blood-brain barrier
BCL6	B-cell lymphoma 6
BCR	B-cell receptor
BEC	Brain endothelial cell
B-LCL	B-lymphoblastoid cell line
BSA	Bovine serum albumin
BTK	Bruton's tyrosine kinase
CBLB	Casitas B-lineage lymphoma proto-oncogene b
CCR	C-C motif chemokine receptor
CD	Cluster of differentiation
CDMS	Clinically definite MS
cDNA	Complementary DNA (deoxyribonucleic acid)
CIITA	Class II major histocompatibility complex transactivator
CIS	Clinically isolated syndrome
CLEC16A	C-type lectin domain containing 16A
CLIP	class-II associated invariant chain peptides
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTL	Cytotoxic T lymphocyte
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
EDSS	Expanded disability status scale
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FOXP3	Forkhead box P3
FSC	Forward scatter

GC	Germinal center
GFP	Green fluorescent protein
GILT	Gamma-interferon-inducible lysosomal thiol reductase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association study
HC	Healthy control
HLA	Human leukocyte antigen
IFI30	Interferon-gamma-inducible protein 30
IFNGR	Interferon-gamma receptor
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IRF1	Interferon regulatory factor 1
JAK	Janus kinase
Li	Invariant chain (CD74)
LMP	Late membrane protein
LPS	Lipopolysaccharides
LTα	Lymphotoxin-alpha
MARCH1	Membrane associated ring-CH-Type Finger 1
MBP	Myelin basic protein
MEC	Medical ethics committee
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MIIC	MHC class II peptide-loading compartment
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Multiple sclerosis
MZ	Marginal zone
NF-κB	Nuclear factor-kappa B
NM	Naive mature
OCB	Oligoclonal band
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PPMS	Primary progressive MS
qPCR	quantitative PCR
RA	Rheumatoid arthritis

RNA	Ribonucleic acid
RRMS	Relapsing-remitting MS
RT	Room temperature
SD	Standard deviation
SEM	Standard error of the mean
SHM	Somatic hypermutations
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SPMS	Secondary progressive MS
SPPL2A	Signal peptide peptidase-like 2A
SSC	Side scatter
STAT	Signal transducer and activator of transcription
SYK	Spleen tyrosine kinase
T-bet	T-box transcription factor (TBX21)
TCR	T-cell receptor
TD	T-cell dependent
T_{FH}	T follicular helper cell
T_{H}	T helper cell
TI	T-cell independent
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
T_{REG}	Regulatory T cell
TYK2	Tyrosine kinase 2
VCAM	Vascular cell adhesion protein 1
VDRE	Vitamin D response element
VLA-4	Very late antigen-4 (Integrin α4β1)

SUMMARY

Multiple sclerosis (MS) is a chronic immune-mediated disease of the central nervous system (CNS). Various pro-inflammatory immune cells mediate local pathology. CD20 depletion therapies and genome-wide association studies (GWAS) have shown that the contribution of B cells to the pathogenesis of MS is indisputable. The strong and rapid beneficial clinical effects of anti-CD20 treatment in MS patients has revealed that B cells serve as antigen-presenting rather than antibody-producing cells in the periphery. Although pro-inflammatory T_H-cell responses are significantly reduced in these patients, the exact underlying antibody-independent functions of B cells in MS are poorly understood. B cells activate T_H cells through the presentation of antigens, expression of costimulatory molecules and secretion of cytokines. In this thesis, we aimed to uncover genes and pathways that are functionally altered in human B cells and potentially contribute to MS. To study this, we made use of gene silencing and functional assays related to B- and T_H-cell interaction and took advantage of recent GWAS data, different MS cohorts and the effects of immunosuppressive conditions and treatment on disease activity.

The identification of functional B-cell subsets that can preferentially migrate into the CNS and contribute to local inflammation is of considerable interest. In **chapter 2**, we studied the triggers, development and CNS infiltration capacity of human B cells in the context of MS. We found that CXCR3-expressing B cells are selectively enriched in the CNS of MS patients, while being decreased in the periphery. Treatment of MS patients with natalizumab, a drug that blocks lymphocyte recruitment to the CNS, resulted in selective accumulation of $CXCR3^+IgG1^+$ B cells in the periphery. Furthermore, we demonstrated that these $CXCR3^+IgG1^+$ B cells have an enhanced capacity to transmigrate across the blood-brain barrier *in vitro*. Finally, we showed that integrating interferon gamma (IFN-γ) and pathogen-associated Toll-like receptor 9 (TLR9) signals are critically involved in the development of these brain-homing B cells in MS.

HLA class II (HLA-II) is the major genetic risk factor for MS development, however little is known about how B cell-intrinsic HLA-II expression is regulated during MS disease onset. In **chapter 3**, we studied the impact of a minor MS- and autoimmunity-associated risk allele, *CLEC16A,* on HLA-II antigen presentation pathway in B cells. We demonstrated that *CLEC16A* contributes to this tightly coordinated pathway in B cells by influencing both B-cell receptor-mediated antigen uptake and the biogenesis of HLA-II peptide-loading compartments. In primary B cells, *CLEC16A* was only induced under class-II associated chain peptides (CLIP)-stimulating conditions *in vitro* and was predominantly expressed in $CLIP^{high}$ naive populations. Consistently, *CLEC16A* levels associated with the presentation of CLIP-loaded HLA-II molecules by human B-cell lines and primary B cells. This association

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was not found in B cells from the blood of early MS patients, showing abundant expression of surface CLIP and impaired co-regulation with *CLEC16A*.

In **chapter 4**, we assessed a pro-inflammatory cytokine that is functionally associated with the precursor of CLIP (CD74) termed the macrophage migration inhibitory factor or MIF. We demonstrated that MIF and MIF receptor CD74 were downregulated, while MIF receptor CXCR4 was upregulated on B cells from early onset MS patients. This was shown for CIS patients who will rapidly develop MS as well as for clinically definite MS patients. *In vitro* experiments supported the inverse regulation of MIF/CD74 and CXCR4 expression in B cells *ex vivo*. Functionally, MIF signalling via CD74 controlled their pro-inflammatory capacity and proliferation, while signalling via CXCR4 regulated their sensitivity to Fas-mediated apoptosis. The observed $CXCR4^{high}CD74^{low}$ B-cell phenotype in early MS blood points to the presence of more immature B cell populations with senescent features. This may contribute to the increased ability of naive B cells to survive peripheral tolerance checkpoints in MS.

Pregnancy is a natural modifier of disease activity in MS patients, but the underlying mechanisms remain elusive. In **chapter 5**, we studied how peripheral B-cell differentiation is regulated during pregnancy-associated low and high relapse risk periods in patients with MS. Reduced proportions of transitional B cells were seen from the first to the third trimester, which significantly increased again in the early postpartum period. Although frequencies were modestly reduced, memory B cells showed increased immunoglobulin (Ig) expression and a more germinal center (GC) light zone-related chemokine receptor expression profile ($CXCR4^{low}CXCR5^{high}CCR6^{high}CXCR3^{high}$) in postpartum versus paired third trimester samples. This profile implied an increased potential of these B cells to interact with T_{FH} cells that are located within GC light zones. Because such memory B cells are prone to infiltrate the CNS, we assessed whether this phenotype may also be related to their capacity to further differentiate into antibody-secreting cells. Indeed, under T_{FH}-like *in vitro* conditions, especially postpartum memory B cells of MS patients were highly capable of differentiating into Ig-secreting ($CD138^{+}$) plasma cells.

In **chapter 6**, we discussed where and how the interaction between B and T cells are expected to play a role in MS. During B-cell development, peripheral B cells escape from tolerance checkpoints, possibly as the result of impaired control by chronically exhausted or genetically altered regulatory T cells. Subsequently, B cells interact with IFN-γ-producing effector T_H cells in germinal centers of lymphoid organs to create a feedforward loop, after which highly pathogenic subsets break through blood-CNS barriers and are locally reactivated to cause MS pathology. Although definite proof is still lacking, these pathogenic events are likely mediated by an interplay between persistent infections such as EBV and genetic risk variants. Together, these factors may alter the selection, differentiation and pathogenic features of B- and T-cell subsets.

We conclude this thesis with **chapter 7**, in which we further elaborated on specific alterations in functional B-cell pathways in MS. This involves altered B-cell antigen processing and presentation via HLA-II molecules, T cell-derived IFN-γ and organization within GCs to trigger pathogenic B cells as well as their capacity to infiltrate and further mature in the CNS. We also proposed future studies such as the use of CRISPR/Cas9 technology to edit and study the effect of one single nucleotide change as well as weighted genetic risk scores to assess the overall impact on B cells. Moreover, we discussed the design of more targeted therapy that inhibit the development or function of pathogenic B cells, while leaving other subsets unharmed. In this thesis, we have shown that CXCR3^{+} (T-bet^{+}) memory B cells are ideal candidates for such targeting, being potent antigen-presenting cells and prone to infiltrate the CNS in MS patients.

SAMENVATTING

Multipele sclerose (MS) is een chronische immuun-gemedieerde ziekte van het centraal zenuwstelsel (CZS). Verschillende pro-inflammatoire immuun cellen mediëren lokale pathologie. CD20 depletie therapieën en genoom-brede associatiestudies (GWAS) hebben aangetoond dat de bijdrage van B cellen aan de pathogenese van MS onbetwistbaar zijn. De sterke en snelle effecten van anti-CD20 behandeling in MS patiënten heeft laten zien dat B cellen fungeren als antigeen-presenterende in plaats van antilichaam-producerende cellen in de periferie. Hoewel de pro-inflammatoire T_H-cel reacties significant verminderd zijn in deze patiënten, zijn de exacte onderliggende antilichaam-onafhankelijke functies van B cellen in MS niet goed begrepen. B cellen kunnen T_H cellen activeren door middel van antigeen presentatie, expressie van co-stimulerende moleculen en uitscheiding van cytokines. Onze doelstelling van dit proefschrift was om genen en signaalroutes die functioneel veranderd zijn in humane B cellen en die mogelijk bijdrage aan MS te ontdekken. Om dit te onderzoeken hebben de expressie van genen uitgeschakeld en andere functionele proeven gerelateerd aan de functie van B- en T_H cel interacties uitgevoerd. Hierbij hebben we gebruik gemaakt van recente GWAS data, verschillende MS cohorten, de effecten van immunosuppressieve condities en behandelingen van ziekteactiviteit.

De identificatie van de functionele B-cel subgroep die preferentieel migreert naar het CSZ en bijdraagt aan de lokale ontsteking is van aanzienlijk belang. In **hoofdstuk 2** hebben we de impulsen, ontwikkeling en CZS infiltratie capaciteit van humane B cellen in de context van MS onderzocht. Hier hebben we gevonden dat B cellen die CXCR3 tot expressie brengen selectief verrijkt zijn in het CZS van MS patiënten, terwijl deze verminderd zijn in de periferie. Behandeling van MS patiënten met natalizumab, een medicijn dat de migratie van lymfocyten naar het CZS remt, resulteerde in selectieve ophoping van $CXCR3^+IgG1^+$ B cellen in de periferie. Bovendien hebben we aangetoond dat deze $CXCR3^+IgG1^+$ B cellen een verhoogde capaciteit hebben om te migreren over de bloed-brein barrière *in vitro*. Tot slot laten we zien dat de integratie van de interferon gamma (IFN-γ) en pathogeen-geassocieerde Toll-like receptor 9 (TLR9) signalen een cruciale rol spelen in de ontwikkeling van deze brein-geassocieerde B cellen in MS.

HLA klasse II (HLA-II) is de grootse genetische risicofactor voor MS ontwikkeling, echter is er maar weinig bekend over hoe B-cel intrinsieke HLA-II expressie gereguleerd wordt tijdens MS ziekte ontwikkeling. In **hoofdstuk 3** hebben we onderzoek gedaan naar de impact van een minor MS- en auto-immuun-geassocieerde risico allel, *CLEC16A*, op de HLA-II antigeen presentatie route in B cellen. We hebben aangetoond dat *CLEC16A* bijdraagt aan deze strikt gecoördineerde route in B cellen door zowel B-cel receptor-gemedieerde antigeen opname en de biogenese van HLA-II peptide-beladingscompartimenten te beïnvloeden. In primaire B cellen werd *CLEC16A* alleen geïnduceerd onder klasse-II-geassocieerde keten

peptiden (CLIP)-stimulerende condities *in vitro* en kwam voornamelijk tot expressie in CLIP^{hoge} naïeve populaties. Daarnaast associeerden de *CLEC16A* expressie niveaus met de presentatie van CLIP-geladen HLA-II moleculen door humane B cellijnen en primaire B cellen. Deze associatie werd niet gevonden met B cellen in het bloed van vroege MS patiënten, waarin CLIP hoog tot expressie komt en er dus een verstoorde co-regulatie is met *CLEC16A*.

In **hoofdstuk 4** hebben we een pro-inflammatoire cytokine onderzocht die functioneel geassocieerd is met de voorloper van CLIP (CD74) genaamd de macrofaag migratie inhibitie factor of in het kort MIF. We hebben aangetoond dat MIF en zijn receptor CD74 verminderd tot expressie kwam, terwijl een andere MIF receptor CXCR4 verhoogd tot expressie kwam op B cellen van patiënten met een vroege ontwikkeling naar MS. We hebben dit laten zien voor zowel klinisch geïsoleerd syndroom (CIS) patiënten die in de toekomst snel MS ontwikkelde, als voor klinisch definitief MS (CDMS) patiënten. *In vitro* experimenten ondersteunde de omgekeerde regulatie van MIF/CD74 en CXCR4 expressie in B cellen *ex vivo*. Functioneel hebben we laten zien dat MIF signalering via CD74 de pro-inflammatoire capaciteit en proliferatie van B cellen reguleerde, terwijl de signalering via CXCR4 de sensitiviteit voor Fas-gemedieerde apoptose reguleerde. De geobserveerde CXCR4^{hoog}CD74^{laag} B cel fenotype in het bloed van vroege MS patiënten wijst naar de aanwezigheid van een meer immature B cel populatie met senescente kenmerken. Dit draagt mogelijk bij aan het verhoogde vermogen van naïeve B cellen om perifere tolerantie controlepunten te overleven in MS.

Zwangerschap veranderd ziekteactiviteit in MS patiënten op een natuurlijke manier, maar het onderliggende mechanisme hiervan blijft ongrijpbaar. In **hoofdstuk 5** hebben we onderzocht hoe perifere B-cel differentiatie gereguleerd wordt gedurende zwangerschap-geassocieerde lage en hoge relapse risicoperiodes in patiënten met MS. We vonden verlaagde frequenties van transitionele B cellen in de derde trimester versus de eerste trimester van de zwangerschap, welke weer significant verhoogde in de vroege postpartum fase. Hoewel de proporties van geheugen B cellen gematigd verlaagd waren, was de immunoglobuline expressie significant verhoogd. Verder vertoonde deze geheugen B cellen een meer kiemcenter ('germinal center'; GC) lichte zone-gerelateerd chemokine receptor expressie profiel (CXCR4^{laag}CXCR5^{hoog}CCR6^{hoog}CXCR3^{hoog}) in postpartum versus derde trimester samples. Dit profiel impliceerde een verhoogde potentie van deze B cellen om in aanraking te komen met T_{FH} cellen welke gevestigd zijn binnen de lichte zones van een GC. Omdat dergelijke geheugen B cellen geneigd zijn om het CZS te infiltreren, onderzochten we of dit fenotype wellicht ook gerelateerd was aan de capaciteit om verder te differentiëren in antilichaam-afscheidende (plasma) cellen. Hier vonden we inderdaad dat onder T_{FH}-lijkende *in vitro* condities, voornamelijk postpartum geheugen B cellen van MS patiënten zeer capabel waren om te differentiëren naar (CD138^{+}) plasmacellen.

In **hoofdstuk 6** hebben we besproken waar en hoe de interactie tussen B en T cellen mogelijk een rol speelt in MS. Tijdens de ontwikkeling van een B-cel ontsnappen perifere B cellen aan tolerantie controlepunten, wat mogelijk een gevolg is van verminderde controle door chronisch vermoeide of genetisch veranderde regulatoire T cellen. Vervolgens kunnen deze B cellen een interactie aangaan met IFN-γ-producerende T_H cellen in GCs van lymfatische organen om een voorwaartskoppeling te maken, waarbij zeer pathogene subgroepen door de bloed-CZS barrière kunnen breken en lokaal gereactiveerd worden om MS pathologie te veroorzaken. Hoewel dit voor een deel nog hypothetisch is, denken wij dat deze pathogene gebeurtenissen mogelijk gemedieerd worden door een wisselwerking van aanhoudende infecties zoals EBV en genetische risico variaties. Samen veranderen deze factoren mogelijk de selectie, differentiatie en pathogene kenmerken van B- en T-cel subgroepen in MS patiënten.

We eindigen dit proefschrift met **hoofdstuk 7**, waarin we verder ingaan op specifieke veranderingen in functionele B-cel signaleringsroutes in MS. Dit betreft gewijzigde B-cel antigeen verwerking en presentatie via HLA-II moleculen, de rol van T-cel-afkomstige IFN-γ, de organisatie binnen GCs welke pathogene B cellen activeren en hun capaciteit om het CZS te infiltreren en daar lokaal verder te differentiëren. Ook bespreken we mogelijke toekomstige studies zoals het gebruiken van CRISPR/Cas9 technologie om een enkele nucleotide te veranderen en te onderzoeken evenals het gebruiken van 'afgewogen' (weighted) genetische risico scores om de totale impact op B cellen te onderzoeken. Daarnaast bespreken we de ontwikkeling van meer doelgerichte therapieën welke de ontwikkeling of functie van pathogene B cellen kan remmen, terwijl andere subgroepen onaangetast blijven. In dit proefschrift hebben we aangetoond dat $CXCR3^+$ ($T\text{-}bet^+$) geheugen B cellen ideale kandidaten zijn voor desbetreffende doelgerichte therapieën, aangezien dit effectieve antigeen-presenterende cellen zijn welke een verhoogde capaciteit hebben om te migreren naar het CZS in MS patiënten.

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CURRICULUM VITAE

Liza Rijvers was born on June 4, 1991 in Breda, the Netherlands. In 2012 she obtained the Bachelor of Applied Science in Biomedical Laboratory Research and Forensic Laboratory Research at Avans University in Breda. In 2014 she obtained her Master of Science in Molecular Medicine at Erasmus University in Rotterdam. She started her PhD in October 2014 at the Department of Immunology at Erasmus Medical Center, under supervision of Dr. Marvin M. van Luijn and Prof. Dr. Rogier Q. Hintzen. Unfortunately Rogier passed away, in which Prof. Dr. Peter D. Katsiskis and Prof. Dr. Peter A.E. Sillevis Smitt took over his supervision. In November 2020 she started her training as resident clinical chemistry and currently works at the Gelre Hospital in Apeldoorn (under supervision of Dr. Jeroen van Suijlen).

Liza Rijvers is geboren op 4 juni 1991 in Breda, Nederland. In 2012 behaalde zij haar Bachelor of Applied Science in Biomedisch Laboratorium Onderzoek en Forensisch Laboratorium Onderzoek aan Avans hogeschool te Breda. In 2014 behaalde zij haar Master of Science in Moleculaire Geneeskunde aan Erasmus Universiteit te Rotterdam. Hierna begon zij als PhD student op de afdeling Immunologie van het Erasmus Medisch Centrum te Rotterdam, onder de supervisie van Dr. Marvin M. van Luijn en Prof. Dr. Rogier Q. Hintzen. Na het overlijden van Rogier namen Prof. Dr. Peter D. Katsiskis en Prof. Dr. Peter A.E. Sillevis Smitt zijn supervisie over. In november 2020 is zij begonnen als AIOS klinische chemie en momenteel werkzaam in Gelre ziekenhuizen te Apeldoorn (onder de supervisie van Dr. Jeroen van Suijlen).

Werkervaring

2020 - heden **Gelre ziekenhuizen, afdeling Klinische Chemie en Hematologie**
AIOS klinische chemie
Dr. Jeroen van Suijlen & Dr. Janine van Hulstein

2018 - 2020 **Erasmus MC, afdeling Immunologie**
Post-doc Neuro-Immunology Brain groep
Dr. Marvin M. van Luijn

2014 - 2020 **Erasmus MC, afdeling Immunologie**
PhD student Neuro-Immunology Brain groep
Dr. Marvin M. van Luijn & Prof. Dr. Rogier Q Hintzen †

Stage

2013-2014 **Erasmus MC, afdeling Immunologie**
Afstudeerstage MSc Molecular Medicine
Dr. Jorn J. Heeringa & Dr. Menno C. van Zelm

2012-2013 **Erasmus MC, afdeling Vrouwenziektes en gynaecologie**
Stage MSc Molecular Medicine
Dr. Marten van der Zee & Dr. Leen Blok

2011-2012 **VUMC, afdeling Cardiovasculaire pathologie**
Afstudeerstage BaSc Biomedisch laboratorium onderzoek
Dr. Mark P.V. Begieneman & Prof. Dr. Hans W.M. Niessen

Opleiding

2020 – heden **Gelre ziekenhuizen, Apeldoorn**
AIOS klinische chemie

2014- heden **SMBWO, Utrecht**
Immunoloog

2014-2020 **Erasmus MC, Rotterdam**
Postgraduate school MolMed

2012-2014 **Erasmus MC, Rotterdam**
Master of Science, Molecular Medicine

2009-2012 **Avans Hogeschool, Breda**
Bachelor of Applied Science, Biomedisch laboratorium onderzoek
Forensisch laboratorium onderzoek

2003-2009 **Markenhage college, Breda**
VWO (Atheneum)

PHD PORTFOLIO

Summary of PhD training and teaching

L. Rijvers

Dept. of Immunology

Research school:	Postgraduate Molecular Medicine
PhD period:	October 2014 – December 2020
Promotor:	Prof. Dr. P.D. Katsikis
Promotor:	Prof. Dr. P.A.E. Sillevis Smitt
Copromotor:	Dr. M.M. van Luijn

PhD training

In Depth Courses

2013	Molmed Course
2014	Research integrity
2014	BD flow cytometry course basic and advanced
2014	Basic genetics
2014	In vivo imaging (OJC)
2015	Advanced immunology
2015	Introduction into data-analysis (NIHES)
2015	SPSS
2016	Presenting Skills for junior researcher
2016	Biomedical English writing course
2017	Biobusiness summerschool (outside EMC)

Seminars and Workshops

2014-2018	Journal club at the department of Immunology
2014-2020	Seminars and Minisymposia at the department of Immunology

Presentations (oral)

2016	International conference on autoimmunity, Leipzig, Germany
2016, 2017	Annual multiple sclerosis research days, Stichting MS research, The Netherlands
2016, 2017	B cell research meeting, Erasmus MC, The Netherlands
2016, 2017, 2018	Department meeting, Erasmus MC, The Netherlands
2017	Annual Meeting/Winter school of the Dutch Society of Immunology (NVVI), Noordwijkerhout, The Netherlands

2018	5th European Congress of Immunology (ECI), Amsterdam, The Netherlands

(Inter)national conferences

2014, 2015, 2017	Annual Meeting/Winter school of the Dutch Society of Immunology (NVVI), Noordwijkerhout, The Netherlands
2015 – 2018	Annual Molmed day, Rotterdam, The Netherlands
2015 – 2018	Annual multiple sclerosis research days, Stichting MS research, The Netherlands
2016	International conference on autoimmunity, Leipzig, Germany
2016	NVVI (Dutch Society of Immunology) and BSI (Britisch Society of Immunology joint meeting, Liverpool, UK
2017	EMBO meeting Antigen processing and presentation, Salamanca, Spain
2017	EMBO meeting; To-B or not to-B: B cells in health and disease, Girona, Spain
2018	5th European Congress of Immunology (ECI) Amsterdam,The Netherlands

Grants and awards

2016	NVVI (Dutch Society of Immunology), travel grant for NVVI and BSI joint meeting, Liverpool, UK
2016	Erasmus Trustfonds travel grant for Congress of autoimmunity, Leipzig, Germany
2017	Erasmus Trustfonds travel grant for EMBO meeting; To-B or not to-B: B cells in health and disease, Girona, Spain
2017	EMBO travel grant for EMBO meeting; Antigen processing and presentation, Salamanca, Spain
2018	Poster award of Postgraduate school Molecular Medicine (Molmed), Rotterdam, The Netherlands
2018	ECI Travel grant for the ECI meeting, Amsterdam, The Netherlands

Teaching

2014-2018	Supervising 1st and 2nd year Medical students' practicum 'Immunology'
2015-2016	Supervising MSc internship 'Master Science Technology and Health' (France)

2017	Supervising MSc internship 'Master Infection and Immunity student' (EMC)
2017-2018	Supervising MSc internship 'Master Neuroscience and Cognition' (UMC Utrecht)
2017	Co-supervising BSc internship 'HLO-student'
2017	Supervising MSc literature study 'Master Infection and Immunity student' (UMC Utrecht)

LIST OF PUBLICATIONS

Publications within thesis

Malou Janssen* and **Liza Rijvers***, Steven C. Koetzier, Annet F. Wierenga-Wolf, Marie-José Melief, Jamie van Langelaar, Tessel F. Runia, Rinze Neuteboom, Joost Smolders, and Marvin M. van Luijn
Pregnancy-induced effects on memory B-cell development in multiple sclerosis
Manuscript submitted 2020

Liza Rijvers, Marie-José Melief, Jamie van Langelaar, Roos M. van der Vuurst de Vries, Annet F. Wierenga-Wolf, Steven C. Koetzier, John J. Priatel, Tineke Jorritsma, S. Marieke van Ham, Rogier Q. Hintzen, and Marvin M. van Luijn
The role of autoimmunity associated gene CLEC16A in the B cell receptor-mediated HLA-II pathway
Journal of Immunology 2020. July;205: 945-956

Jamie van Langelaar, **Liza Rijvers**, Joost Smolders, and Marvin M. van Luijn
B and T cells driving multiple sclerosis: identity, mechanisms and potential triggers
Frontiers in Immunology 2020. May;11:760

Jamie van Langelaar* and **Liza Rijvers***, Malou Janssen, Annet F. Wierenga-Wolf, Marie-José Melief, Theodora A. Siepman, MD, Helga E. de Vries, Peter-Paul A. Unger, S. Marieke van Ham, Rogier Q. Hintzen, and Marvin M. van Luijn
Induction of Brain-Infiltrating T-bet–Expressing B Cells in Multiple Sclerosis
Annals of Neurology 2019. Aug;86(2):264-278

Liza Rijvers, Marie-José Melief, Roos M. van der Vuurst de Vries, Maeva Stéphant, Jamie van Langelaar, Annet F. Wierenga-Wolf, Jeanet M. Hogervorst, Anneke J. Geurts-Moespot, Fred C. G. J. Sweep, Rogier Q. Hintzen, and Marvin M. van Luijn
The macrophage migration inhibitory factor pathway in human B cells: tight control and dysregulation in multiple sclerosis
European Journal of Immunology 2018. Nov;48: 1861-1871

Other publications

Malou Janssen, Arlette L Bruijstens, Jamie van Langelaar, YuYi Wong, Annet F. Wierenga-Wolf, Marie-José Melief, **Liza Rijvers**, E. Daniëlle van Pelt, Joost Smolders, Beatrijs H. Wokke, and Marvin M. van Luijn
Naive B cells in neuromyelitis optica spectrum disorders: impact of steroid use and relapses
Brain Communications. 2020; Nov;2(2):fcaa197

Jorn J. Heeringa, **Liza Rijvers**, Nicolette J. Arends, Gertjan J. Driessen, Sandra G. Pasmans, Jacques J. M. van Dongen, Johan C. de Jongste, and Menno C. van Zelm
IgE-expressing memory B cells and plasmablasts are increased in blood of children with asthma, food allergy and atopic dermatitis
Allergy. 2018; Jun;73(6):1331-1336

Mark P.V. Begieneman, Reindert W. Emmens, **Liza Rijvers**, Linde Woudstra, Walter J. Paulus, Bela Kubat, Alexander B.A. Vonk, Albert C. van Rossum, Diana Wouters, Sacha Zeerleder, Marieke van Ham, Casper G. Schalkwijk, Hans W.M. Niessen, and Paul A.J. Krijnen
Myocardial infarction induces atrial inflammation that can be prevented by C1-esterase inhibitor
Journal of Clinical Pathology. 2016; Dec;69:1093-1099

Mark P.V. Begieneman, Ellis ter Horst, **Liza Rijvers**, Elisa Meinster, René Leen, Jeannette E. Pankras, Jan Fritz, Bela Kubat, René J. P. Musters, André B. P. van Kuilenburg, Jan Stap, Hans W. M. Niessen, and Paul A. J. Krijnen
Dopamine induces lipid accumulation, NADPH oxidase-related oxidative stress and a pro-inflammatory status of the plasma membrane in H9C2 cells
American Journal of Physiology - Heart and Circulatory Physiology, 2016; Nov;311: H1097-H1107

Mark P. Begieneman, Reindert W. Emmens, **Liza Rijvers**, Bela Kubat, Walter J. Paulus, Alexander B.A. Vonk, Lawrence Rozendaal, P. Stefan Biesbroek, Diana Wouters, Sacha Zeerleder, Marieke van Ham, Stephane Heymans, Albert C. van Rossum, Hans W.M. Niessen, and Paul A.J. Krijnen
Ventricular myocarditis coincides with atrial myocarditis in humans
Cardiovascular Pathology, 2016; Apr;25:141-148

Mark P.V. Begieneman, **Liza Rijvers**, Bela Kubat, Walter J. Paulus, Alexander B.A. Vonk, Albert C. van Rossum, Casper G. Schalkwijk, Wim Stooker, Hans W.M. Niessen, and Paul A.J. Krijnen

Atrial fibrillation coincides with increased intravascular depositions of the advanced glycation end-product Nε-(carboxymethyl) lysine in the atria of the heart

The American Journal of Pathology. 2015; Aug;185:2096-2104