B and T cell-mediated central nervous system demyelinating disease:

underlying mechanisms and clinical perspectives

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The research for this thesis was performed within the framework of the Erasmus MC Postgraduate School Molecular Medicine.

The studies described in this thesis were performed at the Department of Immunology and the Department of Neurology, Erasmus MC, Rotterdam, the Netherlands.

The studies were financially supported by the Dutch MS Research Foundation and Zabawas.

The printing of this thesis was supported by Erasmus MC.

ISBN: 978-94-91811-28-9

Cover design: Karien van Langelaar
Lay-out: Bibi van Bodegom & Daniëlle Korpershoek
Printing: Ridderprint | www.ridderprint.nl

B and T Cell-mediated Central Nervous System Demyelinating Disease:
Underlying mechanisms and clinical perspectives

B en T cel-gemedieerde demyeliniserende ziekte van het centrale zenuwstelsel:
Onderliggende mechanismen en klinische perspectieven

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rector magnificus

Prof.dr. F.A. van der Duijn Schouten

and in accordance with the decision of the Doctoral Board
The Public defense will be held on

Tuesday 16 March 2021 at 10:30 hrs

by

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Chapter 1

General introduction

GENERAL INTRODUCTION

Every day the human body encounters various pathogens such as bacteria and viruses. If these pathogens break through the initial physical and chemical barriers of the body, the immune system is recruited to eliminate them. To achieve this, the immune system uses tightly regulated innate and adaptive defense mechanisms. Rapid recognition and elimination of the pathogen is mediated by the innate immune response, while the adaptive immune response is slower but more specific and generates immunological memory for a quicker response if re-exposure occurs. The intricate balance between effector and regulatory immune cells ensures an optimal immune response. This balance also prevents immune responses against self-antigens through a phenomenon called immune tolerance. However, the interplay between intrinsic (genetic) and extrinsic (environmental) factors likely causes breakdown of self-tolerance, eventually resulting in autoimmune disease. Adaptive immunity (B and T cells) plays a fundamental, but yet incompletely understood role in patients with multiple sclerosis (MS) and neuromyelitis optica spectrum disorder (NMOSD). MS and NMOSD are inflammatory and demyelinating diseases of the central nervous system (CNS) that are mediated by autoimmune-related processes. In this chapter, we introduce how B and T cells develop and interact in healthy individuals and what could trigger particular subsets to become pathogenic instead of protective in such patients. This sets the stage for the current thesis, which addresses the underlying mechanisms and clinical impact of human B and T cells on inflammatory CNS demyelinating disease.

ADAPTIVE IMMUNITY

B and T cells initially develop in the bone marrow and originate from hematopoietic stem cells (HSCs) committed to the lymphoid lineage [1]. Depending on the cytokine milieu and distinct transcription factors [1-3], lymphoid progenitor cells will either remain in the bone marrow to mature into B cells or migrate to the thymus to mature into T cells. During these processes, highly specific and unique B- and T-cell receptors are generated, making it possible to recognize a large variety of antigens. Both central and peripheral tolerance checkpoints are exploited to prevent recognition of self-antigens and to promote the development of functional subsets with non-self-reactive receptors [4]. Such B and T cells recirculate in the blood until an antigen is encountered within lymphoid organs. Upon encounter with antigens, B and T cells are activated and differentiate into effector and memory cells in order to remove the pathogen through antibody-mediated humoral and cytotoxic cellular immunity [5, 6]. To reach these effector and memory stages, B and T

cells undergo a series of well-regulated developmental steps, which are further explained below.

B-cell development and effector functions

In the bone marrow, HSC-derived precursors develop into immature B cells with a B cell receptor (BCR) that is generated by random gene recombination of their immunoglobulin (Ig) heavy and light chain loci, known as V(D)J rearrangements [7, 8]. Two main central tolerance mechanisms ensure that immature B cells expressing high affinity BCRs for self-antigens are removed. Receptor editing occurs when a self-reactive BCR is expressed during the early stages of B-cell development and certifies that this specificity is lost through a second round of V(D)J rearrangements [9]. This mechanism involves the removal of an autoreactive BCR by deleting the self-reactive light-chain gene and replacing it with another sequence. If receptor editing fails, clonal deletion in the bone marrow removes self-reactive B cells [9]. The cells that survive these checkpoints exit the bone marrow and enter the circulation as transitional B cells ($CD38^{high}CD24^{high}IgM^{+}IgD^{+}$), which then further differentiate into naive mature B cells ($CD38^{-/dim}IgM^{+}CD27^{-}IgD^{+}$; Fig. 1) with a functional BCR [7, 8]. At this particular stage, peripheral tolerance mechanisms including apoptosis, anergy and T regulatory cell (Treg)-mediated suppression are important to control autoreactive B cells that have escaped negative selection in the bone marrow [10].

Within secondary lymphoid organs such as the lymph nodes, mature naive B cells encounter antigens via the BCR, internalize, process and present these antigens on human leukocyte antigen (HLA) class II molecules to $CD4^{+}$ T cells [11]. This can initiate either germinal center (GC)-dependent or –independent differentiation of naive B cells into memory B cells and antibody-secreting cells (ASCs; Fig. 1) [12, 13]. In GC-dependent responses B cells receive signals from $CD4^{+}$ T follicular helper (Tfh) cells via CD40 ligand (CD40L[CD40]), CD28 (CD80/CD86) and interleukin (IL)-21 to undergo clonal expansion, class switch recombination and somatic hypermutations of the VH genes [6]. During class switch recombination, the constant region of the BCR Ig heavy chain is replaced by other isotypes that have varying properties and functions. Somatic hypermutations take place at the variable regions of both the Ig heavy and light chains and comprises of single-nucleotide exchanges, deletions and point mutations [14]. Both these processes increase the affinity of B cells for antigens. Somatic hypermutations in the GC can lead to formation of self-reactive B cells as well, however the tolerance mechanisms involved here to regulate their removal remains poorly understood [15]. Strong HLA-II antigen presentation and co-stimulation via CD40L/CD40 are key processes for maintaining peripheral tolerance [9, 16]. B cells that survive peripheral tolerance checkpoints and enter GCs develop into class switched IgG^{+}, IgA^{+} or IgE^{+} memory B cells and long-lived plasma cells ($CD38^{high}CD27^{high}CD138^{+}$) [17, 18]. There is also generation of non-class switched IgM^{+} memory B cells [19, 20]. This can occur

in a GC-independent manner via the marginal zone in the spleen giving rise to natural effector B cells (CD27^{+}IgM^{+}IgD^{+}) or in a GC-dependent manner that results in the development of 'IgM-only' (CD27^{+}IgM^{+}IgD^{-}) B cells (Fig. 1) [20]. Furthermore, GC-independent responses can lead to the generation of short-lived plasmablasts (CD38^{high}CD27^{high}) when B cells interact with CD4^{+} T cells in extra-follicular regions [12, 13]. Memory B cells function to act as antigen-presenting cells (APCs) and secrete pro- or anti-inflammatory cytokines [21, 22]. Whereas, ASCs consist of both short-lived plasmablasts and long-lived plasma cells

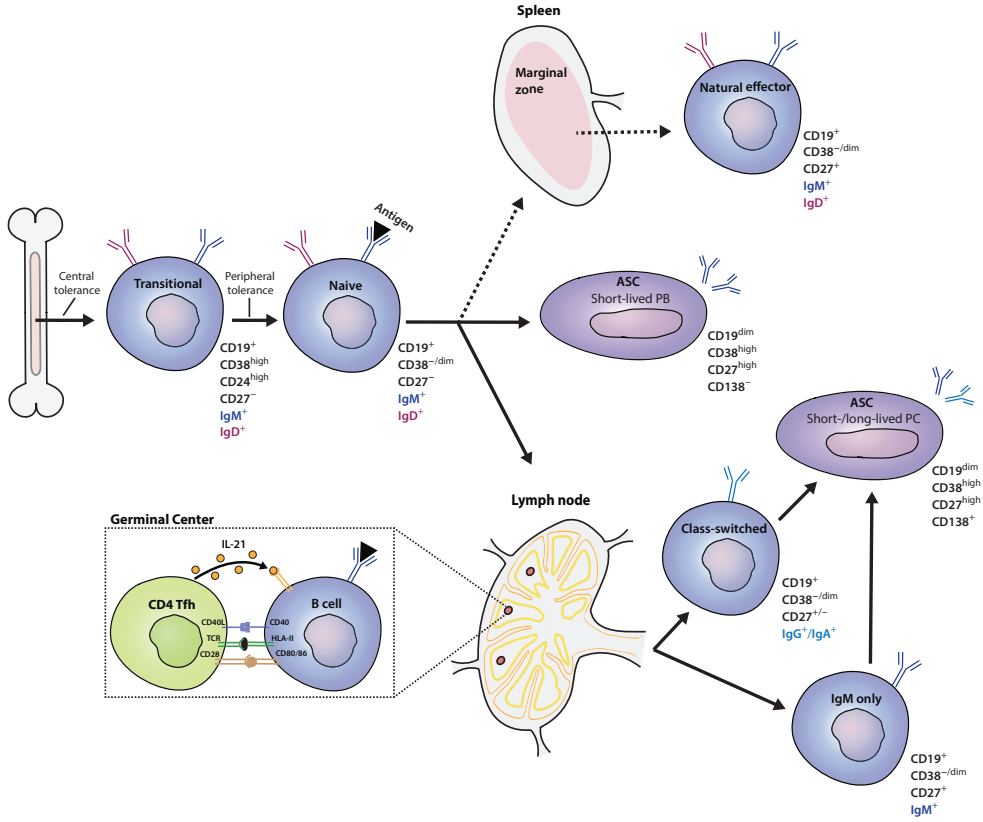

Figure 1. Peripheral B-cell development.
Immature B cells that have survived central tolerance egress from the bone marrow into the circulation as transitional B cells (CD38^{high}CD24^{high}IgM^{+}IgD^{+}). When an antigen is encountered, naive mature B cells (CD38$^{-/dim}$CD27^{-}IgM^{+}IgD^{+}) can take three pathways of differentiation. Outside the germinal center (GC), B cells are able to directly mature into antibody-secreting cells (ASC; i.e. short-lived plasmablasts [PB; CD38^{high}CD27^{high}CD138^{-}]). Natural effector B cells (CD27^{+}IgM^{+}IgD^{+}) develop in the marginal zone of the spleen and are triggered in a GC-independent manner [20]. Inside the GC, B cells are induced by T follicular helper (Tfh) cells via HLA class II, co-stimulatory molecules (CD40-CD40L, CD80/CD86-CD28) and IL-21 to differentiate into IgM-only/class-switched memory B cells as well as antibody-secreting cells (short- and long-lived plasma cells [PC; CD38^{high}CD27^{high}CD138^{+}]).

[23] that produce specific antibodies to neutralize the pathogen, activate the complement system and mediate antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis [24].

Besides for BCR signaling, B-cell intrinsic Toll-like receptor (TLR) activation also plays a role in shaping antibody responses against pathogens [25, 26]. TLRs directly sense microbes by recognizing pathogen-associated molecular patterns [25]. Human B cells express TLR1, TLR6, TLR7, TLR9 and TLR10 [25, 27, 28]. TLR1 and TLR6 are present at the plasma membrane and sense extracellular microbes, whereas TLR7 and TLR9 are localized in endosomal compartments and respond to pathogen-derived nucleic-acids such as single-stranded RNA and CpG-ODN (also referred to as CpG-DNA), respectively [29, 30]. BCR and CD40 stimulation enhance TLR expression on GC B cells and induce proliferation, somatic hypermutations and class switching before developing into high affinity ASCs [27, 31]. TLR9 is of particular interest since *in vitro* studies show that B cell differentiation via CpG-ODN requires additional BCR, CD40 or cytokine signaling [26, 32]. This seems only to be the case in humans and not in mice [33, 34]. Through this interaction, TLRs cooperate with antigen-specific signals to generate optimal B-cell responses after an infection [25]. TLR signaling is also able to enhance the APC function of B cells [35] as well as their secretion of a wide range of inflammatory cytokines depending on the triggers [36, 37].

T-cell development and effector functions

Within the thymus, HSC-derived T-cell precursors (i.e. thymocytes) further mature by undergoing rounds of positive and negative selection, and T-cell receptor (TCR) gene rearrangement [5, 38]. However, thymic deletion does not eliminate all self-reactive T cells. After leaving the thymus, these cells are further kept in check via intrinsic (ignorance, anergy, phenotype skewing, apoptosis) and extrinsic (Tregs, tolerogenic dendritic cells [DCs]) peripheral tolerance mechanisms [39]. These central and peripheral mechanisms both contribute to regulate or eliminate autoreactive T cells which are generated in the thymus by the stochastic nature of TCR rearrangement. In the end, non-autoreactive naive T cells enter secondary lymphoid organs for antigen-driven activation [38]. Here, naive T cells are primed by DCs to differentiate into effector and memory subsets. There are two major types of T cells: $CD4^+$ T helper (Th) and $CD8^+$ cytotoxic T cells (CTLs). Based on the type of cytokines produced, $CD4^+$ Th cells can be classified into functionally distinct subpopulations. Th1 cells are required to generate an optimal $CD8^+$ CTL response [40] and mount an immune response against intracellular bacteria and viruses [41]. Th2 cells are involved in removing extracellular parasites and helminthes, while Th17 cells normally respond to extracellular bacteria and fungi [5]. The capacity of macrophages and B cells to respond to extracellular antigens is stimulated by Th1 and Th2 cells, respectively [41]. Furthermore, $CD4^+$ Th cells induce antibody responses depending on the pathogens

presented. Th1 typically induces IgG antibody responses, Th2 promotes IgE and Th17 cells are able to trigger all subclasses including IgM, IgA, IgG and IgE [42, 43]. Also important in driving germinal center and antibody responses are Tfh cells, which are mainly found within follicles and produce IL-21 that induces such responses [44, 45].

$CD4^+$ Th cell differentiation involves the interplay of certain cytokines and transcription factors (Fig. 2). Th1 cells are induced by IL-12 and interferon gamma (IFN-γ), which activate downstream signaling molecules signal transducer and activator of transcription (STAT)4 and STAT1 respectively [5, 46]. The T-box transcription factor (T-bet) is the master regulator of Th1 development and enhances IFN-γ production [47]. IFN-γ secretion creates a feedforward loop where IFN-γ induces STAT1 and T-bet expression which in turn leads to increased production of IFN-γ [5, 48]. Th2 cells are induced by IL-4. IL-4-mediated STAT6 expression enhances the master transcription factor GATA-binding protein 3 (GATA3) [5, 49]. The development of Tfh cells involves IL-6 and IL-21 that drive STAT3 and B cell lymphoma 6 (BCL-6) expression [5].

Th17 differentiation is more complex and involves three distinct phases, including 1) differentiation, 2) self-amplification and 3) stabilization [5]. Th17 cell development is induced by transforming growth factor beta (TGF-β), IL-1β and IL-6, IL-21 or IL-23 [5, 50]. These cytokines activate downstream STAT3 signaling, which induces the master transcription factor retinoic acid receptor-related orphan gamma t (RORγt). Interestingly, IL-1β together with IL-23 induces both T-bet and RORγt expression in murine and human Th17 cells [51, 52]. Th17 cells are known to be highly polyfunctional, being able to additionally express transcription factors that are typical for Th1. These differences in signaling pathways and transcription factors result in distinct expression of chemokine receptors and cytokines, which can be used to define the phenotype of Th cells (Fig. 2). While Th1 and Th2 cells can be simply defined by their classical chemokine receptor profiles, Th17 cells are more heterogeneous and consist of different phenotypes depending on the chemokine receptor combinations used to define them. C-C chemokine receptor (CCR)6-negative Th cells consist of Th1 cells that are positive for C-X-C chemokine receptor (CXCR)3 and Th2 cells that express CCR4 [53, 54]. Th17 cells are positive for CCR6 and can be further defined based on CXCR3 expression into Th1-like Th17 cells ($CCR6^+CXCR3^+$). Th17 cells also express CCR4 that can be used to subdivide these cells into Th17.1 ($CCR6^+CXCR3^+CCR4^{-/dim}$) and Th17 double positive (DP; $CCR6^+CXCR3^+CCR4^+$) cells (Fig. 2) [55]. The expression of these receptors reflects the pro-inflammatory cytokines produced by each Th subset. Human Th17 ($CCR6^+CXCR3^-CCR4^+$) cells produce high levels of IL-17A and lack IFN-γ, while GM-CSF is moderately expressed [55]. This is in contrast to mice where IL-17A is typically co-expressed with GM-CSF [56]. Th17.1 ($CCR6^+CXCR3^+CCR4^{-/dim}$) cells secrete high levels of IFN-γ and GM-CSF but only low levels of IL-17A [55], while Th17 DP ($CCR6^+CXCR3^+CCR4^+$) cells produce equally low amounts of these cytokines.

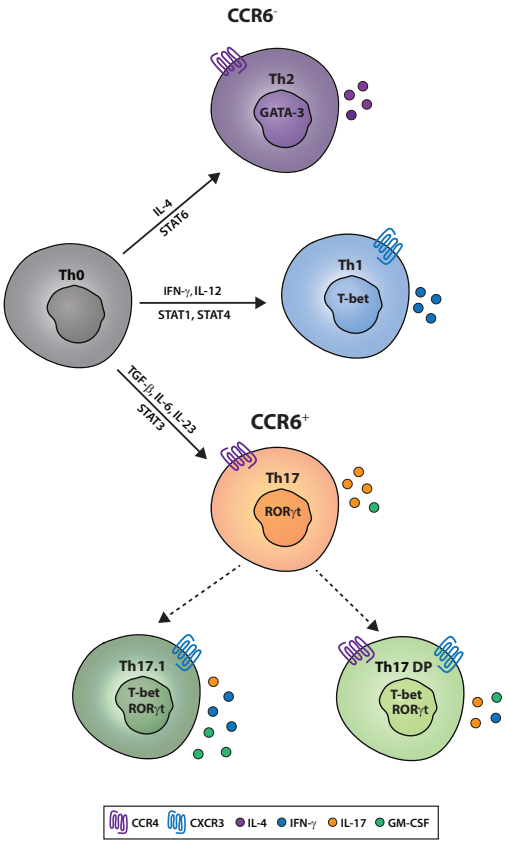

Figure 2. Functional characteristics of human CCR6$^-$ and CCR6$^+$ effector/memory Th cells.
CD4$^+$ Th0 cells differentiate into distinct effector Th subsets based on the cytokine milieu that triggers certain transcription factors. Transcription factor, chemokine receptor and cytokine profiles can also be used to classify these Th subsets. Both Th1 (CXCR3$^+$) and Th2 (CCR4$^+$) cells do not express CCR6. CCR6$^+$ cells bear three major subsets: Th17(CCR4$^+$), Th17.1 (CXCR3$^+$CCR4$^{-/dim}$) and Th17 double-positive (DP; CXCR3$^+$CCR4$^+$) cells [55]. Each of these subsets has its own signature cytokine profile.

MULTIPLE SCLEROSIS

General features

MS is a heterogeneous chronic inflammatory demyelinating disease of the CNS. The pathological hallmark of MS is lesions within different CNS compartments including the brain, optic nerve and spinal cord [57, 58]. These lesions are initiated by immune cell-mediated inflammation and contribute to demyelination and axonal loss [59]. Neurodegeneration is the main cause of clinical disability in MS patients [60]. The diagnosis is based on the detection of lesions disseminated in space and time using MRI and

the presence of oligoclonal bands (immunoglobulins, Ig) within the cerebrospinal fluid (CSF) that is obtained through lumbar puncture [61]. MS affects approximately 2.5 million people world-wide and is common in young adults between 20 and 40 years of age [60, 62]. This disease is more frequent in women than in men with a ratio of 2.3:1 [63].

Disease course

The clinical and pathological heterogeneity of MS makes prognosis and treatment difficult [60, 64]. Clinically isolated syndrome (CIS) refers to the first neurological symptoms that last at least 24 hours and are suggestive of inflammatory demyelinating diseases of the CNS [65-67]. Approximately 60% of CIS patients will experience subsequent relapses and convert to clinically definite MS (Fig. 3) [65, 68]. Relapsing-remitting MS (RRMS) is the most common subtype of MS, which is found in approximately 85% of patients and is defined by episodes of neurological dysfunction (relapses) followed by periods of relative clinical stability (remission) [60]. This subtype is characterized by waves of CNS-infiltrating immune cells that contribute to demyelination during a relapse [60]. The remission phase of RRMS is defined by remyelination. Eighty percent of RRMS patients eventually convert to secondary progressive MS (SPMS), which is characterized by gradual increases in neurological deficits with reduced numbers of inflammatory relapses. There is a lack of biomarkers to accurately predict the conversion of CIS to RRMS and RRMS to SPMS. Approximately 10% of MS patients show a gradual neurological decline already from disease onset onwards [60]. This subtype is termed primary progressive MS (PPMS; Fig. 3).

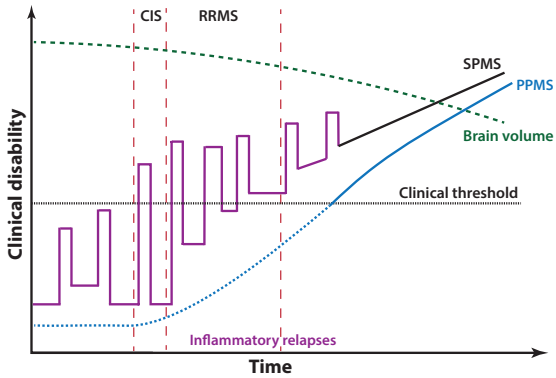

Figure 3. The heterogeneous disease course of MS.
Symptoms for MS appear once the inflammatory relapses go beyond the clinical threshold [60]. The first clinical representation of MS is called clinically isolated syndrome (CIS), which transitions into relapsing-remitting MS (RRMS) after subsequent neurological attacks (relapses). RRMS is characterized by episodes of neurological dysfunction followed by remission. Secondary progressive MS (SPMS) evolves from RRMS, while primary progressive MS (PPMS) starts at disease onset. The progressive phase of the disease course involves gradual worsening of clinical disability [60].

Treatment

Over the last few decades, there have been remarkable advances in the treatment of MS (extensively reviewed and summarized in [69]). Most of these therapies target the immune system and networks involving B and T cells [69, 70]. Immunosuppressive drugs such as glucocorticoids are used to treat acute exacerbations in MS patients but are not disease-modifying [71]. First-line diseases-modifying drugs include dimethylfumarate, teriflunomide, interferon β1a/b and glatiramer acetate [70]. Second-line disease-modifying treatments show the strongest anti-inflammatory effects, which are especially seen for alemtuzumab, ocrelizumab and natalizumab [69, 72]. The efficacy, tolerability and safety profile differs greatly across these treatments [70]. The beneficial effects are often accompanied by serious side effects, which is particularly the case for second-line treatments. For example, clinical concerns during the use of natalizumab include the risk of progressive multifocal leukoencephalopathy (PML) [73] and severe rebounds of MS activity following treatment discontinuation [74]. Furthermore, these treatments are effective in dampening relapses, but are not able to prevent or stop the transition into progressive disease [70, 75]. Since, relapse rates are associated with disease progression, it is crucial to unravel the (adaptive) immune mechanisms driving MS disease activity, especially during the early phases of the disease in order to allow earlier and thereby more effective treatment.

Etiology: genetic and environmental factors

Although the exact cause of MS is still unknown, both genetic and environmental factors have been described to increase the risk of developing MS. Genetic variation accounts for approximately 30% of the overall risk and large genome-wide association studies have now identified 233 different genetic regions that associate with MS [76]. Results from these studies underscore the role of disturbed adaptive immune responses in MS, which overlap and point to mechanisms shared with other autoimmune diseases [77]. The majority of gene variants associated with MS encode for proteins that are involved in functional pathways specifically affecting B and $CD4^+$ T cells [78]. The strongest genetic association with MS is HLA-DRB1*1501 [79]. Individuals who are carriers of the HLA-DRB1*1501 allele have an approximately three times higher risk of developing MS [69, 80].

Environmental factors such as low vitamin D, diet, sex hormones, infections and smoking can contribute to MS susceptibility [60, 63, 81]. Even though each of these factors increases the risk of MS, infection with the Epstein-Barr virus (EBV) is one of the best established contributors [82]. Individuals with high anti-EBV antibody titers or a history of infectious mononucleosis have an increased risk of developing MS [83-85]. Almost all MS patients are seropositive for EBV, which is the case for 90% of the healthy population [84, 86, 87]. The latter implies that EBV alone is not sufficient but rather interacts with genetic

risk loci to cause MS. Consistently, the presence of the HLA-DRB1*1501 allele associates with increased levels of anti-EBV nuclear antigen (EBNA)1 IgG [88].

Immunopathology: B and T cells

Memory B and T cells are triggered in the periphery to infiltrate and contribute to local inflammation in the CNS of MS patients [89-91]. Pro-inflammatory lymphocytes can enter the CNS through three main routes, i.e. the blood-brain barrier (BBB), the blood-cerebrospinal fluid barrier (BCSFB) at the choroid plexus and the blood-meningeal barrier [92, 93]. After their migration across the BBB, lymphocytes first enter the perivascular space to interact with local APCs such as macrophages [92, 94]. Re-activated populations then have to cross the glia limitans to infiltrate the CNS parenchyma [92, 94]. The BBB and BCSFB are thought to be dysfunctional, which is especially seen during the early phase of MS [57, 60, 95, 96]. The exact mechanisms underlying the breakdown of BBB and BCSFB integrity are incompletely understood. Differential expression of pro-inflammatory cytokines, chemokine receptors and integrins produced by infiltrating lymphocytes are thought to facilitate this process in MS [57, 97]. This can be either through activation of brain endothelial or choroid plexus epithelial cells leading to upregulation of adhesion molecules or through disruption of tight junctions between these cells [96, 98-100]. $CD4^+$ T cells are thought to initiate this damage [99, 101, 102] and subsequently allow the recruitment of other potentially pathogenic immune cells into the CNS to cause damage to myelin sheaths. These cells can also infiltrate the CNS through the BCSFB at the choroid plexus [97]. The successful use of current treatment modalities in MS further highlight the role of B and T cells in the pathogenesis. For example, B-cell depletion (anti-CD20) therapies significantly reduce disease activity and even slows down progression in MS patients. This is probably caused by reduced T-cell activation, as a result of the lack of antigen presentation, rather than antibody production by B cells [103]. Natalizumab is a monoclonal antibody that binds to α4β1 integrin (VLA-4) preventing T and B cells from binding to the vascular cell adhesion molecule (VCAM)-1 [104], thereby blocking their migration into the CNS. Yet, these therapies target the bulk lymphocyte population and therefore have severe side effects. Earlier targeting of the exact Th and B cell subsets contributing to disease activity may circumvent this.

T cells in MS

Based on human and experimental autoimmune encephalomyelitis (EAE) studies, the original view of MS is that increased activation or impaired regulation of effector T cells primarily mediate inflammatory relapses [57]. $CD4^+$ T cells are found deeper within lesions and $CD8^+$ T cells are mostly found at the edges of lesions [95, 105]. Although $CD8^+$ T cells outnumber $CD4^+$ T cells within the CNS of late-stage MS patients [106], there are several

lines of evidence that $CD4^+$ T cells contribute to MS disease onset [104, 107]. Consistently, an abundant number of $CD4^+$ T cells are also visible in pre-active lesion sites [108], suggesting an involvement of these cells in the early stages of lesion formation. Recently, it was demonstrated that in contrast to $CD8^+$ T cells, brain-associated $CD4^+$ T-cell TCR clonotypes are reduced in MS blood, indicating selective recruitment or, alternatively, local clonal expansion in the CNS [109]. Naive $CD4^+$ T cells already seem to be more activated in CIS patients who rapidly develop CDMS, suggesting an increased ability to differentiate into pathogenic subsets [110]. A potential cause of this increased activation is the lack of Treg control in the periphery [57, 111]. Tregs were shown to be abnormally enriched but functionally impaired in MS [112-115]. This phenomena is associated with decreased FOXP3 expression [114]. It is therefore thought that pro-inflammatory $CD4^+$ Th cells, triggered by APCs such as B cells or myeloid cells (macrophages, dendritic cells and microglia), escape Treg control and enter the brain.

Both Th1 and Th17 cells are associated with MS (Fig. 4) [104]. Prior to the identification of Th17 cells, Th1 cells were thought to be the critical autoreactive T-cell subset because IFN-γ induces EAE and is found in active MS lesions [116-118]. However, later studies challenged the involvement of Th1 cells by showing that genetic deletion of IFN-γ lead to increased severity of EAE [69, 119], indicating a protective role for IFN-γ instead. This controversy and identification of Th17 cells lead to their detection in the draining lymph nodes of mice with EAE, as well as in peripheral blood and CNS infiltrates of patients with MS [107, 118, 120]. Th17 cells have also been shown to be enriched in the CSF of MS patients during a clinical relapse compared to patients in remission [121] and can directly induce neuronal dysfunction in the CNS [122]. Interestingly, Th17 cells do not only produce their signature cytokine, IL-17 (as explained in the section T-cell development and effector functions), but have been found to co-produce IFN-γ and/or GM-CSF depending on the cytokine milieu [123]. This is particularly true for myelin-reactive $CCR6^+$ memory Th cells in MS patients. Furthermore, GM-CSF is an emerging cytokine that plays a crucial role in MS disease pathogenesis. The fact that $CCR6^+$ cells responding to myelin produce different types of cytokines under diverse conditions indicates that further research is needed to define distinct subsets in order to understand their exact role in MS and to better predict and treat the clinical course.

B cells in MS

A first clue that B cells contribute to MS immunopathogenesis came from studies that identified oligoclonal bands in the CSF of MS patients [94, 124]. Oligoclonal bands are used as a diagnostic tool in MS and are present in the CSF of 90% of patients [125]. More recent studies identified meningeal follicle-like structures containing B cells and plasma cells close to cortical lesions of MS patients [126-128], which correlate to the degree of cortical

demyelination [129]. Furthermore, similar B cell clones have been found in the periphery and CNS of MS patients [130, 131], indicative of their ability to migrate across the BBB and produce antibodies intrathecally (Fig. 4). Plasmablasts or plasma cells express little to no CD20 and as such are not targeted by anti-CD20 B-cell depletion therapies, which results in no effect on the amount of oligoclonal bands present within the CSF [57, 91]. This along with reduced T cell activation by such therapies indicates B cells can function as APCs in the periphery of MS patients (Fig. 4). Consistently, in mice, the antigen-presenting and not the antibody-producing function of B cells is essential for EAE induction [91]. Similarly, in MS patients, memory B cells have been shown to activate and induce auto-proliferation of CNS-infiltrating IFN-γ-producing Th cells in an HLA-DRB1*1501-dependent manner [89]. B cells from MS patients also have an increased ability to produce pro-inflammatory cytokines, such as IL-6, GM-CSF, TNFα and lymphotoxin-α (LTα), which may contribute to local inflammation and damage [103, 132-134]. Which peripheral mechanisms are required for the development of CNS-infiltrating pathogenic B cells in MS patients remain elusive.

In contrast to other autoimmune diseases, only peripheral and not central tolerance checkpoints for B cells are defective in MS, which is accompanied by increased frequencies

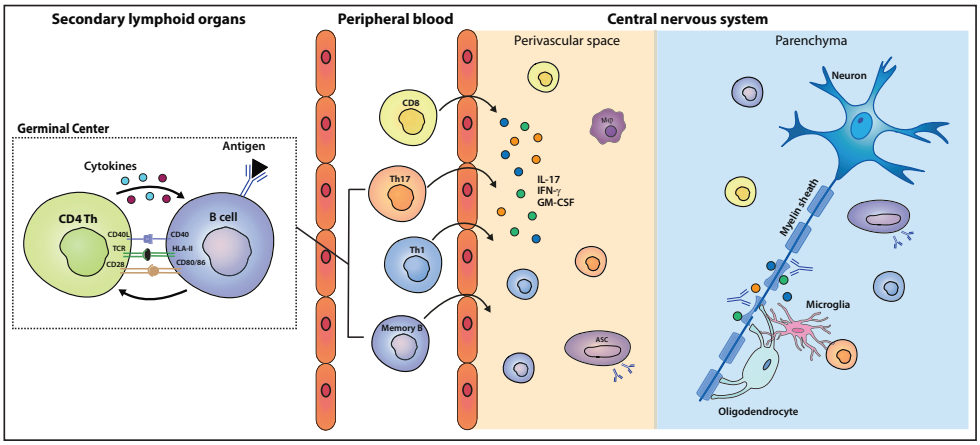

Figure 4. Model of peripheral triggering of CNS-infiltrating B and T cells as underlying mechanism in early MS.

In secondary lymphoid organs, $CD4^+$ T helper (Th) and B cells interact to form germinal centers. In MS, yet unknown antigens are recognized and taken up by B cells, processed and presented via HLA-II molecules to $CD4^+$ T cells. Together with costimulatory and cytokine signals, this causes a feedforward loop in which both B and T cells are activated. In early MS, pathogenic B- and T-cell subsets migrate across a dysfunctional blood-brain barrier into the perivascular space and likely become reactivated to cross the glia limitans into the CNS parenchyma. These cells can also cross the blood-CSF and blood-meningeal barrier to enter the CNS. Once in the CNS B- and T-cell subsets produce pro-inflammatory cytokines that trigger demyelination along with other cells such as microglia. $CD8^+$ T cells are also present in the CNS to induce local damage, although this is thought to be less during early MS. B cells develop into antibody-secreting cells (ASCs) to probably further mediate local pathology.

of potentially autoreactive naive populations in the blood [112, 135, 136]. Similar to $CD4^+$ Th cells, this may be attributed to the dysfunction of Tregs, allowing naive B cells to survive and develop into pathogenic memory B cells in peripheral lymphoid organs. After their escape from these checkpoints, naive B cells likely interact with Th cells in GCs to develop into memory populations capable of infiltrating the MS brain (Fig. 4). Recent evidence from autoimmune mice show that autoreactive B cells are triggered by IFN-γ, which is likely produced by Tfh cells [18]. Moreover, pathogenic B-cell responses in autoimmune diseases such as systemic lupus erythematosus are enhanced after IFN-γ- and virus-mediated induction of T-bet- and CXCR3-expressing B cells [137-139]. Such IgG-switched $T\text{-}bet^+$ B cells show increased antiviral responsiveness as well [140, 141]. However, in MS patients less is known about the signals coming from $CD4^+$ Th cells to induce the development of such B cell subsets.

EBV-related B- and T-cell responses in MS

EBV is a human DNA herpesvirus that establishes a lifelong latency within resting memory B cells [142]. During an acute infection, EBV is spread through saliva and infects naive B cells in the tonsils via binding of viral glycoproteins gp350/220 with CD21 and gp42/gH/gL with HLA-II expressed on B cells [142-144]. To accomplish latency in memory B cells, the virus uses a series of programs that drive B cells towards a GC response in a both antigen- and T cell-independent manner. Latent membrane protein (LMP)2A and LMP1 resemble signals coming from the BCR and CD40 receptor [142, 145]. In addition to their regulation of GC responses, [146], recent evidence shows that LMP2A and LMP1 can synergize with BCR and CD40 signaling as well [147].

Although definite evidence for EBV as a causal factor in MS is lacking, there are many hypotheses proposed on how EBV is involved in the immunopathogenesis of MS [148]. These include 1) molecular mimicry through cross-reactivity [149], 2) bystander activation and 3) infection of autoreactive B cells in the periphery [142, 150]. The first hypothesis has been favored for many years and suggests that EBV antigens prime T cells, which cross-react with and instead attack cells expressing CNS antigens [151]. In line with this, IFN-γ-producing $CD4^+$ Th cells in the blood of MS patients have been shown to respond to both myelin and EBV antigens [83, 87]. Also IFN-γ-producing T cells in MS CSF and brain tissue show increased EBV specificity and responses to EBV-infected B cells [152-155]. The second hypothesis suggests that immune cells respond to EBV infection and cause bystander damage to the CNS in the process of trying to eliminate the virus [142]. The third hypothesis postulates that EBV causes MS by infecting autoreactive B cells in genetically susceptible individuals [142, 144]. Here, the hypothesis is that EBV-infected memory B cells develop and escape $CD8^+$ CTL control to infiltrate the CNS and produce (auto)antibodies locally [142, 145]. Chronic viral stimulation of autoreactive B cells can in turn enhance

EBV-specific T-cell responses [150]. Moreover, EBNA1-specific IgG antibodies are predictive for early disease activity [156] and are present in CSF from MS patients [152, 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 [158-163].

NEUROMYELITIS OPTICA SPECTRUM DISORDER

General features

NMOSD is a rare, but severe inflammatory demyelinating disease of the CNS, which mostly affects the spinal cord, optic nerve and brainstem [94]. Approximately 80-90% of patients have relapses of optic neuritis and myelitis, most of which worsen over several days with slow recovery thereafter [164]. NMOSD has an estimated incidence and prevalence of 0.05-0.40 and 0.1-4.4 cases per 100,000 individuals [94, 165]. The mean age at onset ranges from 32-45 [165]. NMOSD is more common in females with a range of 66-88% of the patient population [165].

Initially, NMOSD was thought to be a clinical variant of MS. However, in 2004, aquaporin-4 (AQP4), a membrane-bound water channel expressed on astrocytes, was identified as a target antigen in approximately 75% of NMOSD patients [94, 166]. The detection of AQP4 antibodies allows NMOSD to be distinguished from MS [166]. Recent studies indicate that within the subgroup of AQP4-IgG seronegative NMOSD patients, about 30-40% have antibodies specific for myelin oligodendrocyte glycoprotein (MOG) [167, 168]. MOG antibody-associated disorder (MOGAD) most frequently presents as acute disseminated encephalomyelitis (ADEM) in children under seven years and as optic neuritis in older children and adults [169, 170]. Currently, the diagnosis of AQP4-IgG-positive NMOSD and MOGAD is based on clinical manifestations, neuroimaging and serology [171]. Both anti-AQP4 and anti-MOG antibodies are of the IgG1 subtype and are more abundant in the serum than in CSF of these patients [166, 172]. Patients with MOGAD are predominantly males, have fewer relapses and show an improved recovery as compared to AQP4-seropositive NMOSD patients [171, 173]. Clinical studies have also suggested reasons to include MOG-IgG-positive patients under the spectrum of NMOSD, but this remains a matter of debate since the exact pathology of disease is unknown [171]. For both prognosis and treatment decisions, it is important to understand the relation between the immunopathogenesis and relapse occurrence in these patient groups. This is exemplified by the fact that effective MS treatments such as interferon-β and natalizumab can exacerbate NMOSD [174-176]. Steroids such as prednisolone are often used to dampen acute relapses, but does not prevent disease progression in both NMOSD and MS patients [173]. The exact cause of NMOSD is even less clear than in MS.

Immunopathology: B and T cells

NMOSD is considered to be an autoimmune disease driven by autoantibodies that target astrocytes and/or oligodendrocytes in the CNS [94, 177-179]. Not much is known about how B cells are triggered, interact with T cells and develop into ASCs to cause local damage in this spectrum of diseases. It is highly likely that such specific ASCs are already induced in the periphery (Fig. 5) [94]. In contrast to MS, both central and peripheral B cell tolerance defects are present in patients with NMOSD, indicating that large numbers of autoreactive naive B cell clones accumulate in the periphery [180]. Moreover, B cell depletion therapies with anti-CD20 [181, 182] and -CD19 [183] monoclonal antibodies are very effective in treating NMOSD. However, treatment response does not always correlate with reduced AQP4-specific antibody titers in the periphery [184]. Whether AQP4- or MOG-specific antibodies are pathogenic or represent a bystander or epiphenomenon remains unclear [185], and together with B cell depletion therapy outcomes could suggest that similar to MS, also antibody-independent B-cell mechanisms are involved in the pathology of NMOSD and MOGAD. In a few studies, increased frequencies of AQP4-specific Th17 cells have been found in the peripheral blood of NMOSD patients [186, 187]. AQP4-specific Th cells are also able interact with B cells and induce differentiation of ASCs within the peripheral lymph nodes [94]. Subsequently, peripherally produced anti-AQP4 or anti-MOG antibodies migrate into the CNS (Fig. 5) [188-190]. Within the brain, AQP4 is located at the foot of the astrocytes, which are CNS-resident cells that function to protect neurons and control the BBB and blood flow [173]. Binding of anti-AQP4 antibodies to astrocytes results in complement- and cell-mediated damage (Fig. 5) [173]. This largely involves the recruitment of eosinophils and neutrophils [191]. The destruction of astrocytes is a primary event in NMOSD that eventually leads to a lack of support to myelin sheaths on neurons [173]. MOG, is a well-established autoantigen in EAE [192], which is expressed on the outermost lamellae of myelin sheaths and the cell body and processes of oligodendrocytes. Binding of anti-MOG antibodies to these structures causes demyelination (Fig. 5) [193]. Similarly, in a small number of encephalitis patients who are seropositive for anti-MOG antibody, demyelination by myelin-specific macrophages has been described [194, 195]. A role for complement-mediated demyelination in MOGAD remains unclear. Although complement is present in CNS lesions of MOGAD, MOG-specific antibodies have a limited ability to activate complement-mediated demyelination as shown both *in vitro* [185, 196] and *in vivo* [197].

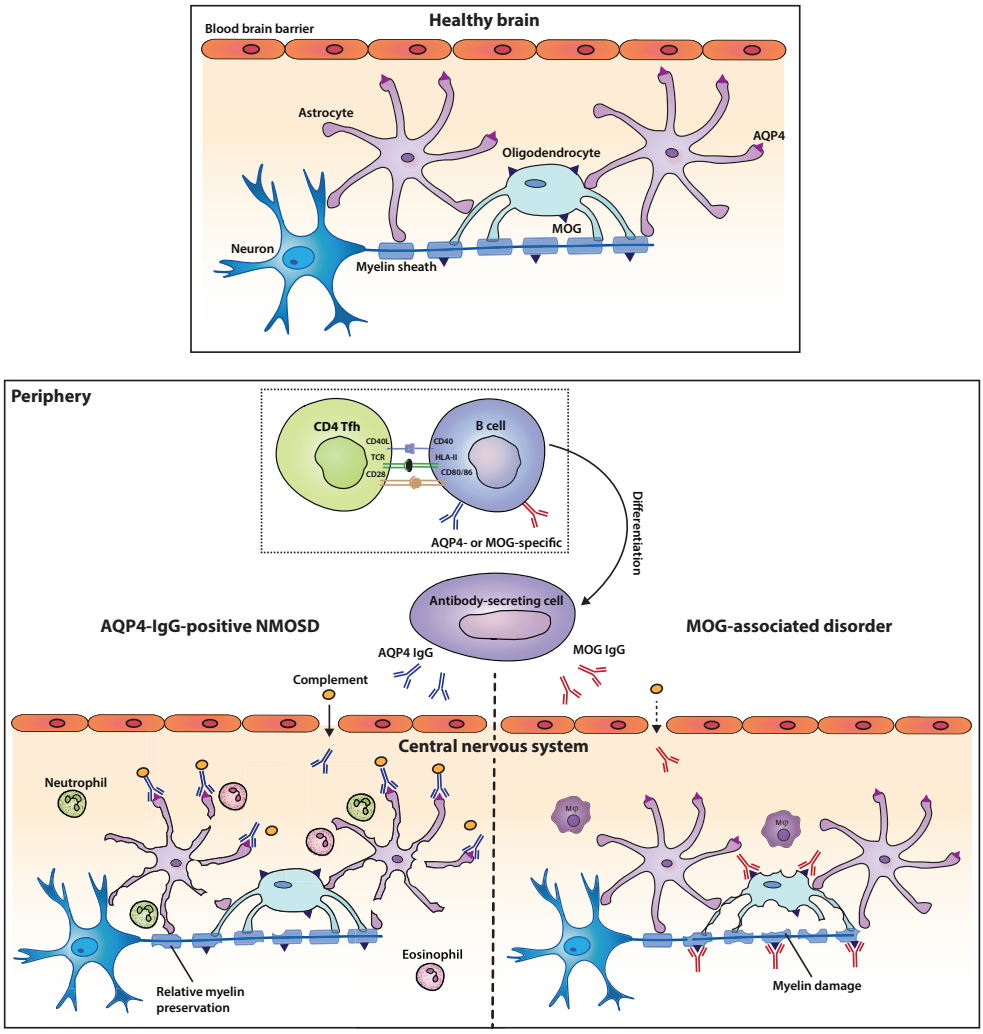

Figure 5. Immunopathogenesis of AQP4-IgG-positive NMOSD and MOGAD.
The first part illustrates the different CNS-resident cells involved in neuronal support and maintenance in the healthy brain. The second part demonstrates a model of what pathogenic events may occur to induce AQP4-IgG-positive NMOSD and MOGAD. In AQP4-associated NMOSD, AQP4 IgG enter the CNS and bind to its antigen and induces astrocyte damage. During initial disease there is relative myelin preservation but eventually lack of astrocyte support induces myelin damage. In contrast, MOGAD involves MOG IgG binding to its antigen on oligodendrocytes and causes demyelination. Adapted from Whittam et al., Journal of Neurology 2017 [198].

THE SCOPE OF THIS THESIS

B and $CD4^+$ Th cells are critically involved in the pathogenesis of CNS demyelinating diseases such as MS and NMOSD. How human B- and $CD4^+$ Th cell populations develop in the periphery in order to drive disease activity remains poorly understood. In this thesis, we set out to unravel the identity, mechanisms and triggers of disease-relevant lymphocyte subsets in MS and NMOSD patients.

In **Chapter 2,** we studied human $CCR6^+$ Th memory cells in detail and determined which Th17 effector phenotypes and functions correlate with early MS disease activity. The chemokine receptors CXCR3 and CCR4 were used to delineate subsets with high (Th17), dim (Th17 DP) and low (Th17.1) IL-17A levels in the blood (both pre- and post-natalizumab), CSF and brain tissue of MS patients. The expression of Th1 and Th17 transcription factors (T-bet, RORγt) and cytokines (IFN-γ, GM-CSF, IL-17A) by these cells were assessed to pinpoint their pathogenic potential. To further shed light on the differences in CNS-homing capacity of these Th17 subsets, we compared their recruitment to the CSF between patients with early MS and other types of neurological diseases. This capacity was related to their sensitivity to glucocorticoids as standard treatment of acute MS activity through the expression of multidrug resistance (MDR)1 and glucocorticoid receptor (GR) (**Chapter 3)**. MDR1 was used as a marker to detect glucocorticoid-resistant Th17.1 cells in MS brain compartments (*both ex vivo* and *in situ*).

Chapter 4 focuses on which and how functional B-cell subsets are triggered to infiltrate the CNS of MS patients. We assessed both their *in vitro* differentiation and CNS infiltration potential, as well as their *ex vivo* recruitment to MS brain compartments. IFN-γ and TLR9 ligand CpG-ODN were used as triggers of naive and memory B cells in GC-like differentiation systems. In **Chapter 5**, the impact of EBV infection on the induction of B cells was assessed using PBMCs from MS patients who have received autologous bone marrow transplantation and patients treated with natalizumab. A highly sensitive qPCR was used to measure EBV DNA copy numbers in sorted B-cell subsets.

Since naive B cells have also been put forward as key contributors to NMOSD pathogenesis, we compared both *ex vivo* and *in vitro* naive B-cell differentiation between NMOSD and MOGAD patients with and without steroid treatment (**Chapter 6**), which is commonly used to dampen acute relapses. Results were compared to B cells from matched healthy controls and MS patients. Finally, in **Chapter 7**, the results obtained from this thesis are discussed and integrated with current knowledge into a model of how B and T cells could play a role in disease activity in patients with MS and NMOSD.

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Chapter 2

T helper 17.1 cells associate with multiple sclerosis disease activity: perspectives for early intervention

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Brain 2018;141;1334-1349

ABSTRACT

Interleukin-17-expressing $CD4^+$ T helper 17 (Th17) cells are considered as critical regulators of multiple sclerosis disease activity. However, depending on the species and pro-inflammatory milieu, Th17 cells are functionally heterogeneous, consisting of subpopulations that differentially produce interleukin-17, interferon-gamma and granulocyte macrophage colony-stimulating factor. In the current study, we studied distinct effector phenotypes of human Th17 cells and their correlation with disease activity in multiple sclerosis patients. Th memory populations single- and double-positive for C-C chemokine receptor 6 (CCR6) and CXC chemokine receptor 3 (CXCR3) were functionally assessed in blood and/or cerebrospinal fluid from a total of 59 clinically isolated syndrome, 35 untreated and 24 natalizumab-treated patients with relapsing-remitting multiple sclerosis, and nine patients with end-stage multiple sclerosis. Within the clinically isolated syndrome group, 23 patients had a second attack within 1 year and 26 patients did not experience subsequent attacks during a follow-up of >5 years. Low frequencies of T helper 1 (Th1)-like Th17 ($CCR6^+CXCR3^+$), and not Th17 ($CCR6^+CXCR3^-$) effector memory populations in blood strongly associated with a rapid diagnosis of clinically definite multiple sclerosis. In cerebrospinal fluid of clinically isolated syndrome and relapsing-remitting multiple sclerosis patients, Th1-like Th17 effector memory cells were abundant and showed increased production of interferon-gamma and granulocyte macrophage colony-stimulating factor compared to paired $CCR6^+$ and $CCR6^-$ $CD8^+$ T cell populations and their blood equivalents after short-term culturing. Their local enrichment was confirmed *ex vivo* using cerebrospinal fluid and brain single-cell suspensions. Across all pro-inflammatory Th cells analyzed in relapsing-remitting multiple sclerosis blood, Th1-like Th17 subpopulation T helper 17.1 (Th17.1; $CCR6^+CXCR3^+CCR4^-$) expressed the highest very late antigen-4 levels and selectively accumulated in natalizumab-treated patients who remained free of clinical relapses. This was not found in patients who experienced relapses during natalizumab treatment. The enhanced potential of Th17.1 cells to infiltrate the central nervous system was supported by their predominance in cerebrospinal fluid of early multiple sclerosis patients and their preferential transmigration across human brain endothelial layers. These findings reveal a dominant contribution of Th1-like Th17 subpopulations, in particular Th17.1 cells, to clinical disease activity and provide a strong rationale for more specific and earlier use of T cell-targeted therapy in multiple sclerosis.

INTRODUCTION

Multiple sclerosis (MS) is mediated by effector T cells trafficking from the periphery into the central nervous system (CNS) to trigger local inflammation, demyelination and neurodegeneration [1]. Although current T cell-directed treatment attenuates disease activity, it often causes serious complications and does not prevent disease progression in MS [2]. To improve treatment efficacy and risk management, more in-depth insight into human effector T cells during MS onset is warranted. In the earliest clinical presentation of MS, clinically isolated syndrome (CIS), increased peripheral $CD4^+$ T cell activation is linked to the occurrence of a second attack [3]. However, substantial knowledge has been gained about specific human T helper (Th) functions, and the exact nature of the pro-inflammatory Th subsets involved in MS is incompletely understood.

Both Th1 and Th17 cells are known to be encephalitogenic, but use distinct transmigration routes to enter the CNS. In experimental autoimmune encephalomyelitis, Th1 cells preferentially migrate into the spinal cord, while Th17 cells mainly infiltrate the brain [4]. This is facilitated by their differential expression of pro-inflammatory cytokines, chemokine receptors and integrins [5-7]. Interleukin-17 (IL-17) and C-C chemokine receptor 6 (CCR6) are key determinants for Th17 transmigration across the blood-brain barrier [8]. IL-17 is generally considered as the signature cytokine produced by CCR6-positive Th17 cells. However, this greatly underestimates Th17 effector function, since subpopulations also (co-)produce interferon-gamma (IFN-γ) and granulocyte macrophage colony-stimulating factor (GM-CSF). Next to IL-17, also IFN-γ and GM-CSF are strongly produced by myelin-specific CCR6-positive Th cells in MS [7, 9]. Th17 polyfunctionality is differently regulated between species, as reflected by the antagonistic regulation of IL-17 and GM-CSF expression in human compared to murine Th cells [10-12]. Particularly GM-CSF produced by Th cells is implicated as a critical mediator of MS onset [13, 14].

The surface expression of another chemokine receptor, CXCR3, defines Th17 cells with Th1-like features [15]. CCR6 and CXCR3 expression on $CD4^+$ T cells is controlled by transcription factors RORγt and T-bet, respectively, which were originally associated with IL-17/IFN-γ double-production [15]. However, recent findings demonstrate more heterogeneous IL-17, IFN-γ and GM-CSF expression profiles in Th17 cells, depending on the inflammatory milieu [16]. Besides CCR6 and CXCR3, also the presence of the α4β1 integrin very late antigen-4 (VLA-4), which is abundant on Th17 cells in MS cerebrospinal fluid (CSF) [17], determines T-cell transmigration capacities. Anti-VLA-4 monoclonal antibody natalizumab is currently one of the most effective therapies in MS, but relapses are still encountered after one year in about one-third of treated patients [18]. Understanding which distinct pro-inflammatory Th subsets are targeted by natalizumab will help to better predict treatment response in MS [19].

Here, blood and CSF samples from CIS and both untreated and natalizumab-treated relapsing-remitting MS (RRMS) patients were assessed for distribution, memory phenotype, activation and pro-inflammatory capacity of Th17 subsets. We reveal that IFN-γ-/GM-CSF-producing ($CCR6^+CXCR3^+$), but not IL-17-producing ($CCR6^+CXCR3^-$) Th17 effector cells are key regulators of MS onset. A Th1-like Th17 subpopulation termed Th17.1 ($CCR6^+CXCR3^+CCR4^-$) is selectively targeted by natalizumab in MS patients who remained free of clinical relapses. This work supports the design and early use of therapeutic strategies against Th17.1 cells to prevent relapses in MS.

MATERIALS AND METHODS

Patients

Characteristics of patients and controls in the screening cohorts are summarized in Table 1. Main experimental results were confirmed using additional cohorts (Supplementary Table 1). All CIS and RRMS patients as well as controls were included at Erasmus MC (Rotterdam, The Netherlands), which is a national tertiary referral center for patient with MS (MS Center ErasMS). All primary material was collected between 2007 and 2017.

For blood analyses, we selected 23 patients with CIS who did not experience a second clinical attack for at least 5 years of follow-up (CIS-CIS) and 26 CIS patients who were diagnosed with clinically definite MS (CDMS) within 1 year after CIS (CIS-CDMS) from our prospective cohort. None of these patients were treated with disease-modifying therapies before or at time of sampling. CIS was defined as a first clinical attack of demyelination in the CNS [20]. CDMS diagnosis was made when a patient experienced two attacks with clinical evidence of two separate lesions according to the Poser criteria [21]. A relapse was defined as sub-acute worsening of existing symptoms, or new symptoms after at least 30 days of improvement or stable disease [22]. Fatigue scores were acquired at time of the first attack using the self-administered Krupp's Fatigue Severity Scale (FSS), as shown previously [23]. Anti-EBNA1 IgG levels were determined in plasma using a well-validated chemiluminescent assay and analyzer (Liaison XL; both Diasorin, Saluggia, Italy) according to manufacturers' instructions at our local referral center for virus diagnostics (Erasmus MC).

Patients with RRMS were diagnosed according to the McDonald 2010 criteria [24]. Blood Th cell analyses were performed for 31 treatment-naive RRMS patients, as well as for 24 RRMS patients before the start and after both 6 and 12 months of natalizumab therapy. The median time between the last clinical attack and first administration of natalizumab was 2.8 months [interquartile range (IQR): 1.7-6.3]. Seventeen of these patients (70.8%) were treated with disease-modifying therapy before initiation of natalizumab (14 with

Table 1. Characteristics of patients and controls in screening cohorts

Blood, *ex vivo*					
Cohort	**HC**	**CIS-CIS**	**CIS-CDMS**	**RRMS, no treatment[a]**	**RRMS, NAT treatment[a]**
Patient, n	19	16	16	18	17[b]
Gender, female, n (%)	16 (84)	11 (69)	13 (81)	15 (83)	12 (71)
Age in years, median (IQR)[c]	45 (35-49)	36 (27-40)	33 (28-37)	46 (37-50)	38 (30-46)[d]
Follow-up time in years, median (IQR)	NA	6.8 (6.2-7.3)	4.1 (3.1-5.7)	NA	NA
Disease duration in months, median (IQR)[e]	NA	2.0 (1.3-3.1)	2.0 (1.2-3.0)	120 (48-193)	92 (48-160)[d]
≥9 lesions on T_2-weighted images at baseline, n (%)	NA	3 (19)	10 (63)	NA	NA
CSF/brain, *ex vivo*				**CSF, TCC**	
Cohort[f]	**Early MS**	**Late MS**	**Late NDC**	**Early MS**	**Late MS**
Patient, n (paired blood)	4 (4)	5 (5)	2 (2)	10 (4)	7 (7)[g]
Gender, female, n (%)	2 (50)	5 (100)	1 (50)	10 (100)	5 (71)
Age in years, median (IQR)[c]	32 (18-41)	62 (44-72)	78 (NA)	33 (23-38)	70 (60-82)
Follow-up time in years, median (IQR)	0.3 (0.3-0.5)	NA	NA	1.5 (0.6-5.5)	NA
Disease duration in months, median (IQR)[e]	3.8 (2.7-5.2)	NA	NA	3.8 (1.0-22.4)	NA
PMD in hours, median (IQR)	NA	8.5 (8.4-9.2)	6.1 (NA)	NA	8.6 (8.3-9.3)
pH CSF, median (IQR)	NA	6.3 (6.3-6.7)	6.5 (NA)	NA	6.5 (6.3-6.7)

[a]RRMS according to the McDonald (2010) criteria.
[b]Fourteen patients were included for in-depth analysis of Th17 subpopulations in blood. For three patients, Th subsets were only used for analysis of pro-inflammatory cytokine expression.
[c]At the time of sampling.
[d]At the time of pre-treatment sampling.
[e]Time from CIS diagnosis to sampling.
[f]Samples obtained from either CIS and RRMS patients ('early') or deceased patients with MS and non-demented control subjects ('late').
[g]Three patients were also used for *ex vivo* CSF/brain T cell analysis.
HC = healthy control; MS = multiple sclerosis; NA = not applicable or available; NAT = natalizumab; NDC = non-demented control; PMD = post-mortem delay; TCC = T cell culture.

IFN-β, one with both dimethylfumarate and fingolimod, one with glatiramer acetate and one with mitoxantron).

CSF with and without paired blood samples were obtained from 14 patients with early-stage MS (ErasMS) and nine with late-stage MS (Netherlands Brain Bank, Amsterdam). In the early-stage MS group, 10 patients were CIS at the time of lumbar puncture and four patients were diagnosed with RRMS within 6 months before sampling. The median time between sampling and the last clinical attack was 2.8 months (IQR: 1.3-5.8). Additional autopsied brain tissues were obtained from five patients with late-stage MS and two non-demented control subjects (Netherlands Brain Bank). All study protocols were

approved by the medical ethics committee of the Erasmus MC (Rotterdam) and VUmc (Amsterdam, The Netherlands). Written informed consent was obtained from all included patients and controls.

Mononuclear cell isolation from blood, CSF and brain tissue

Blood from patients and matched controls was collected using Vacutainer CPT tubes (BD Biosciences, Erembodegem, Belgium) containing sodium heparin. Peripheral blood mononuclear cells (PBMC) were isolated according to manufacturer's instructions. After centrifugation, cells were taken up in RPMI 1640 (Lonza, Verviers, Belgium) containing 40% fetal calf serum (FCS) and 20% dimethyl sulfoxide (Sigma-Aldrich, Saint-Louis, MO) and stored in liquid nitrogen until further use. Surplus CSF of patients with early-stage MS was obtained through lumbar puncture for diagnostic purposes. Blood and CSF samples from patients with late-stage MS were acquired post-mortem through heart puncture and ventricle drainage, respectively. Collection tubes with CSF were centrifuged for 10 min at 500g. Paired blood and blood from buffy coats were diluted in phosphate-buffered saline (PBS), after which mononuclear cells were isolated by density gradient centrifugation using Ficoll® Paque Plus (GE Healthcare, Freiburg, Germany). CSF and blood mononuclear cell fractions were resuspended in RPMI 1640 containing 10% heat inactivated human AB serum (Sanquin, Rotterdam, The Netherlands) and 1% Pen/Strep (Lonza) and left to rest at 37°C until further use. Brain tissue samples were processed and single-cell suspensions were obtained as described previously [25].

Short-term CSF and blood T-cell cultures

Short-term culturing of CSF-derived T cells was required to obtain sufficient cell numbers for fluorescence-activated cell sorting (FACS) and intracellular cytokine staining of the Th subsets of interest. CSF and blood T cells were cultured as previously described [26]. In short, mononuclear cell fractions were treated for 13 to 15 days with γ-irradiated feeder cells (10×10^6 PBMC and 1×10^6 EBV^- B-cell lines HAL-02 and RS-411), phytohemagglutinin-L (1 ng/ml; Sigma-Aldrich), IL-2 (25 U/ml; Erasmus MC) and IL-15 (12.5 ng/µl; Miltenyi Biotec, Leiden, The Netherlands) in RPMI 1640 containing L-glutamine (Lonza), 1% Pen/Strep and 10% heat-inactivated human AB serum. IL-2 and IL-15 were added every 3 to 4 days. Post-mortem CSF samples were re-stimulated using the same protocol.

Antibodies and flow cytometry

Multicolor flow cytometric analysis was performed using the following fluorochrome-labeled monoclonal anti-human antibodies: CD3 BV785 (SK7), CD8 FITC (SK1), CD45RA APC-H7 (HI100), HLA-DR FITC and BB515 (G46-6), VLA-4 BV711 and APC (9F10), CD45RO PerCP-Cy5.5 (UCHL1), CD25 BV605 and APC-R700 (2A3), CD226 BB515 (DX11), MCAM

PerCP-Cy5.5 (P1H12), PSGL-1 APC (KPL-1), GM-CSF BV421 and PE-CF594 (BVD2-21C11; all BD Biosciences), CD4 BV510 (OKT4), CD38 BV711 and PerCP-Cy5.5 (HIT2), CXCR3 BV421, PE-Cy7 and APC (G025H7), CCR6 PE (G034E3), CCR7 PE-CF594 (150503), CCR4 PE-Cy7 and PE-Dazzle (L291H4), CD161 BV605 (HP-3G10), IFN-γ BV421 and BV711 (4S.B3), and IL-17A APC (BL168; all Biolegend, London, UK). Cells were stained for 30 min at 4°C, measured with an LSRII-Fortessa flow cytometer and analyzed using FACSDiva 6.1.2 software (both BD Biosciences). Th1, Th17 and Th1-like Th17 cells were defined based on markers CCR6 and CXCR3 with and without the use of CCR4. For analyses without CCR4, total $CD4^+$ T cells were subdivided into $CCR6^-CXCR3^+$ (Th1), $CCR6^+CXCR3^-$ (Th17) and $CCR6^+CXCR3^+$ (Th1-like Th17) subsets. In each Th subset, the proportion of effector memory ($CCR7^-CD45RA^-$) and central memory ($CCR7^-CD45RA^+$) cells was analyzed. CCR4 was used as a marker to discriminate $CCR6^-CXCR3^+CCR4^-$ (Th1), $CCR6^+CXCR3^-CCR4^+$ (Th17), $CCR6^+CXCR3^+CCR4^-$ (Th17.1) and $CCR6^+CXCR3^+CCR4^+$ (Th17 double-positive, DP) subpopulations [27].

Intracellular cytokine staining

Th1 ($CCR6^-CXCR3^+$), Th17 ($CCR6^+CXCR3^-$), Th1-like Th17 ($CCR6^+CXCR3^+$), as well as $CCR6^-$ and $CCR6^+$ $CD8^+$ T cells were sorted from blood and CSF T cell memory pools ($CD3^+CD25^{-/int}CD45RO^+CD45RA^-$) using a BD FACSAria™ III cell sorter. Prior to isolation of Th memory subsets from buffy coats, $CD4^+$ cells were purified from the mononuclear cell fraction using CD4 microbeads and the autoMACS Pro Separator (both Miltenyi Biotec). Cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 1:2000) and ionomycin (1:500; both Sigma-Aldrich) for 5 h. GolgiStop™ (1:1500; BD Biosciences) was added during the last 2.5 h of stimulation. Stimulated cells were fixed and permeabilized using the BD Cytofix/Cytoperm™ kit (BD Biosciences) according to the provided protocol, and stained for IFN-γ, GM-CSF and IL-17A within the same tube.

RNA isolation and quantitative PCR

Sorted T-cell subsets were washed with PBS and resuspended in RNA lysis solution with 1% 2-ME. Total RNA was extracted using the GenElute™ Total RNA Purification kit (Sigma-Aldrich) and treated with DNase I (Invitrogen, Carlsbad, CA). Complementary DNA (cDNA) was synthesized from total RNA using a reaction mix containing Tris-aminomethane (200 mM), KCl (500 mM), $MgCl_2$ (0.2 M; Sigma-Aldrich), DTT (100 mM; Invitrogen), random hexamers (50 μM; Invitrogen), oligo(dT) 15 primer (100 μg/ml; Promega, Madison, WI), dNTP mix (10 mM; Promega), RNAsin® (40 U/μl; Promega) and superscript™ II (200 U/μl; Invitrogen). After incubation at 42°C for 50 min and inactivation at 99°C for 3 min, cDNA was diluted and stored at -20°C until use. For quantitative PCR, 0.2 μM forward and reverse primer (Sigma-Aldrich), 10 μM probe (Universal Probe Library; Roche Applied Science, Penzberg, Germany) and diluted cDNA were added to Taqman® Universal PCR Master Mix.

Target gene expression was measured using optimal primer/probe assays and Taqman® 7900HT (Applied Biosystems, Foster City, CA). We used the following thermal cycle protocol: 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. CT values were analyzed using SDS 2.4.1 software (Applied Biosystems). Expression levels of target genes were normalized using 18S rRNA as a reference. Primer sequences are provided in Supplementary Table 2.

T-cell transmigration assays

$CD4^+CD25^{-/int}$ memory T cells depleted from naive populations ($CCR7^+CD45RA^+$) were sorted by FACS and added at 2×10^5 cells/well to 3 µm pore size transwell plates (Corning, Amsterdam, The Netherlands). Migration of Th17 subsets towards medium or CXCL10 (900 ng/ml; R&D Systems, Abingdon, UK) was analyzed after 3 h incubation at 37°C using FACS. To assess trans-endothelial migration of Th17 subsets, migration experiments were performed across confluent monolayers of human brain endothelial cells (hCMEC/D3 cell line) [28] on collagen-coated 5 µm pore size Transwell® plate, as previously described [29]. In this system, 5×10^5 Th memory cells were added per well and migration was assessed after 4 h.

Statistical analyses

Statistical analyses were carried out using Graphpad Prism Software, version 5.04. We used the two-tailed Mann-Whitney U test to compare two independent groups and the Wilcoxon matched-pairs signed rank test to compare samples of the same persons. Correlations were tested using Spearman's rank. A logistic regression model was used to correct for MRI measurements in the multivariate analyses. Experimental data are depicted as mean and standard error of the mean. P-values < 0.05 were considered significant.

RESULTS

Low frequencies of Th1-like Th17 and not Th17 effector cells in CIS blood associate with rapid MS onset

To search for pro-inflammatory Th subsets that are critically involved in early diagnosis of CDMS, we used peripheral blood mononuclear cells at time of CIS from age- and gender-matched patients who remained monophasic for at least 5 years (CIS-CIS, n = 16) and from patients who experienced a second attack within 1 year (CIS-CDMS, n = 16; Table 1). Flow cytometric analysis of $CD4^+$ T cells showed decreased Th1-like Th17 ($CCR6^+CXCR3^+$) frequencies in the CIS-CDMS group compared to the CIS-CIS group (median: 5.9% versus 11.2%, $p = 0.011$; Fig. 1A). After correction for lesion load on MRI at baseline, using a logistic regression model, the association remains significant (OR: 0.78 per percent increase in

Th1-like Th17; $p = 0.026$). In CIS-CDMS, additional reductions in effector memory (EM) to central memory (CM) ratios were found for Th1-like Th17 (mean: 0.30 versus 0.50, $p = 0.005$; Fig. 1B). Similar but less strong reductions were observed for Th1 ($CCR6^{-}CXCR3^{+}$; 10.0% versus 12.5%, $p = 0.070$ and mean EM/CM ratio: 0.21 versus 0.29, $p = 0.021$). Frequencies and EM/CM ratios for Th17 ($CCR6^{+}CXCR3^{-}$) did not differ between CIS-CIS and CIS-CDMS (Fig. 1A and B). Th subset distribution in CIS patients was not affected after stratification for methylprednisolone treatment in the last 3 months prior to sampling (data not shown). Finally, Th1-like Th17 EM/CM ratios in CIS blood inversely correlated to anti-EBNA1 IgG titers ($p = 0.013$; Fig. 1C) and fatigue ($p = 0.001$; Fig. 1D), which were reported as independent predictive markers for early CDMS diagnosis [23, 30].

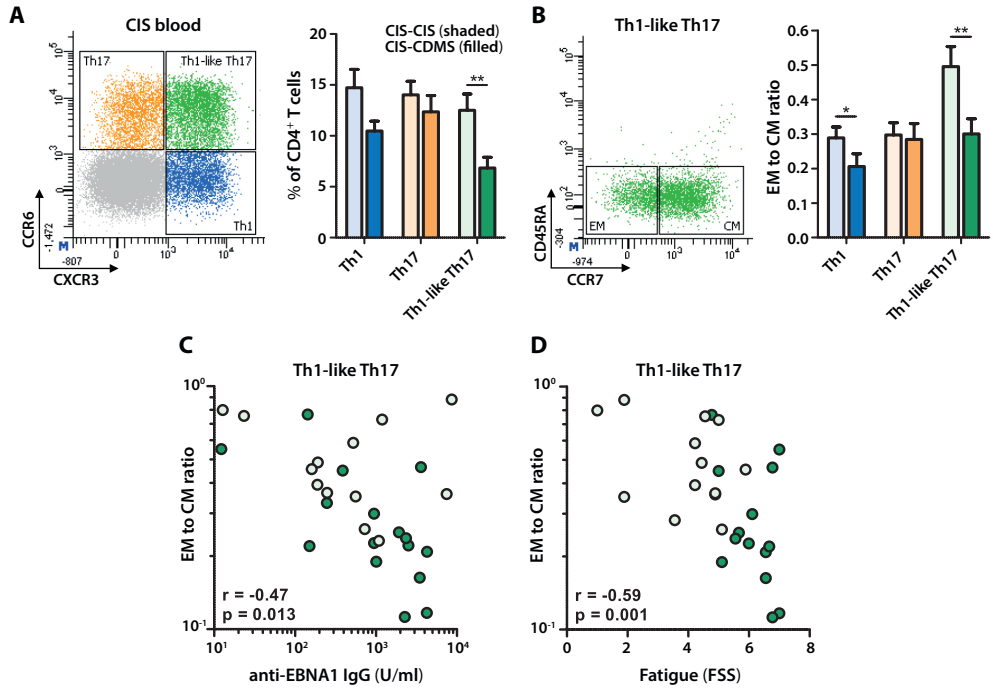

Figure 1. Reduction of Th1-like Th17 effector cells in the blood of CIS patients with short time to CDMS. CIS patients were selected based on blood sampling within 4 months after diagnosis and time between CIS and CDMS. 'CIS-CDMS' patients were diagnosed with CDMS within 1 year (n = 16; filled bars), while 'CIS-CIS' patients were not diagnosed with CDMS for at least 5 years (n = 16; shaded bars). $CD4^{+}$ T cells in the blood were compared for (**A**) Th1 ($CCR6^{-}CXCR3^{+}$), Th17 ($CCR6^{+}CXCR3^{-}$) and Th1-like Th17 ($CCR6^{+}CXCR3^{+}$) cell distribution, as well as (**B**) effector memory (EM; $CCR7^{-}CD45RA^{-}$) to central memory (CM; $CCR7^{+}CD45RA^{-}$) cell ratios within each of these subsets, as determined by flow cytometry. Th1-like Th17 effector to central memory cell ratios were correlated to reported predictors of early CIS to CDMS transition, anti-EBNA1 IgG blood titer (**C**) and fatigue severity scale (FSS; **D**). * $p < 0.05$; ** $p < 0.01$.

Effector populations of highly activated Th1-like Th17 cells are reduced in blood after MS diagnosis

To verify that these selective differences in Th subsets are associated with MS diagnosis, we explored total frequencies of blood Th1 EM and Th1-like Th17 EM cells in treatment-naive RRMS patients (n = 18, Table 1), and age-/gender-matched healthy controls (n = 19). Strongly reduced frequencies were found for both these subsets in RRMS (median: 1.1% and 0.7%) compared to CIS-CIS (1.9%, $p < 0.001$ and 2.8%, $p < 0.0001$ respectively) and healthy controls (2.8%, $p < 0.001$ and 3.3%, $p < 0.0001$ respectively; Fig. 2A). These frequencies did not significantly differ between the RRMS and CIS-CDMS (1.0% and 1.3%, respectively) group. In RRMS, a significant proportion of blood Th1-like Th17 cells was positive for both CD38 and HLA-DR (Fig. 2B), indicating a highly activated phenotype after MS diagnosis. This was not seen for Th1 cells (Fig. 2B). These data suggest that Th1-like Th17 effector cells are selectively activated in the periphery and recruited to the CNS during MS onset.

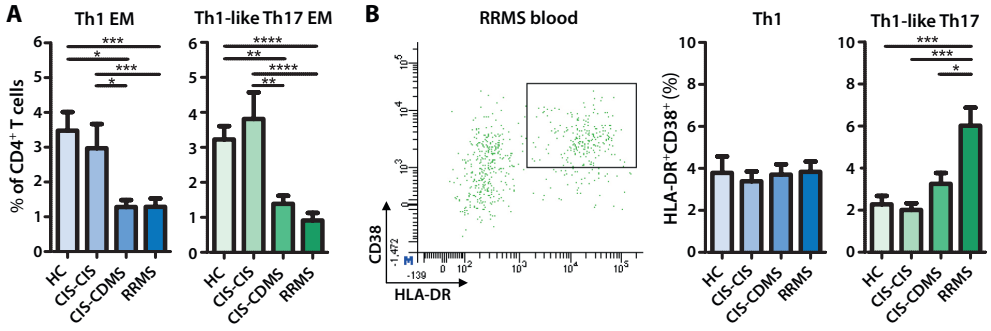

Figure 2. Th1-like Th17 effector cells are highly activated and less present in the blood after MS diagnosis. (**A**) Th1 and Th1-like Th17 effector memory (EM) frequencies in $CD4^+$ T cells from CIS-CIS (n = 14) and CIS-CDMS (n = 16) as well as RRMS (n = 18) and both age- and gender-matched healthy control (HC; n = 19) blood. (**B**) Highly activated fractions of blood Th1 and Th1-like Th17 cells in CIS-CIS (n = 14), CIS-CDMS (n = 16) and RRMS (n = 18) patients as well as HC (n = 19), as defined by co-expression of late T-cell activation markers HLA-DR and CD38. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Predominant expression of T-bet, RORγt, IFN-γ and GM-CSF, but not IL-17A by activated blood Th1-like Th17 cells

Human $CCR6^+CD4^+$ T cells are not only strong producers of IL-17, but also express IFN-γ and GM-CSF [9, 27]. To explore how these pro-inflammatory cytokines are co-regulated in our phenotypically defined Th1-like Th17 ($CCR6^+CXCR3^+$) cells, T-bet and RORγt, as well as IFN-γ, GM-CSF and IL-17A expression was compared to paired Th1 ($CCR6^-CXCR3^+$) and Th17 ($CCR6^+CXCR3^-$) populations from healthy blood donors. Th1-like Th17 expressed both *TBX21*/T-bet and *RORC*/RORγt mRNA at higher levels than Th1 ($p = 0.016$ and $p = 0.004$)

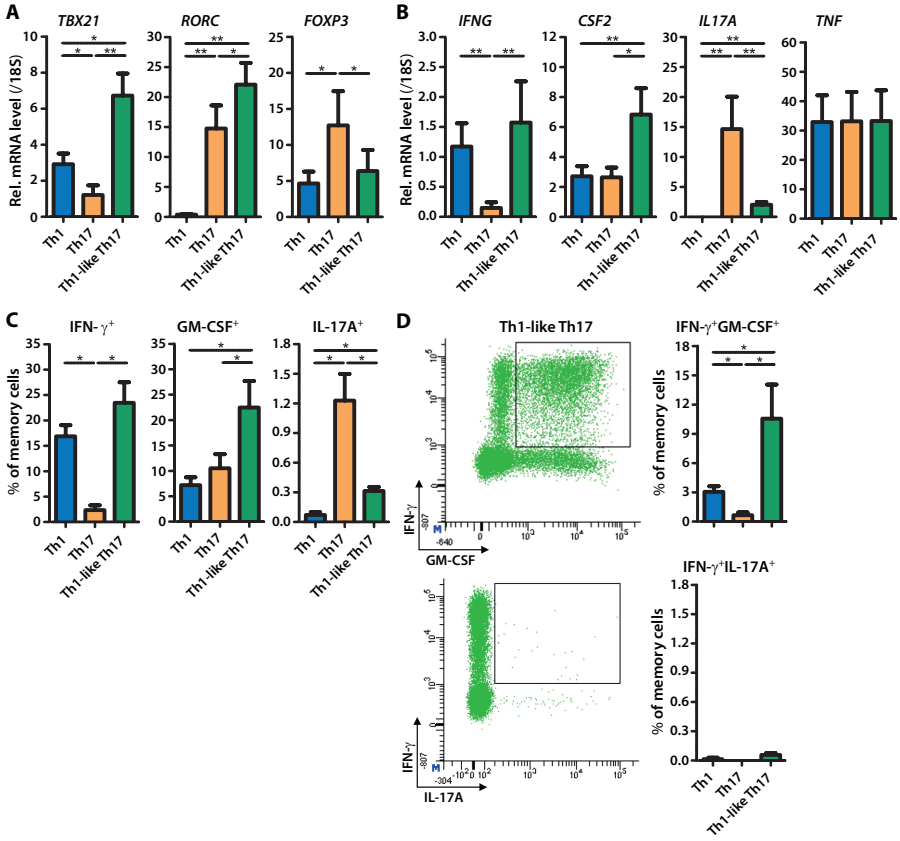

Figure 3. Blood Th1-like Th17 cells predominantly express T-bet and RORγt, and are high IFN-γ and GM-CSF, but low IL-17A producers.

Buffy coats from nine healthy blood donors were used to assess gene expression of *TBX21*, *RORC* and *FOXP3* (**A**), as well as *IFNG*, *CSF2*, *IL17A* and *TNF* (**B**) in sorted Th1 ($CCR6^-CXCR3^+$), Th17 ($CCR6^+CXCR3^-$) and Th1-like Th17 ($CCR6^+CXCR3^+$) memory populations. Cells were stimulated with PMA and ionomycin and mRNA levels were measured using quantitative PCR. (**C**) Flow cytometric analysis of intracellular IFN-γ, GM-CSF and IL-17A expression in PMA- and ionomycin-stimulated Th1, Th17 and Th1-like Th17 memory cells from the same blood donors (n = 7). (**D**) Representative dot plots and quantification of co-expression of IFN-γ with GM-CSF and IL-17A in Th1-like Th17 memory cells (n = 7). * $p < 0.05$; ** $p < 0.01$.

and Th17 ($p = 0.008$ and $p = 0.039$, respectively; Fig. 3A). *IFNG*/IFN-γ mRNA levels were similar between Th1 and Th1-like Th17, while *CSF2*/GM-CSF mRNA levels in Th1-like Th17 were higher than in Th1 ($p = 0.008$) and Th17 ($p = 0.020$). Th1-like Th17 cells only moderately expressed *IL17A* mRNA (Fig. 3B). Differences in IFN-γ, GM-CSF and IL-17A expression were verified at the protein level (Fig. 3C). The percentage of GM-CSF-positive cells was 2- to 3-fold higher in Th1-like Th17 than in Th1 and Th17. IL-17A-positive cells were ~4-fold less present in Th1-like Th17 compared to Th17. At the single-cell level, IFN-γ was mainly

co-expressed with GM-CSF and not with IL-17A in Th1-like Th17 cells (Fig. 3D). These cytokine profiles were the same for Th subsets from CIS and RRMS blood (data not shown). *TNF* expression was comparable between Th1, Th17 and Th1-like Th17 subsets (Fig. 3B). CD226 was higher, while CD25 and FoxP3 were lower expressed by Th1-like Th17 as compared to Th17 (Fig. 3A and Supplementary Table 3), which supported their pro-inflammatory potential [31, 32]. Th1-like Th17 cells also showed sustained CD161 expression (Supplementary Table 3), reflecting an ex-Th17 phenotype [33].

Pro-inflammatory Th1-like Th17 cells are abundant in CSF T cell cultures of MS patients

To explore the local pro-inflammatory capacity of Th1-like Th17 cells in early MS, CSF (n = 10) and paired blood (n = 4) T cell subsets from early-stage MS patients (Table 1) were analyzed after short-term culturing. Th1 and Th1-like Th17 were the main populations in the CSF $CD4^+$ T cell pool (Fig. 4A). Proportions of Th1-like Th17 were higher than those of Th1 ($p = 0.020$) and their equivalents in blood (2- to 3-fold increase; Fig. 4B). Similar results were obtained with EM/CM ratios, which were high for both subsets but most prominent in Th1-like Th17 cells in CSF (Fig. 4C). Within the total CSF T cell pool, Th1-like Th17 cells were enriched and co-produced more IFN-γ and GM-CSF compared to $CCR6^-$ and $CCR6^+$ $CD8^+$ T cells, and paired blood counterparts (Fig. 4D to G). The percentage of IFN-γ-positive cells was increased in CSF Th1-like Th17 versus Th1 (Fig. 4E). The enrichment of Th1-like Th17 in CSF compared to blood (Fig. 4A and B) was also found in T cell cultures from late-stage MS patients (n = 7, $p = 0.016$; Table 1 and Fig. 4H), suggesting that Th1-like Th17 recruitment to the CNS also occurs at later stages of the disease.

Ex vivo Th1-like Th17 cells are enriched in the CNS and accumulate in the blood after natalizumab treatment

To confirm their recruitment to the CNS, we compared *ex vivo* Th1 and Th1-like Th17 frequencies in single-cell suspensions of 10 brain tissues and paired CSF and blood samples of five patients with late-stage MS (Table 1, Fig. 5A and B). Th1 and Th1-like Th17 cells were over-represented, while Th17 cells were hardly seen in MS brain tissues and CSF, in contrast to blood. The enrichment of Th1-like Th17 was also found in CSF, but was less in brain tissues from two non-demented controls (Fig. 5B), suggesting that enhanced infiltration of Th1-like Th17 cells into the brain parenchyma is associated with MS [34-36].

In addition to chemokine receptors and pro-inflammatory cytokines [6, 7], adhesion molecules play a key role in migration of peripheral Th cells into the CNS, including VLA-4, MCAM and PSGL-1 [37]. Interestingly, VLA-4 but not MCAM and PSGL-1 was the most abundant on Th1-like Th17 cells (Supplementary Table 3). In patients with RRMS (n = 14), blood Th1-like Th17 proportions were elevated after 6 months of treatment with natalizumab

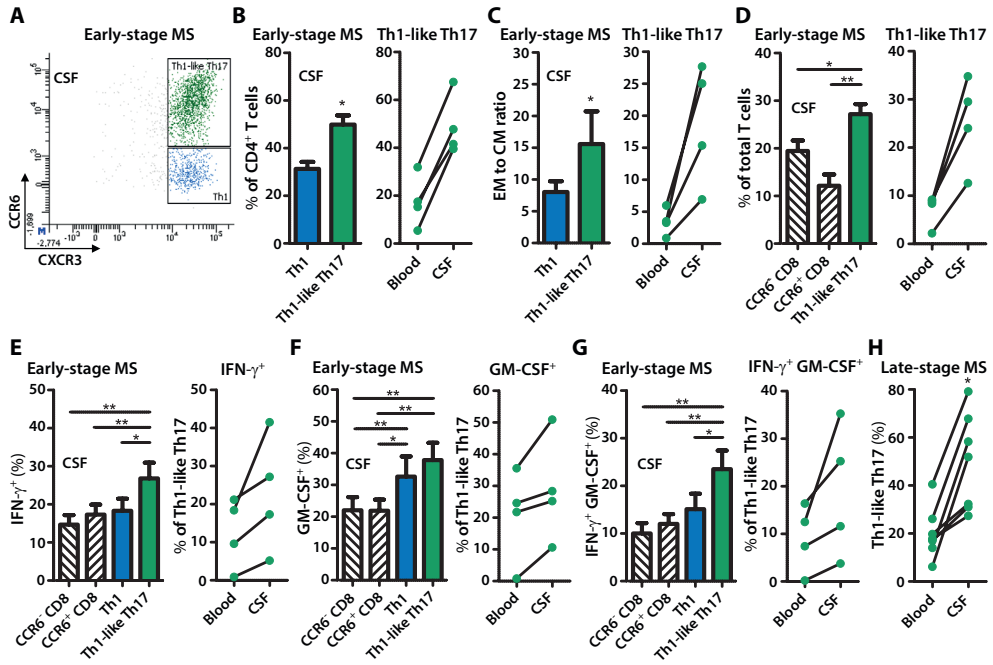

Figure 4. Prevalence of pro-inflammatory Th1-like Th17 cells in CIS and MS CSF compared to blood T cell cultures.

(**A-D**) Presence of Th1-like Th17 ($CCR6^+CXCR3^+$) subsets in short-term CSF T cell cultures from 10 early-stage MS patients (CIS, n = 7; RRMS, n = 3). CSF Th1-like Th17 were compared to Th1 cells and their equivalents in blood for percentages in the total $CD4^+$ T cell pool (**A** and **B**) and for effector memory (EM) to central memory (CM) ratios (**C**) from the same patients. Similar analyses were performed for CSF Th1-like Th17 and both $CCR6^-$ and $CCR6^+$ $CD8^+$ T cell subsets within the total T cell pool (**D**). These T cell subsets were separated, stimulated with PMA and ionomycin and assessed for intracellular expression of (**E**) IFN-γ, (**F**) GM-CSF and (**G**) IFN-γ with GM-CSF. For each analysis, Th1-like Th17 subsets were compared between paired CSF and blood T cell cultures. (**H**) Th1-like Th17 frequencies in T cell cultures from paired blood and CSF of late-stage MS patients (n = 7). * $p < 0.05$; ** $p < 0.01$.

(anti-VLA-4 monoclonal antibody; median pre- versus post-treatment: 7.7% versus 10.4%, $p = 0.006$; Fig. 5C and Table 1). Th1-like Th17 cells did not show differences in EM/CM ratio (data not shown), but their activation state (see also Fig. 2B) was significantly reduced after natalizumab therapy (Fig. 5D). Th1-like Th17 showed increased capacity to produce IFN-γ and GM-CSF in post- versus pre-treatment blood samples (Fig. 5E and F). These results show that the effects of natalizumab in MS are associated with an accumulation of Th1-like Th17 cells in the blood.

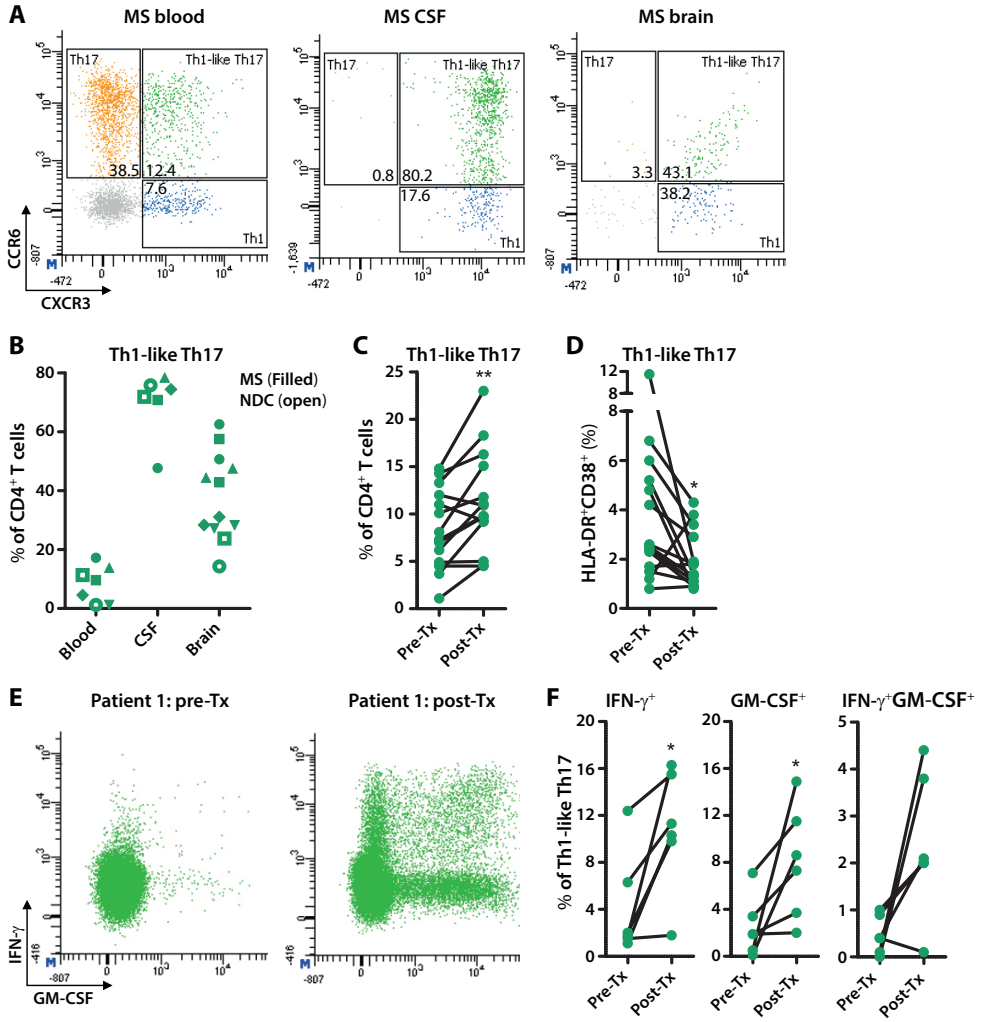

Figure 5. Th1-like Th17 recruitment to the CNS and targeting by natalizumab in MS patients.
(**A**) Presence of Th1 ($CCR6^{-}CXCR3^{+}$), Th17 ($CCR6^{+}CXCR3^{-}$) and Th1-like Th17 ($CCR6^{+}CXCR3^{+}$) cells in single-cell suspensions from brain tissue, CSF and blood of a patient with MS, as determined by FACS. (**B**) Th1-like Th17 frequencies in 10 brain tissues and paired CSF and blood samples from five different MS patients (filled shapes). Similar analyses were performed for two non-demented controls (NDC; open shapes). Each shape represents a different donor. For Th1-like Th17 cells in MS blood, frequencies (n = 14; **C**), activation (n = 14; **D**) as well as pro-inflammatory capacities (n = 6; **E** and **F**) were determined before and 6 months after natalizumab treatment. T-cell activation was assessed by surface expression of both HLA-DR and CD38. To determine their pro-inflammatory capacity, Th1-like Th17 memory cells were isolated from pre- and post-treatment blood, stimulated with PMA and ionomycin, and stained for intracellular IFN-γ and GM-CSF. * $p < 0.05$; ** $p < 0.01$.

Targeting of VLA-4^{high} Th17.1 cells by natalizumab in MS patients who respond to treatment

To assess the selectivity of natalizumab effects on pro-inflammatory Th populations in MS patients, CCR4 was included as a surface marker in our flow cytometric panels for subdivision of Th1-like Th17 into recently described pathogenic Th17.1 (IFN-γ^{high}GM-CSF^{high}IL-17^{low}) and Th17 double-positive (DP; IFN-γ^{low}GM-CSF^{low}IL-17^{int}) subpopulations [27, 38]. Th17.1 (CCR6^{+}CXCR3^{+}CCR4^{-}) frequencies were significantly increased in RRMS blood samples after both 6 and 12 months of treatment (median: 5.3% and 6.1%) versus pre-treatment (3.7%; n = 14, both p = 0.0002; Table 1; Fig. 6A and B). No significant differences were found in Th1 (CCR6^{-}CXCR3^{+}CCR4^{-}), Th17 (CCR6^{+}CXCR3^{-}CCR4^{+}) and Th17 DP (CCR6^{+}CXCR3^{+}CCR4^{+}) cells (Fig. 6A and B). Importantly, this accumulation of Th17.1 was most pronounced in natalizumab-treated patients who were free of clinical relapses (n = 9; pre-treatment, 3.8% versus 6m post-treatment, 6.5% and 12m post-treatment, 6.8%; p = 0.008 and p = 0.004, respectively). As compared to patients who had relapses during treatment (n = 5; pre-treatment, 3.2% versus 6m post-treatment, 4.0% and 12m post-treatment, 4.1%; Fig. 6C). The accumulation of Th17.1 cells in the blood of clinical responders was validated using a second cohort (Supplementary Table 1; Supplementary Fig. 1A).

In pre-treated RRMS blood, VLA-4 surface expression on Th17.1 (mean MFI: 2603) was the highest of all pro-inflammatory Th subsets analyzed, including Th1 (MFI: 1328, p = 0.001), Th17 (MFI: 1255, p = 0.0001) and Th17 DP (MFI: 2038, p = 0.002; Fig. 6D and Supplementary Fig. 1B). After natalizumab treatment, VLA-4 was downregulated on all subsets analyzed, but this was most prominent for Th17.1 (mean reduction: 6m post-treatment, 56%, 12m post-treatment, 58%), as compared to Th1 (6m post-treatment, 52%, p = 0.038; 12m post-treatment, 54%, p = 0.005), Th17 (6m post-treatment, 37%, p = 0.003; 12m post-treatment, 38%, p = 0.0009) and Th17 DP (6m post-treatment, 49%, p = 0.002; 12m post-treatment, 50%, p = 0.002; Fig. 6E). This indicates that Th17.1 cells are preferentially targeted by natalizumab treatment, preventing their transmigration into the CNS of MS patients.

Pathogenic Th17.1 cells have a superior capacity to transmigrate into the CNS in early MS

To study the CNS transmigration potential of Th17.1 in MS further, we performed different *in vitro* transwell migration assays using total Th memory cell fractions. Th17.1 was the main Th17 subpopulation migrating across transwell filters towards inflammatory mediator CXCL10 [39] (Fig. 7A and B). No migration was observed towards medium only (data not shown). Particularly Th17.1 cells did show spontaneous transmigration across human brain endothelial layers (hCMEC/D3), which was enhanced under CXCL10-attracting conditions (Fig. 7C and D).

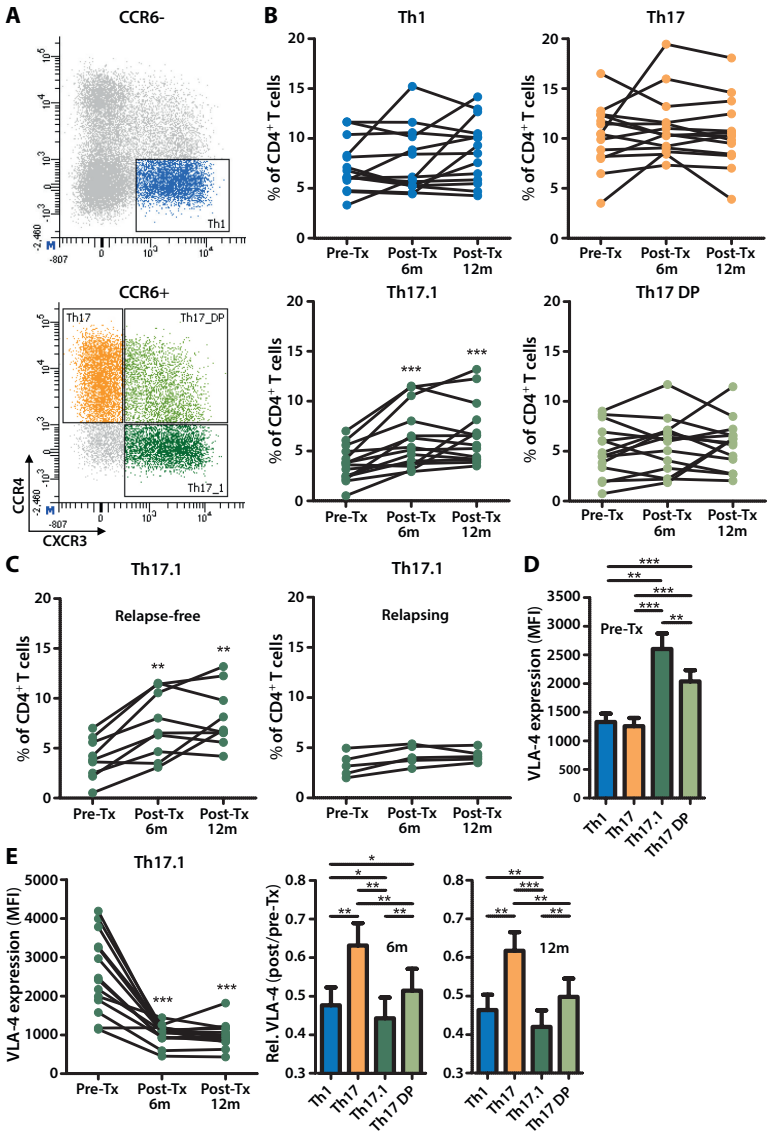

Figure 6. Selective accumulation of Th17.1 cells in natalizumab-treated MS patients who do not experience clinical relapses.

Using CCR4 as an additional marker, Th1-like Th17 cells were subdivided into Th17.1 ($CCR6^+CXCR3^+CCR4^-$) and Th17 DP ($CCR6^+CXCR3^+CCR4^+$) subsets and analyzed in natalizumab-treated RRMS patients by flow cytometry (**A**). Th1 ($CCR6^-CXCR3^+CCR4^-$), Th17 ($CCR6^+CXCR3^-CCR4^+$), Th17.1 and Th17 DP cells were monitored in pre- and both 6 and 12 months post-treatment blood samples (n = 14; **B**). Th17.1 proportions were separately evaluated in relapse-free (n = 9) and relapsing (n = 5) treatment groups (**C**). VLA-4 surface expression levels were determined on these Th subpopulations before (**D**) and both 6 and 12 months after (**E**) natalizumab treatment. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. MFI = mean fluorescent intensity; Tx = treatment.

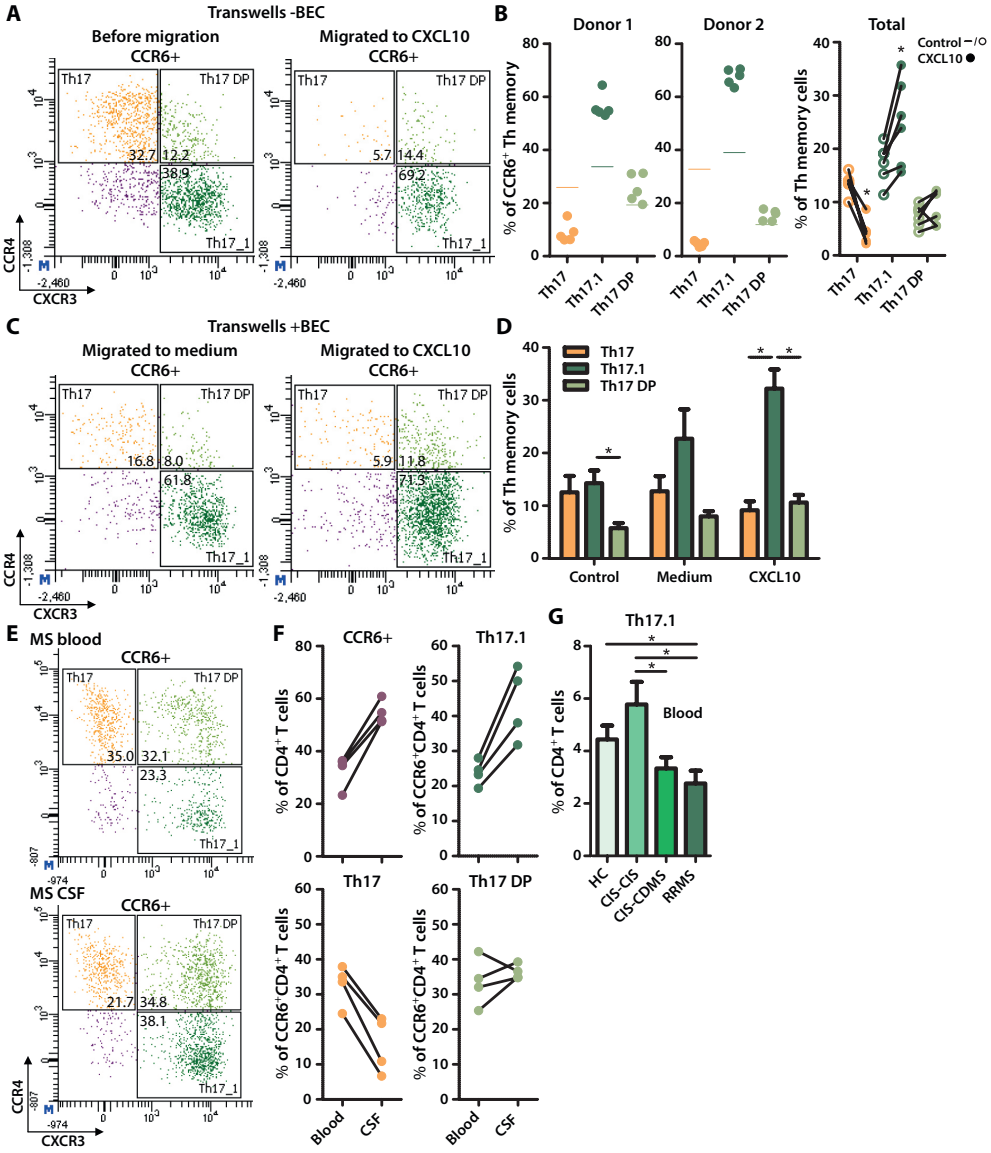

Figure 7. Enhanced CNS transmigration potential of Th17.1 cells and their recruitment to CSF in early MS. Total Th memory cells were sorted from healthy blood and used to assess the *in vitro* transmigration capacities of Th17, Th17.1 and Th17 DP cells across transwell membranes (**A** and **B**; n = 6) and monolayers of human brain endothelial cells (BEC; **C** and **D**, n = 4) towards CXCL10. Each experiment was performed in quintuplicate. Th17 subset distribution was assessed before ('control') and after migration towards medium or CXCL10 using FACS. (**E** and **F**) *Ex vivo* Th17, Th17.1 and Th17 DP frequencies of CCR6+ Th cells in paired CSF and blood from four early MS patients. (**G**) The presence of Th17.1 cells in blood samples from 21 CIS-CIS, 26 CIS-CDMS and 13 treatment-naive RRMS patients, as well as 12 healthy controls (HC), as determined by FACS. * $p < 0.05$.

In addition, *ex vivo* flow cytometric analysis revealed an enrichment of Th17.1 versus Th17 and Th17 DP cells in CSF versus paired blood samples from four patients with early-stage MS (three CIS and one RRMS; Table 1; Fig. 7E and F). Consistently, lowered Th17.1 frequencies were found in the blood from 26 CIS-CDMS versus 21 CIS-CIS patients (p = 0.019), as well as 13 RRMS patients versus 12 matched healthy controls (p = 0.031; Fig. 7G and Supplementary Table 1). Both Th17.1 and Th17 DP cells were abundant in CSF compared to blood from late-stage MS patients (Supplementary Fig. 2).

Finally, to confirm that Th17.1 is a distinct pro-inflammatory Th17 subset, we evaluated the expression of key regulators of Th17 differentiation and pathogenicity. Along with VLA-4 (see also Fig. 6D), CD161, CD226, *ABCB1* (MDR1), *IL23R*, *STAT4*, *FCMR* (TOSO) and *GZMB* (granzyme B; all upregulated), as well as CD25 and *BATF* (downregulated) were discriminative markers for Th17.1 (Supplementary Table 3 and Supplementary Fig. 3). The abundant expression of T-bet, RORγt, IFN-γ and GM-CSF in Th17.1 cells (Supplementary Fig. 4) confirmed the pronounced Th1 features of this Th17 subset [27].

Collectively, these data demonstrate the propensity of Th17.1 cells to recruit to the CNS and mediate disease activity in early MS.

DISCUSSION

In this study, we demonstrate that IFN-γ- and GM-CSF-expressing Th1-like Th17 ($CCR6^+CXCR3^+$) cells are selectively associated with early disease activity in patients with MS. During disease onset, highly activated and effector memory Th1-like Th17 cells are markedly reduced in the peripheral blood and represents the main pro-inflammatory T-cell population within CSF. This local recruitment seemed to be preferentially targeted by natalizumab treatment to prevent subsequent MS relapses, since a Th1-like Th17 subpopulation termed Th17.1, and no other Th subsets, predominantly accumulated in the blood of relapse-free patients. The current work provides in-depth insights into the pro-inflammatory capacity of distinct $CCR6^+$ Th subpopulations during the course of MS [9], and offers new possibilities to fine-tune currently approved T-cell directed treatment for patients with MS.

The use of both CCR6 and CXCR3 as discriminating markers for Th17 cells does not only reflect their pro-inflammatory state, but also their capability to migrate into local inflammatory sites. Previous studies on Th17 cells in experimental autoimmune encephalomyelitis and MS primarily focused on single expression of CCR6 [9], or IL-17, which is increased in blood and is further upregulated in CSF during a relapse [17]. Here, we demonstrated that additional expression of CXCR3 subdivides human $CCR6^+$ Th17 into high ($CXCR3^-$) and low ($CXCR3^+$) producers of IL-17A. In these $IL\text{-}17^{low}$ producers, which were over-represented in

early-stage MS CSF compared to blood T cell cultures, GM-CSF is the major pro-inflammatory cytokine expressed together with IFN-γ. This is likely caused by their elevated levels of T-bet, and not RORγt, as previously reported for human Th cells [11]. The association of Th1-like Th17 (T-bet-dependent) and not Th17 (RORγt-dependent) with a short time to CDMS diagnosis is supported by the expression of T-bet, and not RORγt in $CD4^+$ T cells during rapid MS onset [40]. Th1-like Th17 cells were also highly activated after MS diagnosis, which links to the important role of $CD4^+$ T-cell activation in CIS progression [3]. This suggests that during MS disease onset, the loss of T regulatory function [41] results in the activation of peripheral Th1-like Th17 subsets, which infiltrate the CNS to mediate local inflammation. Indeed, memory Th cells of relapsing MS patients were more capable of differentiating into Th1-like Th17 cells, albeit co-producing IFN-γ and IL-17 [35]. These Th cells were cultured in the presence of IL-23, prompting RORγt and subsequently IL-17 expression [11]. In our CCR6- and CXCR3-based approach, we defined pro-inflammatory cytokine profiles of Th17 and Th1-like Th17 populations directly from the blood. This could explain why we identified IFN-γ/GM-CSF- and not IL-17-producing Th17 cells as the most pro-inflammatory subset in early MS, and also agrees with the minimal influence of IL-17 and strong impact of GM-CSF on experimental autoimmune encephalomyelitis induction [13, 42, 43]. For proper analysis of cytokine production by Th1-like Th17 cells in CSF, we had to add IL-2 to short-term T cell cultures, inducing GM-CSF expression [11]. Th1-like Th17 subsets co-produced more IFN-γ and GM-CSF than other T cell subsets in CSF and their counterparts in blood. Our finding that pro-inflammatory Th1-like Th17 and especially Th17.1 cells were highly enriched in CSF of patients with early-stage MS is in line with their reduced frequencies in the blood (this study), and the increased CSF CD4 to CD14 ratios in CIS patients with a short time to CDMS [44]. Consistent with *in situ* observations in MS brain tissue [35], a small fraction of blood and CSF Th1-like Th17 and Th17.1 cells did co-produce IFN-γ and IL-17, but this was considerably less than their co-production of IFN-γ and GM-CSF. Besides Th17.1, Th17 DP ($IL\text{-}17^{int}$) cells were also enriched in CSF of patients with late-stage MS, suggesting that local IL-17 production is mainly involved in disease progression. Nevertheless, the predominance of Th1-like Th17 cells in MS CSF and brain tissues as observed in this study corresponds to more recent findings that CNS inflammation in MS is largely mediated by infiltrating IFN-γ- and not IL-17-producing Th cells [34, 36, 45]

Th1-like Th17 cells contain several features promoting their selective intrusion into the CNS, although local Th17 plasticity cannot be completely ruled out [46]. Th1-like Th17 cells produce high levels of IFN-γ, triggering CXCL10 expression by endothelial cells to favor CXCR3-mediated migration into the CNS [36, 47], and thereby MS disease activity [48], which is supported by our *in vitro* and *ex vivo* transmigration results. Additional expression of GM-CSF by this subset may further dysregulate the blood-brain barrier, as described for monocytes [49]. Prior to their extravasation, Th17 cells make use of distinct molecules

involved in the rolling on and adhesion to endothelial cells, which are activated by pro-inflammatory cytokines and chemokines [37, 50, 51]. One of these molecules is the α4β1-integrin VLA-4, which is targeted by natalizumab to cause a strong reduction of lymphocytes in MS CSF [52]. In addition to previous work [53], we now show that only a particular Th1-like Th17 subpopulation termed Th17.1 accumulates in the blood from MS patients who clinically respond to natalizumab treatment. These selective effects may thus be useful for predicting freedom from MS activity [19], and understanding the potential lethal MS rebounds that occur in patients who have to stop this treatment due to increased risk of progressive multifocal leukoencephalopathy (PML) [54, 55]. MS rebounds are characterized by a rapid influx of pro-inflammatory cells into the CNS to cause excessive inflammation, potentially resulting in PML-immune reconstitution inflammatory syndrome (IRIS) [56]. Although not proven yet, the marked accumulation of Th17.1 in natalizumab-treated MS blood puts forward their transmigration into CNS as a critical process during these complications. Out of all pro-inflammatory Th subsets defined by CCR6, CXCR3 and CCR4, Th17.1 revealed the strongest VLA-4 surface expression levels in MS blood, which explains their restricted targeting by natalizumab. Consistent with our results, VLA-4 levels were found to be higher on Th17 than on Th1 cells in MS patients, probably mediating their trafficking into the CNS [17]. However, when we compared individual Th17 subpopulations, i.e. CCR6^{+} Th17 (CXCR3^{-}CCR4^{+}; IL-17^{high}), Th17 DP (CXCR3^{+}CCR4^{+}; IL-17^{dim}) and Th17.1 (CXCR3^{+}CCR4^{-}; IL-17^{low}) [27], VLA-4 surface expression seemed to be inversely associated with their ability to produce IL-17, as also described for mice [57]. The predominant expression of VLA-4 on Th17.1 cells closely parallels the dependence of IFN-γ- and not IL-17-producing Th cells on this integrin for their entry into the CNS during experimental autoimmune encephalomyelitis [57, 58]. However, adhesion molecules other than VLA-4 must be taken into account for alternative transmigration routes of pro-inflammatory Th17 cells as well [50], especially considering the rebound effects after natalizumab discontinuation in MS.

This cross-sectional study exemplifies that a more refined evaluation of chemokine surface receptors, pro-inflammatory cytokines and adhesion molecules is warranted to better understand the contribution of human Th1 and Th17 to MS and other autoimmune and neuroinflammatory diseases. Based on CCR6/CXCR3, IFN-γ/GM-CSF and VLA-4 expression, we identify Th1-like Th17 as a clinically relevant CD4^{+} T cell population during disease onset and treatment in MS patients. Future work on the localization and antigen specificity of these subsets in human brain lesions will be critical to determine their local impact on myelin and axonal loss in MS. The prominent association of Th1-like Th17 cells, in particular Th17.1, with MS activity suggests the possibility for more specific T cell-targeted therapies, and pleads for further assessment of the use of natalizumab earlier in the disease course of MS [59].

FUNDING

This work was made possible by the support of the Dutch MS Research Foundation (15-490d MS). We thank the Zabawas Foundation for additional financial support.

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SUPPLEMENTAL DATA

Supplementary Table 1. Characteristics of patients and controls in additional cohorts

Blood, *ex vivo*					
Cohort	**HC**	**CIS-CIS**	**CIS-CDMS**	**RRMS, no treatment**	**RRMS, NAT treatment**
Patient, n	12	21[a]	26[a]	13	9[b]
Gender, female, n (%)	8 (67)	15 (71)	21 (81)	10 (77)	5 (56)
Age in years, median (IQR)[c]	33 (28-48)	35 (29-39)	33 (27-36)	45 (37-54)	28 (21-43)[d]
Follow-up time in years, median (IQR)	NA	7.0 (6.1-7.3)	3.7 (2.5-5.8)	NA	NA
Disease duration in months, median (IQR)[e]	NA	2.4 (1.3-3.8)	2.6 (1.4-3.4)	113 (38-130)	33 (24-57)[d]
≥9 lesions on T_2-weighted images at baseline, n (%)	NA	6 (29)	13 (50)	NA	NA

[a]14 CIS-CIS and 16 CIS-CDMS were also included in the screening cohorts (see Table 1).
[b]2 natalizumab-treated RRMS patients were also used for analysis of pro-inflammatory cytokine expression only (Table 1).
[c]At the time of sampling.
[d]At the time of pre-treatment sampling. RRMS according to the McDonald 2010 criteria.
[e]Time from CIS diagnosis to sampling.
HC = healthy control; MS = multiple sclerosis; NA = not applicable or available; NAT = natalizumab.

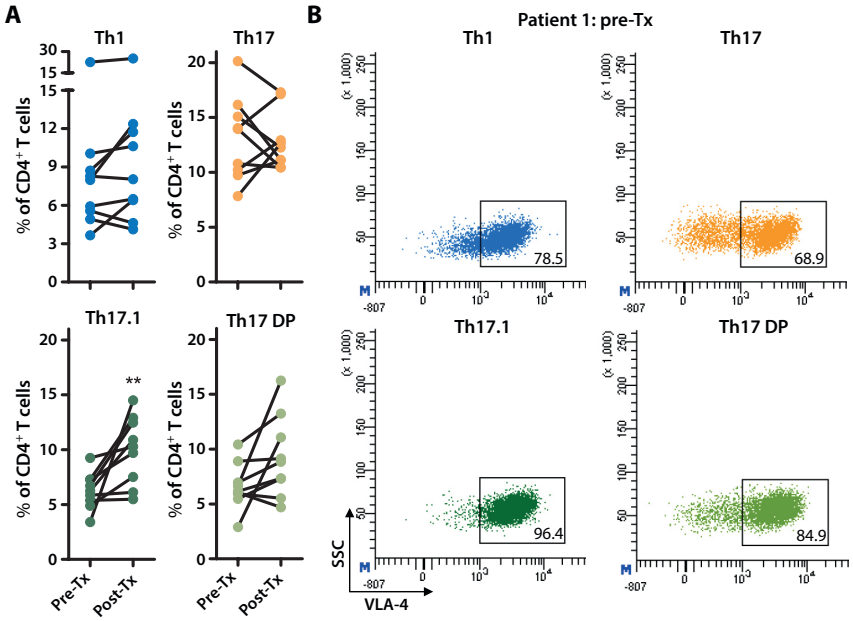
Supplementary Table 2. qPCR primer sequences

Gene	Forward primer	Reverse primer
ABCB1	GGAAATTTAGAAGATCTGATGTCAAAC	CACTGTAATAATAGGCATACCTGGTC
BATF	ACACAGAAGGCCGACACC	CTTGATCTCCTTGCGTAGAGC
CSF2	TCTCAGAAATGTTTGACCTCCA	GCCCTTGAGCTTGGTGAG
FCMR	GAACCTTCCTGCCATCCA	GAGCCATAGTCCAGTGCTCTC
FOXP3	ACCTACGCCACGCTCATC	TCATTAAGTGTCCGCTGCT
GZMB	CGGTGGCTTCCTGATACAA	CCCCAAGGTGACATTTATGG
IFNG	GGCATTTTGAAGAATTGGAAAG	TTTGGATGCTCTGGTCATCTT
IL17A	TGGGAAGACCTCATTGGTGT	GGATTTCGTGGGATTGTGAT
IL23R	CCTGGCTCTGAAGTGGAATTA	GGCTATTACTGCATCCCATTG
RORC	AGAAGGACAGGGAGCCAAG	CAAGGGATCACTTCAATTTGTG
STAT4	CCAATGGGAGTCTCTCAGTAGAA	TGTGACAGCCCTCATTTCCT
TBX21	GTCCAACAATGTGACCCAGA	AAAGATATGCGTGTTGGAAGC
TNF	CAGCCTCTTCTCCTTCCTGAT	GCCAGAGGGCTGATTAGAGA

Supplementary Table 3. Expression of surface markers on distinct Th subpopulations

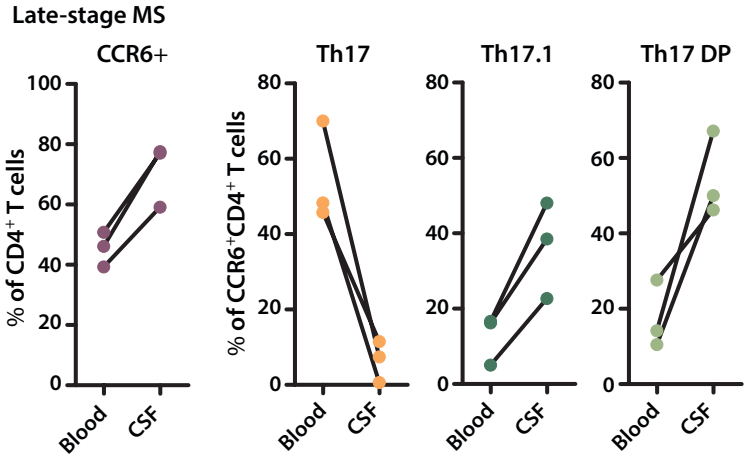
Surface marker	Cohort	CD4+ Th subpopulations						
CD25	n=10	6-3+	6+3-	6+3+		6-3+4-	6+3-4+	6+3+4-	6+3+4+
% positive cells	HC	9.6 ± 0.8	28.1 ± 3.0	19.8 ± 1.7		6.9 ± 0.7	33.9 ± 3.1	13.9 ± 1.8	24.8 ± 2.0
	RRMS	10.3 ± 0.7	31.1 ± 1.7	20.3 ± 1.7		7.9 ± 1.0	36.6 ± 1.3	11.9 ± 1.0	26.1 ± 1.9
MFI positive	HC	1303 ± 23	1519 ± 41	1347 ± 83		1193 ± 30	1572 ± 49	1082 ± 17	1592 ±
	RRMS	1375 ± 55	1521 ± 84	1476 ±		1221 ± 46	1578 ± 96	1069 ± 19	1639 ±
CD161									
% positive cells	HC	20.8 ± 3.0	45.8 ± 2.4	53.7 ± 2.5		22.8 ± 3.6	46.4 ± 2.3	63.0 ± 2.7	47.7 ± 2.7
	RRMS	17.5 ± 2.9	44.4 ± 2.9	46.7 ± 3.5		20.3 ± 3.6	42.8 ± 3.0	56.1 ± 2.8	42.0 ± 3.5
MFI positive	HC	2074 ±	2695 ±	2764 ±		2153 ±	2694 ±	2931 ±	2517 ±
	RRMS	2023 ± 79	2595 ± 90	2638 ±		2094 ± 63	2504 ±	2820 ±	2356 ±
CD226									
% positive cells	HC	89.2 ± 1.4	91.3 ± 1.1	96.8 ± 0.6		87.3 ± 2.1	92.4 ± 1.0	98.5 ± 0.3	95.7 ± 0.7
	RRMS	89.2 ± 1.7	92.1 ± 1.0	96.4 ± 0.7		88.0 ± 2.7	92.2 ± 1.0	98.1 ± 0.5	95.4 ± 0.8
MFI positive	HC	3411 ±	3504 ±	4464 ±		3014 ±	3839 ±	4291 ±	4674 ±
	RRMS	3080 ±	3396 ±	4086 ±		2743 ±	3570 ±	4036 ±	4138 ±
VLA-4									
% positive cells	HC	83.4 ± 2.1	75.1 ± 1.9	90.8 ± 1.3		86.6 ± 2.0	70.4 ± 2.4	96.2 ± 0.7	85.5 ± 1.8
	RRMS	83.9 ± 1.4	76.3 ± 2.0	90.6 ± 1.2		87.4 ± 1.8	71.8 ± 2.1	96.2 ± 0.7	85.3 ± 1.8
MFI positive	HC	2268 ±	2309 ±	3597 ±		2092 ±	2548 ±	3622 ±	3586 ±
	RRMS	2108 ±	2409 ±	3283 ±		1959 ±	2644 ±	3342 ±	3192 ±
MCAM									
% positive cells	HC	0.9 ± 0.2	8.6 ± 0.9	4.6 ± 0.7		0.3 ± 0.1	9.8 ± 0.9	3.9 ± 0.6	4.9 ± 0.5
	RRMS	1.0 ± 0.1	8.6 ± 0.3	4.2 ± 0.3		0.4 ± 0.1	9.6 ± 0.5	3.0 ± 0.4	4.7 ± 0.4
MFI positive	HC	504 ± 20	555 ± 13	528 ± 11		525 ± 38	552 ± 11	519 ± 15	537 ± 16
	RRMS	506 ± 10	548 ± 10	507 ± 7		650 ± 63	545 ± 9	513 ± 13	508 ± 9
PSGL-1									
% positive cells	HC	92.0 ± 2.0	95.8 ± 0.9	97.3 ± 0.6		90.6 ± 2.3	97.0 ± 0.6	97.2 ± 0.5	97.6 ± 0.7
	RRMS	95.8 ± 0.5	97.6 ± 0.3	98.4 ± 0.2		95.1 ± 0.6	98.2 ± 0.2	98.1 ± 0.2	98.7 ± 0.3
MFI positive	HC	4758 ±	5863 ±	6163 ±		4960 ±	6432 ±	6126 ±	6216 ±
	RRMS	4682 ±	6322 ±	6219 ±		4817 ±	6741 ±	5990 ±	6332 ±

*Patients and controls were treatment-naive, age-/gender-matched and part of the cohorts described in Supplementary Table 1. None of these markers showed differences in expression between patients and controls.

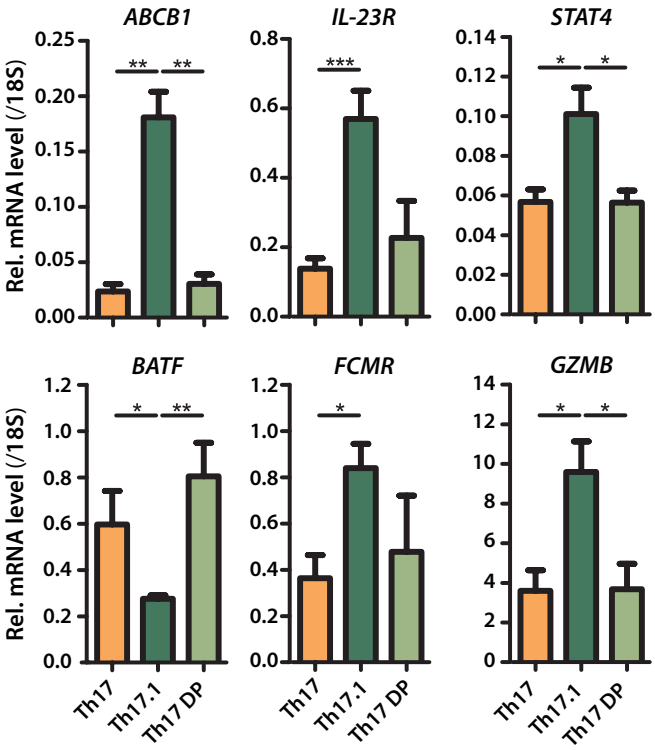


Supplementary Figure 1.

(**A**) Validation of Th17.1 accumulation in the blood of nine MS patients after natalizumab treatment. Frequencies of Th1 ($CCR6^-CXCR3^+CCR4^-$), Th17 ($CCR6^+CXCR3^-CCR4^+$), Th17.1 ($CCR6^+CXCR3^+CCR4^-$) and Th17 DP ($CCR6^+CXCR3^+CCR4^+$) cells within the $CD4^+$ T cell pool were compared pre- and 12m post-treatment using multicolor flow cytometry. (**B**) Representative gating and percentages of $VLA\text{-}4^+$ cells within Th1, Th17, Th17.1 and Th17 DP subpopulations in pre-treatment MS blood.

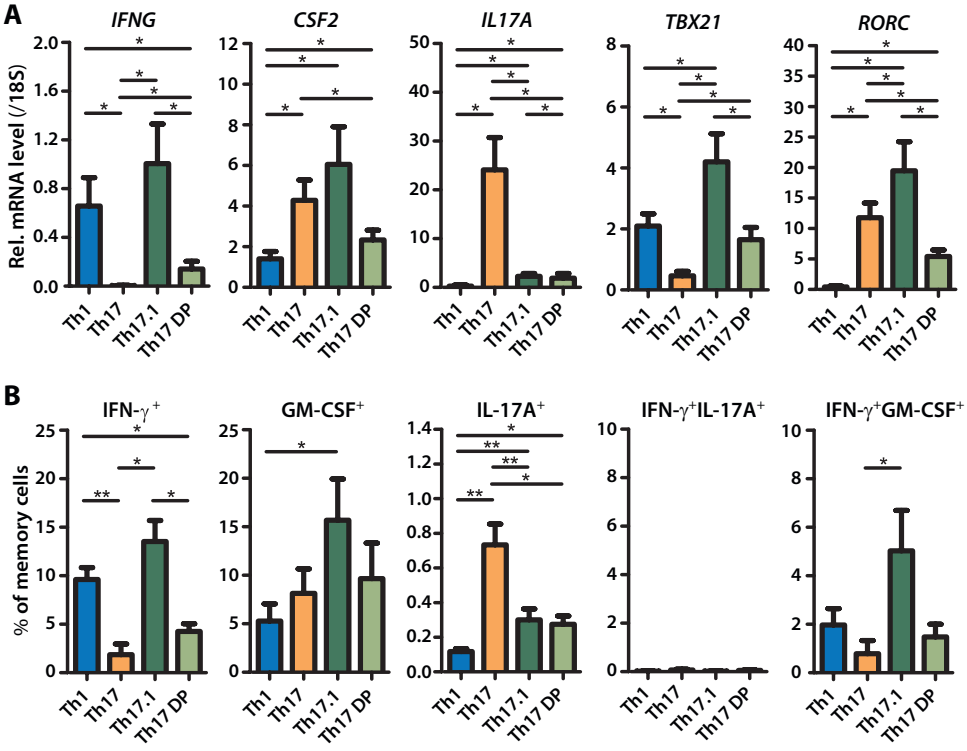


Supplementary Figure 2. Flow cytometric analysis of Th17 subpopulations in paired *ex vivo* CSF and blood samples of three late-stage MS patients.



Supplementary Figure 3. Validation of Th17.1 as a distinct Th17 subset based on the expression of key genes involved in Th17 differentiation and pathogenicity.

We sorted memory Th17 ($CCR6^+CXCR3^-CCR4^+$), Th17.1 ($CCR6^+CXCR3^+CCR4^-$) and Th17 DP ($CCR6^+CXCR3^+CCR4^+$) cells of seven healthy blood donors and compared the relative expression levels of *ABCB*1 (MDR1), *IL23R*, *STAT4*, *BATF*, *FCMR* (TOSO) and *GZMB* (granzyme B). For *FCMR* and *GZMB* expression analyses, Th subsets were activated with anti-CD3/CD28 abs for 24 h.



Supplementary Figure 4. Validation of Th17.1 as a distinct Th17 subset based on the expression of Th1- and Th17-associated pro-inflammatory cytokines and transcription factors.

Th1 (CCR6$^-$CXCR3$^+$CCR4$^-$), Th17 (CCR6$^+$CXCR3$^-$CCR4$^+$), Th17.1 (CCR6$^+$CXCR3$^+$CCR4$^-$) and Th17 DP (CCR6$^+$CXCR3$^+$CCR4$^+$) cells were sorted from seven healthy blood donors and compared for mRNA (**A**) and protein (**B**) expression of IFN-γ (*IFNG*), GM-CSF (*CSF2*), IL-17A (*IL17A*), T-bet (*TBX21*) and RORγt (*RORC*).

Chapter 3

Brain-homing CD4$^+$ T cells display glucocorticoid-resistant features in multiple sclerosis

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Neurology, Neuroimmunology and Neuroinflammation 2020;7

ABSTRACT

Background: Although glucocorticoids can shorten acute relapses of multiple sclerosis (MS), subsequent exacerbations and disease progression are not prevented. A reduced glucocorticoid sensitivity of CNS-homing pathogenic T helper (Th) cells could contribute to these limited effects in MS.

Objective: In this study, we analyzed whether features of glucocorticoid resistance can be utilized to delineate disease-relevant Th cells in early MS.

Methods: The expression of key determinants of glucocorticoid sensitivity, multidrug resistance protein 1 (MDR1/*ABCB1*) and glucocorticoid receptor (GR/*NR3C1*), was investigated in pro-inflammatory Th subsets and compared between natalizumab-treated MS patients and healthy individuals. Blood, CSF and brain compartments from MS patients were assessed for the recruitment of glucocorticoid-resistant Th subsets using FACS, qPCR, immunohistochemistry and immunofluorescence.

Results: An MS-associated Th subset termed Th17.1 showed a distinct glucocorticoid-resistant phenotype as reflected by high MDR1 and low GR expression. This expression ratio was further elevated in Th17.1 cells that accumulated in the blood of MS patients treated with natalizumab, a drug that prevents their entry into the CNS. Pro-inflammatory markers CCR6, IL-23R, IFN-γ and GM-CSF were increased in MDR1-expressing Th17.1 cells. This subset predominated the CSF of early MS patients, which was not seen in paired blood or in the CSF from patients with other inflammatory and non-inflammatory neurological disorders. The potential of MDR1-expressing Th17.1 cells to infiltrate brain tissue was confirmed by their presence in MS white matter lesions.

Interpretation: This study reveals that glucocorticoid resistance coincides with preferential CNS recruitment of pathogenic Th17.1 cells, which may hamper the long-term efficacy of glucocorticoids in early MS.

INTRODUCTION

Early multiple sclerosis (MS) is characterized by waves of brain-infiltrating immune cells that drive inflammation within the central nervous system (CNS), resulting in demyelination and eventually neurodegeneration [1]. Glucocorticoids are broad-spectrum immunosuppressive drugs that are used as standard regimen to dampen acute exacerbations in MS [2]. Although glucocorticoids effectively relieve clinical symptoms, these drugs do not halt subsequent exacerbations or disease progression [3]. Interestingly, blood cells of relapsing-remitting MS (RRMS) patients show reduced sensitivity to glucocorticoids [4, 5], which offers a limited window of opportunity for inducing long-term efficacy of this MS treatment. To achieve this, more insights into the underlying mechanisms of glucocorticoid insensitivity are required in early MS.

Upon binding its ligand, the glucocorticoid receptor (GR/*NR3C1*) hyperphosphorylates, forms dimers with other nuclear receptors and translocates into the nucleus [2]. Within the nucleus, these dimers recognize GR elements in promoter regions or interact with tran-scription factors to transactivate or repress pro-inflammatory gene expression [2]. Apart from binding to the GR, glucocorticoids can be excreted out of the cell by the multidrug resistance protein 1 (MDR1/*ABCB1*) [6].

In experimental autoimmune encephalitis (EAE) mice, the therapeutic efficacy of glucocorticoids depends on the suppression of CNS recruitment of pro-inflammatory $CD4^+$ T helper (Th) cells [7, 8]. Since glucocorticoids are unable to induce long-term protective effects in MS, it is tempting to speculate that certain pathogenic Th cells avoid glucocorticoid-mediated suppression and thereby contribute to recurrent disease activity [9]. C-C chemokine receptor 6 (CCR6) expression on memory Th cells promotes their recruitment into the CNS and is essential for the induction of EAE [10, 11]. In RRMS patients, blood-derived $CCR6^+$ and not $CCR6^-$ memory Th cells show increased pro-inflammatory capacity in response to myelin peptides [12]. Previously, our group revealed that a human pathogenic $CCR6^+$ Th subset commonly termed Th17.1 ($IL\text{-}17^{low}IFN\text{-}\gamma^{high}GM\text{-}CSF^{high}$) is associated with early MS [13-17]. Interestingly, we and others found that Th17.1 cells from healthy individuals express high levels of *ABCB1* [13, 15], which encodes for MDR1 and links to the reduced glucocorticoid sensitivity found in RRMS patients [4].

In this study, we addressed how MDR1 and GR are expressed amongst memory Th subsets including Th17.1 and whether this coincides with their potential to infiltrate the CNS of early MS patients.

MATERIALS AND METHODS

Patients and sampling

Treatment-naive clinically isolated syndrome (CIS) and RRMS patients were diagnosed based on the McDonald 2017 criteria and included at the MS Center ErasMS, Erasmus MC. We collected cerebrospinal fluid (CSF) and blood samples from these as well as alternatively diagnosed patients (Supplementary Table 1). Post-mortem blood, meninges and white matter tissues were obtained from autopsied MS donors (Netherlands Brain Bank, Amsterdam; Supplementary Table 1) and processed as previously described [18]. Natalizumab-treated MS patients were treated for 12 months before blood sampling. The use of primary material and experimental procedures were approved by the medical ethics committee of each respective center.

Antibodies and flow cytometry

Flow cytometry was performed using fluorescently labeled anti-human monoclonal antibodies (Supplementary Table 2). For MDR1 surface staining, cells were taken up in RPMI 1640 containing 2% fetal calf serum (FCS) and 25 µM Cyclosporin A (Sigma-Aldrich, St Louis, MO, USA) or 1:500 absolute ethanol (Merck, Schiphol-Rijk, The Netherlands) as a vehicle control. Subsequently, MDR1 (Biolegend, London, UK) antibody was added and cells were incubated for 20 min at 37˚C and 5% CO_2. In all experiments, viable cells were analyzed using Fixable Viability Stain 700 (BD Biosciences, Erembodegem, Belgium) or the Fixable Viability Dye eFluor 520 (Thermo Fisher Scientific, Landsmeer, The Netherlands). Other surface markers were stained as previously described [13]. Cells were measured using the LSRII-Fortessa or FACSAria-III flow cytometer and analyzed using FACSDiva software (Version 8.0.1; BD Biosciences).

Cell sorting

$CD4^+$ cells were isolated from healthy donor blood (Sanquin, Amsterdam, The Netherlands) using CD4 microbeads and the autoMACS Pro Separator (both Miltenyi Biotec, Bergisch Gladbach, Germany). From these cells, $CCR6^+$ memory Th populations ($CD3^+CD4^+CD8^-CD25^{low}CD45RA^-CCR6^+$) were isolated using a FACSAria-III machine. $CCR6^+$ memory subsets were defined based on differential expression of CXCR3 and CCR4; Th17 ($CCR6^+CXCR3^-CCR4^+$), Th17 double-positive (DP; $CCR6^+CXCR3^+CCR4^+$) and Th17.1 ($CCR6^+CXCR3^+CCR4^{-/dim}$) [14].

RNA isolation and quantitative PCR

RNA isolation, complementary DNA synthesis and real-time quantitative PCRs were performed as previously described [13]. Primer-probe sets were designed using the

Universal ProbeLibrary (Roche Applied Science, Penzberg, Germany). Apart from *IFNG* and *CSF2*, which were measured after Phorbol 12-myristate 13-acetate and ionomycin stimulation, gene expression was measured in unstimulated cells [13]. An overview of all used primer sequences can be found in Supplementary Table 3. RNA samples containing less than 75 ng total RNA were excluded from the analysis.

In vitro proliferation assay

Frozen buffy-derived $CD4^+$ T cells were thawed and labeled with 0.075 µM CellTrace CFSE according to the manufacturer's instructions (Thermo Fisher Scientific). After washing, Th17 and Th17.1 memory cells were purified using FACS, as described above. Sorted Th17 and Th17.1 cells were plated at 250.000 cells/ml and activated with aCD3/CD28 dynabeads (1:5; Thermo Fisher Scientific) for 3 days. Cells were cultured in RPMI 1640 (Thermo Fisher Scientific) supplemented with 1% Penicillin/Streptomycin (Lonza, Verviers, Belgium), 5% human AB serum (Sanquin) and 75 µM methylprednisolone sodium succinate (Pfizer, Capelle a/d IJssel, The Netherlands) or a vehicle control. To compare differences in proliferation, the frequencies of viable, CFSE-negative cells were analyzed using flow cytometry.

Rhodamine efflux and MDR1 shift assay

We combined a rhodamine efflux and MDR1 shift assay to measure both the efflux potential and MDR1 expression of each Th memory subset [15, 19]. PBMCs were taken up in RPMI 1640 containing 2% FCS and 0.5 µg/ml Rhodamine 123 (Rh123) or 1:2000 absolute ethanol as a vehicle control (both Sigma-Aldrich) at a concentration of $1\times10E^7$ cells/ml and were incubated on ice for 30 min in the dark. Cells were washed with the same culture media and taken up in media containing 25 µM Cyclosporin A or 1:500 absolute ethanol. Next, MDR1 antibody was added for 2h at 37˚C and 5% CO_2, after which cells were stained with Th subset-defining markers as described above.

Immunohistochemistry and multiplex immunofluorescence

Immunohistochemistry was performed with an automated, validated and accredited staining system Ventana Benchmark ULTRA using the optiview universal DAB detection Kit (both Ventana Medical Systems, Oro Valley, AZ, USA). In brief, after deparaffinization and heat-induced antigen retrieval, 7 µm thick brain sections were incubated with the antibody of interest for 32 min. This was followed by a hematoxylin II counter staining for 12 min and the addition of a blue coloring reagent for 8 min according to the manufacturer's instructions (both Ventana Medical Systems). To assess MDR1 expression on Th cells, immunofluorescent staining for MDR1 and CD4 was performed using the automated multiplex platform Benchmark Discovery (Ventana Medical Systems). In short, brain sections were deparaffinized and antigen-retrieved with cell conditioning (CC) 1 (Ventana Medical

Systems) for 32 min. Tissue slides were incubated with MDR1 antibody for 32 min at 37˚C followed by detection with FAM (Roche Applied Science). Antibody denaturation was performed using CC2 (Ventana Medical Systems) for 8 min at 100˚C. Subsequently, CD4 antibody was incubated for 32 min at 37˚C followed by detection with Cy5 (Roche Applied Science). Finally, slides were washed in PBS and mounted with Vectashield containing DAPI (Vector laboratories, Peterborough, UK). For detailed information of the used antibodies, see Supplementary Table 2.

Statistical analysis

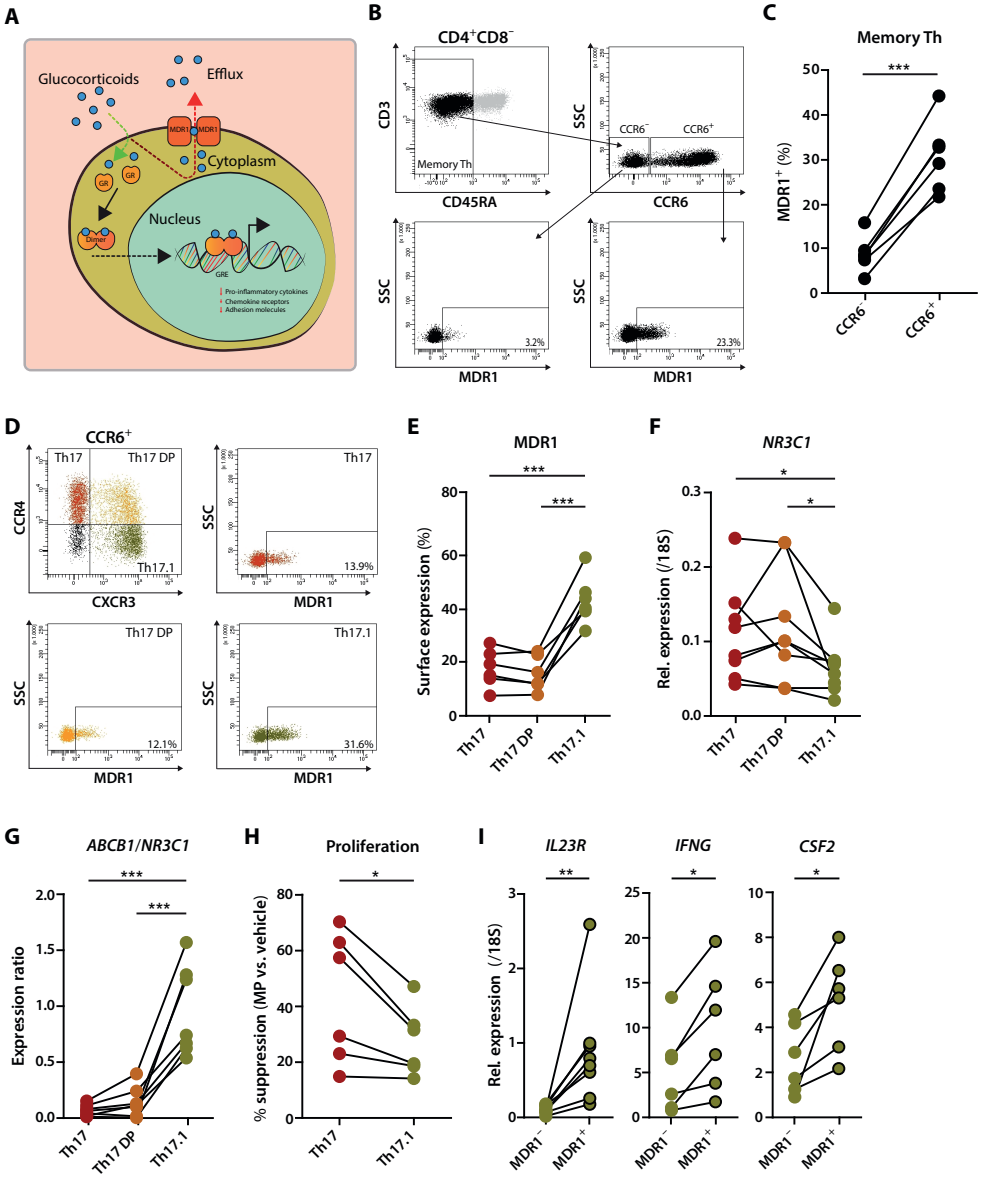
Statistical tests were performed using GraphPad Prism 5 software and are described in each figure legend. Results are displayed as the mean, standard error of the mean or as a box & whiskers plot. For all tests, a p value of < 0.05 (*) was considered significant.

RESULTS

High ABCB1 and low NR3C1 expression defines glucocorticoid-resistant Th17.1 cells with enhanced pro-inflammatory capacity

Glucocorticoid sensitivity is determined by the expression of *ABCB1* (MDR1) and *NR3C1* (GR) (Fig. 1A). Using a MDR1 shift assay, we found that MDR1 was predominantly expressed on CCR6$^+$ versus CCR6$^-$ memory Th cells within the blood of healthy individuals ($p < 0.001$; Fig. 1B and C), indicating that high MDR1 expression is not associated with Th1 cells. After

Figure 1 (right page). High *ABCB1* and low *NR3C1* expression in Th17.1 cells from healthy blood donors. (**A**) Simplistic illustration of glucocorticoid regulation within an immune cell. Glucocorticoids diffuse through the plasma membrane and bind to the glucocorticoid receptor *(NR3C1/*GR*)* within the cytoplasm. Upon binding, GRs form dimers and translocate into the nucleus to regulate pro-inflammatory gene expression. However, glucocorticoids can also be transported out of the cell by the multidrug resistance protein 1 (*ABCB1/*MDR1). (**B**) Representative FACS plot displaying the gating strategy and expression of MDR1 for CCR6$^-$ and CCR6$^+$ memory Th (CD3$^+$CD4$^+$CD8$^-$ CD45RA$^-$) cells. CD25^{high} (Treg) cells were excluded from our analyses. (**C**) Frequencies of MDR1-expressing cells within paired CCR6$^-$ and CCR6$^+$ memory Th populations from healthy blood donors (n = 6). (**D**) Representative gating of functionally distinct CCR6$^+$ Th memory cells (Th17, Th17 DP and Th17.1) and surface expression of MDR1. (**E**) Frequencies of MDR1-expressing cells within each CCR6$^+$ Th memory subset (n = 6). Relative expression of *NR3C1* (**F**), as well as their *ABCB1/NR3C1* ratios (**G**) were analyzed for paired Th17, Th17 DP and Th17.1 cells from healthy blood using qPCR (n = 7-8). (**H**) *In vitro* glucocorticoid response of paired Th17 and Th17.1 cells from healthy donors (n = 6). Cells were stimulated with aCD3/CD28 in the presence of methylprednisolone (MP; 75 μM) or a vehicle (control) for 3 days and analyzed by flow cytometry. (**I**) *IL23R* (IL-23 receptor), IFNG (IFN-γ) and CSF2 (GM-CSF) expression relative to the housekeeping gene *18S* between MDR1$^+$ versus MDR1$^-$ Th17.1 cells from the same healthy donors (n = 7-8). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Data were compared using paired t tests (**C**, **H** and **I**), repeated measures one-way ANOVA with a Newman-Keuls multiple comparison test (**E**, **F** and **G**) and Wilcoxon paired test (**I**).


A
Glucocorticoids
Efflux
MDR1
MDR1
Cytoplasm
GR
GR
Nucleus
GR
GRE
Pro-inflammatory cytokines
Chemokine receptors
Adhesion molecules
B
CD4+CD8−
CD3
Memory Th
CD45RA
SSC
CCR6−
CCR6+
CCR6
SSC
3.2%
MDR1
SSC
23.3%
MDR1
C
Memory Th
***
MDR1+ (%)
50
40
30
20
10
0
CCR6−
CCR6+
D
CCR6+
Th17
Th17 DP
Th17.1
CCR4
CXCR3
Th17
13.9%
SSC
MDR1
Th17 DP
12.1%
SSC
MDR1
Th17.1
31.6%
SSC
MDR1
E
MDR1
***
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Surface expression (%)
80
60
40
20
0
Th17
Th17 DP
Th17.1
F
NR3C1
*
*
Rel. expression (/18S)
0.3
0.2
0.1
0.0
Th17
Th17 DP
Th17.1
G
ABCB1/NR3C1
***
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Expression ratio
2.0
1.5
1.0
0.5
0.0
Th17
Th17 DP
Th17.1
H
Proliferation
*
% suppression (MP vs. vehicle)
80
60
40
20
0
Th17
Th17.1
I
IL23R
**
Rel. expression (/18S)
3
2
1
0
MDR1−
MDR1+
IFNG
*
25
20
15
10
5
0
MDR1−
MDR1+
CSF2
*
10
8
6
4
2
0
MDR1−
MDR1+

subdivision of CCR6$^+$ Th cells into functionally distinct subsets based on CXCR3 and CCR4 surface expression [13, 14], MDR1 was abundant on Th17.1 (IL-17^{neg}IFN-γ^{high}GM-CSF^{high}) compared to Th17 (IL-17^{high}IFN-γ^{neg}GM-CSF^{dim}) and Th17 DP (IL-17^{dim}IFN-γ^{low}GM-CSF^{dim}) cells from the same blood donors ($p < 0.001$; Fig. 1D and E). Subsequently, we sorted these populations and analyzed co-expression of MDR1 (*ABCB1*) with GR (*NR3C1*). *NR3C1* was selectively downregulated in Th17.1 cells ($p < 0.05$; Fig. 1F), resulting in strongly elevated *ABCB1/NR3C1* expression ratios ($p < 0.001$; Fig. 1G). *In vitro* experiments showed that MDR1^{high}GR^{low} Th17.1 cells were less sensitive to glucocorticoids in comparison to paired MDR1^{low}GR^{high} Th17 cells ($p < 0.05$; Fig. 1H). Pro-inflammatory and Th17.1-associated genes IL-23 receptor (*IL23R*), IFN-γ (*IFNG*) and GM-CSF (*CSF2*) [13, 14] displayed elevated expression in MDR1$^+$ versus MDR1$^-$ fractions of Th17.1 ($p < 0.01$ versus $p < 0.05$ respectively; Fig. 1I). These findings show that Th17.1 cells have a distinctive glucocorticoid-resistant phenotype, which probably contributes to their role in MS disease activity [13].

Th17.1 cells trapped in the blood of natalizumab-treated MS patients show increased ABCB1 and reduced NR3C1 expression

In our previous study, Th17.1 cells were found to selectively accumulate in the blood from MS patients who clinically responded to natalizumab treatment [13]. This peripheral entrapment makes it possible to analyze the glucocorticoid resistance profile of Th17.1 cells that normally infiltrate the CNS during pathogenesis. After sorting of these and other CCR6$^+$ memory Th cells from the blood, we found selectively increased *ABCB1* expression in Th17.1 cells from 11 RRMS patients who clinically responded to natalizumab treatment versus 9 age- and gender-matched healthy controls ($p < 0.05$; Fig. 2A). This was not found in patients who experienced clinical relapses despite natalizumab therapy (non-responders; n = 6; Fig. 2A). *NR3C1* was reduced in all CCR6$^+$ Th subsets analyzed from these patients, which was only significant in non-responders and mainly found in Th17.1 (Fig. 2A). As a result, *ABCB1*/*NR3C1* expression ratios were enhanced especially in natalizumab responders compared to healthy controls (Fig. 2A). Similar results were obtained when analyzing frequencies of MDR1$^+$ Th17.1 cells ($p < 0.05$), whereas their Rh123 efflux potential remained unchanged between the groups (Fig. 2B and C). CSF-homing marker CCR6 was higher expressed on MDR1$^+$ versus MDR1$^-$ Th17.1 cells from the blood of natalizumab-treated patients ($p < 0.0001$), which was not seen for CXCR3 (Fig. 2D). These data show that glucocorticoid-resistant Th17.1 cells have a phenotype associated with preferential recruitment to the CSF in MS patients.

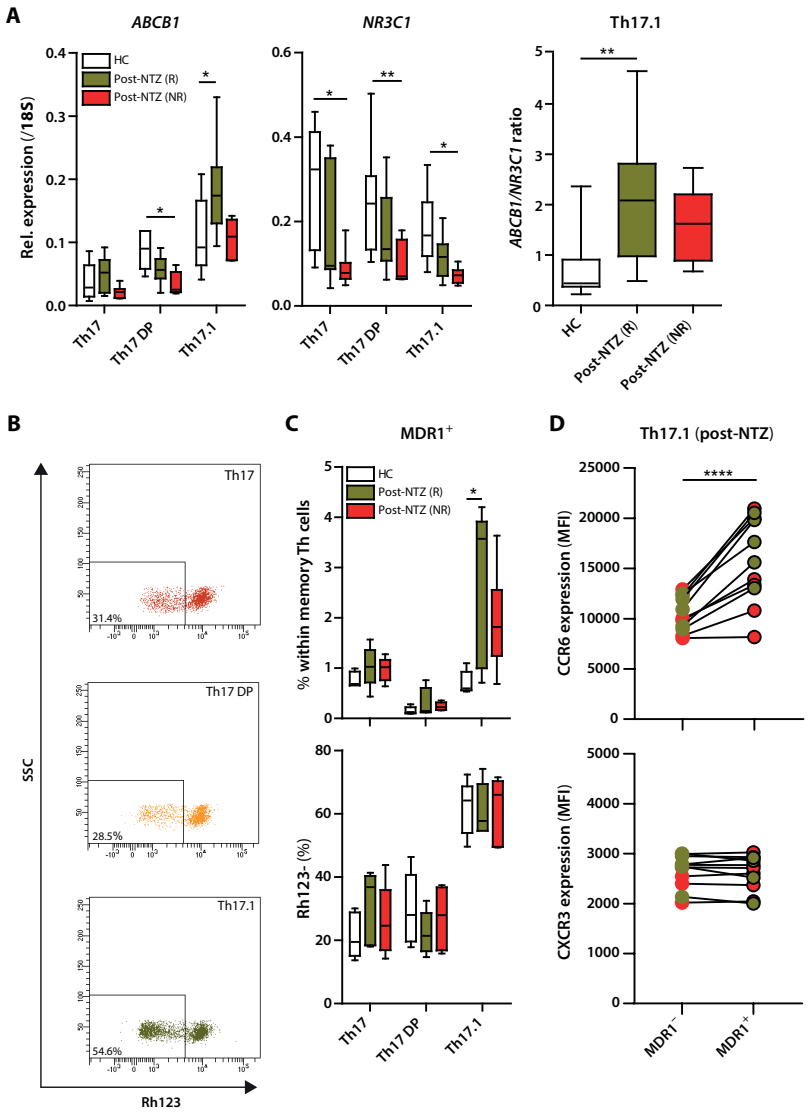

Figure 2. Increased frequencies of MDR1+ Th17.1 cells in the blood of natalizumab-treated MS patients compared to healthy controls.

(**A**) *ABCB1*, *NR3C1* and *ABCB1/NR3C1* expression ratios for blood Th17, Th17 DP and Th17.1 cells of MS patients who clinically responded (n = 10-11) or did not respond (n = 5-7) to natalizumab, as well as age- and gender-matched healthy controls (n = 6-9). (**B**) Representative FACS plot showing Rh123 efflux in Th17, Th17 DP and Th17.1 cells. (**C**) Frequencies of MDR1+ Th17, Th17 DP and Th17.1 cells within memory Th cells (top) and percentages of Rh123-negative cells within each subset (bottom) for 11 natalizumab-treated MS patients and 5 healthy controls. (**D**) CCR6 and CXCR3 expression (MFI) on MDR1- versus MDR1+ Th17.1 cells from 11 natalizumab-treated MS patients. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. Data were analyzed using Kruskal-Wallis with a Dunn multiple comparison test (**A** and **C**) and paired t tests (**D**).

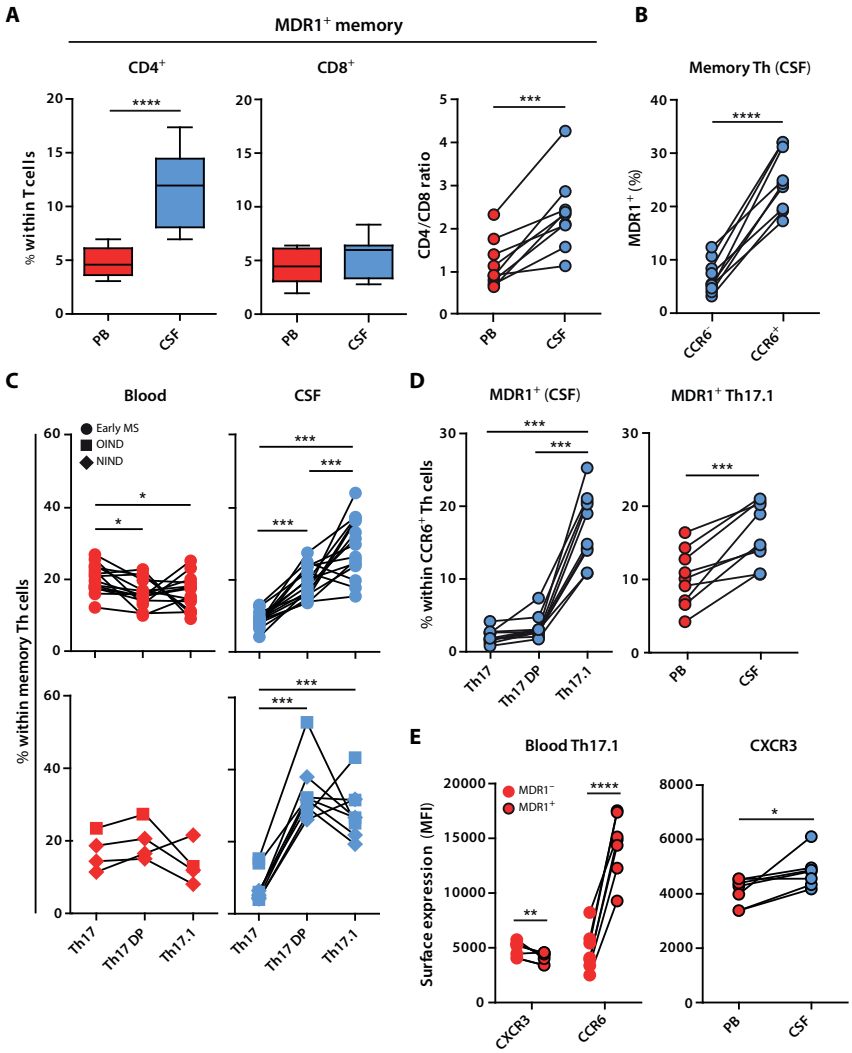

Figure 3. Selective enrichment of MDR1⁺ Th17.1 cells in the CSF of treatment-naive early MS patients.
(**A**) Percentages and ratios of MDR1-expressing CD4⁺ and CD8⁺ memory fractions within CD3⁺ T cells in the blood and CSF from early MS patients (n = 9). (**B**) Percentages of MDR1-expressing cells within paired CCR6⁻ versus CCR6⁺ memory Th cells from early MS CSF (n = 9). (**C**) Top panel: percentages of CCR6⁺ subsets within the memory Th compartment in the blood (left panel, n = 14) and CSF (right panel, n = 15) from early MS patients. Bottom panel: same items displayed for patients with other inflammatory neurological disorders (OIND) or non-inflammatory neurological disorders (NIND) (n = 4 for blood and n = 8 for CSF). (**D**) Percentages of MDR1⁺ CCR6⁺ Th subsets in the CSF (left) and MDR1⁺ Th17.1 frequencies in the CSF versus blood (right) from early MS patients (n = 9-10). (**E**) CCR6 and CXCR3 expression levels (MFI) on paired MDR1⁺ versus MDR1⁻ Th17.1 cells from early MS patients (n = 7). CXCR3 expression (MFI) on CSF- and blood-derived MDR1⁺ Th17.1 cells from early MS patients (n = 7). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Data were analyzed using paired t tests (**A, B, D** and **E**) and repeated measures one-way ANOVA with a Newman-Keuls multiple comparison test (**C** and **D**).

MDR1+ Th17.1 cells are enriched in CSF of early MS patients and not in patients with other neurological disorders

Ex vivo flow cytometric analysis of paired blood and CSF samples from treatment-naive early MS patients (n = 9, Supplementary Table 1) revealed a significant rise in MDR1-expressing CD4+ ($p < 0.0001$) and not CD8+ T memory cells, resulting in increased CD4/CD8 ratios ($p < 0.001$) within the CSF (Fig. 3A). Similar to blood cells of natalizumab-treated MS patients (Fig. 2D), CCR6+ Th memory cells expressed higher levels of MDR1 compared to CCR6- fractions in MS CSF ($p < 0.0001$; Fig. 3B). Th17.1 predominated the CSF of early MS patients when compared to Th17 and Th17 DP cells (n = 15, Supplementary Table 1 and $p < 0.001$, Fig. 3C). This selective enrichment was not found in paired blood samples, or in the CSF of 8 patients with other inflammatory or non-inflammatory neurological disorders (Supplementary Table 1 and Fig. 3C). The predominance of Th17.1 cells in early MS CSF was even more apparent when analyzing MDR1-expressing proportions ($p < 0.001$; Fig. 3D). Within the blood of these patients, CCR6 and not CXCR3 was enriched on MDR1+ compared to MDR1- Th17.1 cells ($p < 0.0001$ and $p < 0.01$; Fig. 3E). MDR1+ Th17.1 cells in CSF showed higher CXCR3 levels than their blood counterparts ($p < 0.05$; Fig. 3E). The enhanced recruitment of MDR1+ Th17.1 cells to MS CSF suggests that this subset has a greater capacity to infiltrate brain tissue to promote local inflammation.

MDR1-expressing CD4+ T cells are recruited to MS brain tissue and show a Th17.1 phenotype

Lastly, we studied whether MDR1 is expressed on CD4+ T cells that have infiltrated the brain of MS patients. We performed *in situ* analyses of post-mortem white matter tissues from 3 MS donors with a high number of perivascular infiltrates. Immunohistochemical analysis for MDR1 and CD4 showed their co-existence in perivascular areas of active lesions (Fig. 4A and B). Besides the expected presence of MDR1 in other types of CNS-resident cells [20], we were able to validate the co-expression of MDR1 with CD4 using confocal microscopy (Fig. 4C). To address how MDR1 was expressed amongst MS brain-infiltrating CCR6+ Th subsets, we analyzed *ex vivo* single-cell suspensions of post-mortem blood, meninges and brain white matter tissue from 8 late-stage MS donors using flow cytometry (Fig. 4D and E). In contrast to Th17 and Th17 DP cells, frequencies of MDR1+ Th17.1 cells were significantly increased in brain tissue compared to blood ($p < 0.001$) and meninges ($p < 0.05$; both Fig. 4E). Although not significant, Th17.1 cells also seemed to be more present in the meninges than in the blood and were more abundant than Th17 DP cells in brain tissue. Overall, these findings demonstrate that MDR1 expression marks a subset of CNS-homing Th17.1 cells and support a reduced glucocorticoid sensitivity of putatively pathogenic CD4+ T cells in MS.

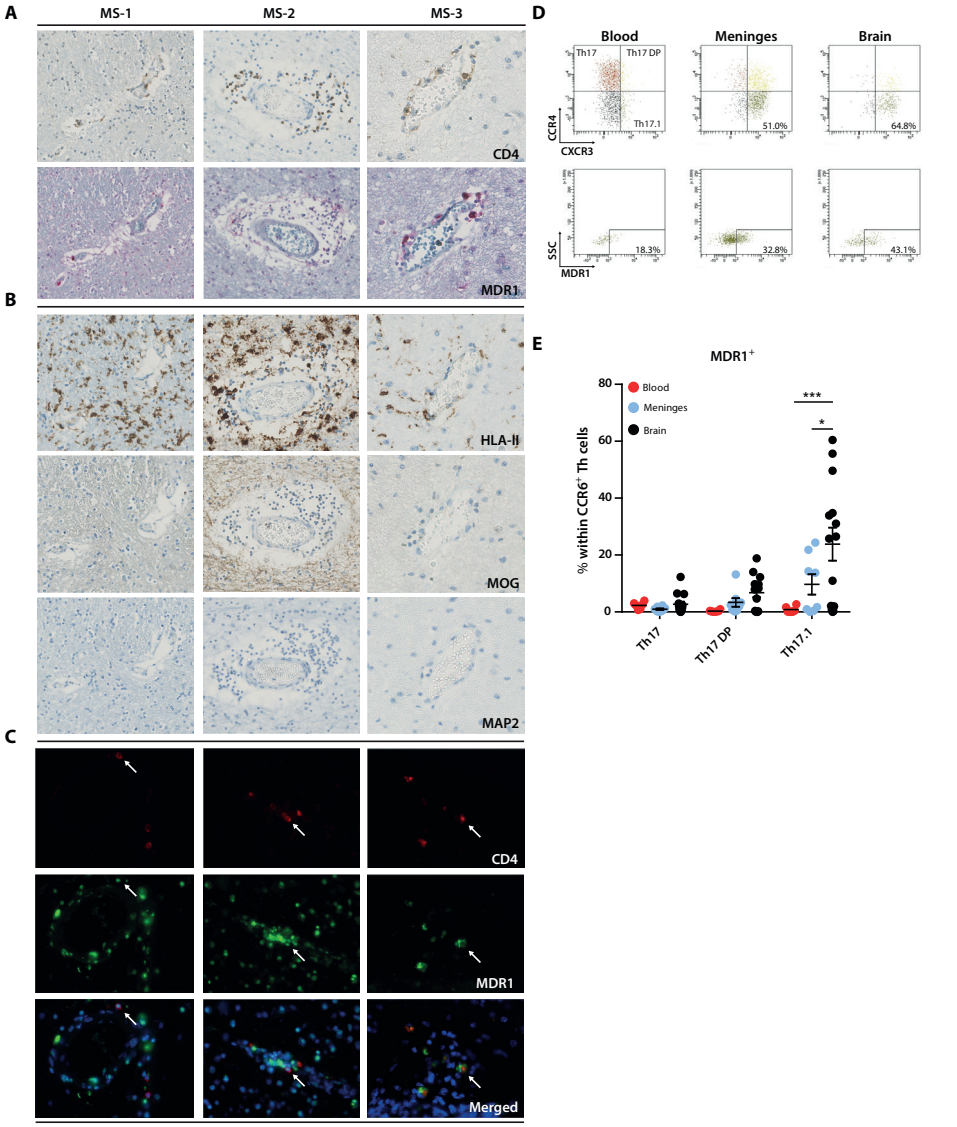

Figure 4. Presence of MDR1⁺ Th17.1 cells in post-mortem white matter tissue of MS patients.
Representative immunohistochemical staining for CD4 and MDR1 (**A**) as well as HLA-II, MOG and MAP2 (**B**) in formalin-fixed, paraffin-embedded white matter tissues from 3 MS patients. (**C**) Co-expression of CD4 (red) and MDR1 (green) in DAPI (blue)-positive cells in the same tissues, as determined by immunofluorescence staining. (**D**) Representative gating of *ex vivo* CCR6⁺ Th subsets and MDR1⁺ fractions of Th17.1 ($CCR6^{+}CXCR3^{+}CCR4^{-/dim}$) in the blood, meninges and brain tissue from an MS donor. (**E**) *Ex vivo* frequencies of MDR1⁺ Th17, Th17 DP and Th17.1 cells in post-mortem blood, meninges and brain tissues from 8 late-stage MS donors, as determined by FACS. A total of 14 brain tissues from 8 donors were analyzed. * $p < 0.05$ and *** $p < 0.001$. Data in **E** were compared using a non-repeated measurement two-way ANOVA with a post-hoc Bonferroni multiple comparison test.

DISCUSSION

In sharp contrast to late-stage MS, early phases of MS are characterized by increased numbers of $CD4^+$ and not $CD8^+$ T cells in the CSF [18, 21]. Since glucocorticoids are used to dampen acute MS relapses, we focused on glucocorticoid resistance features amongst different pathogenic $CD4^+$ Th cell subsets. Here, we reveal that Th17.1 cells display a unique glucocorticoid-resistant (*ABCB1*^{high}*NR3C1*^{low}) phenotype, which is accompanied by a selective enrichment within the CSF of early MS and not alternatively diagnosed patients. The fact that this glucocorticoid-resistant, pathogenic $CD4^+$ Th subset also localizes within MS white matter lesions supports our notion that Th17.1 and particularly MDR1 is a promising target for predicting and inducing glucocorticoid treatment efficacy during early MS. To our knowledge, this is the first study that links glucocorticoid resistance to the brain-infiltrating capacity of human Th subsets in a neurological disease such as MS.

Several factors may be responsible for the selective upregulation of *ABCB1* (MDR1) and downregulation of *NR3C1* (GR) in Th17.1 cells. A pathogenic feature of MS is chronic activation of the hypothalamus–pituitary–adrenal (HPA) axis, resulting in elevated levels of the natural glucocorticoid cortisol [5]. It has been shown that *in vivo* administration of glucocorticoids induces MDR1 on peripheral blood lymphocytes [22], suggesting that the elevated cortisol levels in MS patients could give rise to the glucocorticoid-resistant phenotype of Th17.1. Chronic inflammation was also found to upregulate MDR1 expression on lymphocytes via activation of the signal transducer and activator of transcription 3 (STAT3) protein [23]. Consistently, STAT3 drives the expression of RAR-related orphan nuclear receptor γt (RORγt), which inhibits glucocorticoid action [24] and is highly abundant in Th17.1 cells [13]. This probably also explains our finding that the $MDR1^+$ subset of Th17.1 expresses high levels of GM-CSF, which is directly controlled by RORγt [25]. In addition, IL-2 is not only an important trigger of GM-CSF in Th memory cells from MS patients [26], but is also able to reduce GR expression [27]. In EAE, macrophage migration inhibitor factor (MIF) induced glucocorticoid resistance of Th cells via upregulation of T-box transcription factor (T-bet) [28], another Th17.1-associated transcription factor [13]. In parallel with this, we found that T-bet-dependent IFN-γ was upregulated in $MDR1^+$ Th17.1 cells. Together with the downregulation of GR under high glucocorticoid conditions [29], these studies indicate that the glucocorticoid-resistant phenotype of Th effector cells such as Th17.1 may be induced by combined effects of a hyperactive HPA axis and chronic inflammation in MS patients. Alternatively, a GR-repressive isoform (GRβ) may play a role in glucocorticoid resistance [30]. However, the synthetic glucocorticoid methylprednisolone is used for the treatment of acute exacerbations in MS patients and is unable to bind to GRβ sufficiently, thus failing to repress the signaling isoform GRα [30, 31]. GRα could also be differentially

regulated at the post-transcriptional level, but remains challenging to analyze on the protein level in primary Th cells.

Apart from the role of MDR1 and GR in glucocorticoid resistance, both molecules also play an important role in the induction of neuroinflammation. Increased GR signaling in pathogenic T cells protects from CNS autoimmunity, while deletion of GR abrogates this phenomenon in EAE [8, 32]. Deletion of *ABCB1* resulted in a similar protective phenotype [33], likely because of its additional role in the excretion of pro-inflammatory cytokines [34]. These studies suggest that both low GR and high MDR1 expression in brain-homing Th effector cells such as Th17.1 drive neuroinflammation. Due to their involvement in both glucocorticoid resistance and induction of local pathology, selective targeting of these molecules in Th17.1 cells may enhance glucocorticoid efficacy in MS. To further support this hypothesis, the relationship between MDR1^{+} Th17.1 frequencies in the CNS of MS patients and glucocorticoid efficacy should be explored. Unfortunately, we had no access to the required patient material. A recent study showed that low vitamin D levels associate with glucocorticoid-resistant relapses in MS patients [35]. Although controversial results were obtained from clinical trials with respect to disease activity, increasing vitamin D levels improved glucocorticoid efficacy and suppressed EAE induction in mice via Th-cell intrinsic upregulation of *NR3C1* [35]. Since vitamin D also reduces CCR6 expression on human Th cells [17], this steroid hormone may be exploited to enhance the therapeutic efficacy of glucocorticoids in MS by sensitizing Th17.1 cells. Selective targeting of MDR1 is promising due to its role in glucocorticoid resistance, pro-inflammatory cytokine excretion as well as trafficking of T cells across the blood-brain barrier [36], but should also be taken with care since it is expressed by many other cells. In-depth analysis of Th17.1 cells by currently available single-cell platforms would reveal additional targets that can be used for the design of small-molecule therapeutics. Finally, the predominance of Th17.1 in the CSF of early MS and not in alternatively diagnosed patients may set the stage for its use as a biomarker to predict rapid disease onset. To address this, the local presence and features of Th17.1 cells should be compared between larger numbers of MS and control subgroups in the near future.

ACKNOWLEDGMENT

This study was performed within the Erasmus Postgraduate School Molecular Medicine. We are thankful to our FACS operators Harm de Wit and Peter van Geel for sorting. We thank all patients and healthy individuals for donating biological material. The authors would like to dedicate this article to Professor Rogier Q. Hintzen, who passed away on May 15, 2019. We will continue his research in our MS Center with the same drive and passion as he did.

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SUPPLEMENTAL DATA

Supplementary Table 1. Patient characteristics

Peripheral blood	***Ex vivo* analysis**		***In vitro* analysis**[a]		
Cohort	**HC**	**RRMS, NTZ-treated**[b,c]	**HC**	**RRMS, NTZ-treated**[b,c]	
Individuals, n	9	18	5	11	
Females, n (%)	4 (44)	13 (72)	3 (60)	8 (72)	
Age in years, median (range)[d]	47 (23-53)	36 (19-53)	46 (29-62)	36 (19-49)	
Disease duration in months, median (range)[e]	NA	100 (12-202)	NA	59 (12-151)	
CNS compartment	**CSF, *ex vivo***			**Brain/meninges, *ex vivo***	**Brain tissue, *in situ***
Cohort	**CIS**[b,h]	**RRMS**[b,h]	**NIND/OIND**[f,h]	**MS (post-mortem)**	**MS (post-mortem)**
Patient, n (paired blood)	4 (4)	11 (10)	8 (4)	8 (5)	3
Females, n (%)	2 (50)	5 (45)	5 (63)	6 (86)	2 (67)
Age in years, median (range)[d]	38 (25-42)	32 (18-40)	57 (26-68)	65 (51-70)	49 (48-58)
Follow-up time in months, median (range)	11 (1-22)	6 (2-56)	NA	NA	NA
Disease duration in months, median (range)[e]	3 (0-4)	3 (0-55)	NA	NA	NA
PMD in hours, median (range)[g]	NA	NA	NA	7:08 (5:10-8:20)	09:20 (08:30-10:45)

[a]Rhodamine efflux and MDR1 shift assay.
[b]Diagnosis according to the McDonald 2017 criteria.
[c]*Ex vivo* analyses: 11 clinical responders and 7 clinical non-responders. *In vitro* analyses: 5 clinical responders and 6 clinical non-responders.
[d]At the time of sampling.
[e]Time from CIS or if applicable RRMS diagnosis to sampling.
[f]Diagnosis: myelopathy, ulnar neuropathy, Neuro-Behçet's disease, neurosarcoidosis, B-cell lymphoma.
[g]Depicted as hour:minutes.
[h]Patients did not receive glucocorticoids prior to sampling.

CIS = clinically isolated syndrome; RRMS = relapsing-remitting multiple sclerosis; HC = healthy controls; NIND = non-inflammatory neurological diseases; OIND = other inflammatory neurological diseases; NTZ= natalizumab; NA = not applicable or available; PMD = post-mortem delay.

Supplementary Table 2. Used antibodies for *ex vivo* and *in situ* analysis

Ex vivo			
Marker	**Clone**	**Fluorescent label**	**Supplier**
CCR4	L291H4	PE-Cy7, PE-Dazzle 594	Biolegend
CCR6	G034E3	PE	Biolegend
CCR7	150503	PE-CF594	BD Biosciences
CD243 (MDR1)	UIC2	APC	Biolegend
CD25	2A3	BV605	BD Biosciences
CD3	SK7	AF700, APC-H7, BV785	Biolegend
CD4	OKT4	BV510	Biolegend
CD45	HI30	PerCP-Cy5.5	BD Biosciences
CD45RA	HI100	APC-H7, BV711	BD Biosciences
CD45RO	UCHL1	BV711	BD Biosciences
CD8	SK1	FITC, PerCP-Cy5.5	BD Biosciences
CXCR3	G025H7	APC, BV421, PE-Cy7	Biolegend
VLA-4	9F10	BV711	BD Biosciences
In situ			
Marker	**Clone**	**Host species**	**Supplier**
CD4	SP35	Rabbit	Ventana
HLA-II	CR3/43	Mouse	DAKO
MAP2	HSM5	Mouse	Pierce
MDR1	UIC2	Mouse	Genetex
MOG	Z12	Mouse	Kind gift from prof. dr. S. Amor, VUmc

Supplementary Table 3. Used primer sets for qPCR

Gene name	Protein name	Forward/Reverse	5'-3' sequence
ABCB1	MDR1	Forward	GGAAATTTAGAAGATCTGATGTCAAAC
		Reverse	CACTGTAATAATAGGCATACCTGGTC
NR3C1	GR	Forward	TTCAAAAGAGCAGTGGAAGGA
		Reverse	TTTCTTCGAATTTTATCGATGATG
IL23R	IL-23R	Forward	CCTGGCTCTGAAGTGGAATTA
		Reverse	GGCTATTACTGCATCCCATTG
IFNG	IFN-γ	Forward	GGCATTTTGAAGAATTGGAAAG
		Reverse	TTTGGATGCTCTGGTCATCTT
CSF2	GM-CSF	Forward	TCTCAGAAATGTTTGACCTCCA
		Reverse	GCCCTTGAGCTTGGTGAG

Chapter 4

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

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Annals of Neurology 2019;86:264-278

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 indicates that certain B cells infiltrate the central nervous system (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, cerebrospinal fluid, 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 the 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 interferon-γ (IFN-γ) reduced the transmigration potential and antigen-presenting 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 (OCBs) 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-γ (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.

SUBJECTS 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 Medical Center (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 2018. All patients gave written informed consent, and study protocols were

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

CNS = central nervous system; MS = multiple sclerosis; HC = healthy control; NAT = natalizumab, Tx = treatment; IQR = interquartile range; NA = not applicable.

approved by the medical ethics committee of the Erasmus Medical Center (Rotterdam) and VU University Medical Center (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 1h at 37°C, after which the meninges were filtered through a cell strainer (45µm) 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 min 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 pH 7.4 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[b]
CD4	BV510	OKT4	Biolegend
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
CD46d (VLA-4)	APC	9F10	BD Biosciences
CCR4	PE-Cy7	L291H4	Biolegend
CCR6	PE	G024E3	Biolegend
CXCR3	BV605 and APC	G025H7	Biolegend
CXCR5	APC-R700	RF8B2	BD Biosciences
IgA	FITC	IS11-8E10	Miltenyi Biotech[c]
IgD	PE and PE-CF594	IA6-2	BD Biosciences
IgG	APC-H7	G18-145	BD Biosciences
IgG1	PE	HP6001	Southern Biotech[d]
IgG2	AF488	HP6002	Southern Biotech
IgM	BV510	MHM-88	Biolegend
T-bet	PE-Cy7	4B10	Biolegend
Fixable viability dye (FVS 700)	AF700		BD Biosciences

[a]Biolegend, London, UK
[b] BD Biosciences, Erembodegem, Belgium
[c]Miltenyi Biotech, Leiden, The Netherlands
[d]Southern Biotech via ITK diagnostics, Uithoorn, The Netherlands

Human B-cell migration assays

Flow-activated cell sorting (FACS)-sorted $CD27^-$ and $CD27^+$ memory $CD19^+$ B cells from buffy coat-derived PBMCs were placed on 96 wells permeable transwell plates with a 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, $2,5\text{-}5\times10^5$ 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 medium 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% (2 mM) 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 (magnetic activated cell sorted). B cells (1×10^5) and T cells (0.5×10^5) 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, Bergisch Gladbach, Germany), 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 (10×10^3 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 $20\text{-}25\times10^3$ 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, Uden, The Netherlands) were coated overnight with anti-human IgG1 monoclonal capture antibody (1µg/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% (vol/vol) 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 (Winooski, VT) Synergy 2. 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, San Diego, CA) and compared using two sided Mann-Whitney U tests, Wilcoxon matched-pairs signed rank test, 1- or 2-way analysis of variance with Tukey post hoc test, Friedman paired with Dunn post hoc test, and Spearman correlation coefficients (as indicated in each figure legend). Experimental data are depicted as the mean ± standard error of the mean (SEM). Prior to statistical analyses, datasets were tested for normal distribution. Probability values <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 chemoattractants 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]; brain tissue, 2,889 [26-18,050]. The frequency of CXCR3⁺, and not CXCR5⁺ or CCR6⁺ B cells was strongly increased in *ex vivo* cell suspensions from MS brain tissues ($p < 0.0001$), meninges ($p = 0.0003$), and CSF ($p < 0.0001$) compared to blood (Fig. 1B). CXCR3-expressing T cells, including Th17.1, were also enriched in the CNS compartments of these donors (data not shown), supporting our recent observations [20].

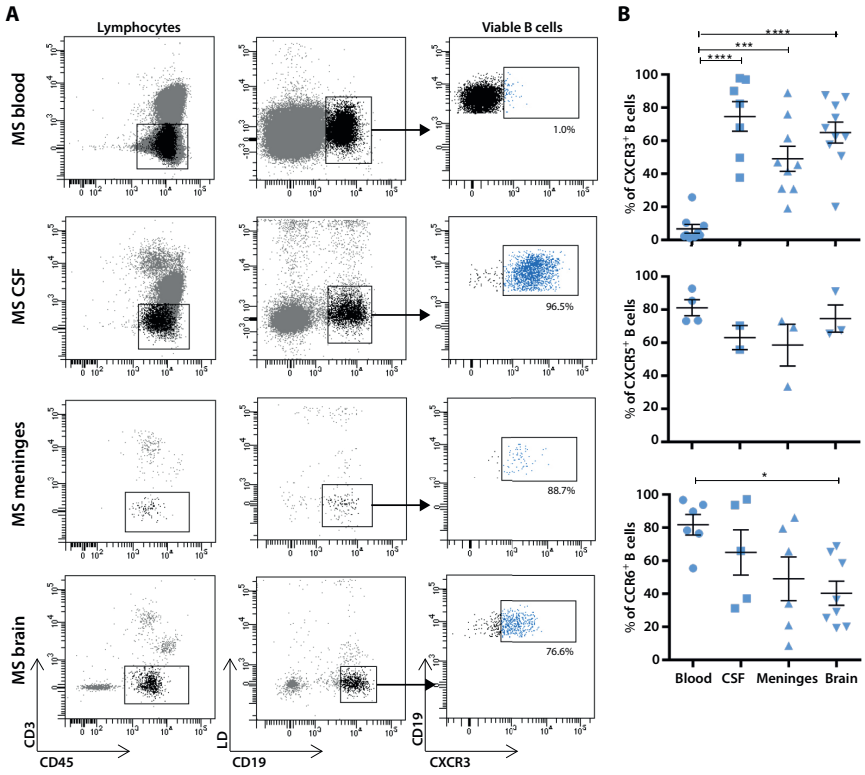


Figure 1. CXCR3⁺ B cells are abundant in the CNS compared to blood of MS patients.

(**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 <25 viable B cells were excluded from these analyses. Data are presented as the mean ± SEM. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. The p values for **B** were calculated by a 1-way analysis of variance test. LD = live/dead (for detection of viable cells).

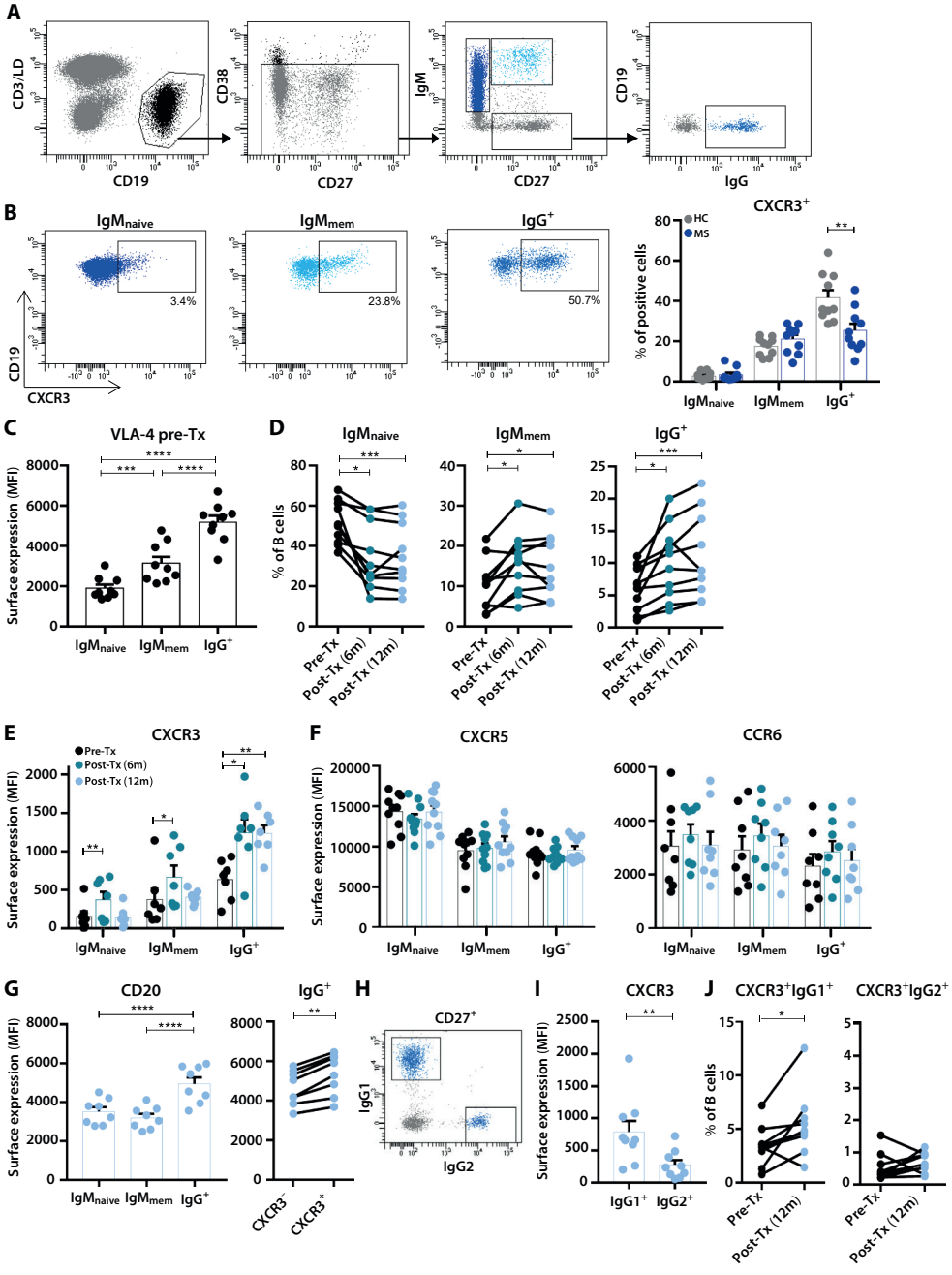
Reduced frequencies of CXCR3$^+$IgG(1)$^+$ B cells in the blood of MS patients are abrogated after natalizumab treatment

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), whereas 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 the blood of MS patients both 6 and 12 months after versus before 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 (Table 1; data not shown). Notably, CD20 expression levels were increased on IgG$^+$ B cells and higher on CXCR3$^+$ compared to CXCR3$^-$ counterparts in the blood of natalizumab-treated MS patients (Fig. 2G).

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 (HC; 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 MS patient blood before and after natalizumab treatment (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 < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. The p values were calculated by Mann-Whitney U (**B**), 2-way analysis of variance (**C** and **G**), Friedman paired (**D-F**), and Wilcoxon matched-pairs signed rank (**G, I** and **J**) tests. MFI = mean fluorescence intensity; Tx = treatment; LD = live/dead (for detection of viable cells).


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
IgG1+
IgG2+
J
CXCR3+IgG1+
CXCR3+IgG2+

Because 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 [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 OCBs in MS patients [28].

IFN-γ promotes CXCR3 expression and CD4$^+$ T-cell activation by human T-bet$^+$ B cells under T follicular helper-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 (Tfh) 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. Because antigen uptake by B cells is limited 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

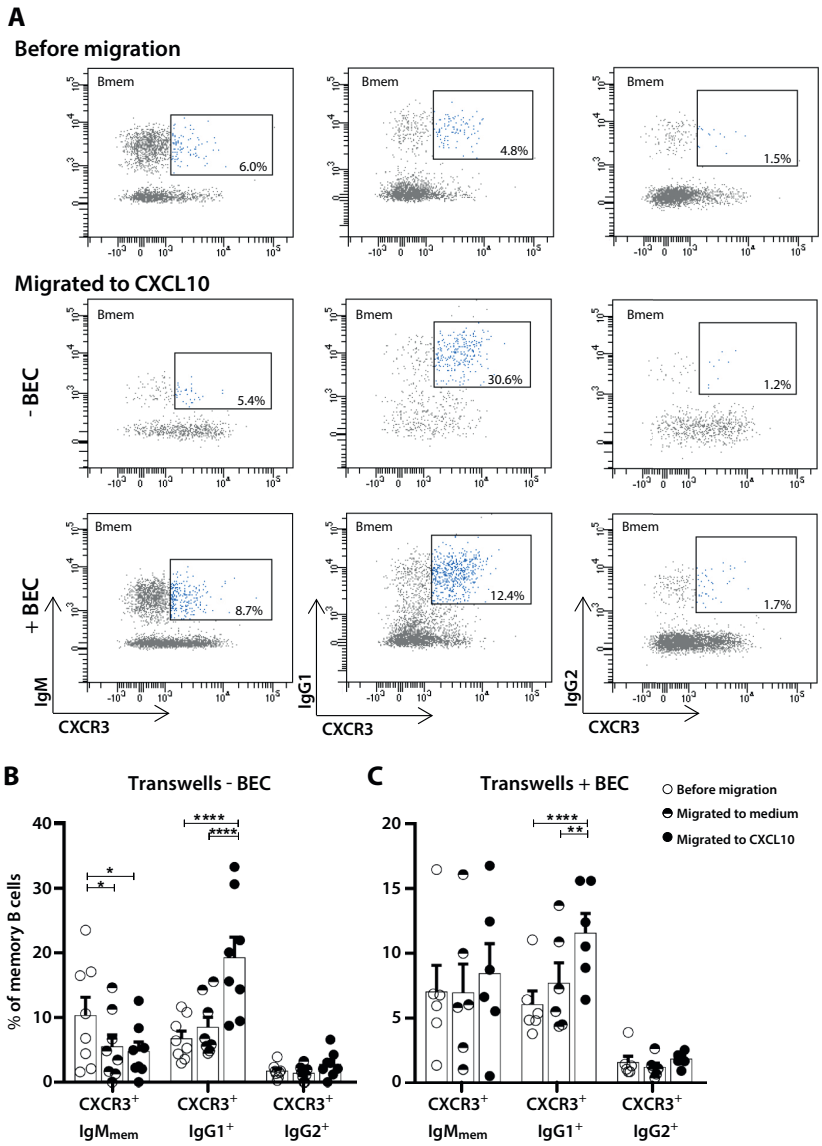


Figure 3. Enhanced migration of CXCR3⁺IgG1⁺ B cells across transwell filters and human brain endothelial monolayers *in vitro*.

Sorted memory B cells from healthy donor blood were assessed for selective *in vitro* transmigration towards CXCL10. (**A**) Representative FACS plots and (**B** and **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 to 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. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. The p values were calculated by 2-way analysis of variance (**B** and **C**).

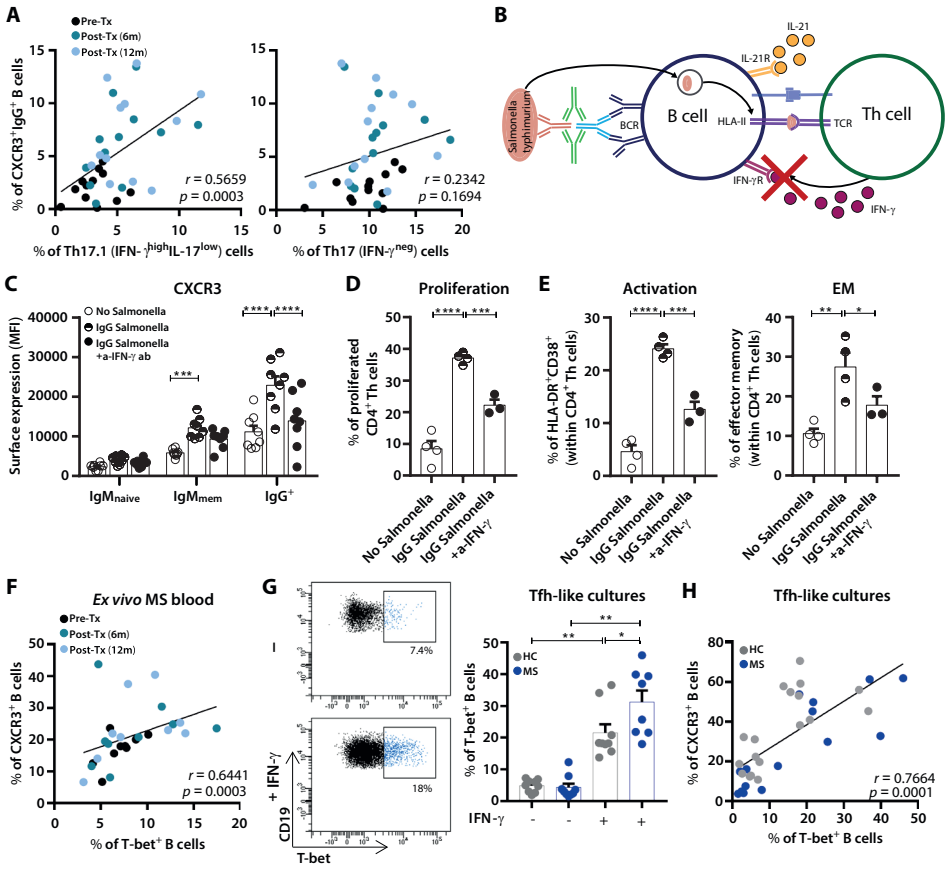


Figure 4. T helper 1 cytokine IFN-γ is a major trigger of CXCR3⁺(T-bet⁺) B-cell differentiation in MS.
(A) Correlation of *ex vivo* CXCR3⁺IgG⁺ B cells with Th17.1 (IFN-γ^{high}IL-17^{low}) and Th17 (IFN-γ^{neg}) cells in MS blood before and after natalizumab treatment (pre-Tx and post-Tx; n = 12). (B) Experimental model of *Salmonella*-primed autologous B- and T-cell co-cultures. (**C-E**) B cells from healthy donor blood were primed with *S. typhimurium* through BCR crosslinking using a tetrameric antibody complex, as described in Subjects and 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** and **E**). These experiments were performed in two independent experiments and in duplicate for (**C**) four and (**D** and **E**) two different blood donors. (**F**) Correlation of surface CXCR3 and intracellular T-bet expression in *ex vivo* B cells of MS patients before and after natalizumab treatment (pre-Tx and post-Tx; n = 9). (**G** and **H**) Total B cells from the blood of MS patients (n = 9) and both age-and gender-matched healthy controls (HC; n = 10) were cultured *in vitro* under Tfh-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. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. The *p* values were calculated by 2-way analysis of variance (ANOVA) (**C**), 1-way ANOVA (**D** and **E**) and Wilcoxon matched-pairs signed rank (**G**) tests. The correlation coefficients and p values for **A, F** and **H** were calculated by Spearman rank. m = months; MFI = mean fluorescence of intensity; TCR = T-cell receptor.

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 Tfh-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), whereas 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 Tfh-like B-cell cultures

Besides IFN-γ, TLR9 ligand CpG-DNA has also 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 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

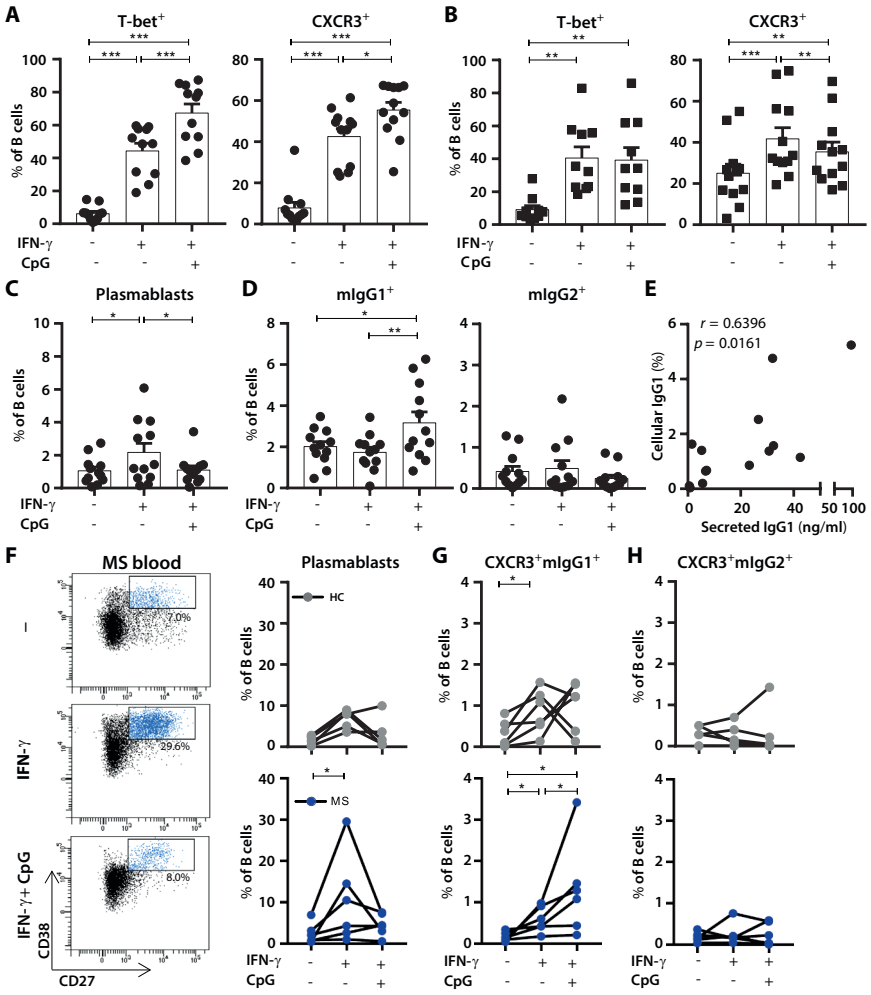


Figure 5. IFN-γ induces plasmablast differentiation, whereas both IFN-γ and CpG-DNA further upregulate T-bet and trigger IgG1 switching in B cells of MS patients.

(**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 Tfh-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) are shown. 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 was 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 (HC; n = 6; grey dots) were cultured under the same Tfh-like conditions and analyzed for (**F**) plasmablast (CD38^{high}CD27$^+$) and (**G** and **H**) CXCR3$^+$mIgG1$^+$ and CXCR3$^+$mIgG2$^+$ B-cell differentiation after 11 days of culture. Data are presented as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. 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.

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. 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 Tfh-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]. Although 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 clarification of how they may contribute to propagating local injury responses are 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. 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]. Although 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 Tfh 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 Tfh1 cells in germinal centers can trigger development of such B cells. In MS patients, B cells were found to express higher T-bet levels under Tfh1-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. Because 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 Tfh1 cells, innate TLR signaling is also 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 Tfh-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, Epstein-Barr virus-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-induced 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 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 finding that antigen-specific B cells are potentially 1,000 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

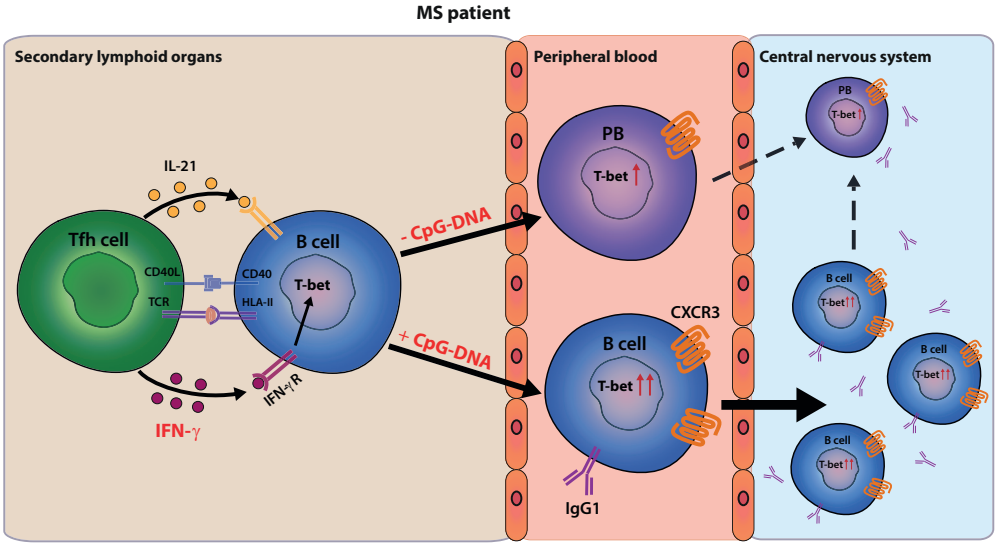


Figure 6. IFN-γ and TLR9 signaling upregulate T-bet in peripheral B cells, likely driving CXCR3-mediated recruitment and IgG1 production in the CNS of MS patients.
Our findings suggest that in the secondary lymphoid organs of MS patients, IFN-γ triggers naive B cells to differentiate into T-bet-expressing populations in a Tfh-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.

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 (jakinibs) [49] and the TLR/myD88 pathway [50] are already used in 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.

ACKNOWLEDGEMENT

This work was made possible by the support of the Dutch MS Research Foundation (14-875 MS) and partly by Erasmus MC (Mrace grant; both to M.M.v.L.). This research was performed within the framework of the Erasmus Postgraduate School Molecular Medicine. We thank P. van Geel and H. de Wit for FACS sorting.

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Chapter 5

The association of Epstein-Barr virus infection with CXCR3^{+} B-cell development in multiple sclerosis: impact of immunotherapies

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European Journal of Immunology 2020; Epub ahead of print

ABSTRACT

Epstein-Barr virus (EBV) infection of B cells is associated with increased multiple sclerosis (MS) susceptibility. Recently, we found that CXCR3-expressing B cells preferentially infiltrate the central nervous system of MS patients. In chronic virus-infected mice, these types of B cells are sustained and show increased antiviral responsiveness. How EBV persistence in B cells influences their development remains unclear. First, we analyzed *ex vivo* B-cell subsets from MS patients who received autologous bone marrow transplantation (n = 9), which is often accompanied by EBV reactivation. The frequencies of non-class-switched and class-switched memory B cells were reduced at 3-7 months, while only class-switched B cells returned back to baseline at 24-36 months post-transplantation. At these time points, EBV DNA load positively correlated to the frequency of $CXCR3^+$, and not $CXCR4^+$ or $CXCR5^+$, class-switched B cells. Second, for $CXCR3^+$ memory B cells trapped within the blood of MS patients treated with natalizumab (anti-VLA-4 antibody; n = 15), latent EBV infection corresponded to enhanced *in vitro* formation of anti-EBNA1 IgG-secreting plasma cells under germinal center-like conditions. These findings imply that EBV persistance in B cells potentiates brain-homing and antibody-producing $CXCR3^+$ subsets in MS.

INTRODUCTION

The success of anti-CD20 therapy puts forward B cells as important players of autoimmune diseases including multiple sclerosis (MS) [1]. The strongest infectious risk factor for MS is the Epstein-Barr virus (EBV) [2, 3], a DNA γ-herpes virus that infects and persists within B cells using a series of programs that mimic germinal center (GC) differentiation signals [4]. In MS, one of the hypotheses is that EBV remains latent within pathogenic memory B cells that invade the central nervous system (CNS) [3, 5]. Once in the CNS, pathogenic memory B cells are likely re-activated and further develop into antibody-secreting plasma cells to mediate local inflammation [1]. IL-21 and CD40L are the main triggers of plasma cell differentiation during a GC response [6, 7]. However, in murine models of autoimmune diseases this response seems to be dysregulated in the presence of IFN-γ [8].

IFN-γ triggering of B cells induces the T-box transcription factor T-bet, which drives the surface expression of CXC chemokine receptor (CXCR)3 [9]. Recently, our group found that CXCR3-expressing B cells are abundant in the MS CNS and selectively accumulate in the blood of MS patients treated with natalizumab (anti-VLA-4 mAb; NTZ) [10], an effective drug that prevents their infiltration into the CNS. Moreover, naive B cells of untreated MS patients preferentially developed into CXCR3-expressing plasmablasts under IL-21-, CD40L- and IFN-γ-inducing conditions *in vitro* [10]. Interestingly, in mice, CXCR3(T-bet)-expressing B cells do not only show increased antiviral responses, but are also more sustained during chronic viral infections [11]. How EBV infection contributes to the development of pathogenic memory B cells is poorly understood.

Here, we used two distinct MS cohorts to determine the association of B-cell EBV DNA load with CXCR3+ memory B-cell development. First, memory B-cell subsets were studied *ex vivo* from MS patients who received autologous bone marrow transplantation (BMT), which is often accompanied with EBV reactivation [12, 13]. Second, GC-like plasma cell differentiation was explored *in vitro* for memory B cells trapped within the blood of NTZ-treated MS patients, who are known to experience a massive local influx of EBV-infected B cells after treatment cessation [14].

MATERIALS AND METHODS

Patients

Characteristics of patients used in this study are summarized in Supplementary Table 1. For the analysis of EBV infection in relation to *ex vivo* B-cell subsets, we used 9 MS patients who had undergone autologous BMT as previously described [15]. In short, CD34+ stem cells were isolated from bone marrow and cryopreserved until use. These patients obtained

a vigorous T cell depletion regimen consisting of ATG (anti-thymocyte globulin) and total body irradiation before treatment with the preserved stem cells [15]. For each patient, thawed peripheral blood mononuclear cells (PBMCs) were analyzed before and both 3-7 and 24-36 months after BMT. For the analysis of EBV load in relation to $CXCR3^+$ B-cell differentiation *in vitro*, we used thawed PBMCs of MS patients treated with NTZ (anti-VLA-4 antibody; 1-4y post-treatment) [10]. These patients were both age- and gender-matched with healthy controls. Study protocols were approved by the medical ethics committee of the Erasmus Medical Center (Rotterdam, The Netherlands) and all patients gave written informed consent [15, 16].

Cell isolation, antibodies and flow cytometry

PBMCs were isolated from blood of patients using Ficoll®-Paque Plus (GE Healthcare, Freiburg, Germany) and density centrifugation or with vacutainer CPT® tubes (BD Biosciences, Erembodegem, Belgium) and stored in liquid nitrogen until use as earlier reported [10]. FACS was performed on PBMCs using monoclonal antibodies as indicated in Supplementary Table 2. Cells were stained extracellularly for 30 min at 4°C. Prior to extracellular staining, cultured memory B cells were labelled with a viability stain (FVS700) for the analysis of viable cells. All measurements were performed on a LSRII-Fortessa machine and analyzed using FACS Diva software, version 8.0.1 (both BD Biosciences). B-cell subsets were separated using a FACSAria III cell sorter (BD Biosciences) for qPCR analysis or *in vitro* cultures.

DNA isolation and EBV load qPCR

DNA was extracted using a GenElute™ Mammalian Genomic DNA Miniprep Kit according to the manufacturer's instructions (Sigma-Aldrich via Merck, Kenilworth, New Jersey, USA). EBV DNA load was determined using a multiplex RQ-PCR assay using a FAM-labeled probe specific for *BALF5* (the EBV DNA polymerase; Eurogentec, Liège, Belgium) and a VIC-labeled probe specific for reference gene beta-2 microglobulin (*B2M*; Thermo Fisher Scientific, Landsmeer, The Netherlands) [17, 18]. Primer and probe sequences and used concentrations are shown in Supplementary Table 3. We added 5 µl (200 ng) DNA to 20µl reaction mixture containing primers and probes for *BALF5* and *B2M* supplemented with TaqMan™ Universal Master Mix II, no UNG (Applied Biosystems™, Thermo Fisher Scientific). Samples were run on a QuantStudio™ 5 machine (Applied Biosystems™, Thermo Fisher Scientific) using the following thermal cycle protocol: 10 min at 95°C followed by 50 cycles of 15 s at 95°C and 1 min at 60°C.

EBV genome copies per 1×10^6 cells were calculated using standard curves for *BALF5* and *B2M* as analyzed and generated by the QuantStudio™ Design and Analysis software version 1.4.1. The standard curves were made by serial dilutions of Namalwa DNA [17]

containing 40^4 (stock concentration: 132 ng/µl DNA; assuming that 1 cell contains 6.6 pg DNA and each Namalwa cell contains two integrated EBV genomes), 10^4, 10^3, 200, 40, 10, 4 and 1 EBV genome per µl H_2O. The quantity of EBV DNA in sorted B cells was extrapolated from these curves using CT values measured in each experiment. All samples were measured in duplicate.

In vitro memory B-cell differentiation assay

Sorted $CD27^+$ memory B cells were cultured under GC-like conditions as previously described [10]. In short, irradiated 3T3-CD40L cells were seeded on flat-bottom 96-well plates. We added 5×10^4 memory B cells per well in the presence of human recombinant IL-21 (50 ng/ml; Thermo Fisher Scientific) with and without human recombinant IFN-γ (50 ng/ml; Peprotech/Bio-Connect, Huissen, The Netherlands). After 6 days of culture, supernatants were collected and stored at -80°C until use for an EBNA1 IgG enzyme-linked immunosorbent assay (ELISA). The cultured cells were removed and extracellularly stained using flow cytometry as described above.

Anti-EBNA1 IgG ELISA

A commercially available ELISA kit (IBL international, Hamburg, Germany) was used to quantify anti-EBNA1 IgG antibodies in memory B-cell culture supernatants. 50 µL of each supplied standard and culture supernatants were added to the plate and incubated for 60 min at room temperature (RT). After washing, samples were incubated with rabbit peroxidase-conjugated IgG for 30 min at RT. Each well was incubated with TMB substrate solution for 20 min at RT and the reaction was stopped with TMB solution. Optical densities were measured at 450nm using a BioTek Synergy 2 machine (Winooski, Vermont, USA). Data were analyzed by following the manufacturer's instructions. A cut-off value for culture supernatants was determined based on the average from cultured naive mature B cells (n = 8). Results above ≥1.59 U/ml were considered positive, which was the case for 13 out of 21 samples.

Statistical analysis

Statistical analyses on datasets were carried out using GraphPad Prism Software, version 8 (GraphPad Software, San Diego, CA) and indicated in figure legends. Data are depicted as the mean ± standard error of the mean and probability values <0.05 were considered significant.

RESULTS AND DISCUSSION

EBV load corresponds to CXCR3 expression in class-switched memory B cells of BMT-treated MS patients

To address the specific relation between EBV load and CXCR3$^+$ B-cell induction, we explored the distribution, EBV load and chemokine receptor profiles of *ex vivo* B-cell subsets within the blood of 9 MS patients after autologous BMT (Supplementary Table 1) [15]. The proportions of transitional (CD38^{high}CD27$^-$) B cells were increased at 3-7 months and reduced to baseline levels at 24-36 months, while those of naive mature (CD38$^{-/dim}$IgM$^+$CD27$^-$) B cells remained unaltered post-BMT (Fig. 1A and B). Both Ig class-switched (IgM$^-$IgD$^-$) and non-class-switched (IgM$^+$CD27$^+$) memory B cells were decreased at 3-7 months, and only class-switched B cells returned to baseline levels at 24-36 months post-BMT (Fig. 1A and B). After purifying these subsets, high EBV copy numbers were measured for class-switched B cells at 3-7 months post-BMT (Fig. 1C), at the timeframe in which low numbers of memory B cells are present in the blood. This is in line with previous studies using allogeneic stem cell transplants [19, 20]. EBV levels in class-switched B cells were increased for 5 patients and reduced for 4 patients when comparing 3-7 months post- to pre-BMT samples. These changes in EBV load strongly corresponded to the expression of CXCR3 (Fig. 2A and B). Taking all individual samples into account, we found a positive correlation between EBV load and CXCR3$^+$, but not CXCR4$^+$ or CXCR5$^+$ fractions of class-switched B cells (Fig. 2C and D). Despite the lower levels of EBV, similar trends were observed in non-class-switched B cells (Supplementary Fig. 1A). In contrast to non-class-switched B cells, EBV load positively correlated to the expression of activation markers CD69 and CD95 in class-switched B cells (Supplementary Fig. 1B). Notably, three out of four BMT-treated patients whose memory B cells showed decreased EBV and CXCR3 levels, showed stable or improved disability scores during 36 months follow-up as reflected by expanded disability status scale (EDSS) score and ambulatory index (AI, Fig. 2E) [15]. In BMT-treated patients with an increase in memory B cell EBV and CXCR3 levels, an increased disability was observed in four out of five and five out of five participants, respectively.

Overall, these findings implicate that the high EBV levels found after autologous BMT in MS patients induces the development of CXCR3$^+$ memory B cells and is possibly related to clinical worsening. CXCR3 upregulation through EBV infection may be caused by a disturbed immune system [21], and contribute to the increased recruitment of pathogenic B cells and the formation of follicle-like structures in the MS CNS [10, 22].

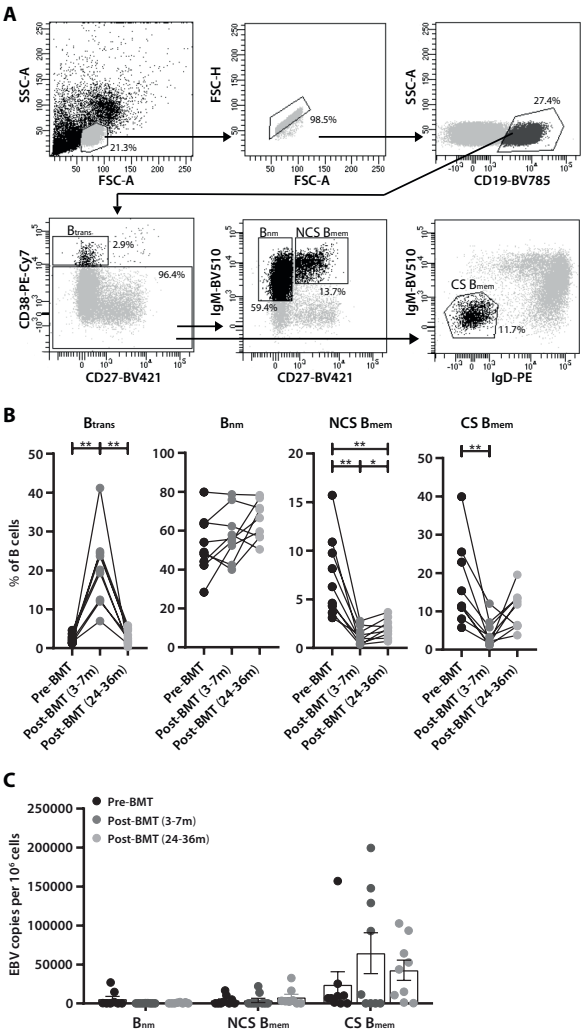

Figure 1. Reconstitution and EBV load of B-cell subsets in the blood of autologous BMT-treated MS patients.

(**A**) FACS gating strategy used to define transitional ($CD38^{high}CD27^{-}$; B_{trans}), naive mature ($CD27^{-}IgM^{+}$; B_{nm}), non-class-switched memory ($CD27^{+}IgM^{+}$; NCS B_{mem}) and class-switched memory ($IgM^{-}IgD^{-}$; CS B_{mem}) within blood $CD19^{+}$ B cells. (**B**) Quantification of B-cell subset frequencies in the blood of MS patients before (black dots) and both 3-7 months (dark grey dots) and 24-36 months (light grey dots) after receiving autologous BMT (paired samples; n = 9; Supplementary Table 1). (**C**) EBV DNA load in sorted B_{nm}, NCS B_{mem} and CS B_{mem} cells before and both 3-7 and 24-36 months after BMT (n = 9; copies / 1×10^{6} cells). FACS data were measured and B cell subsets were sorted in five independent experiments, with paired time points for 1-2 patients per experiment. EBV DNA load was determined in three independent experiments, with paired time points for 3-5 patients per experiment. Data are presented as the mean ± SEM. ** p <0.01 and * p <0.05. The p values in **B** were calculated by repeated measures one-way analysis of variance with Tukey post hoc test.

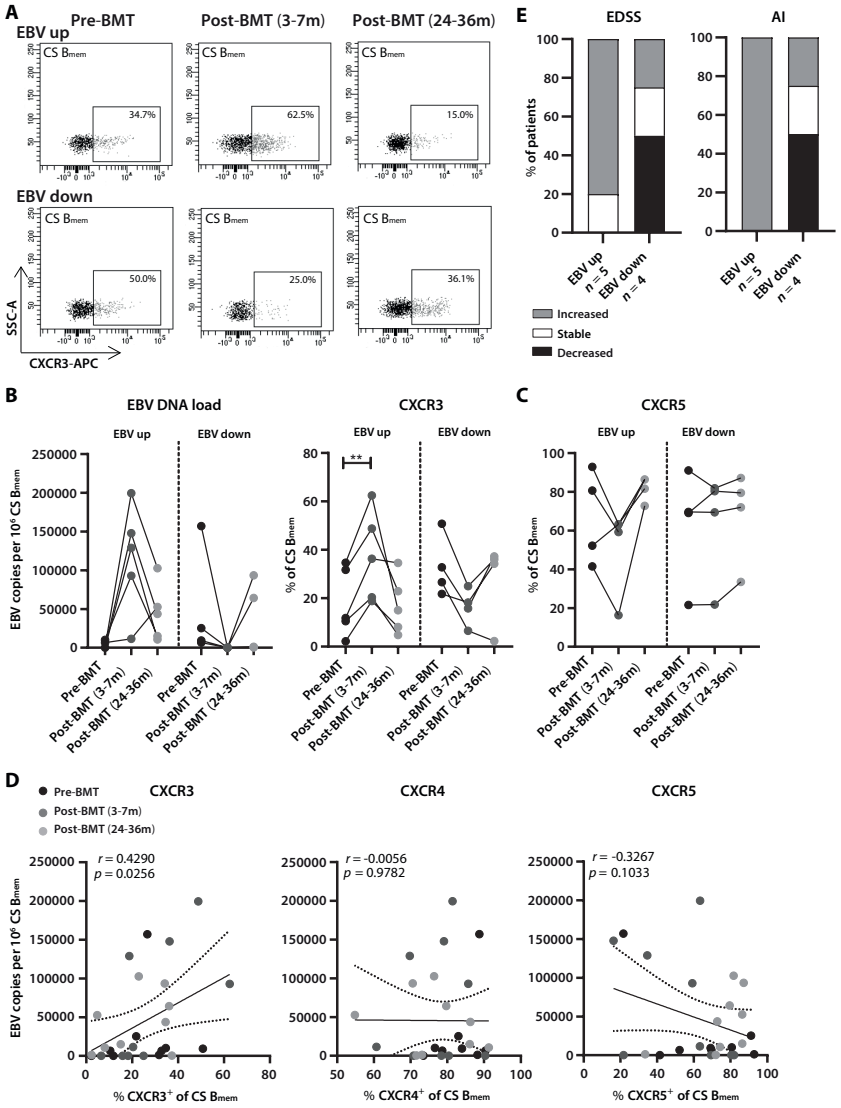

Figure 2. The association between EBV load and chemokine receptor expression in class-switched B cells of BMT-treated MS patients.

(**A**) Representative FACS plots of CXCR3-expressing CS B_{mem} cells from an MS patient before and both 3-7 months and 24-36 months after autologous BMT. EBV copy numbers and frequencies of $CXCR3^+$ (**B**) as well as $CXCR5^+$ (**C**) fractions were determined for CS B_{mem} cells of 9 BMT-treated MS patients. (**D**) EBV copy numbers were correlated to $CXCR3^+$, $CXCR4^+$ and $CXCR5^+$ fractions of CS B_{mem} cells. Data were collected in the same number of experiments as depicted in Figure 1. (**E**) EDSS and AI changes after BMT treatment (Δ pre- vs 36 months post-BMT) for patients showing increased (n = 5) and decreased (n = 4) memory B-cell EBV loads. ** $p < 0.01$. The p values were calculated by repeated measures one-way analysis of variance with Tukey post hoc test (**B** and **C**) and correlation coefficient by Pearson rank (**D**).

EBV^{high} memory B cells of NTZ-treated MS patients preferentially develop into CXCR3$^+$ plasma cells

EBV mainly persists within GC-derived memory B cells, which subsequently differentiate into antibody-secreting plasma cells [4]. After NTZ treatment, MS patients show increased numbers of CXCR3$^+$ memory B cells in the blood, which are normally recruited and further differentiate into plasma cells in the CNS [10, 23]. Hence, blood samples from these patients offer a unique opportunity to assess the relation between latent EBV infection and plasma cell development of potentially CNS-infiltrating memory B cells. To determine this, we screened EBV load in different blood samples from 15 MS patients treated with NTZ for 1 to 4 years. Of these patients, one showed an increase, one a decrease and the remainder a stable EDSS-score over the course of 36 months after sampling (Supplementary Table 1). Ten samples with >1000 copies per 1×10^6 cells (EBV^{high}) and eight samples with <500 copies per 1×10^6 cells (EBV^{low}) were selected for our *in vitro* differentiation study. CD27$^+$ memory B cells were cultured under IL-21- and CD40L-stimulating (GC-like) conditions for 6 days and differentiation into plasma cells (CD38^{high}CD27^{high}CD138$^+$) was compared with and without the addition of IFN-γ (Fig. 3A and B). Under these circumstances, total and CXCR3$^+$ plasma cells were found to be more induced in the EBV^{high} versus the EBV^{low} group, which was most pronounced in cultures containing IFN-γ ($p = 0.023$ and $p = 0.011$, respectively; Fig. 3C and D). For 9 out of 10 samples from the EBV^{high} group, CXCR3$^+$ plasma cell frequencies were higher under IFN-γ-inducing conditions than those from a healthy reference group (n = 6; dotted line), which was the case for 2 out of 6 samples from the EBV^{low} group. B-cell EBV load correlated to CXCR3$^+$ plasma cell outgrowth in IL-21- and IFN-γ-induced samples (Fig. 3E). This agrees with a mouse study showing that enhanced CXCR3 expression results in aberrant plasma cell development within GCs [9]. Both surface CXCR3 and intracellular T-bet levels were further triggered by IFN-γ during these cultures, but were not different between EBV^{high} and EBV^{low} groups (Supplementary Fig. 2A). Finally, the ability of *in vitro*-induced plasma cells to secrete anti-EBNA1 IgG positively correlated with the expression of CXCR3 (Fig. 3F) and T-bet (Supplementary Fig. 2B).

CONCLUDING REMARKS

In this study, we reveal that high EBV load is associated with 1) early emergence of CXCR3$^+$ class-switched memory B cells from autologous BMT-treated MS patients, and 2) enhanced *in vitro* generation of CXCR3$^+$ plasma cells from memory B cells trapped in the blood of NTZ-treated MS patients. Although we did not show the dependence of memory B-cell development on CXCR3 in these settings, there is a clear relation between CXCR3 expression and GC-like B-cell development [9] and recruitment to the CNS [24]

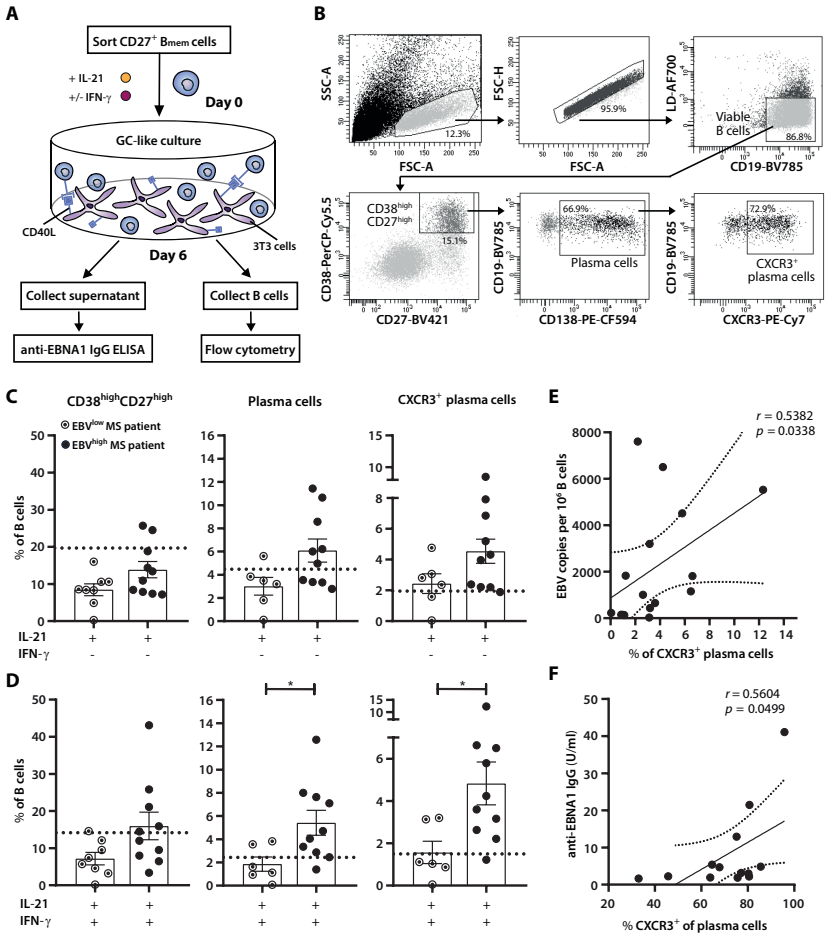

Figure 3. *In vitro* plasma cell formation of memory B cells from NTZ-treated MS patients with different B-cell EBV load.

(**A**) Experimental setup of the GC-like memory B-cell differentiation assay. $CD27^+$ memory B cells (B_{mem}) were sorted from different blood samples of a total of 15 NTZ-treated MS patients (Supplementary Table 1) and cultured under IL-21-/CD40L-inducing (GC-like) conditions for 6 days. (**B**) Representative gating strategy for the analysis of *in vitro*-differentiated plasma cells within viable $CD19^+$ cells after 6 days of culturing. Plasma cell development was analyzed for NTZ-treated MS patients with high and low B-cell EBV load (n = 6-10) under conditions with and without IFN-γ (**C** and **D**). Dotted lines indicate the mean frequencies of each population analyzed for simultaneous *in vitro* cultures with memory B cells of 6 age- and gender-matched healthy controls. *In vitro* cultures and FACS data were collected in six independent experiments, with 1-2 EBV^{high} and 1-2 EBV^{low} MS patients, as well as 1 healthy control per experiment. (**E**) Correlation between EBV copy numbers and fractions of *in vitro*-induced $CXCR3^+$ plasma cells. (**F**) Correlation between anti-EBNA1 IgG secretion and CXCR3 surface expression by *in vitro*-differentiated plasma cells. anti-EBNA1 IgG was measured in four independent experiments and each sample was measured in duplicate. Data are presented as the mean ± SEM. * $p < 0.05$. The p values were calculated by Mann-Whitney U (**D**) and correlation coefficients by Spearman rank (**E** and **F**) tests.

in virus-infected mice. Based on our findings, EBV load is related to the development of CXCR3⁺ memory B cells into plasma cells, while the expression of CXCR3 by these plasma cells seems to enhance their ability to secrete anti-EBNA1 antibodies. This may not only link to the predictive value and intrathecal detection of anti-EBV antibodies [25, 26], but also add to the ongoing debate about the local presence of EBV-infected B cells in MS [27].

ACKNOWLEDGEMENTS

The authors would like to dedicate this article to the memory of Professor Rogier Q. Hintzen, who passed away on May 15, 2019. We thank Harm de Wit and Peter van Geel for FACS sorting. We thank the patients and healthy controls for donating blood for this study. This work was financially supported by the Dutch MS research foundation (14-875 MS and 15-490d MS). This research was performed within the framework of the Erasmus Postgraduate School Molecular Medicine.

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SUPPLEMENTAL DATA

Supplementary Table 1. Demographics of MS patients included in this study

***Ex vivo* B-cell analysis**							
BMT-treated MS patient[a]	**Age (years)[b]**	**Gender**	**Disease duration (months)[c]**	**EDSS**		**AI**	
				Pre-BMT	**36m post-BMT**	**Pre-BMT**	**36m post-BMT**
1	47	M	60	6.5	6.5	4	6
2	44	F	48	6.5	8	6	9
3	31	F	60	5.5	4.5	4	3
4	37	F	60	6	5.5	4	4
5	50	F	48	5.5	6.5	3	6
6	41	F	24	6	6	6	5
7	23	M	72	6	8	5	9
8	34	F	36	5	6.5	3	6
9	34	M	144	6	7	4	7
***In vitro* B-cell analysis**							
NTZ-treated MS patient	**Age (years)[b]**	**Gender**	**Disease duration (months)[c]**	**EDSS**		**AI**	
				Pre-NTZ	**36m post-NTZ**		
1	41	F	44	3.5	3.5	NA	
2	38	M	41	3.5	3.5	NA	
3	36	F	104	4	4	NA	
4	46	F	46	3	3	NA	
5	44	F	5	3.5	3.5	NA	
6	46	M	88	3.5	3.5	NA	
7	21	M	39	2.5	2.5	NA	
8	29	M	124	4.5	4.5	NA	
9	33	F	131	6.5	6.5	NA	
10	25	F	79	1.5	NA[d]	NA	
11	37	F	109	6.5	6.5	NA	
12	34	F	45	2.5	2.5	NA	
13	49	F	192	5	4	NA	
14	27	M	2	4.5	4.5	NA	
15	28	M	11	2.5	4	NA	

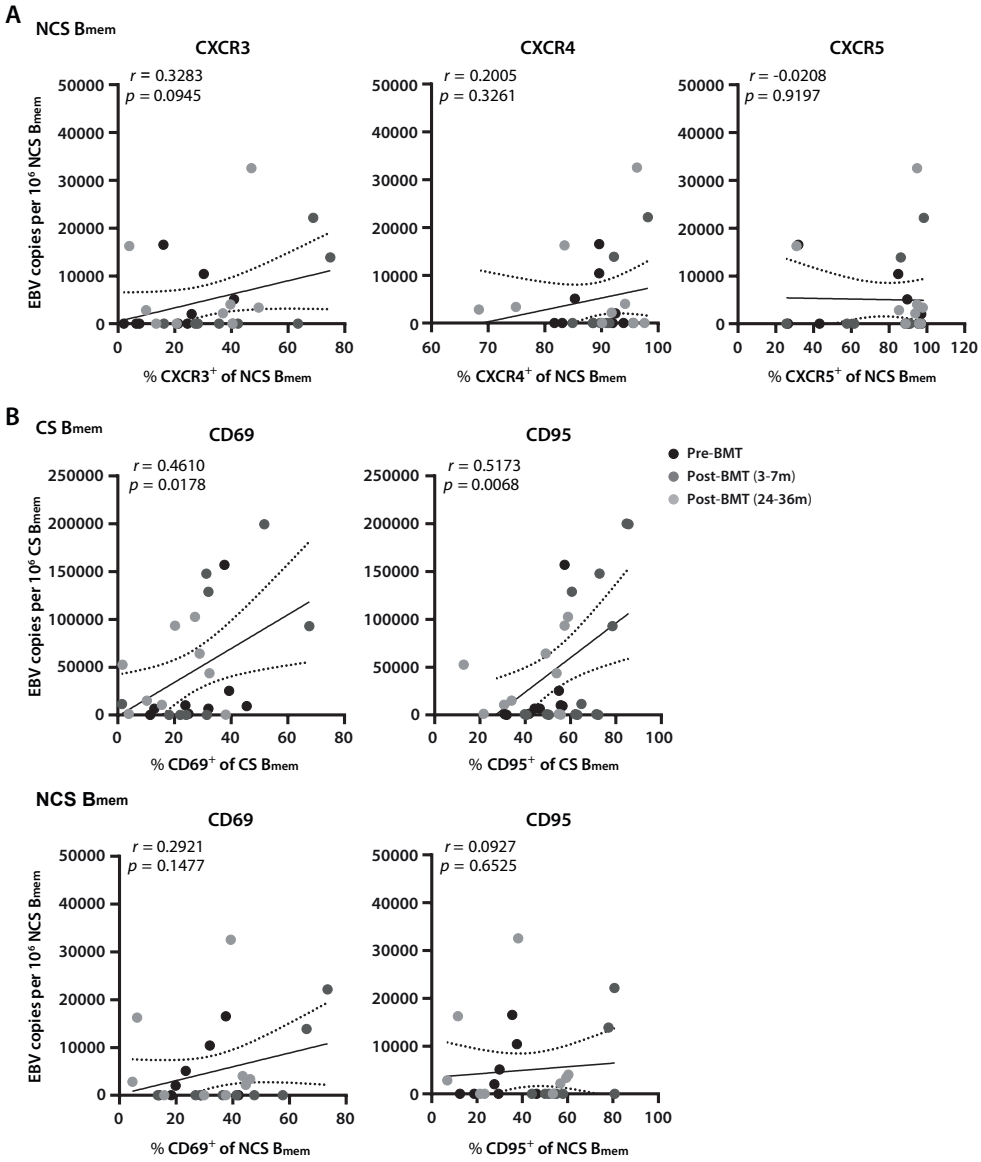
[a]Secondary progressive MS patients who had received an autologous bone marrow transplantation [15].
[b]At start of treatment.
[c]Time from MS diagnosis to start of treatment.
[d]This patient discontinued natalizumab at 24 months of treatment.
BMT = bone marrow transplant; MS = multiple sclerosis; EDSS = expanded disability status score; AI = ambulatory index; NTZ = natalizumab; NA = not applicable.

Supplementary Table 2. Monoclonal antibodies used for FACS analysis

Antibody marker	Fluorochrome	Clone	Company
CD3	FITC	SK7	BD Biosciences
CD19	BV785	HIB19	BD Biosciences
CD27	BV421	M-T271	BD Biosciences
CD38	PE-Cy7 and PerCP-Cy5.5	HIT2	Biolegend
CD69	APC-R700	FN50	BD Biosciences
CD95	BV605	DX2	Biolegend
CD138	PE-CF594	MI15	BD Biosciences
CXCR3	BV605, APC, PE-Cy7	G025H7	Biolegend
CXCR4	PE-CF594	12G5	BD Biosciences
CXCR5	PerCP-Cy5.5	RF8B2	BD Biosciences
IgD	PE	IA6-2	BD Biosciences
IgM	BV510	MHM-88	Biolegend
Fixable viability dye	AF700		BD Biosciences

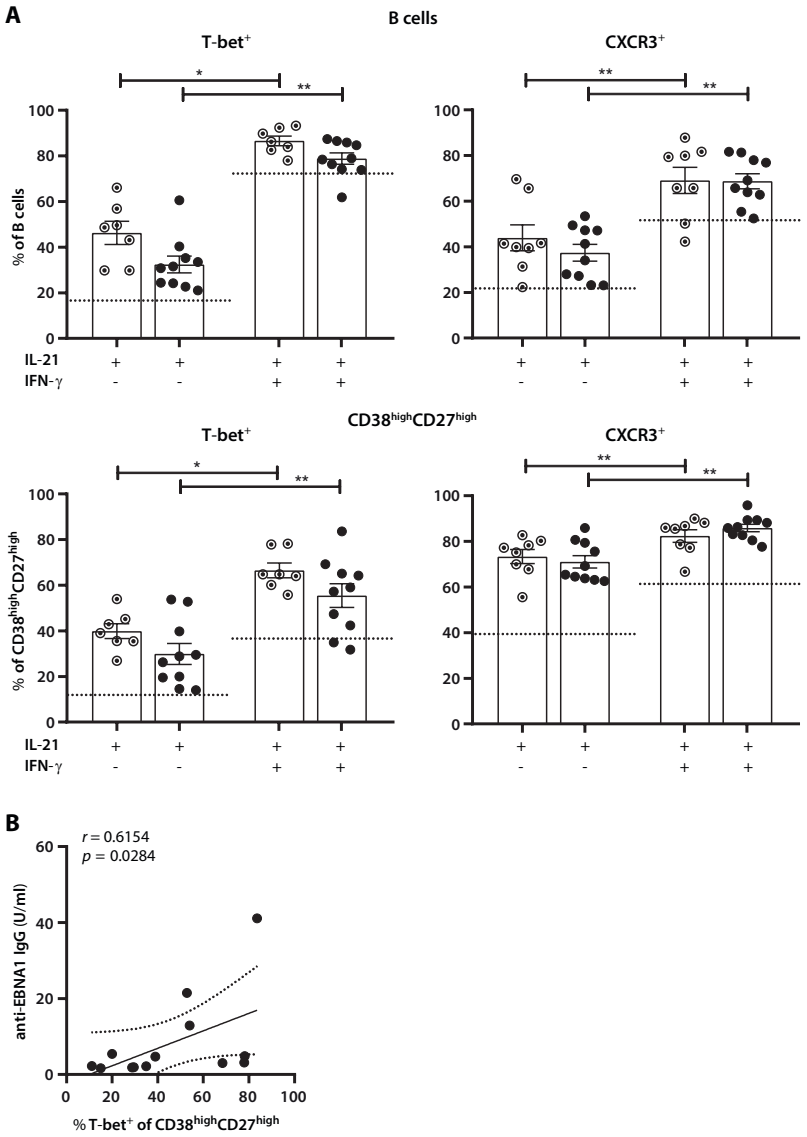
Supplementary Table 3. Primers and probes used for qPCR

BALF5	
Forward primer (10 μM)	CTTTGGCGCGGATCCTC
Reverse primer (10 μM)	AGTCCTTCTTGGCTAGTCTGTTGAC
Probe (5 μM)	(FAM)-CATCAAGAAGCTGCTGGCGGCC-(TAMRA)
B2M	
Forward primer (3 μM)	GGAATTGATTTGGGAGAGCATC
Reverse primer (4 μM)	CAGGTCCTGGCTCTACAATTTACTAA
Probe (5 μM)	(VIC)-AGTGTGACTGGGCAGATCATCCACCTTC-(TAMRA)



Supplementary Figure 1. Correlations of EBV load to chemokine receptor expressions on NCS B_{mem} cells and activation markers on CS and NCS B_{mem} cells.

EBV copy numbers were correlated to CXCR3, CXCR4 and CXCR5 surface expression in NCS B_{mem} cells (**A**), and to surface expression of CD69 and CD95 in both CS and NCS B_{mem} cells (**B**) of 9 BMT-treated MS patients. Data were collected in the same number of experiments as depicted in Figure 1. The *p* values and correlation coefficients were calculated by Pearson rank test.



Supplementary Figure 2. T-bet and CXCR3 expression is induced by IFN-γ and anti-EBNA1 IgG levels correlate to T-bet expression in CD38^{high}CD27^{high} cells during *in vitro* cultures.

(**A**) Surface expression of T-bet and CXCR3 on total CD19^{+} B cells and CD38^{high}CD27^{high} cells were analyzed for NTZ-treated MS patients with high (n = 10) and low (n = 7) B-cell EBV load cultured under GC-like conditions with and without IFN-γ. Dotted lines indicate the mean frequencies of T-bet and CXCR3 analyzed for age-/gender-matched healthy controls (n = 6). (**B**) Correlation between anti-EBNA1 IgG and the surface expression of T-bet on CD38^{high}CD27^{high} cells. Data were collected in the same number of experiments as depicted in Figure 3. Data are presented as the mean ± SEM. ** p <0.01 and * p <0.05. The p values were calculated by Wilcoxon matched-pairs signed rank (**A**) and correlation coefficients by Spearman rank (**B**) tests.

Chapter 6

Naive B cells in neuromyelitis optica spectrum disorders: impact of steroid use and relapses

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Brain Communications 2020; Epub ahead of print

ABSTRACT

Neuromyelitis optica spectrum disorders (NMOSD) are a group of rare, but severe autoimmune diseases characterized by inflammation of the optic nerve(s) and/or spinal cord. Although naive B cells are considered key players by escaping central tolerance checkpoints, it remains unclear how their composition and outgrowth differ in patients with NMOSD. Under complete treatment-naive circumstances, we found that naive mature/transitional B-cell ratios were reduced in the blood of 10 patients with aquaporin-4 (AQP4) IgG-positive disease (NMOSD) as compared to 11 both age- and gender-matched healthy controls, 8 patients with myelin oligodendrocyte glycoprotein-IgG-associated disorder (MOGAD) and 10 multiple sclerosis patients. This was the result of increased proportions of transitional B cells, which were the highest in NMOSD patients with relapses and strongly diminished after corticosteroid treatment. For purified naive mature B cells of 7 NMOSD and MOGAD patients with relapses, TLR9 ligand synergized with IFN-γ to enhance plasmablast formation during germinal center-like cultures. This was not seen for 11 patients without relapses and 9 healthy controls. In the NMOSD group, *in vitro* plasmablast formation corresponded to total and anti-AQP4 IgG secretion, of which the latter only for relapsing cases. These data demonstrate that naive B-cell homeostasis is different and targeted by corticosteroids in NMOSD patients. This also supports further exploration of naive B cells for use in TLR9-dependent *in vitro* platforms in order to predict NMOSD activity.

INTRODUCTION

Neuromyelitis optica spectrum disorders (NMOSD) are rare and convey a range of severe clinical presentations caused by inflammation with preferential involvement of the optic nerves and spinal cord [1]. Although the exact cause of these divergent presentations remains poorly understood, the dominant role of the B-cell lineage is undisputed [2]. In approximately 75% of NMOSD patients, IgG antibodies are found that target the neuronal water channel protein aquaporin-4 (AQP4) [3]. Furthermore, 30-40% of AQP4-IgG-negative NMOSD patients test positive for antibodies against myelin oligodendrocyte glycoprotein (MOG) [4, 5], which are associated with a distinct entity termed MOG-IgG-associated disorder (MOGAD).

There are significant differences in clinical features between AQP4-IgG-positive NMOSD and MOGAD [6-8], including a higher frequency of and worse recovery from relapses in AQP4-IgG-positive NMOSD. Relapses are commonly treated with corticosteroids in both entities. To prevent relapses, AQP4-IgG-positive NMOSD patients and relapsing patients with MOGAD are usually treated with maintenance therapy. Currently, no biomarkers are available to accurately predict relapses and guide treatment decisions. This could be due to the fact that previous studies on the immunopathogenesis of NMOSD primarily used patients treated with corticosteroids or other maintenance therapy, thereby possibly obliterating disease-relevant B-cell subsets.

Recent findings reveal that AQP4-specific B cells are already present in naive populations that escape early tolerance checkpoints [9, 10]. Normally, self-reactive clones are counterselected during early B-cell development in the bone marrow (central tolerance) and during subsequent maturation of transitional into naive mature B cells after entering the circulation (peripheral tolerance). In patients with AQP4-IgG-positive NMOSD, naive mature B cells escape both these checkpoints and likely develop into antibody-secreting cells in a germinal center-dependent manner [9-11]. In systemic autoimmune disease, which coexists in ~20% of AQP4-IgG-positive NMOSD patients [12], IL-21, IFN-γ and TLR9-ligand CpG-ODN serve as key triggers of autoreactive germinal center B cells expressing T-box transcription factor T-bet [13, 14].

In this study, we aimed to define the impact of AQP4-IgG serostatus, steroid treatment and relapse occurrence on naive B-cell development in NMOSD. The composition of the naive B-cell pool within the blood was compared between NMOSD, MOGAD and MS groups with and without corticosteroid treatment, as well as matched healthy controls. Furthermore, naive B-cell outgrowth into (anti-AQP4 or -MOG) IgG-secreting plasmablasts was explored *in vitro* for patients with and without relapses during T-bet-inducing, germinal center-like cultures.

MATERIALS AND METHODS

Participants

We included 10 treatment-naive AQP4-IgG-positive NMOSD patients [15] and 8 treatment-naive patients with MOGAD (all with optic neuritis and/or transverse myelitis). These patients did not get immune suppressive therapy before blood sampling; no steroids within 1 month and no other maintenance treatment within 3 months. Additionally, 5 patients with AQP4-IgG-positive NMOSD and 4 patients with MOGAD were included who received corticosteroids (i.e. oral prednisone or intravenous methylprednisolone) within 1 month prior to sampling. In the corticosteroid-treated MOGAD group, 2 patients had an NMOSD phenotype, 1 patient presented with acute disseminated encephalomyelitis and 1 patient was diagnosed with encephalitis. None of the patients included in this study received therapy with prolonged immune suppressive activity such as anti-CD20 or any other B cell-directed monoclonal antibodies before sampling. Patient characteristics are shown in Table 1.

Ex vivo B-cell subset frequencies were compared to age- and gender-matched treatment-naive multiple sclerosis (MS) patients (n = 10) as well as healthy controls (n = 20). As a reference group for corticosteroid-treated patients, we included 8 clinically isolated syndrome (CIS) patients treated with methylprednisolone within 1 month before sampling and diagnosed according to the McDonald 2017 criteria. For the NMOSD and MOGAD groups, serum was collected at the same time as peripheral blood mononuclear cells. An NMOSD relapse was defined as a new episode of disease activity at least 3 months separated from the previous disease episode. All subjects gave written informed consent, and the study was approved by the medical ethics committee of Erasmus MC.

Total IgG ELISA

IgG concentrations in supernatants were determined by ELISA using flat-bottom 96-well half-area plates (Corning, Tewksbury, USA) coated overnight at 4°C with goat anti-human Ig (1 mg/ml; Southern Biotech, Birmingham, USA). Plates were washed with PBS/0.05%Tween-20 to remove unbound antibody and blocked with PBS/5%FCS for 2 h at room temperature. Sample and a human IgG standard (Sigma-Aldrich/Merck, Darmstadt, Germany) were added for 1.5 h at room temperature. Subsequently, plates were washed with PBS/0.05%Tween-20 and bound IgG was detected by peroxidase-conjugated goat anti-human IgG (Thermo Fisher Scientific, Landsmeer, The Netherlands). TMB Substrate (Thermo Fisher Scientific) was used to reveal peroxidase activity. The reaction was stopped with H_2SO_4 and OD was measured at 450 nm.

Table 1. Patient characteristics

Cohort	Age[#]/ Gender	Disease location onset + location relapse	Treatment[#*]	Time since first event[#] (months)	Relapse	Serum AQP4-IgG/MOG-IgG level (ΔMFI)[§#]	*Ex vivo* subgroup	Used *in vitro*
AQP4-IgG								
Patient 1	61/F	TM	None	5	No	7469	NMOSD-A	Yes
Patient 2	46/F	TM	None	4	No	20533	NMOSD-A	No
Patient 3	46/M	ON bilateral	None	15	No	545	NMOSD-A	Yes
Patient 4	41/F	ON + ON	None	7	Yes	7644	NMOSD-A	Yes
Patient 5	39/F	TM + TM	None	78	Yes	13365	NMOSD-A	Yes
Patient 6	47/F	ON	None	2	No	15437	NMOSD-A	Yes
Patient 7	27/F	TM	None	46	No	7874	NMOSD-A	Yes
Patient 8	56/F	ON bilateral + ON/BS	None	6	Yes	13736	NMOSD-A	Yes
Patient 9	26/F	BS	None	122	No	7262	NMOSD-A	Yes
Patient 10	36/F	ON	None	0	No	ND	NMOSD-A	Yes
Patient 11	34/F	TM + TM	P	5	Yes	82	CS-treated	No
Patient 12	58/F	TM/ON bilateral	MP + P	2	No	15618	CS-treated	No
Patient 13	50/F	TM + TM	MP	13	Yes	17061	CS-treated	No
Patient 14	19/F	BS + TM	MP + P	6	Yes	14055	CS-treated	No
Patient 15	37/F	BS/ON bilateral	MP + P	4	No	16701	CS-treated	No
MOG-IgG								
Patient 1	52/M	ON	None	10	No	136	NMOSD-M	Yes
Patient 2	40/F	ON bilateral	None	10	No	1234	NMOSD-M	Yes
Patient 3	38/F	ON + ON	None	95	Yes	24237	NMOSD-M	Yes
Patient 4	26/M	ON + ON	None	25	Yes	5099	NMOSD-M	Yes
Patient 5	25/F	TM + ON	None	17	Yes	424	NMOSD-M	Yes
Patient 6	29/M	TM	None	51	No	1200	NMOSD-M	No
Patient 7	56/F	TM	None	18	No	696	NMOSD-M	Yes
Patient 8	25/F	ON bilateral + ON	None	201	Yes	6617	NMOSD-M	No
Patient 9	37/M	TM/ON	MP	1	No	6495	CS-treated	Yes
Patient 10	32/M	E + E	MP	181	Yes	21	CS-treated	No
Patient 11	32/M	ADEM + TM	MP	326	Yes	1681	CS-treated	Yes
Patient 12	26/F	ON bilateral	MP + P	1	No	2058	CS-treated	Yes

[#] At time of sample collection.

* Steroid treatment within 1 month before sampling (all patients were at least not treated with maintenance medication for 3 months).

[§] All AQP4-IgG and MOG-IgG serum titers were measured within the same experiment using ΔMFI.

ADEM = acute disseminated encephalomyelitis; BS = brainstem (area postrema or cranial nerves); E = encephalitis; MP = methylprednisolone (intravenous); ND, not determined; ON = optic neuritis; bilateral = both eyes; P = prednisone (oral); TM = transverse myelitis.

AQP4- and MOG-IgG cell-based assays

For determination of AQP4- and MOG-IgG levels in sera and culture supernatants, standardized cell based assays were used as previously described [5, 6, 16]. In short, either HEK293T transfectants with EGFP-tagged AQP4-M23 or 1:1 mixtures of LN18 cells transfected with and without full length human MOG were incubated with the sample and stained with goat anti-human secondary antibody (IgG labeled with APC; Jackson ImmunoResearch, Amsterdam, The Netherlands). Our AQP4-IgG cell-based assays showed a mean transfection efficiency (GFP^+) of approximately 35%. Mean fluorescence intensity (MFI) representing the amount of AQP4- or MOG-IgG bound to the cell surface was compared between transfected (GFP^+) and untransfected (GFP^-) cells within the same experiment using flow cytometry.

Cell isolation, antibodies and flow cytometry

Peripheral blood mononuclear cells were collected using Vacutainer CPT® tubes containing sodium heparin according to the manufacturer's instructions (BD Biosciences, Erembodegem, Belgium). After centrifugation, cells were taken up in RPMI 1640 (Lonza, Basel, Switzerland) containing 40% fetal calf serum (Lonza) and 20% dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO) and stored in liquid nitrogen until further use. *Ex vivo* naive mature ($CD19^+CD38^{dim/-}CD27^-IgG^-IgA^-$) B cells were purified for *in vitro* cultures using a BD FACSAria III cell sorter. For immunophenotyping, cells were incubated with Fixable Viability Stain 700 (BD Biosciences) for 15 min and monoclonal antibodies for 30 min at 4°C. The following FACS antibodies were used: CD24 (BV605, ML5), CD27 (BV421, M-T271), IgD (PE-CF594, IA6), IgG (APC-H7, G18-145; BD Biosciences), CD19 (BV785, HIB19), CD38 (PE-Cy7, HIT2), IgM (BV510, MHM-88), T-bet (PE-Cy7, 4B10; Biolegend, London, UK) and IgA (FITC, IS11-8E10; Miltenyi Biotec, Bergisch Gladbach, Germany). For intracellular T-bet staining, cells were fixed with 2% paraformaldehyde (Merck, Schiphol-Rijk, the Netherlands) and permeabilized using PBS pH7.4 containing 0.3% BSA and 0.5% saponin (Sigma-Aldrich). All measurements were conducted with an LSRII-Fortessa flow cytometer and analyzed using FACS Diva software, version 8.0.1 (BD Biosciences). For both *ex vivo* and *in vitro* analyses, we first gated on viable $CD19^+$ B cells.

Germinal center-like B-cell differentiation assay

Germinal center-like B cell cultures were performed as recently described [17]. In short, irradiated murine 3T3 fibroblasts expressing human CD40L were co-cultured with sorted naive mature ($CD27^-CD38^{dim/-}IgG^-IgA^-$) B cells in the presence of IL-21 (50 ng/ml; Thermo Fisher Scientific) with and without IFN-γ (50 ng/ml; Peprotech, Huissen, The Netherlands) and CpG-ODN (10 µg/ml; InvivoGen, San Diego, USA). After 11 days of culturing, cells were

stained for flow cytometry and supernatants were stored and analyzed for the presence of AQP4- and MOG-IgG.

Statistical analysis

Statistical analysis was performed using Graphpad Prism Software, version 5.04. Kruskal-Wallis and Dunn's post-hoc tests were performed for comparing multiple groups. Mann-Whitney U-tests were used for comparing two groups. Paired datasets were assessed using Wilcoxon signed-rank tests. Correlations between variables were tested using the Spearman rank or Pearson coefficients, depending on results of the D'Agostino & Pearson omnibus normality test. Percentages and MFI were displayed as the mean. p values < 0.05 were considered statistically significant.

RESULTS

Naive mature/transitional B-cell ratios are reduced in the blood of treatment-naive AQP4-IgG-positive NMOSD patients

First, we assessed the proportions of naive and memory B-cell subsets in the blood of NMOSD patients without any form of previous immune suppressive treatment. Because the female-to-male ratio is higher in AQP4- than MOG-IgG-associated disease (see Table 1) [5], we selected healthy controls who were age- and gender-matched either to treatment-naive patients with NMOSD (n = 11) or MOGAD (n = 9). The proportion of transitional B cells ($CD38^{++}CD27^{-}$) and not naive mature ($CD38^{dim/-}CD27^{-}IgM^{+}$) B cells [18, 19] was elevated in NMOSD versus healthy controls (mean 16.2% versus 5.6% for transitional B cells and 47.3% versus 41.5% for naive mature B cells, respectively), which resulted in lowered naive mature/transitional B-cell ratios (Fig. 1A and B). This classification of naive B cells was confirmed using IgD and CD24 (Supplementary Fig. 1). No differences were seen between the MOGAD and healthy control group (Fig. 1C and D). Two out of three NMOSD patients with relapses showed extremely high frequencies of transitional B cells (35% and 44% of the total B-cell pool, Fig. 1B). Naive mature/transitional B-cell ratios were lower in NMOSD compared to MOGAD ($p < 0.01$; Fig. 1F).

Germinal center-independent natural effector ($CD38^{dim/-}CD27^{+}IgM^{+}IgD^{+}$) memory B cells [20] were significantly reduced in the NMOSD versus healthy control group (lowest in two relapsing cases with the highest percentage of transitional B cells; Supplementary Fig. 2A and B). A similar trend was found in the MOGAD group (Supplementary Fig. 2B). The proportions of germinal center-dependent IgM-only B cells ($CD38^{dim/-}CD27^{+}IgM^{+}IgD^{-}$) and IgG^{+} (both $CD27^{+}$ and $CD27^{-}$) B cells [20] (Supplementary Fig. 2B) or plasmablasts ($CD38^{++}CD27^{++}$; Supplementary Fig. 3A), did not differ between groups. None of the

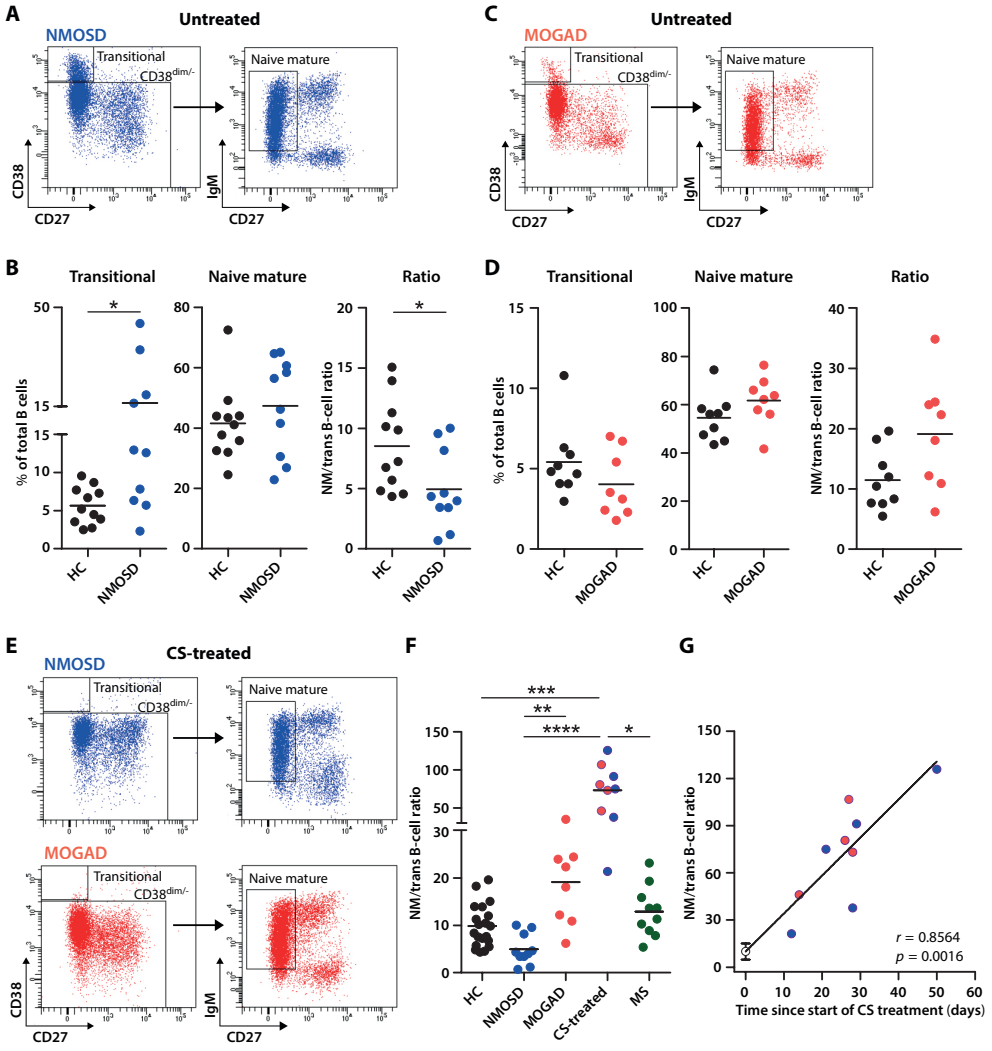

Figure 1. Transitional and naive mature B-cell frequencies in the blood of different NMOSD, MOGAD, MS and healthy control groups.

Representative gating, proportions and ratios of transitional ($CD38^{++}CD27^{-}$) and naive mature ($CD38^{dim/-}CD27^{-}$) B cells from blood of treatment-naive patients with NMOSD (n = 10; **A,B**) or MOGAD (n = 8; **C,D**). The fractions of transitional and naive mature B cells and their ratios were compared to a separate age- and gender-matched healthy control group (for NMOSD, n = 11; for MOGAD, n = 9). (**E**) Gating example for the detection of transitional and naive mature B cells in the blood from corticosteroid-treated patients with NMOSD or MOGAD (CS-treated). (**F**) Naive mature/transitional B-cell ratios in the blood of treatment-naive NMOSD or MOGAD, CS-treated NMOSD or MOGAD (n = 9), treatment-naive MS (n = 10) and healthy control (HC; n = 20) groups. (**G**) Correlation of naive mature/transitional B-cell ratios to time since start of CS treatment in NMOSD and MOGAD patients

populations and ratios correlated to AQP4- or MOG-IgG serum levels (Table 1) in the NMOSD groups (data not shown).

Corticosteroid treatment corresponds to increased naive mature/ transitional B-cell ratios in AQP4- and MOG-IgG-positive disease

To study the impact of corticosteroids as a standard treatment of acute relapses, we compared our results to B-cell subsets from the blood of 9 AQP4-IgG or MOG-IgG-positive patients who only received corticosteroids and no other forms of immunosuppressive treatment. In this group, naive mature/transitional B-cell ratios were significantly elevated (fold change versus NMOSD: 14.8, $p < 0.0001$; Fig. 1E and F), which correlated positively with time since start of corticosteroid treatment ($r = 0.86$ and $p = 0.002$, Fig. 1G). These elevated ratios were the result of an almost complete absence of transitional B cells (Fig. 1E, Supplementary Fig. 4). The association of corticosteroid treatment with transitional and not naive mature B cells was confirmed in an additional cohort of patients with a clinically isolated syndrome (Supplementary Fig. 5). In the steroid-treated NMOSD and MOGAD group, the proportion of natural effector B cells was similar to healthy controls and correlated to time since start of treatment ($r = 0.71$, $p = 0.02$; Supplementary Fig. 2C and D). Corticosteroid treatment did not affect plasmablast, IgM-only and IgG^+ ($CD27^-/CD27^+$) B-cell frequencies (Supplementary Fig. 3B and data not shown).

TLR9 ligand synergizes with IFN-γ to promote naive mature B cell to plasmablast formation only for AQP4- or MOG-IgG-positive patients with relapses

To assess how the B-cell germinal center-like differentiation pathway is regulated in NMOSD, we purified naive mature B cells from the peripheral blood of 9 AQP4-IgG-positive patients, 9 MOG-IgG-positive patients and 9 healthy controls and cultured these populations in the presence of IL-21 and CD40L-3T3 cells with and without IFN-γ and TLR9 ligand CpG-ODN. The percentage of viable $CD38^{++}CD27^{++}$ plasmablasts was analyzed after 11 days using flow cytometry (Fig. 2A). To further substantiate the clinical relevance of this model, we explored the functional association of *in vitro*-generated plasmablasts with disease activity within the continuum of relapse risk.

For all tested subjects, IFN-γ induced the development of plasmablasts (Fig. 2B), which was similar between the groups. However, in both NMOSD and MOGAD patients with relapses (n = 7), the addition of CpG-ODN to IFN-γ-containing cultures significantly increased plasmablast formation (Fig. 2B; mean, IFN-γ: 19.8%, IFN-γ + CpG: 34.9%, $p = 0.0156$). The opposite was found in patients without relapses (n = 11; IFN-γ: 15.8%; IFN-γ + CpG: 12.8%, $p = 0.0137$). Although disease duration was longer for relapsing compared to non-relapsing patients (median 43 vs 17 months, respectively), relapsing patients

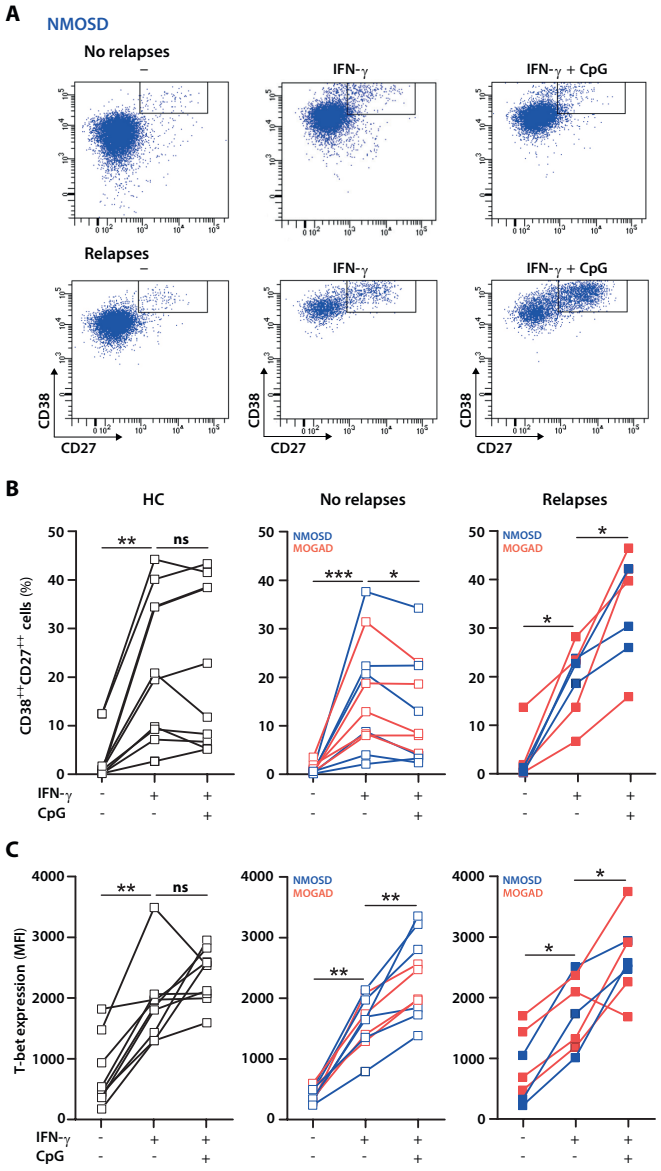

Figure 2. ***In vitro*** **plasmablast outgrowth for naive mature B cells from subgroups with and without relapses under different germinal center-like conditions.**

(**A**) Representative gating of viable plasmablasts ($CD38^{++}CD27^{++}$) cultured from naive mature B cells of an NMOSD patient with and without relapses. Cells were triggered with CD40L-3T3, IL-21, IFN-γ and/or TLR9 ligand CpG-ODN for 11 days. Both the percentage of *in vitro*-generated plasmablasts (**B**) and intracellular T-bet expression (**C**) were determined for cultured naive mature B cells from the blood of NMOSD or MOGAD subgroups with (n = 7) or without relapses (n = 11), as well as healthy controls (n = 9). For 1 patient with MOGAD, we only obtained sufficient cell numbers to analyze plasmablast frequencies and not T-bet expression.

experienced their first relapse within a median time of 10 months since onset. In 6 out of 7 relapsing patients, immune suppressive treatment was initiated after the first relapse, whereas 6 out of 11 monophasic patients were treated from onset onwards. For naive mature B cells of healthy controls, these frequencies were identical for both conditions (IFN-γ: 20.9%, IFN-γ + CpG: 20.4%). Intracellular T-bet levels were upregulated by IFN-γ and further induced by CpG-ODN (Fig. 2C). Since this was comparable between the groups, other factors probably mediate the observed effect of CpG-ODN on *in vitro* plasmablast formation. The proportions of *ex vivo* B-cell subsets and plasmablasts did not differ between the groups with or without relapses (Supplementary Fig. 6).

In both the NMOSD and MOGAD group, *in vitro* secretion of total IgG was significantly increased after the addition of CpG-ODN (Fig. 3A and Supplementary Fig. 7). For the NMOSD group, this positively correlated with *in vitro* plasmablast formation (Fig. 3B). The increase in both plasmablast formation and IgG secretion was the most pronounced in the 2 NMOSD patients with relapses (Fig. 3B). We detected anti-AQP4 IgG in naive mature B-cell culture supernatants of all 3 relapsing but in none of 6 non-relapsing NMOSD patients (Fig. 3C). Anti-AQP4 IgG secretion was enhanced by CpG-ODN for 2 out of 3 relapsing patients; for one relapsing case with very high levels in culture (ΔMFI, IFN-γ: 24442, IFN-γ + CpG: 34725, negative control: 33) and one relapsing case with very low levels in culture (ΔMFI, IFN-γ: 57, IFN-γ + CpG: 94, negative control: 54). In culture supernatants of naive mature B cells of 4 relapsing and 4 non-relapsing patients with MOGAD, anti-MOG IgG could not be detected (Fig. 3C; ΔMFI, positive control: 7322). In this group, total IgG levels did not correlate with *in vitro* plasmablast formation (Fig. 3B).

DISCUSSION

AQP4-IgG production by peripheral B cells is an important driver of NMOSD [21-23]. Since central B-cell tolerance mechanisms are defective in NMOSD [10, 24], the selection of AQP4-specific B cells is already disturbed at the earliest stage in the bone marrow. We now show that the composition and functional outgrowth of circulating naive B cells is different in NMOSD patients, which seems to be linked to corticosteroid treatment, relapse occurrence and AQP4-IgG secretion.

The selective enrichment of transitional B cells in the blood of AQP4-IgG-positive NMOSD patients is likely caused by higher fractions of poly- and autoreactive clones that escaped selection within the bone marrow [10]. We now find that transitional B cells are almost completely absent in the blood of corticosteroid-treated patients. Since the patients described by Cotzomi et al. received immunotherapy including corticosteroids, AQP4-specific B cells and other unique clones may have been missed in their analysis. This

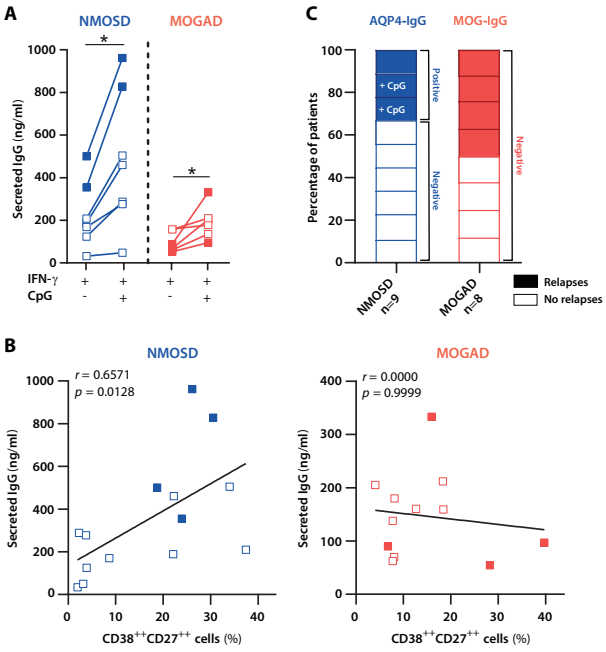

Figure 3. IgG secretion by *in vitro*-generated plasmablasts using naive mature B cells from subgroups with and without relapses.

(**A**) Total IgG secretion was measured in germinal center-like cultures with naive mature B cells from patients with NMOSD (n = 7) or MOGAD (n = 6; 11 days). In each subgroup, two patients had relapsing disease (open symbols). Data were compared between IFN-γ- and both IFN-γ- and CpG-ODN-inducing conditions (**A**) and correlated to *in vitro* plasmablast ($CD38^{++}CD27^{++}$) formation (**B**). (**C**) Detection of anti-AQP4-IgG and -MOG-IgG in naive mature B-cell culture supernatants (11 days) for patients with NMOSD or MOGAD. AQP4-IgG levels were elevated in IFN-γ-containing cultures with CpG-ODN for 2 NMOSD patients with relapses.

effect of steroids on transitional B cells is probably explained by their lack of multidrug resistance receptor 1 [25], a glycoprotein that pumps a wide range of substances including corticosteroids out of the cell [26]. Transitional B cells were abrogated by steroid treatment in patients with NMOSD and MOGAD. This correlated to time since start of treatment in clinically isolated syndrome patients as well, implicating that corticosteroids have a generic impact on these early B-cell emigrants.

In a previous study, the proportion of circulating $CD27^{+}$ memory B cells was found to be reduced in NMOSD patients [11]. We find that frequencies of germinal center-independent $CD27^{+}IgM^{+}IgD^{+}$ memory B cells are lower in NMOSD patients and recovered after corticosteroid treatment. These data suggest that AQP4-specific naive mature B cells preferentially enter the germinal center to undergo proliferation and somatic hypermutation. Consistent with this, under germinal center-like conditions *in vitro,* AQP4-specific plasmablast development occurred more for naive than memory B cells and did not require

antigen [9], and somatic hypermutation was shown to be essential for generating AQP4-specific antibodies [10]. We did not find any increase in circulating memory B-cell subsets, including those lacking CD27 expression [11]. This implies that within germinal centers, AQP4-specific naive B cells develop into plasmablasts rather than memory B cells. Indeed, some studies showed that *ex vivo* circulating plasmablasts are expanded in NMOSD, which seemed to be irrespective of steroid usage [11, 27]. In the current study, no differences in *ex vivo* $CD38^{++}CD27^{++}$ plasmablasts were found, similar to the observations by Wilson et al. The vulnerability of plasmablasts for freeze-thaw cycles [28] can be a confounding factor, although this was similar for all subjects in this study. Another factor is that plasmablasts probably further mature and reside within the bone marrow or inflamed tissues to produce AQP4-specific antibodies [9, 29].

Whereas the ability of *ex vivo* plasmablasts to produce AQP4-IgG is highly controversial [9, 27], the development of naive B cells into antibody-secreting cells has become widely accepted. In the study by Cotzomi et al., none of the recombinant anti-AQP4 IgGs that reverted back to unmutated precursors were able to bind to AQP4, indicating that naive mature B cells need to enter germinal centers to develop into anti-AQP4 IgG producers. Recently, we found that IFN-γ induces naive mature B cells to develop into plasmablasts during germinal center-like cultures [17]. The same is true for naive mature B cells of NMOSD patients (this study). However, in contrast to MS patients [17], the addition of CpG-ODN had an inducing effect on *in vitro* plasmablast outgrowth for NMOSD patients with recorded relapses. This difference in *in vitro* naive mature B-cell outgrowth is likely related to the impaired central B-cell tolerance found in NMOSD and not in MS [10]. Based on the data in this study it is tempting to speculate that the elevated frequencies of (autoreactive) transitional B cells in patients with a high relapse risk causes preferential development of naive mature B cells into AQP4-IgG-secreting plasmablasts within secondary lymphoid organs. However, our findings should be interpreted cautiously due to low patient numbers and inherent differences in the clinical course between NMOSD, MOGAD and MS.

CpG-ODN alone is known to suppress [30], but synergizes with IFN-γ to potentiate autoreactive $T\text{-}bet^{+}$ B cells [31]. T-bet expression does not seem to mediate the difference in IFN-γ- and CpG-ODN-induced plasmablast induction *in vitro* between relapsing and non-relapsing NMOSD groups. An intriguing scenario may be that IFN-γ enhances IL-6 production by B cells [32], leading to TLR9 upregulation [33]. TLR ligation has been previously associated with the activation of transcription factor X-box binding protein 1 (XBP-1; [34], which enhances IgG secretion [35] and links to the IL-6-driven survival of AQP4-IgG-secreting plasmablasts [27].

To our knowledge, this is the first study that assessed the phenotype and responsiveness of naive B cells from NMOSD patients without immunomodulatory therapy and compared these to MOGAD, MS and healthy control groups. The impact of steroids on

transitional but not on naive mature B cells may be a mechanistic explanation why NMOSD relapses recur after discontinuation of corticosteroids. Our findings provide a rationale for exploring the underlying mechanisms of naive B-cell development in patients with active or stable disease. This study also supports further assessment of CpG-ODN-mediated outgrowth of naive B cells under germinal center-like, IFN-γ-containing *in vitro* conditions as a new approach to predict NMOSD relapses.

ACKNOWLEDGMENTS

We would like to thank all patients and controls for donating their blood. We thank Ruth Huizinga for sharing her experience with the IgG ELISA. The authors would like to dedicate this manuscript to the memory of Prof. Rogier Q. Hintzen, who passed away on May 15, 2019.

FUNDING

Dutch MS Research Foundation (15-490d MS).

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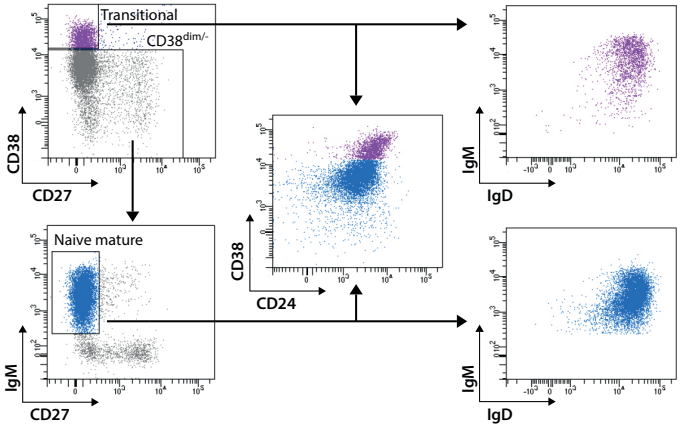
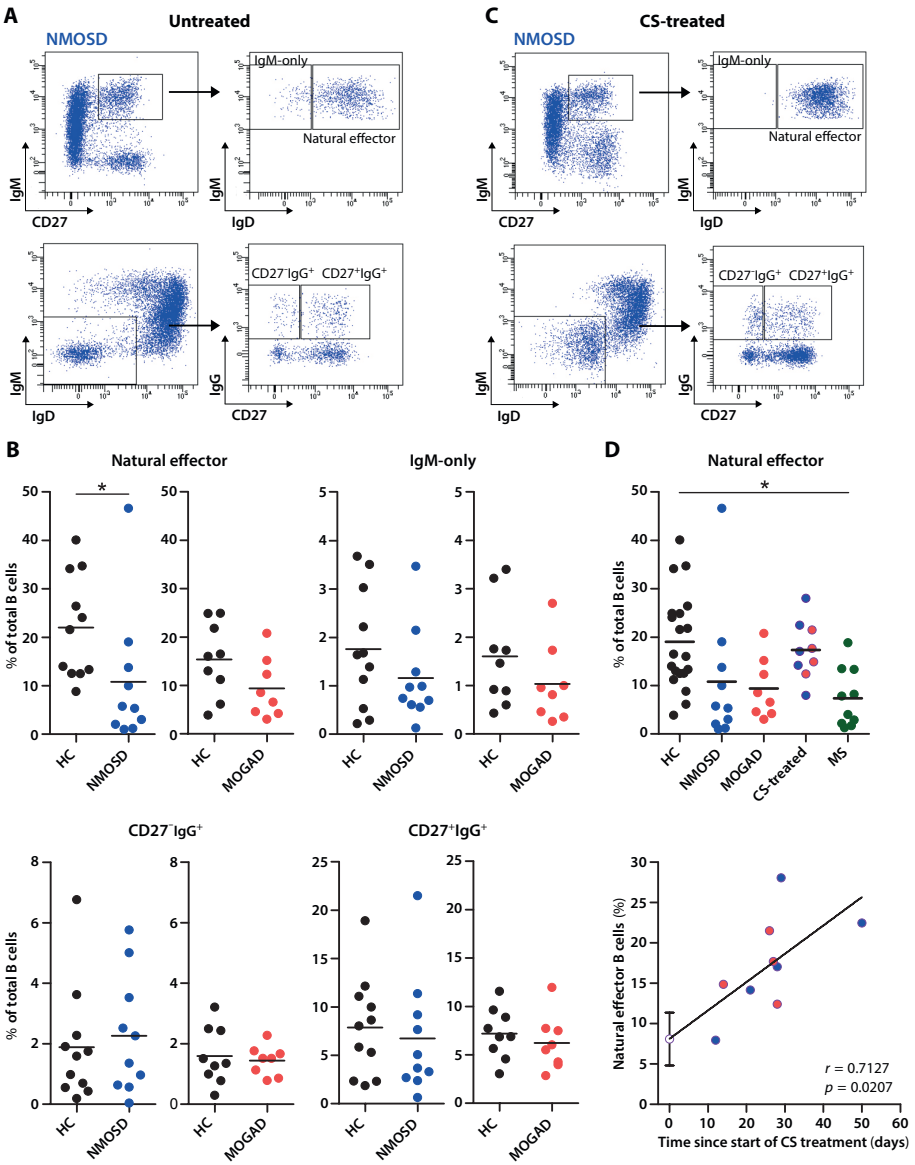
SUPPLEMENTAL DATA

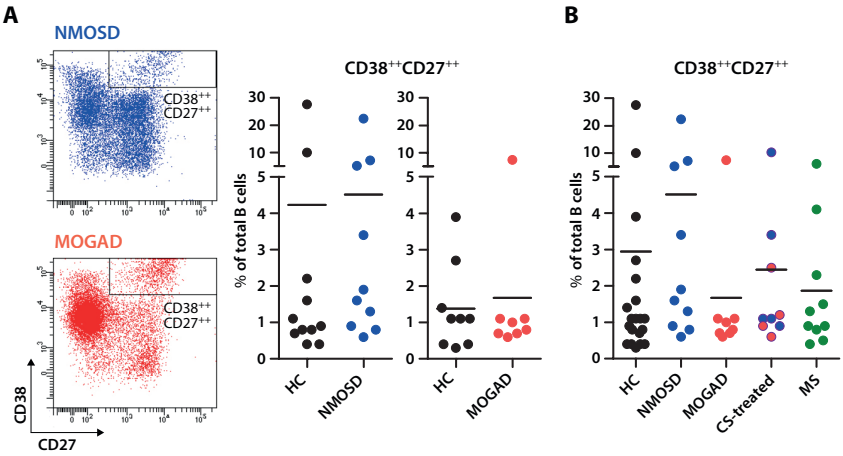


Supplementary Figure 1. Expression of CD24, IgD and IgM on transitional and naive mature B cells in the blood.



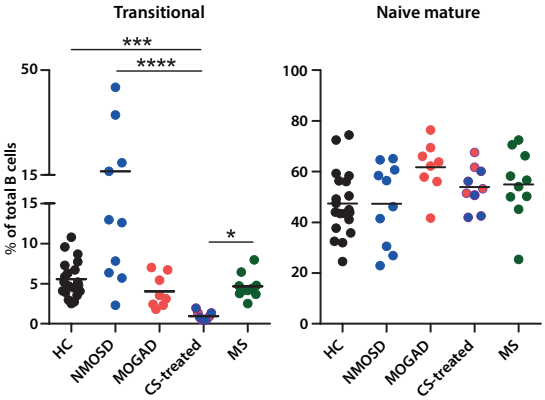
Supplementary Figure 2. Presence of *ex vivo* memory B-cell subsets in the blood of untreated and CS-treated NMOSD, MOGAD, MS and HC subgroups.

Gating strategy for the identification of IgM-only ($CD27^+IgM^+IgD^-$), natural effector ($CD27^+IgM^+IgD^+$) and IgG^+ (both $CD27^-$ and $CD27^+$) memory B cells from the blood of a treatment-naive (**A**) and a corticosteroid (CS)-treated (**C**) AQP4-IgG positive NMOSD patient. Fractions were compared between NMOSD (n = 10) and MOGAD (n = 8) and age- and gender-matched healthy controls (HC) for each group (for NMOSD, n = 11; for MOGAD, n = 9; **B**). (**D**) Fractions of natural effector memory B cells in CS-naive NMOSD, MOGAD, MS, and HC, as well as CS-treated NMOSD and MOGAD groups and their correlation to time since start of CS treatment.



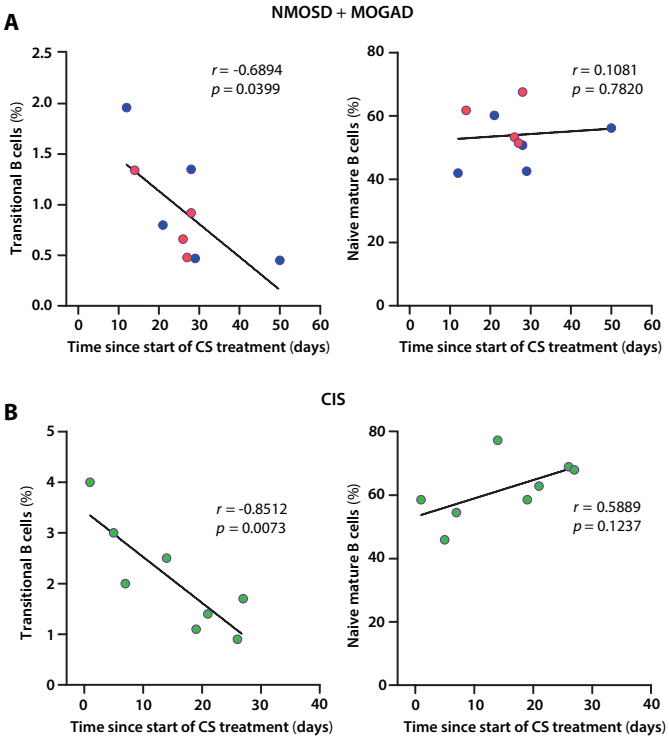
Supplementary Figure 3. Frequencies of *ex vivo* plasmablasts in the blood of untreated and CS-treated NMOSD, MOGAD, MS and HC subgroups.

Representative gating and frequencies of circulating plasmablasts ($CD38^{++}CD27^{++}$) in treatment-naive (**A**) and corticosteroid (CS)-treated (**B**) NMOSD and MOGAD patients. Fractions of *ex vivo* plasmablasts within the total $CD19^{+}$ pool were compared between CS-naive NMOSD (n = 10), CS-naive MOGAD (n = 8), CS-treated NMOSD or MOGAD (n = 9), MS (n = 10) as well as age- and gender-matched HC (for NMOSD, n = 11; for MOGAD, n = 9) groups.



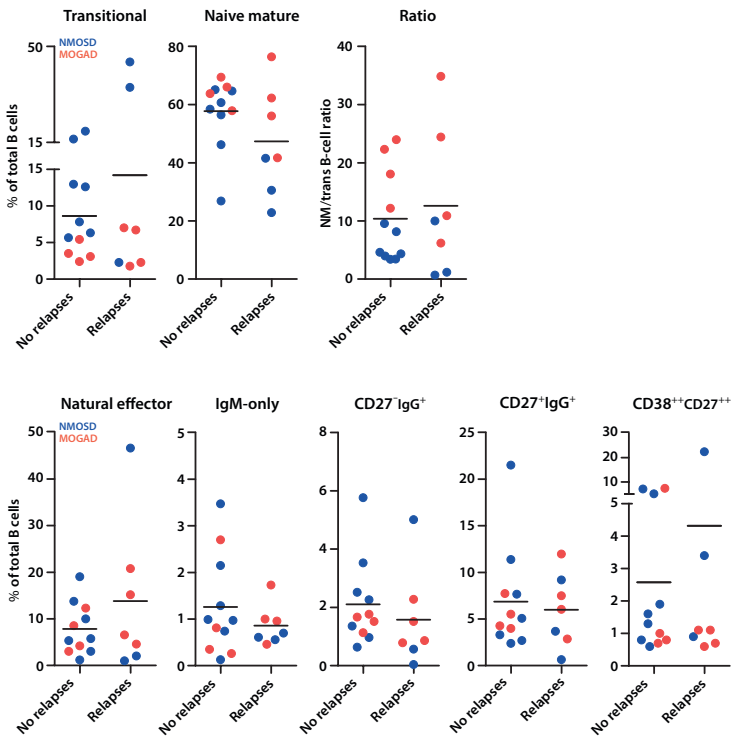
Supplementary Figure 4. Composition of the naive B-cell pool in the blood of untreated and CS-treated NMOSD, MOGAD, MS and HC subgroups.

Proportions of circulating transitional and naive mature B cells within the total $CD19^{+}$ pool were assessed in CS-naive NMOSD (n = 10), CIS-naive MOGAD (n = 8), CS-treated NMOSD or MOGAD (n = 9), MS (n = 10) and HC (n = 20) groups.



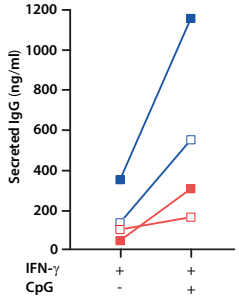
Supplementary Figure 5. The impact of CS treatment on the presence of transitional and naive mature B cells in the blood.

Proportions of circulating transitional and naive mature B cells were correlated to time since start of CS treatment in NMOSD or MOGAD (n = 9; **A**) and clinically isolated syndrome (CIS, n = 8; **B**) groups.



Supplementary Figure 6. Presence of *ex vivo* B-cell subsets in relapsing and non-relapsing NMOSD and MOGAD subgroups.

Proportions of naive and memory B-cell subsets as well as plasmablasts ($CD38^{++}CD27^{++}$) were analyzed in the blood using FACS and compared between CS-naive NMOSD (blue) and MOGAD (red) patients with and without relapses.



Supplementary Figure 7. Validation of the differences in IgG secretion by *in vitro*-generated plasmablasts between NMOSD and MOGAD subgroups.

Plasmablasts were generated from naive mature B cells of a relapsing (solid box) and non-relapsing (open box) NMOSD and MOGAD patient under IFN-γ and/or CpG-ODN-inducing germinal center-like conditions. IgG secretion was compared after 11 days.

Chapter 7

General discussion

Partly adapted from "B and T cells driving multiple sclerosis: identity, mechanisms and potential triggers". Frontiers in Immunology. 2020; 11:760.

GENERAL DISCUSSION

In multiple sclerosis (MS) and neuromyelitis optica spectrum disorder (NMOSD), pathogenic lymphocytes are triggered in the periphery to infiltrate the central nervous system (CNS) and cause local inflammation and demyelination. Anti-CD20 therapy has proven to be clinically successful in both MS [1-3] and NMOSD [4, 5]. Although this underscores the fact that B cells play a key role in these CNS demyelinating diseases, the exact triggers, subsets and effector mechanisms contributing to differences in the pathogenesis are unclear. Defects in early B-cell tolerance checkpoints may already skew the differentiation of pathogenic B cells within the periphery. In MS, peripheral tolerance is flawed, while disturbances in both central and peripheral tolerance mechanisms are seen in aquaporin 4 (AQP4)-IgG-positive NMOSD [6, 7]. Furthermore, the impact of B-cell depletion therapies on the antigen-presenting cell function of B cells indicates that interaction with T cells is an important driver of disease activity in MS [1, 8], which may also account for NMOSD. This interaction prompts the development of disease-inducing B and T cells through several mechanisms including antigen presentation, co-stimulation and cytokine production [4, 6]. Additionally, pathogenic B cells may exert their effects through secretion of autoantibodies. In contrast to MS, where the target of (auto)antibodies remain largely unknown, the vast majority of NMOSD patients have antibodies in the blood that are specific against AQP4 and myelin oligodendrocyte glycoprotein (MOG), both of which are expressed in the CNS [9].

In addition, disease-inducing B and T cells are likely generated through increased responsiveness to certain pathogenic triggers. The type and extent to which these cells respond to such triggers could influence differential pathogenesis in MS and NMOSD. The strongest known pathogen associated with MS development is the Epstein-Barr virus (EBV), a human DNA herpesvirus that infects B cells [10, 11]. However, evidence for this association with MS mostly comes from epidemiological studies [11-13] and it remains incompletely understood how EBV is involved in the immunopathogenesis [14, 15]. EBV infection is a risk factor for many other types of autoimmune diseases, including systemic lupus erythematosus (SLE) and Sjogren's syndrome [10]. Interestingly, these systemic autoimmune diseases coincide with AQP4-IgG-positive disease in a subgroup of NMOSD patients [16]. Although a recent serological study has demonstrated possible persistence and active replication of EBV in NMOSD [17], there is currently no proof that EBV infection plays a role in this disease.

In MS, the infiltration of pro-inflammatory 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 [18-20]. 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 [21], it is crucial to understand which exact cells invade the CNS and what triggers these cells to instigate local pathology. Similarly, in NMOSD, it is important to understand if similar triggers in the periphery enable the formation of plasmablasts/plasma cells and how this affects their production of disease-inducing antibodies that are recruited to the CNS.

In this chapter, we integrate the findings in this thesis with current knowledge on which and how lymphocyte subsets contribute to disease activity in MS and NMOSD. The development of such subsets probably involves one or more of the following processes: 1) peripheral escape of pathogenic B cells, 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 (with the focus on MS). For MS, we aimed to understand how both B and $CD4^+$ T cells develop into CNS-homing subsets. To assess this, we performed in-depth analyses of their phenotype in several CNS compartments and compared functional characteristics in the blood from well-defined patient groups. For NMOSD, we focused on how naive B cells as key players in the disease develop into antibody-producing cells and whether this differs amongst serological and corticosteroid-treated subgroups including MS.

IMPAIRED PERIPHERAL CONTROL OF PATHOGENIC B CELLS DURING CNS DEMYELINATING DISEASE

In diseases such as MS and NMOSD, the crosstalk between B and T cells is likely disturbed, eventually causing pathogenic instead of protective immunity. This may already start during the early selection of transitional and/or naive mature autoreactive B cells. In **Chapter 6**, we found that in contrast to MS patients and matched healthy controls, the proportion of transitional B cells is enhanced in the blood of treatment-naive AQP4-IgG-positive NMOSD patients, which resulted in a reduced naive mature/transitional B-cell ratio. This was the highest for two patients with relapsing disease. This finding suggests that disturbed B-cell tolerance already occurs early in the bone marrow and only in this NMOSD patient group. In a very recent study [7], Cotzomi et al. showed that both the frequencies of polyreactive transitional and naive mature B cells are increased in the blood of AQP4-IgG-positive NMOSD patients. This indicates that also peripheral tolerance checkpoints are defective in NMOSD [7]. Additionally, we found that corticosteroid treatment of NMOSD patients resulted in a strong reduction in circulating transitional B cells, which was not seen for other subsets (**Chapter 6**). Since steroids are commonly used to treat acute relapses, depletion of transitional B cells may interfere with the development of autoantibody-secreting cells in this disease. In contrast to NMOSD, only peripheral B-cell tolerance seems to be inadequate in MS patients, as reflected by the enhanced frequencies

of polyreactive naive mature but not transitional B cells in the blood [6, 7, 22, 23]. Although the exact cause remains to be determined, the lack of peripheral control of B-cell development in both NMOSD and MS may be explained by chronic T-cell stimulation and/or dysfunction of $CD4^+$ T regulatory cells (Tregs) (Figs. 1 and 2).

Although many theories have been proposed how EBV can influence MS pathogenesis [24], 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 [25, 26]. This, together with the impact of HLA and other risk alleles [27], may result in inappropriate T cell-mediated control of EBV-infected (pathogenic) B cells in genetically susceptible individuals. Consistent with this, peripheral $CD8^+$ cytotoxic T cells (CTLs) show decreased responses to EBV and not to cytomegalovirus (CMV) antigens during the MS course [26]. EBV antigens can also induce Tregs capable of suppressing effector T-cell responses to recall antigens [28], as seen for other persistent infections such as lymphocytic choriomeningitis virus [29, 30].

However, forkhead box P3 ($FOXP3^+$) Tregs have also been described to control infections [31], implying that additional T cell-intrinsic defects are involved. For example, in MS patients, certain Treg populations that are enriched in the blood show increased IFN-γ and reduced FOXP3 levels. This may not only result in less suppression of effector T cells [32-34], but possibly also in an impaired removal of pathogenic B cells, as described for other autoimmune diseases [6, 35, 36]. AQP4-specific Tregs are reduced in the blood of NMOSD patients and have decreased FOXP3 expression, which suggests that such processes also occur in this disease [37, 38]. Especially for MS and NMOSD, the direct impact of functionally defective Tregs on (pathogenic) B-cell development still has to be proven.

THE GERMINAL CENTER AS A B- AND T-CELL POWERHOUSE DRIVING CNS DEMYELINATING DISEASE

Th cells as inducers of pathogenic memory B cells and plasmablasts/plasma cells

After their escape from peripheral tolerance checkpoints, naive B cells possibly interact with $CD4^+$ Th cells in germinal centers (GCs) to develop into memory and/or plasmablast/plasma cells that trigger CNS demyelinating disease through autoimmune-related processes. In MS patients, it is very likely that naive B cells recognize a yet unknown antigen and enter GCs to become pathogenic memory B cells that are prone to infiltrate the CNS. In the CNS, B cells are probably re-activated to further mature into antibody-secreting cells and trigger local inflammation (Fig. 1). Within peripheral lymphoid organs, IL-21 produced by T follicular helper (Tfh) cells is crucial for GC formation and plasma cell differentiation

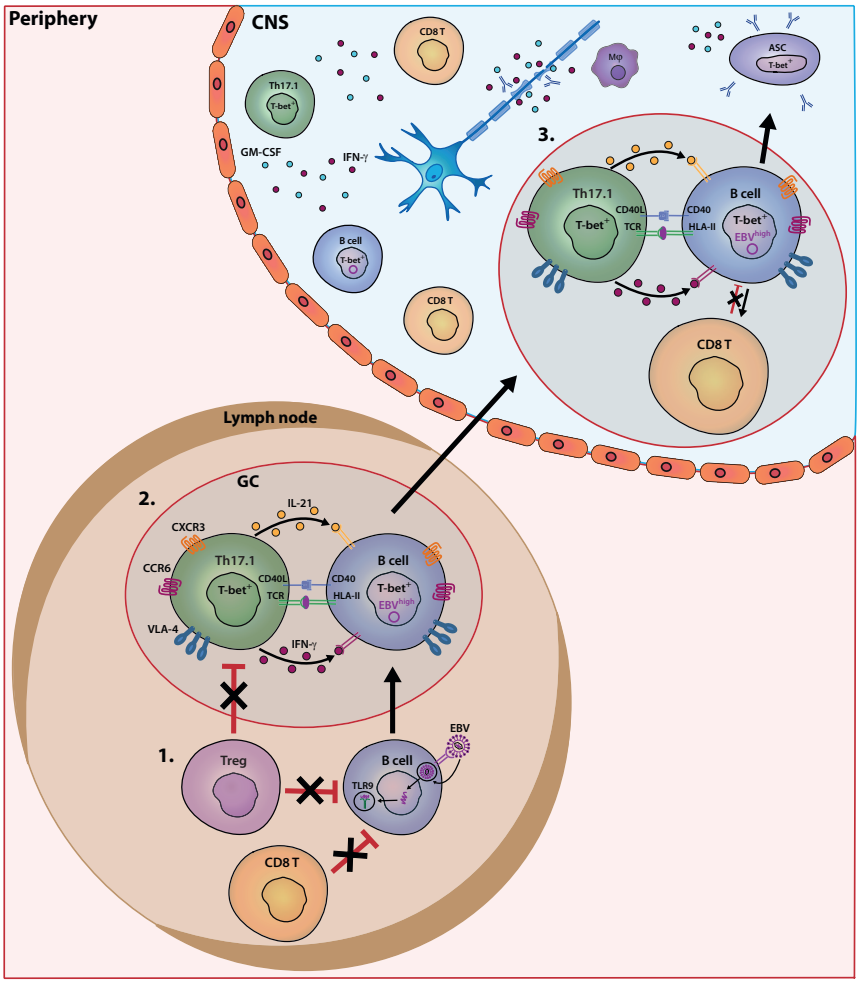


Figure 1. Simplified model of the key pathogenic events involving human B- and T-cell subsets driving MS disease activity.

In MS patients, B- and T-cells interact in the periphery and central nervous system (CNS) to contribute to disease pathogenesis. In this hypothetical model, we put forward three important pathogenic events involving 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 $CD8^+$ CTLs and T regulatory (Treg) cells (**1**). Subsequently, these activated B cells enter germinal centers (GCs) and interact with follicular Th(17.1) cells to further differentiate into pathogenic memory B cells. Under the influence of IL-21, CD40L, IFN-γ and TLR9 and/or HLA-II signaling, B cells develop into CXCR3(T-bet)-expressing memory cells, which in turn activate Th effector cells (**2**). Especially Th17.1 cells infiltrate the CNS of MS patients by distinct expression of chemokine receptors (CXCR3, CCR6), adhesion molecules (VLA-4) as well as pro-inflammatory cytokines (IFN-γ, GM-CSF). (**3**) Within the CNS, Th17.1 cells and CXCR3(T-bet)$^+$ memory B cells probably crosstalk in follicle-like structures, resulting in clonal expansion and eventually inflammation and demyelination. CXCR3(T-bet)$^+$ memory B cells further differentiate into plasmablasts and plasma cells to secrete high numbers of potentially harmful antibodies (oligoclonal bands).

[39]. However, in an autoimmune setting, additional production of IFN-γ by Tfh cells elicits autoimmune GCs, which has become evident from studies in SLE [40-42]. Similarly, Toll-like receptor 9 (TLR9) signaling promotes IgG class-switching and autoantibody production [43, 44]. In mice, both IFN-γ and TLR9 ligation induces the expression of the T-box transcription factor T-bet, which triggers CXC chemokine receptor 3 (CXCR3) [45, 46]. In **Chapter 4**, we demonstrate that naive B cells from MS patients are differentially influenced by IFN-γ and TLR9 ligand CpG-ODN during *in vitro* GC-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, TLR9 triggering was crucial for T-bet and $CXCR3^+$ expression on B cells during IFN-γ-mediated GC-like cultures of naive B cells (**Chapter 4**). This seemed to be more pronounced for naive B cells of MS patients. The synergistic *in vitro* effects of IFN-γ and CpG-ODN on T-bet/CXCR3 induction and B-cell differentiation were not seen when culturing purified memory B cells (**Chapter 4**), indicating that these triggers are important for the fate of naive B cells. Although not studied in this thesis, pathogenic B-cell responses during autoimmunity probably rely on both IFN-γ and TLR-mediated induction of T-bet [41, 46-48]. This is also reflected by the increased pro-inflammatory response of B cells from MS patients to IFN-γ and TLR9 triggering [49]. Previously, B cell-intrinsic deletion of the IFN-γ receptor has been shown to impair autoimmune GC formation and systemic autoimmunity [41]. Ongoing work by our group reveals that in human B cells carrying a MS risk SNP in the coding region of *IFNGR2* [50, 51], signal transducer and activator of transcription (STAT)1 is highly phosphorylated and stimulates T-bet as well as *IRF1* gene expression under IFN-γ-stimulating conditions (Fig. 3). Hence, this suggests that risk variants related to IFN-γ signaling, such as *IFNGR2* but also for example *IFI30*, may contribute to increased responsiveness and functionality of pathogenic B cells. Since T-bet has been shown to regulate IgG class-switching in other autoimmune diseases [44], our results further suggest that IFN-γ and TLR9 work in concert to enhance T-bet-mediated IgG1 class-switching in MS patients.

In NMOSD patients, naive B-cell populations already seem to be specific for AQP4 and develop into AQP4-IgG-secreting cells probably in a germinal center-dependent, but antigen-independent manner within peripheral lymphoid organs (Fig. 2) [7, 52, 53]. Since NMOSD has been associated with other systemic autoimmune diseases [16], IFN-γ and CpG-ODN could potentiate these autoreactive naive B cells to become pathogenic AQP4- or MOG-IgG producing plasmablasts. In contrast to MS, *in vitro* plasmablast formation was enhanced under IFN-γ and CpG-ODN-stimulating GC-like culture conditions using naive mature B cells from NMOSD patients with high disease activity (**Chapter 6**). These differences in outgrowth may be related to the early central tolerance defects found in AQP4-IgG-positive NMOSD, possibly making AQP4-specific naive B cells more prone to develop into plasmablasts. Moreover, the production of total IgG and AQP4-specific IgG was

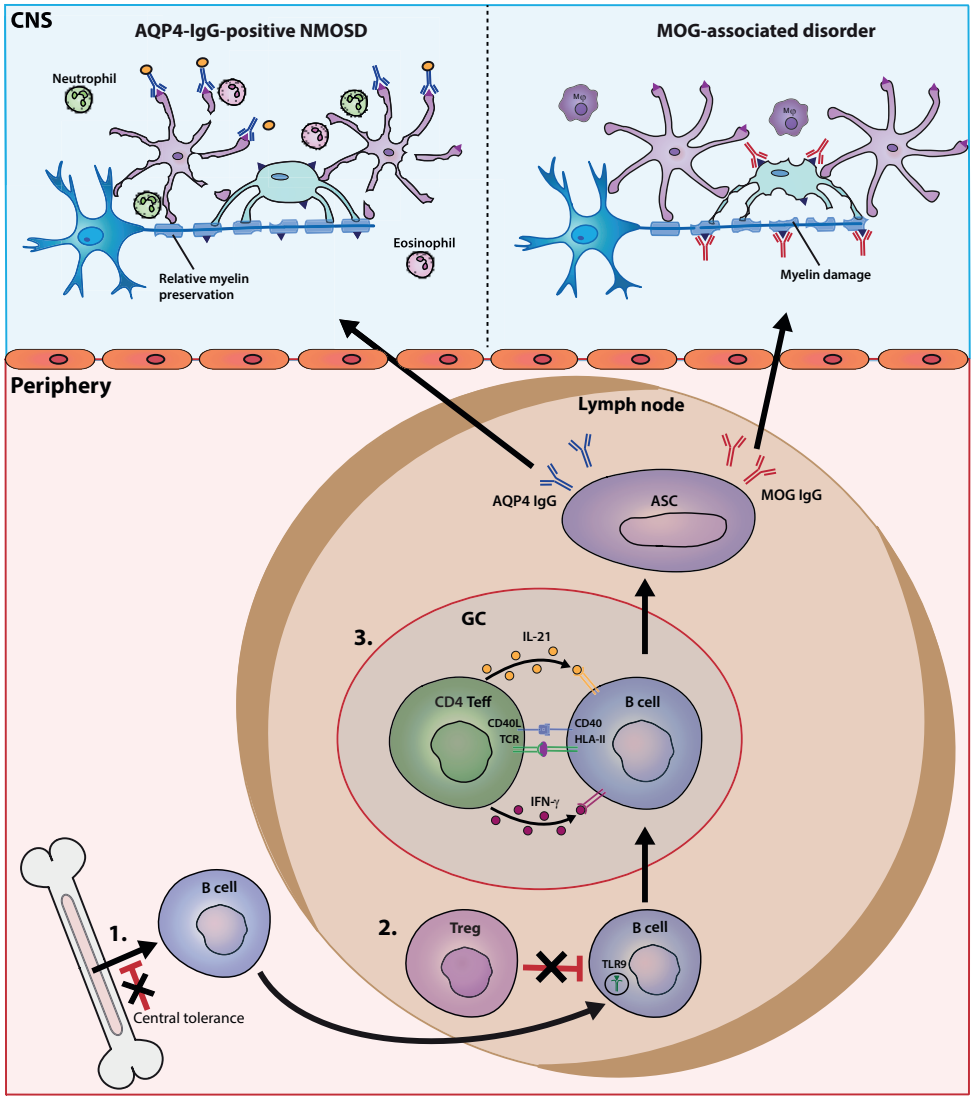


Figure 2. Simplified model of key peripheral processes driving the production of autoantibodies in AQP4-IgG-positive NMOSD and MOGAD.

In NMOSD and MOGAD patients, B- and T-cells interact in the periphery to induce the differentiation of AQP4- or MOG-specific antibodies, which subsequently enter the CNS to mediate local damage. B cells escape both central (**1**) and peripheral (via Tregs; **2**) tolerance checkpoints. These autoreactive naive B cells interact with Th cells in germinal centers to differentiate into AQP4-IgG- or MOG-IgG-producing plasma cells (**3**). AQP4-IgG and MOG-IgG are recruited to the CNS and bind to their respective antigens to mediate disease pathogenesis.

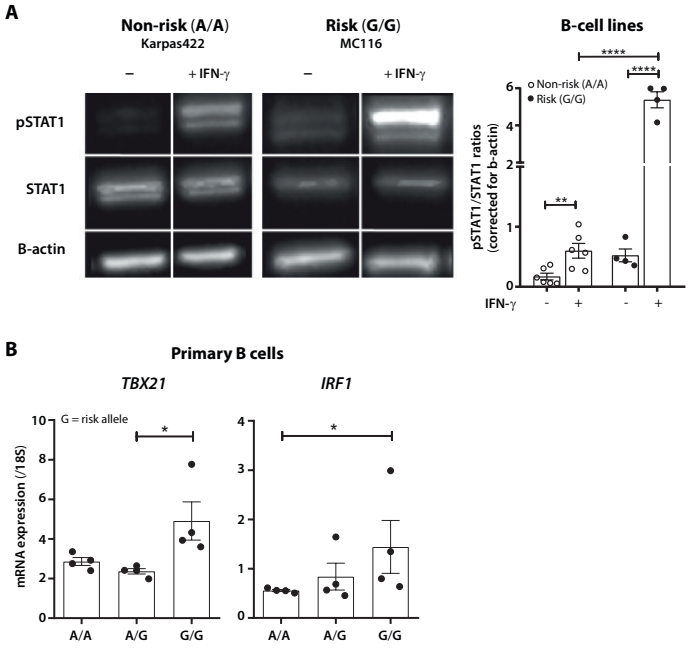


Figure 3. IFN-γ enhances STAT1 phosphorylation in human B cells carrying the coding MS risk variant *IFNGR2*.

(**A**) Western blotting and quantification of phosphorylated STAT1 (pSTAT1) expression in human B-LCLs stimulated with or without IFN-γ for 24 hours. A B cell line (MC116) homozygous for the *IFNGR2* risk allele (G; risk) was compared to a B cell line (Karpas422) that did not contain the risk allele (non-risk). For each B cell line an internal replicate of n = 4-6 was used. (**B**) *TBX21* (T-bet) and *IRF1* gene expression in B cells from clinically isolated (CIS) MS patients stimulated with IFN-γ for 24 hours. CIS patients were grouped based on risk allele carriage (non-risk, A/A; heterozygous risk, G/A; homozygous risk, G/G; n = 4 in each case).

pronounced for AQP4-IgG-positive patients with relapses in GC-like cultures containing IFN-γ and CpG-ODN (**Chapter 6**). This indicates that *in vitro* formed plasmablasts have the ability to produce potentially pathogenic autoantibodies. Intriguingly, for NMOSD patients, the expression of T-bet during such cultures seemed to increase irrespective of whether these patients had relapses or not (**Chapter 6**). An alternative, T-bet-independent, mechanism that could play a role in both IFN-γ and CpG-ODN-driven plasmablast formation in NMOSD could be increased TLR9 expression on naive B cells during relapsing disease [54]. IL-6 may be a pro-inflammatory cytokine driving this expression [54, 55], which is elevated in serum during relapses [56, 57] and promotes plasmablast development [58, 59] and anti-AQP4 antibody production [60] in NMOSD patients. Accumulating evidence shows that the majority of anti-AQP4 antibodies are produced in the periphery and not within the CNS [61, 62]. Subsequently, these autoantibodies infiltrate the CNS to directly induce local damage [63, 64]. Similar events may occur in patients with MOG-IgG-associated

disorder (MOGAD; Fig 2.) [65, 66], although **Chapter 6** does show a first clue that B-cell development is differentially regulated between AQP4-IgG-positive NMOSD and MOGAD. In sharp contrast to MS, we did not find differences in CXCR3 expression on memory B cells of NMOSD or MOGAD patients (data not shown). Therefore, as a result of CXCR3 upregulation, memory B cells are possibly more prone to infiltrate and develop into antibody-producing plasmablasts/plasma cells in the MS CNS.

In **Chapter 4**, we found that CXCR3$^+$ B cells are enriched in CSF, meninges and brain tissues of 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 6- and 12-months after treatment. CXCR3$^+$IgG1$^+$ memory B cells also preferentially migrated towards CXCL10 in human brain endothelial *in vitro* transmigration assays (**Chapter 4**). Together with our *in vitro* culture data, this suggests that in the periphery, naive B cells are triggered by IFN-γ and TLR9 signaling to develop into CNS-infiltrating CXCR3(T-bet)-expressing IgG$^+$ B cells in MS. Notably, CD20 was also found to be enriched on such IFN-γ-inducible CXCR3(T-bet)-expressing IgG$^+$ B cells in MS blood (**Chapter 4**). Since anti-CD20 therapy has been shown to be effective in reducing relapses in MS, T-bet-expressing B cells could potentially be an important therapeutic target.

T-bet does not only elicit IgG class-switching, but also enhances antiviral responsiveness of B cells in mice [44, 45, 67]. Furthermore, in mice, persistent viral infections sustain the development of these types of B cells [46, 67]. To determine whether a persistent virus such as EBV is an additional player in the formation of CXCR3(T-bet)-expressing B cells, we analyzed EBV DNA load and related this to CXCR3 surface expression in blood B cell subsets of MS patients treated with autologous bone-marrow transplantation (BMT; **Chapter 5**). Autologous BMT is often accompanied by EBV reactivation and thus provides a unique setting where the impact of EBV can be followed during B-cell development [68, 69]. We found that increased EBV load associated with enhanced CXCR3 expression in class-switched memory B cells of MS patients treated with autologous BMT (**Chapter 5**). A more pronounced progression of disability was found in the majority of BMT-treated MS patients whose memory B cells showed heightened EBV and CXCR3 levels. This was reflected by an increased expanded disability status scale score as well as ambulation index (**Chapter 5**). EBV is known to reside lifelong within memory B cells [70]. In MS, EBV is hypothesized to infect autoreactive B cells and mimic T-cell help for GC-dependent differentiation into such EBV-infected memory B cells [25, 71-73]. Furthermore, EBV-reactivated B cells have been found to be enriched in lesions of MS patients after natalizumab withdrawal [74], which often result in severe MS rebound affects [75]. Together with our data, this implies that EBV infection could lead to the formation of GC-derived CXCR3$^+$ (EBV^{high}) pathogenic memory B cells capable of entering the CNS, which is possibly related to sustained clinical worsening of MS patients.

B cells as inducers of pathogenic brain-homing memory Th cells

Synchronously, within peripheral GCs, T-bet-expressing memory B cells are ideal candidates to trigger CNS-infiltrating Th cells in MS patients (Fig. 1). In mice, T-bet promotes the antigen-presenting cell function of memory B cells [76]. In **Chapter 4**, we found that blocking of IFN-γ not only interfered with CXCR3 expression on human B cells, but also suppressed human Th cell activation, proliferation and effector memory skewing during autologous co-cultures. It has already been shown that memory B cells in general serve as potent antigen-presenting cells to drive MS disease activity by stimulating brain-homing Th cells [8, 49]. Although no link with T-bet has been described yet, the presence of the major risk locus HLA-DRB1*1501 in B cells seems to be critical for inducing these types of Th cells [8]. These brain-homing Th cells show features of both Th1 and Th17 by co-expressing CCR6 and CXCR3 [8]. In **Chapters 2** and **3**, we explored how such subsets are associated with disease activity and their CNS-infiltrating capacity in MS. In **Chapter 2**, we used peripheral blood from treatment-naive patients with clinically isolated syndrome (CIS, the earliest clinical presentation of MS) and RRMS. We subdivided the CIS group into patients who remained CIS for at least 5 years (CIS-CIS) and patients who rapidly developed clinically definite MS (CIS-CDMS). Th1-like Th17 ($CCR6^+CXCR3^+$) and not Th17 ($CCR6^+CXCR3^-$) cell proportions were reduced, but showed a highly activated phenotype in both CIS-CDMS and RRMS blood. This is supported by a study that shows $CD4^+$ T cells in the periphery are already activated very early in CIS patients with a high risk of developing MS [77]. During this early disease phase, $CD4^+$ T cells are thought to be involved in disrupting the blood-brain barrier (BBB) function [78-80]. The differential expression of chemokine receptors, integrins and pro-inflammatory cytokines by such cells have been argued to mediate this disruption and subsequently allow their infiltration into the CNS [81, 82]. Therefore, in **Chapter 2**, we determined whether Th1-like Th17 cells have the ability to migrate to the CNS by first using CSF paired with blood from CIS and RRMS patients (**Chapter 2**). Although Th1-like Th17 cells were enriched in CSF from these patients, the short-term cultures in the presence of IL-2 used here likely skewed the phenotype of these cells. To eliminate this probable confounder, we next performed *ex vivo* analyses of Th1-like Th17 cells derived from various CNS tissues and confirmed their local enrichment in MS patients (**Chapter 2**).

Chemokine receptors such as CCR6 and CXCR3 are also co-expressed with integrin α4β1 (VLA-4), which allows infiltrating T-cell populations to bind to vascular cell adhesion protein 1 (VCAM-1) on brain endothelial cells [83]. Using peripheral blood of MS patients treated with natalizumab (anti-VLA-4 mAb), we further subdivided Th1-like Th17 into recently described pathogenic Th17.1 and Th17 double-positive (DP) cells based on the differential expression of CCR4 (**Chapter 2**) [84]. Th17.1 ($CCR6^+CXCR3^+CCR4^-$) cells accumulated in the blood of MS patients who clinically responded to natalizumab treatment. This

was not seen for Th17 ($CCR6^+CXCR3^-CCR4^+$), Th17 DP ($CCR6^+CXCR3^+CCR4^+$) or Th1 ($CCR6^-CXCR3^+CCR4^-$) cells. Consistent with this, *ex vivo* analysis showed that Th17.1 cells were enriched in CSF versus paired blood from 4 CIS patients (**Chapter 2**). In **Chapter 3**, we confirmed this enrichment in the CSF of 9 CIS and RRMS patients. Additionally, we showed that Th17.1 is the predominant Th subset in CSF of early MS patients, but not in CSF of patients with other inflammatory or non-inflammatory neurological disorders (**Chapter 3**) and late-stage MS patients (**Chapter 2**). Th17.1 cells also had an increased capacity to infiltrate the brain, as shown in *in vitro* human brain endothelial transmigration assays (**Chapter 2**) as well as *ex vivo* and *in situ* using post-mortem MS brain tissues (**Chapter 3**).

Besides for an increased ability to infiltrate the CNS, Th17.1 cells show pathogenic potential in early MS disease through differential pro-inflammatory cytokine and gene expression profiles. Th17.1 cells have increased co-production of IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) as compared to the classical Th17 cells (**Chapter 2**). Although we see little IL-17A produced by these cells, work by others does indicate that Th17 cells co-express IL-17A and IFN-γ within brain tissues of MS patients [85]. The differences could be due to the fact that in this study, Th cells were cultured in the presence of IL-23 and therefore induces RORγt-mediated IL-17A expression [86]. We directly sorted the different Th subsets from the blood and stimulated these cells with PMA/ionomycin to assess their pro-inflammatory cytokine profiles. Moreover, the pathogenicity of Th17.1 is further exemplified by their dual expression of T-bet and RORγt as well as high levels of genes such as *IL23R*, *ABCB1* (MDR1; multidrug resistance), *FCMR* (TOSO; anti-apoptotic) and *GZMB* (granzyme B; cytotoxicity) (**Chapter 2**) [87-89]. In **Chapter 3,** we demonstrate that Th17.1 cells trapped in the blood of natalizumab-treated MS patients have increased *ABCB1* and reduced *NR3C1* (glucocorticoid receptor) expression. This, along with the enrichment of $MDR1^+$ Th17.1 cells within CSF of early MS patients, indicates that glucocorticoid-resistant Th17.1 cells are recruited to the CNS to mediate early disease activity in MS. Glucocorticoids are broad range immunosuppressive drugs that are used to dampen acute relapses in MS [90]. However, relapses and disease progression are not prevented. In **Chapter 3**, we also found that *ex vivo* Th17.1 cells present in meninges and brain tissues of end-stage MS patients have a glucocorticoid-resistant phenotype ($MDR1^{high}$). This could explain why such therapies may not always be effective and puts forward MDR1 as a potential therapeutic target on these cells to increase efficacy of glucocorticoids.

The fact that granzyme B is abundantly expressed in Th17.1 cells provides new insights into the role of $CD4^+$ cytotoxic cells during MS disease progression [91]. Although the causal MS autoantigen is still unknown, an indirect factor that could be involved in the development of such cytotoxic Th cells in MS is the inadequate control of EBV-infected B cells by $CD8^+$ CTLs [11, 73]. As mentioned above, antiviral $CD8^+$ CTLs become exhausted during persistent viral infections. Indeed, during EBV reactivation, EBV- and not CMV-specific

$CD8^+$ T cell responses are decreased in MS patients [26]. Normally, $CD8^+$ CTL responses are compensated by the induction of $CD4^+$ cytotoxic T cells to keep persistent infections under control [92]. Following EBV infection, $CD4^+$ cytotoxic T cells produce high levels of granzyme B, perforin, IFN-γ and IL-2 [93, 94]. This links to the fact that EBV-specific and myelin-reactive $CD4^+$ T cells from MS patients produce high levels of IFN-γ and IL-2 [95] and strongly respond to B cells presenting myelin peptides [96]. Since CXCR3(T-bet)-expressing B cells are potent antigen-presenting cells [47, 76] and EBV infection alters B cell-intrinsic processing and presentation of endogenous antigens [73], one may speculate that chronic antigen presentation by EBV-infected B cells leads to $CD8^+$ CTL exhaustion. Consistent with this assumption, in **Chapter 5**, we found that increased EBV load in reconstituting memory B cells from BMT-treated MS patients is associated with CXCR3 and not CXCR4 or CXCR5 expression. Furthermore, we show that in MS patient blood, frequencies of ($IFN\text{-}\gamma^{high}IL\text{-}17A^{low}$) Th17.1 cells correlated to $CXCR3^+IgG^+$ B cells (**Chapter 4**). Although not directly proven in the current thesis, we argue that based on these and other studies, EBV^{high} B cells and especially those expressing T-bet and CXCR3 probably escape from control by EBV-specific $CD8^+$ CTLs and interact with IFN-γ-producing Th17.1 cells within germinal centers of peripheral lymphoid organs. This would then promote pathogenic B and $CD4^+$ T cells to infiltrate the CNS and cause local inflammation in MS [97, 98].

LOCAL REACTIVATION AND EFFECTOR FUNCTION OF CNS-INFILTRATING B AND T CELLS IN MS

Although $CD4^+$ Th cells are in general outnumbered by $CD8^+$ CTLs in brain lesions as investigated in autopsy studies [18, 20], 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 [18]. It has also been shown that in contrast to $CD8^+$ CTLs, brain-associated $CD4^+$ Th-cell clonotypes are reduced in MS blood, indicating selective recruitment (as described above) [99]. In addition to this, the fact that $CD4^+$ Th17.1 cells have cytotoxic potential and co-express IFN-γ and GM-CSF (**Chapter 2**) may imply that these cells serve as the first triggers in disrupting the permeability of the BBB during the early stage of MS [100, 101]. This will then allow many other cells including B and $CD8^+$ T cells to infiltrate the CNS (Fig. 1). Similarly, to $CD4^+$ T cells, identical B cell clones have been found in the periphery and different CNS compartments of MS patients [102, 103], supporting their local recruitment [99]. B cells can predominantly be found in the perivascular space and associate with active white matter lesions in autopsy cases with acute and relapsing remitting MS [18] as well as in the meninges [103]. Within the meninges, B- and T cell-rich follicle-like structures are present that localize next to cortical

lesions, presumably mediating progressive loss of neurological function in MS [104, 105]. Interestingly, in MS brain-infiltrating $CD4^+$ T cells are able to express and respond to IL-21 [106], the cytokine that that drives follicular T- and B-cell responses. Furthermore, IFN-γ triggering of B cells promotes ectopic follicle formation in autoimmune mice [40, 41]. Put together, it can at least be assumed that besides perivascular spaces in white matter, the formation of such structures, containing germinal centers, serve as a niche for B cells interacting with IFN-γ-/IL-21-producing $CD4^+$ T (Th17.1) cells to become reactivated in the MS CNS (Fig. 1). We found that both Th17.1 and CXCR3(T-bet)-expressing B cells are enriched in the meninges and brain tissue derived from end-stage MS patients (**Chapters 2, 3** and **4**). Such pathogenic T- and B-cell subsets can lead to local damage in several ways. IFN-γ produced associates with the presence of demyelinating lesions in the CNS [107-109] and activates microglia and macrophages to cause damage to oligodendrocytes [110-112]. The same may be true for pro-inflammatory cytokine GM-CSF, which can be produced by infiltrating Th cells (**Chapter 2**) and B cells [110]. B cells have also been found to have increased production of TNF-α and IL-6 [49, 113], which could add to local lymphocyte activation and inflammation.

Aside from serving as antigen presenters and cytokine producers, the antibody-secreting function of B cells may play an additional role in mediating local inflammation in MS. In **Chapter 4**, we have shown that during GC Tfh-like naive B-cell cultures, IFN-γ drives the differentiation of IgG-producing plasmablasts in MS. Furthermore, high EBV load in memory B cells derived from natalizumab-treated MS patients corresponded to an increased potential to develop into $CXCR3^+$ plasma cells capable of producing anti-EBNA1 IgGs during these same cultures (**Chapter 5**). This suggests that EBV load not only influences their CNS-homing capacity, but also their local differentiation and function as professional antibody-secreting cells (**Chapter 5**). Consistently, both meninges and brain tissues of MS patients are enriched with plasma cells [114, 115] and there is an abundant presence of oligoclonal bands in MS CSF [49, 116]. Also supporting the local differentiation of B cells into antibody-secreting cells is that anti-CD20 therapies in MS target bulk B cells ($CD20^+$) and not plasmablasts/plasma cells ($CD20^-$). Although the (auto)antigen specificity and effector function of locally produced antibodies remain unclear, we have shown that T-bet-expressing B cells display increased IgG1 switching (**Chapter 4**) and thus we argue that IgG secreted by infiltrating T-bet-expressing plasmablasts/plasma cells recognize yet unknown antigens in the CNS to mediate local inflammation in the MS brain [44, 46].

Several antigenic targets have been proposed to contribute to MS pathology. Next to myelin, which is one of the most intensively studied antigens [117], also EBV antigens are considered as major candidates. EBNA1-specific IgG antibodies are predictive for early MS disease activity [118] and are present in CSF from MS patients and not in controls [119, 120]. Interestingly, in **Chapter 5,** we show that CXCR3 and T-bet expression by plasmablasts/

plasma cells correlated with the production of anti-EBNA1-IgG during GC-like memory B-cell cultures. Whether EBV is only recognized in the periphery or also present in the CNS and how this contributes to local pathology in MS is still under debate in the field [15, 74, 121-124]. The fact that EBV load corresponds to brain-homing memory B cells and their ability to develop into anti-EBNA1 IgG-secreting plasma cells *in vitro* at least adds to these controversies. Some studies imply that reactivated B cells in ectopic meningeal follicles [125, 126] present EBV peptides to activate EBNA1-specific $CD4^+$ T cells that are cross-reactive to myelin antigens [95, 127, 128]. Currently, also other candidate B- and T-cell antigenic targets are being proposed to be relevant for MS, such as sperm-associated antigen 16, neurofilament light chain, RAS guanyl-releasing protein 2, αB-crystallin and GDP-l-fucose synthase [8, 129-132]. Whether our identified brain-infiltrating B and Th-cell subsets in MS differentially respond to these candidate antigens remains to be determined in the near future.

CONCLUDING REMARKS

In this chapter, we have summarized the findings within this thesis and discussed potential triggers and mechanisms through which interacting B and T cells could drive the pathogenesis of CNS demyelinating disease. In our presented models, we emphasize the differences in selection and outgrowth of naive B cells between MS and NMOSD subgroups, probably as the result of impaired control by chronically exhausted $CD8^+$ CTLs or functionally impaired Treg cells. The different mechanisms of IFN-γ- and CpG-dependent B-cell differentiation under GC-like conditions may distinguish these two types of CNS demyelinating diseases. Furthermore, the specificity of autoantibodies in the majority of NMOSD patients is known and is thought to arise from autoreactive naive B cells in the periphery. These autoreactive naive B cells likely interact with effector Th cells in peripheral germinal centers to induce the development of plasmablasts/plasma cells, which secrete autoantibodies that enter the CNS to inflict local pathogenesis in NMOSD patients. Although the exact (auto)antigen in MS is unknown, the disease pathology likely involves GC-dependent interaction of B cells with IFN-γ-producing Th cells within lymphoid organs, which creates a feedforward loop resulting in the development of highly pathogenic subsets. Subsequently, these subsets break through blood-CNS barriers and, together with infiltrating $CD8^+$ CTLs, are locally reactivated to cause inflammation and demyelination. Although definite proof is still lacking, these pathogenic events in MS are likely mediated by persistent infections such as EBV. We speculate that at least for this CNS demyelinating disease, EBV^{high} memory B cells escape from peripheral $CD8^+$ CTL-mediated control to induce cytotoxic IFN-γ^{high} Th17.1 development in genetically susceptible individuals.

In turn, Th cell-derived IFN-γ along with pathogen-associated triggers (e.g. EBV and CpG-ODN) enhance CXCR3 and T-bet expression in memory B cells, making them prone to infiltrate, further mature and respond to local antigens in the CNS (Fig. 1).

FUTURE PERSPECTIVES

Although the clinical course of MS and NMOSD is different and complex, both involve B and T cells that are directed at damaging the CNS. This thesis has made advances in identifying the phenotype and triggering of CNS-infiltrating B and T cells in MS (**Chapters 2, 3** and **4**). However, one remaining question is how do the functional programs of Th17.1 and CXCR3(T-bet)$^+$ B cells differ between patient subgroups and controls? Further expansion on the single cell transcriptome and proteome of such subsets from various subgroups of patients (e.g. RRMS vs SPMS vs PPMS) versus controls should be undertaken to answer this question. In addition, isolating these exact subsets and using autologous co-culture systems (as described in **Chapter 4**) with antigens such as myelin or EBV could provide more insights into their antigen-specific interaction. Gaining further knowledge into their functional characteristics will also help to determine whether these subsets can be used as biomarkers to predict onset, progression and/or therapy efficacy. Since TLR9 triggering of naive B cells in NMOSD patients links to relapse occurrence, it would be interesting to see whether similar effects are seen in MS patients with high or low disease activity. Furthermore, the fact that T-bet-expressing B cells are induced with dual IFN-γ and TLR9 stimulation and that T-bet is highly expressed by IFN-γ-producing Th17.1 cells as well suggests that targeting such cells in a 'hit two birds with one stone' approach may be a potential therapeutic strategy to further investigate. Small molecule inhibitors of the IFN-γ signaling (jakinibs) [133, 134] and TLR/myD88 pathway [135] are currently being used for treating other inflammatory diseases. Such targeted treatments may be applied for combined suppression of IFN-γ and TLR signals in B cells, thereby preventing their development as well as their induction of pathogenic T cells in MS.

Another remaining question is whether and how EBV^{high} B cells play a role in the exhaustion of specific CD8$^+$ CTLs and the induction of cytotoxic CD4$^+$ T cells. 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. This includes phenotypical analysis of EBV-specific CTLs using tetramers and exhaustion markers (e.g. CD160, PD-1, CD244, TIM-3, LAG-3) as well as assessing their function (cytokine production, cytotoxicity). Synchronously, EBV DNA load within B cells from patients and controls can be correlated to the exhaustion status of CD8$^+$ CTLs. As mentioned above, under normal circumstances when CD8$^+$ CTLs are exhausted, cytotoxic

$CD4^+$ Th cells compensate to keep viral infections under control [92]. Since such cytotoxic Th populations have been associated with MS progression [91], a third question is whether these show similar characteristics to Th17.1 cells and whether EBV reactivity of such brain-infiltrating Th17.1 cells are enhanced in MS. Similar to $CD8^+$ CTL analysis, tetramer staining could be used to assess the phenotypical characteristics of potentially cytotoxic EBV-specific $CD4^+$ T cells. Alternatively, stimulating bulk $CD4^+$ T cells with EBV peptide mixes can be used to assess their phenotype and cytokine producing capacity.

As for NMOSD patients, it would be interesting to determine whether Th17 cells that are involved in NMOSD [136] show a Th17.1 cell phenotype and are differentially involved in inducing AQP4- or MOG-specific B-cell responses. Firstly, to decipher the distribution and different roles of Th17 (Th17.1, Th17 DP) subsets in NMOSD and MOGAD patients, peripheral blood from these different patient subgroups should be phenotyped to compare such distinct subsets. Secondly, as mentioned for MS, stimulating naive B cells in autologous co-cultures with $CD4^+$ Th cells (or Th17.1 cells) can be used to assess their proliferation and differentiation capacity. Lastly, TLR9 induces plasmablast formation in NMOSD patients with relapses and could potentially be used to predict relapses. Thus, measuring TLR9 expression in B cells along with further investigating the use of CpG-ODN containing GC-like cultures of B cells to predict relapses from different NMOSD subgroups should be done in the near future.

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Addendum

List of abbreviations

English Summary

Samenvatting: Short Dutch Summary

Acknowledgements

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
AQP4	Aquaporin 4
ASC	Antibody secreting cell
BBB	Blood-brain barrier
BCL-6	B-cell lymphoma 6
BCR	B-cell receptor
BMT	Bone marrow transplantation
CDMS	Clinically definite multiple sclerosis
CIS	Clinically isolated syndrome
CD40L	CD40 ligand
CMV	Cytomegalovirus
CNS	Central nervous system
CpG-ODN	CpG oligodeoxynucleotide
CSF	Cerebrospinal fluid
CS	Class-switched
CTL	Cytotoxic T cell
CCR	CC chemokine receptor
CXCR	CXC chemokine receptor
CXCL	CXC chemokine ligand
DP	Double positive
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EBNA1	EBV nuclear antigen 1
EDSS	Expanded disability status scale
ELISA	Enzyme-linked immunosorbent assay
FOXP3	Forkhead box P3
FACS	Fluorescence activating cell sorting
GC	Germinal center
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA-II	Human leukocyte antigen
HSC	Hematopoietic stem cells
IFN-γ	Interferon gamma
IL	Interleukin
Ig	Immunoglobulin
MDR1	Multidrug resistance 1

MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NCS	Non-class-switched
NMOSD	Neuromyelitis optica spectrum disorder
NTZ/NAT	Natalizumab
PBMCs	Peripheral blood mononuclear cells
RQ-PCR	Real-time quantitative PCR
RRMS	Relapsing remitting multiple sclerosis
SLE	Systemic lupus erythematosus
STAT	Signal transducer and activator of transcription
TCR	T-cell receptor
Th	T helper
Tfh	T follicular helper
Treg	T regulatory
TLR	Toll-like receptor
TNF	Tumor necrosis factor
T-bet	T-box transcription factor
VLA-4	Very late antigen-4

SUMMARY

The adaptive immune response plays a central role in both multiple sclerosis (MS) and neuromyelitis optica spectrum disorders (NMOSD). This has become evident from both genetic studies and B-cell depletion therapies in MS (discussed in **Chapter 1**) that show the involvement of both B and $CD4^+$ T helper (Th) cells. B-cell depletion therapies further indicate that the interaction between B and $CD4^+$ Th cells is a key mechanism driving MS disease activity. Additionally, both antibody-dependent and/or -independent functions of B cells contribute to the pathogenesis, which may differ between each disease entity. Deciphering which functionally distinct disease-relevant lymphocyte subsets are triggered to drive MS and NMOSD will help to better predict the early disease course and treatment response in patients.

It is well known that $CD4^+$ effector Th cells play a crucial role in the pathogenesis of MS. However, which exact effector Th subset shows increased pathogenicity in the earliest phases of MS is not fully understood. In **Chapter 2**, we used chemokine receptor expression profiles to study the presence of Th1 ($CCR6^-CXCR3^+$), Th17 ($CCR6^+CXCR3^-$) and Th1-like Th17 ($CCR6^+CXCR3^+$) cells in the peripheral blood of clinically isolated syndrome (CIS) patients who remained CIS for more than 5 years (CIS-CIS) and patients who developed clinically definite MS (CDMS) within 1 year (CIS-CDMS). The results were compared to relapsing remitting MS (RRMS) and matched healthy controls. Selectively reduced percentages of circulating Th1-like Th17 cells were found in CIS-CDMS and RRMS patients. In addition, Th1-like Th17 cells were enriched and co-produced high levels of IFN-γ and GM-CSF in the CSF of these patients. To further define the role of Th1-like Th17 cells, we used CCR4 as a marker to subdivide these populations into Th17 DP ($CCR6^+CXCR3^+CCR4^+$) and Th17.1 ($CCR6^+CXCR3^+CCR4^{-/dim}$). This corresponded to distinct pro-inflammatory cytokine profiles for each subset (Th17: IL-$17A^{high}$GM-CSF^{low}, Th17 DP: IL-$17A^{low}$GM-CSF^{low}IFN-γ^{low}, Th17.1: IFN-γ^{high}GM-CSF^{high}IL-$17A^{low}$). Th17.1 cells showed the highest VLA-4 expression out of all Th subsets evaluated, which coincided with their selective accumulation in the blood of MS patients who clinically responded to natalizumab (anti-VLA-4 mAb) treatment. The fact that Th17.1 cells were prevented from migrating to the central nervous system (CNS) by natalizumab suggests that this subset has preferential transmigration abilities. This was shown in **Chapter 2** by using *in vitro* transmigration assays and analyzing CSF from 4 treatment-naive CIS and RRMS patients *ex vivo*. Besides for transmigration, Th17.1 cells showed enhanced pathogenicity through the expression of *ABCB1* (MDR1), *FCMR* (TOSO) and *GZMB* (granzyme B), which are associated with steroid resistance, anti-apoptosis and cytotoxicity respectively.

MDR1 expression in Th17.1 cells was of particular interest as glucocorticoids are used as a standard treatment for acute MS relapses. This treatment does not prevent recurrent disease activity or disease progression in MS. To further determine whether such Th17.1 cells in MS had reduced sensitivity to glucocorticoids, we compared the expression of MDR1

(*ABCB1*) and glucocorticoid receptor (*NR3C1*) amongst effector Th subsets in early-stage MS patients and controls in **Chapter 3**. Th17.1 cells that were trapped in the blood of RRMS patients treated with natalizumab (**Chapter 2**) showed increased *ABCB1* and reduced *NR3C1* levels as compared to healthy controls. MDR1^{+} Th17.1 cells were confirmed to be enriched in the CSF of 15 treatment-naive CIS and RRMS patients and not in patients with other inflammatory or non-inflammatory neurological disorders. The proportion of these cells were also increased in post-mortem brain tissues compared to blood of 8 late-stage MS patients. These results indicate that the glucocorticoid-resistant phenotype of Th17.1 cells corresponds to their preferential recruitment to the CNS of MS patients.

IFN-γ, which is produced the highest by Th17.1 cells as compared to other Th subsets, has been shown to induce autoimmune germinal centers and CXCR3 expression in B cells from mice. In **Chapter 4**, we gained insight into which peripheral B-cell subsets are capable of infiltrating the MS brain and which peripheral triggers influence their development. First, we phenotyped B cells derived from *ex vivo* post-mortem single-cell suspensions of CNS compartments paired with blood from end-stage MS patients using flow cytometry. We observed an enrichment of CXCR3^{+} B cells in CSF, meninges and brain tissues of these patients. Next, we used blood from MS patients before and after natalizumab treatment to assess whether distinct subsets were prevented from migrating into the CNS. CXCR3^{high}IgG1^{+} memory B cells appeared to accumulate after 6- and 12-months of treatment. Their increased transmigration potential was further confirmed *in vitro* using human brain endothelial monolayers. Lastly, we used an *in vitro* culture system containing IFN-γ and/or CpG-ODN to mimic germinal center responses. IFN-γ and CpG-ODN synergized to enhance CXCR3(T-bet)-expression of differentiating naive and not memory B cells. Both IFN-γ and CpG-ODN-induced T-bet expression was required for IgG1 switching, which correlated to the secretion of IgG1. Altogether, these data indicate that CXCR3-mediated transmigration and local reactivity of memory B cells in the CNS of MS patients may be attributed to IFN-γ and TLR9 triggering of T-bet.

Chronic viral infections have also been described to sustain the development of T-bet^{+} B cells. Epidemiological studies show Epstein-Barr virus (EBV) infection is a strong contributing factor in MS. However, little is known about how such a persistent virus influences the development of CXCR3(T-bet)$^{+}$ B cells. In **Chapter 5**, we used peripheral blood from MS patients treated with bone marrow transplantation (BMT) to first determine whether there is an association between CXCR3 expression and EBV infection in memory B cell subsets. We showed that EBV DNA load correlated with CXCR3, and not CXCR4 or CXCR5 expression in class-switched memory B cells in post-BMT blood samples. Next, we investigated whether high or low EBV DNA load impacted CXCR3^{+} memory B cell development into plasmablasts *in vitro*. Memory B cells from EBV^{high} natalizumab-treated MS patients had an increased ability to differentiate into CXCR3^{+} plasma cells during IFN-γ germinal center-like cultures as compared to EBV^{low} patients and healthy controls. The percentages of CXCR3^{+} plasma cells correlated with EBV DNA load, whereas the expression of CXCR3 on plasma

cells correlated with the production of anti-EBNA1 during these cultures. Although direct proof is not shown, these findings imply that EBV plays a role in the brain-homing capacity of pathogenic B cells and their local production of anti-EBNA1 in MS.

Both central and peripheral B cell tolerance defects have been found in NMOSD, which is in contrast to MS. Since little is known about the composition and outgrowth of such early naive B cells in different subgroups of NMOSD, we determined the proportions of naive mature and transitional B cells within the blood of treatment-naive and corticosteroid-treated AQP4-IgG-positive NMOSD and MOG-IgG-positive (MOGAD) patients as well as treatment-naive MS patients and healthy controls in **Chapter 6**. The ratio of circulating naive mature/transitional B cells was reduced in treatment-naive AQP4-IgG-positive NMOSD compared to other groups. In patients who had undergone steroid treatment, this ratio was increased as a result of decreased frequencies of transitional B cells. In addition, the enhanced naive mature/transitional B cell ratios correlated to time of treatment. To further assess the outgrowth of naive B cells into IgG-secreting plasmablasts, we used the same germinal center-like culture system as in **Chapter 4** to induce plasmablast formation *in vitro*. Stimulation of naive B cells with both IFN-γ and CpG-ODN triggered plasmablast formation in AQP4-IgG-positive NMOSD and MOGAD patients with relapses, which was in contrast to patients without relapses and matched healthy controls. Interestingly, for AQP4-IgG-positive patients, *in vitro* secretion of total IgG was increased for CpG-ODN-containing conditions and was even more elevated in the relapsing cases. Furthermore, AQP4-specific IgG secretion was only found for relapsing patients. These *in vitro* differences were not seen for naive B cells from MOGAD patients. This study indicates that steroid treatment differently affects the proportions of naive and transitional B cells and provides first hints towards the use of TLR9-mediated germinal center-like cultures to predict NMOSD disease activity.

In **Chapter 7**, we brought the results from the above mentioned studies together and highlighted the similarities and differences between MS and NMOSD. Furthermore, we proposed two models of how B and/or Th cells play different roles in the immunopathogenesis of each of these diseases. Primarily, in NMOSD patients, autoreactive B cells that have escaped early tolerance checkpoints interact with Th cells in the periphery to enter germinal centers and develop into AQP4- or MOG-specific IgG producing plasmablasts. These autoantibodies are capable of entering the CNS to induce local damage. In the case of MS, Th17.1 cells possibly interact with B cells within germinal centers in secondary lymphoid organs to produce brain-homing populations that further interact in the CNS to induce local damage.

SAMENVATTING

Het aangeleerde of verworven immuunrespons speelt een centrale rol in zowel multipele sclerose (MS) als neuromyelitis optica spectrum ziekten (NMOSD). Dit blijkt uit resultaten verkregen uit genetisch en B-cel depletie onderzoek in mensen met MS (zie **Hoofdstuk 1**). Hierin is aangetoond dat B en $CD4^+$ T helper (Th) cellen zich anders gedragen in mensen met MS in vergelijking met gezonde individuen. B-cel depletie studies geven verder aan dat de interactie tussen B en $CD4^+$ Th cellen een belangrijke drijfveer is van het ziekteproces in MS. Zowel antilichaam-afhankelijke als onafhankelijke functies van B cellen dragen bij aan de pathogenese van MS en NMOSD, welke kunnen verschillen per ziektebeeld. Wij denken dat het ontcijferen van zowel humane B als $CD4^+$ Th cellen die bepalend zijn voor ziekteactiviteit in MS en NMOSD zal helpen om het beloop van deze ziekten in de toekomst beter te kunnen voorspellen en behandelen.

Het is bekend dat $CD4^+$ effector Th cellen een essentiële rol spelen in de pathogenese van MS. Wat echter niet precies bekend is, is welke effector Th subset zorgt voor verhoogde ziekteactiviteit in de vroege fase van MS. Om dit te achterhalen hebben we chemokine receptor expressie profielen gebruikt om de aanwezigheid van Th1 ($CCR6^-CXCR3^+$), Th17 ($CCR6^+CXCR3^-$) en zogenaamde 'Th1-like' Th17 ($CCR6^+CXCR3^+$) cellen te bestuderen in het perifere bloed van patiënten met een klinisch geïsoleerd syndroom ('CIS'), de vroegste fase van MS (**Hoofstuk 2**). Voor deze studie maakten we onderscheid in patiënten die geen (CIS-CIS) en wel (CIS-CDMS) een tweede neurologische aanval kregen en hiermee de diagnose MS. De resultaten hiervan zijn vergeleken met Th subsets uit relapsing-remitting MS (RRMS) patiënten die overeen kwamen in leeftijd en geslacht met gezonde donoren. De frequenties van circulerende, 'Th1-like' Th17 cellen bleken significant lager in zowel de CIS-CDMS als de RRMS groep. Deze 'Th1-like' Th17 cellen waren verrijkt en produceerden hoge hoeveelheden aan IFN-γ en GM-CSF in het liquor van deze patiënten. Vervolgens gebruikten we CCR4 als derde marker om deze 'Th1-like' Th17 populatie onder te verdelen in 'Th17 DP' ($CCR6^+CXCR3^+CCR4^+$) en 'Th17.1' ($CCR6^+CXCR3^+CCR4^{-/dim}$) cellen. Deze subsets onderscheidden zich op basis van specifieke pro-inflammatoire cytokine profielen (Th17: IL-$17A^{hoog}$IFN-γ^{neg}GM-CSF^{mid}, Th17 DP: IL-$17A^{mid}$IFN-γ^{laag}GM-CSF^{laag}, Th17.1: IL-$17A^{laag}$IFN-γ^{hoog}GM-CSF^{hoog}). De Th17.1 cel bracht VLA-4 het hoogst tot expressie van alle Th subsets die onderzocht werden. Dit kwam overeen met hun selectieve ophoping in het bloed van MS patiënten die klinisch goed reageerden op behandeling met natalizumab (VLA-4 antilcihaam). Het feit dat de Th17.1 cel ophoopt in het bloed van natalizumab-behandelde patiënten suggereert dat deze subset in staat is preferentieel de bloed-brein barrière te passeren. Dit werd in **Hoofdstuk 2** aangetoond door middel van *in vitro* transmigratie assays en *ex vivo* analyses van liquoren verkregen uit 4 onbehandelde CIS en RRMS patiënten. Het ziekte-inducerend vermogen van Th17.1 cellen werd verder gekenmerkt

door selectieve expressie van *ABCB1* (MDR1), *FCMR* (TOSO) en *GZMB* (granzym B), oftewel genen die respectievelijk te maken hebben met resistentie tegen glucocorticoïden, remming van apoptose en cytotoxiciteit.

De expressie van MDR1 in Th17.1 cellen is interessant omdat glucocorticoïd standaard wordt gebruikt als behandeling voor acute aanvallen in MS. Echter, deze behandeling voorkomt niet dat ziekteactiviteit zich herhaalt of dat de ziekte verergert in deze patiënten. Om te bepalen of Th17.1 cellen een verminderde gevoeligheid hebben voor glucocorticoïden en hoe dit relateert aan hun brein-infiltrerend vermogen in MS, hebben we eerst de expressie van zowel MDR1(*ABCB1*) als de glucocorticoïd receptor (*NR3C1*) uit effector Th subsets in patiënten in een vroeg stadium van MS vergeleken met gezonde donoren (**Hoofdstuk 3)**. *ABCB1* werd hoger en *NR3C1* werd lager tot expressie gebracht in Th17.1 cellen die ophoopten in het bloed van natalizumab-behandelde RRMS patiënten (**Hoofdstuk 2**) in vergelijking met gezonde controles. Daarnaast tonen we in dit hoofdstuk aan dat MDR1$^+$ Th17.1 cellen verrijkt zijn in het liquor verkregen uit 15 onbehandelde CIS en RRMS patiënten in tegenstelling tot patiënten met andere inflammatoire en niet-inflammatoire neurologische aandoeningen. De relatieve hoeveelheden van deze cellen waren ook verhoogd in post-mortem hersenweefsel in vergelijking met gepaard bloed van 8 patiënten met eind-stadium MS. Deze resultaten geven aan dat glucocorticoïd-resistente Th17.1 cellen meer in staat zijn het brein van MS patiënten binnen te dringen.

Het feit dat Th17.1 cellen hoge hoeveelheden aan IFN-γ produceren is relevant, omdat in muis modellen is laten zien dat deze cytokine betrokken is bij zowel de ontwikkeling van autoimmuun-gerelateerde kiemcentra als de expressie van T-bet en CXCR3 door B cellen. In **Hoofdstuk 4** laten we zien welke perifere B-cel subsets in staat zijn om het brein van MS patiënten binnen te dringen en welke perifere signalen hun ontwikkeling beïnvloeden. Allereerst hebben we *ex vivo* B cellen uit verschillende breincompartimenten en het bloed van eind-stadium MS patiënten gefenotypeerd met behulp van flow cytometrie. Wij zagen een toename van CXCR3$^+$ B cellen in het liquor, de hersenvliezen en het hersenweefsel ten opzichte van gepaard bloed van deze patiënten. Daarna gebruikten we het bloed van vroege MS patiënten voor en na behandeling met natalizumab om te bepalen of de migratie van dit soort subsets naar o.a. het centrale zenuwstelsel (CZS) geremd werd. Inderdaad, CXCR3$^+$ geheugen B cellen, en met name de IgG1$^+$ populatie die CXCR3 zeer hoog tot expressie brengt, bleken op te hopen in het bloed van deze patiënten na 6 en 12 maanden van behandeling. Het verhoogde brein-infiltrerende vermogen van deze B-cel subsets werd verder *in vitro* bewezen aan de hand van bloed-brein barrière transmigratie assays. Vervolgens hebben we een *in vitro* kweeksysteem gebruikt die een kiemcentrum reactie met dit soort B cellen nabootst. Enkel naïeve en niet geheugen B cellen lieten een verhoogde CXCR3 en T-bet expressie zien na toevoeging van IFN-γ als TLR9 ligand CpG-ODN tijdens hun differentiatie. Deze synergie tussen IFN-γ and CpG-ODN zorgde voor een

verhoogde expressie en uitscheiding van IgG1. Aan de hand van deze data kunnen we concluderen dat voornamelijk CXCR3(T-bet)$^+$ B cellen aangezet worden om het CZS binnen te treden en via IgG1 expressie waarschijnlijk beter kunnen reageren op lokaal aanwezige (maar op dit moment nog onbekende) antigenen.

Muizen studies laten ook zien dat chronische virale infecties in toom worden gehouden door T-bet$^+$ B cellen. Epstein-Barr virus (EBV) infectie is een sterke risicofactor voor het ontwikkelen van MS. EBV houdt zich schuil in de geheugen B cel, maar het is onbekend of dit virus een rol speelt in de ontwikkeling van CXCR3(T-bet)$^+$ B cellen. Aangezien EBV-reactivatie een veelvoorkomend verschijnsel is na beenmergtransplantatie (BMT), gebruikten we het bloed van MS patiënten met een autologe BMT om dit te onderzoeken (**Hoofdstuk 5**). We toonden aan dat 3 tot 7 maanden na BMT, EBV DNA belading in deze cellen positief correleerde met CXCR3 en niet met CXCR4 of CXCR5 expressie op geheugen B cellen. Vervolgens onderzochten we of de mate van EBV belading in geheugen B cellen van invloed was op de differentiatie naar CXCR3$^+$ plasma cellen *in vitro*. Hiervoor maakten we gebruik van de ophoping van CXCR3$^+$ geheugen B cellen die we zagen in het perifere bloed van natalizumab-behandelde MS patiënten (**Hoofdstuk 4**). Geheugen B cellen van patiënten met hoge EBV belading bleken zich meer te ontwikkelen naar CXCR3$^+$ plasma cellen dan die van patiënten met een lage EBV belading en gezonde controles. Het percentage CXCR3$^+$ plasma cellen correleerde met EBV DNA belading, terwijl de expressie van CXCR3 op plasma cellen correleerde met de productie van EBNA1-specifiek IgG. Alhoewel direct bewijs nog geleverd moet worden, geven deze bevindingen eerste aanwijzingen dat een EBV infectie bijdraagt aan het brein-infiltrerend en (EBNA1-specifiek) IgG-producerend vermogen van geheugen B cellen in MS.

In tegenstelling tot MS, worden er in patiënten met NMOSD defecten gevonden in de selectie van autoreactieve B cellen in het beenmerg (centrale tolerantie). Recent is aangetoond dat deze Bcellen in de vroege fase van hun ontwikkeling specificiteit vertonen tegen AQP4, het autoantigeen in een groot deel van NMOSD patiënten. Er is nog weinig bekend over de perifere ontwikkeling van naïeve B cellen in patiënten met AQP4-IgG-positieve NMOSD. In **Hoofdstuk 6** hebben we de relatieve hoeveelheden van transitionele en naïef mature B cellen in het bloed vergeleken tussen zowel onbehandelde als glucocorticoïd-behandelde AQP4-IgG-positieve NMOSD en MOG-IgG-positieve (MOGAD) patiënten, MS patiënten en gezonde controles. De relatieve verhouding tussen naïef mature cellen en transitionele B cellen was verlaagd in onbehandelde NMOSD patiënten. Deze verhouding was juist verhoogd in glucocorticoïd-behandelde patiënten. Dit correleerde met de tijd sinds de start van behandeling en werd veroorzaakt door de afwezigheid van transitionele B cellen in het bloed. Om de uitgroei van deze naïeve B cellen tot antilichaam-producerende cellen te onderzoeken, gebruikten we dezelfde kweeksystemen als in **Hoofdstuk 4** en **5**. Het stimuleren van naïef mature B cellen met zowel IFN-γ als CpG-ODN zette de vorming

van antilichaam-producerende cellen aan. Dit was alleen het geval voor NMOSD en MOGAD patiënten met periodes van aanvallen en niet voor gezonde donoren (**Hoofdstuk 6**) of voor patiënten met MS (**Hoofdstuk 4**). Ditzelfde gold voor de uitscheiding van totaal IgG, met name voor naïef mature B cellen van NMOSD patiënten. Verder werd AQP4-specifiek IgG alleen gedetecteerd tijdens kweken met B cellen van actieve NMOSD patiënten, terwijl MOG-specifiek IgG afwezig bleek in dezelfde kweken voor MOGAD patiënten met en zonder aanvallen. Deze studie laat zien dat de naïeve B-cel populatie selectief beïnvloed wordt door steroïd behandeling en geeft een eerste aanleiding tot verder onderzoek naar het gebruik van dit soort cellen in CpG-ODN-bevattende kweeksystemen om ziekteactiviteit in patiënten met NMOSD te kunnen voorspellen.

De resultaten van de bovengenoemde studies worden bediscussieerd in **Hoofdstuk 7**, met daarin de overeenkomsten en verschillen van B-cel ontwikkeling tussen MS en NMOSD. Op basis hiervan brengen we twee modellen naar voren die laten zien hoe de interactie tussen B en Th cellen mogelijk een rol speelt in de pathogenese van beide ziektes. In het geval van NMOSD, ontsnappen autoreactieve naïeve B cellen aan selectie in het beenmerg, waarna ze een interactie hebben met Th cellen in de kiemcentra van secundaire lymfoïde organen. Hierna ontwikkelen deze cellen zich onder invloed van IFN-γ en CpG-ODN tot plasmablasten/plasma cellen, waarna autoantilichamen zoals AQP4-specifiek IgG het CZS binnendringen om lokale schade te veroorzaken. In het geval van MS, ontsnappen B en Th cellen aan selectie in de periferie om zich te ontwikkelen tot brein-infiltrerende geheugen populaties die zorgen voor de ontstekingen in het CZS.

ACKNOWLEDGEMENTS

From a small village in Lesotho to growing up in Taiwan, returning to South Africa for my Bachelor's degree and finally ending up in The Netherlands. Here I embarked upon a PhD journey I never thought I would begin but have so enjoyed and now it has come to an end. My research during this time has only confirmed the love I have for both the immunology and neuroscience fields with perfectly combining them in a thesis about central nervous system demyelinating diseases. Completing my PhD project would have never been possible without the help and support of many people who I would like to thank below.

Dr. M.M. van Luijn, dear Marvin, while doing my Master's internship under your supervision you asked me whether I ever considered to do a PhD. I told you straight away that I was unsure about this and that it is out of my comfort zone especially because my knowledge on immunology and MS was so little at the time. To which you answered 'I will convince you otherwise', and so you did when you presented me with this very challenging but interesting project about the role of EBV and B cells in MS. Oh, how things have changed over the course of the years with 'small' side projects leading to great publications and ever so slightly changing the course of my project with EBV taking a bit of a backseat. In the end though all my various projects had a connection in one way or another, mainly through CXCR3(T-bet)! Thank you for seeing the potential in me to do a PhD and for mentoring me these last couple of years (and a few more years to come!). You have taught me an immense amount about immunology, flow cytometry and MS. I admire your work ethic and how you seem to so easily and enthusiastically write articles, grants and especially rebuttals. Although, sometimes difficult to understand your line of thoughts, your feedback has helped my writing skills grow with each manuscript presenting its own challenges, yet you guided me through. Your enthusiasm and passion to unravel the different Th and B cell subsets that play a role in MS has inspired and driven me forward and has broadened my own interest to continue with research in the MS field!

Prof. dr. R.Q. Hintzen, dear Rogier, I want to thank you for taking me on as an intern and allowing me to continue my PhD in the MS(NIB) group. I am grateful for your encouragement and, although sometimes confusing, but useful meetings we had throughout my PhD. Just over halfway through my PhD you passed away and couldn't experience the end of this journey. Your extreme drive to solve MS has inspired me (and the NIB group) and I hope through the completion of this thesis that we have answered some of your burning questions. You were always very keen on having a chat about South Africa right before any of our meetings started, in which you always mentioned the beautiful flower gardens and wine routes that you wished to have visited. Although your Africa trip ended up being Tanzania instead of South Africa, I will also always remember the excitement with which you told me about the lions in the tree and immediately showing me the photo of this.

Prof. dr. P.D. Katsikis and Prof. dr. P.A.E. Sillevis Smitt, dear both Peters, thank you very much for your willingness to be my promotors under these extenuating circumstances. I appreciate the time, feedback and help you have both given me in order to complete my thesis book.

I would like to thank my reading (sub)committee members, Prof. dr. Jon Laman, Prof. dr. Bart Jacobs and Prof. dr. Rudi Hendriks for your time and both positive and critical feedback on my thesis manuscript. I am also grateful to Prof. dr. Marieke van Ham, Prof. dr. Inge Huitinga and Prof. dr. Heinz Wiendl for being part of my PhD committee. I look forward to discussing the contents of my thesis with you! Here I would also like to say thank you to Marieke for our collaborations during my project and I hope to continue our collaborations with you in the future. To all other collaborators I would also like to say thank you for your input on our various articles together.

The NIB group (Marvin included), writing one single paragraph to cover all of you will not do you justice so I will try and write a small (crisp and clear) paragraph for each of you as you all played such different roles in helping me through my PhD. Collectively, I can say that having travelled extensively throughout my life, I am so glad I managed to meet a group like you where I feel right at home. All of you were present at my Master's graduation (like a third family) when my parents couldn't be there. You all have also helped me to improve my Dutch through meetings, lunches etc. although I have yet to prove that to you by speaking it in your presence.

Marie-José, from day one we could comfortably chat about many things, including our love for food and trying out various different restaurants (many tips coming from you). Thank you very much for making me feel so at ease in the group, your moral support throughout my PhD and for helping me whenever Annet was unavailable. Being the organizer in the group you were always willing to organize birthday gifts, order antibodies/reagents needed for my experiments and sometimes doing it even on your day off because it was a rush order – this was very much appreciated! You also taught me how to culture and take care of my "babies" in the lab, which at the beginning I kept forgetting about. Our many train rides to and from Delft have also been appreciated, especially if I had a difficult and long day and could let off some steam. During the first COVID-19 lockdown, our visits meant so much to me, especially because it was during the last year of my PhD when I needed a lot of encouragement to get through all the writing. Your support throughout, in many shapes and forms, has definitely helped me reach this end goal!

Annet, you were/are always willing to help me with an experiment no matter how big or small, complicated, or simple. I cannot thank you enough for all the help you have given me over the years both inside and outside of the lab. You have taught me so much in the lab, starting from multicolour flow cytometry all the way to DNA isolation, qPCR and the list goes on! I think it was the time we had to push through 6 weeks of an immense number

of experiments for the Th17.1 Brain paper that I realized we work so well together in the lab, being able to start an experiment and take over so easily from each other whenever needed – like a well-oiled machine. During this time you had my back and supported me through the early mornings, late nights and even having 6 weeks to tackle a new protocol using transmigration assays that we have never used before. I admire your patience and the calmness with which you do each experiment (although sometimes it seems like chaos when last minute changes do happen, sorry for those..). I could not have completed this PhD without your support, experimental insights and even at times help with calculations. I am so happy to have you as my paranymph!

Liza, we have gotten to know each other well, being in the same group, during conferences and the last 2-3 years when we were office roommates. How I enjoyed our brainstorm sessions, chats, laughs, helping each other through difficult times and sharing cute nephew and niece videos. I would also never have found my way in different cities and conference venues without you (I was always following you like a sheep ;)). You have also taught me a lot about B cells and in the end, we could joke and maybe convince the others that B cells are just as important as T cells in MS! We ended up writing our thesis books alongside each other and for this I am grateful, especially for your help, understanding and encouragements through both the good and more difficult times. Thank you for agreeing to be one of my paranymphs! We definitely have to have a nice big celebration when all the restrictions are lifted. I wish you lots of success in your new career path and do not be a stranger!

Steven, where should I begin, maybe with a speedvac tease.. Thank you for providing me with ample material and allowing me to joke with you from time to time, making me laugh each time. You took over from me and expanded our research on Th17.1 cells. I am happy to see that you have been able to gather some nice results and a great publication that confirm our hypothesis (MDR1 rocks!). Thank you for your critical insights into my projects and for less serious coffee moments away from the lab as a small break to escape reality. I wish you success in completing your PhD project. Remember to take a breath now and then, you will get your publications (with almost another publication on the way already - at the time of writing this ;)) and have an awesome thesis book!

Dr. J.J.F.M. Smolders, dear Joost, I still find it difficult to figure out when you are being sarcastic or serious! I do appreciate your enthusiasm and insight into the projects I had ongoing when you joined our group. Also thank you for reading and giving me such positive feedback on my thesis book. Your positive attitude and thoughts on sometimes difficult/negative results has helped me throughout this last year to gain back my own positivity. You have now also peaked my interest on CD8 T cells and I hope to continue some side-line research on them.

To my students, Ilse, Larissa and Caroline, thank you for your help in my various studies!

To the new and old members of the ErasMS center, and to name a few Roos, Yuyi, Arlette, Katelijn, Beatrijs and Dorine, thank you for your contributions to my/our articles. Without your continued supply of patient material and looking up information, these studies would never have been possible. With this I would also like to thank the MS and NMO patients for taking part in our studies! Gijs and Peter-Paul, thank you for your help and collaborations throughout my PhD project. Gijs, especially at the beginning of my PhD, thank you for our conversations and your thoughts about how EBV infection is involved in MS.

The IMN group, Anne, Ruth, Wouter, Willem-Jan and Sander, thank you for all the enjoyable lunch chats, coffee moments and your support on all the different things I have gone through during the last few years inside and outside the lab. In addition to this, we share the same space in the lab and have had many moments to laugh about when doing completely different experiments side by side. Anne, I appreciated the many early mornings when you arrived with your cheerful good morning and sometimes startling me when I was so deep in thought that I didn't realize someone else was in the lab so early!

Fabian, we had many laughs and serious conversations during the course of our PhD projects. Thank you for your encouragement, advice and for showing me how passionate someone can be about the work they do. I wish you success with completing your thesis book, you are almost there!

Manzhi, thank you (Xiè Xiè Ni) for always being honest and saying exactly what you think! I respect the fact that you can carry on with your research while your baby son is in China with your husband, this must be incredibly difficult for you. We started approximately at the same time with our PhDs and are ending very close to each other, thank you for your wisdom and many chats we had! You will accomplish great things in life, and you are almost there as well with your thesis book.

Iris and Dew, both of you are now officially Doctors!!! Dew, I will always respect how hard you worked for your PhD and always with a smile on your face! I also never could understand how you managed to work through the many chats Iris and I had in our office. Iris, as you mentioned in your thesis book, I should also write this to you in Dutch to practice, but as always, my excuse is it is not good enough so I will spare you the grammar and spelling mistakes. I miss our chats very much since you left and also, your support and jokes about me opening the door way too loudly (keeping a tally to remind me that I owe you and Dew sushi when it got over a certain limit!). Thank you for being the friend I needed at every corner throughout my PhD (even when you left you were always a WhatsApp away!) and I know we will continue to be friends outside of our work!

Marie-Joan, you are one of the most bubbly and positive people I know (you remind me a lot of my mom with your personality). I have really enjoyed our occasional chats in your office (thank you for the support) and you could always brighten up my day when we encountered each other in the hallways with your infectious smile!

To the two sort machine operators Harm and Peter, I am very thankful for your help. Almost all my studies required the sort machine in one way or another and I could always rely on your willingness to start early, finish late and sort simple or complicated cell populations!

To all the new and old fellow PhD students, Erika, Javad, Manhaz, Christiaan, Astrid, Olivia, both Martijn's, Emma, Benjamin, Jorn, Paul, Ling, Verna, Ziye, Britt, Christina, Anne, Wida, Xiaofei, Malou, Martine, Marjolein, Wida, thank you each for your individual support and as a group fun PhD retreats!

To the many colleagues at the Department of Immunology (workgroup leaders, Postdocs and technicians), we have in one way or another crossed each other's paths, be it "borrowing" antibodies from diagnostics, lab days, Christmas/New Year's dinners, lunches, birthday celebrations, remaining logged in on analysis computers for long periods of time (sorry for this...). Thank you all in contributing in your own way in supporting me through my PhD project! A special thanks to Jane and Corine for the interest and many chats!

To Bibi and Danielle, I am grateful for your help in formatting my thesis book and for easily adapting the numerous changes I kept throwing your way. This has definitely helped in saving me time!

Last but not least I would like to thank my friends and family outside of academia.

Stephie Houlberg and Rie Kanaya, thank you for your continued support from afar and for your understanding that if I take weeks before responding to your WhatsApp messages or e-mails that you are still important friends to me and that I am thinking of you! I appreciate that our friendships are so strong that when we have not spoken in months or seen each other in years that when we do get to see each other it feels like no time has passed.

Mamma, "ek kan, ek wil, ek sal" – my hele lewe se Ma dit vir my maar ek het dit die meeste waardeer terwyl ek my PhD gedoen het, veral aan die einde. Baie dankie vir Ma se ontsettend baie aanmoediging en positiwiteit deur hierdie "journey" van my. Al verstaan Ma glad nie wat aangaan in die Projek van my nie, Ma vra altyd hoe dit gaan en hoe almal is. Al irriteer dit vir my wanneer Mamma vir my se moenie worrie nie, aan die einde is Ma meestal reg dat dinge altyd sal uitwerk soos dit moet. Pappa, maak nie saak hoe ver my veld van studie/werk afwyk van Pappa s'n nie, Pappa het altyd moeite gedoen om te probeer verstaan presies wat dit is wat ek navors (ten minste het ons projekbestuur in gemeen ☺). Ek moes selfs aanvanklik een of twee keer vir Pappa wys wat my PhD projek behels deur middel van 'n aanbieding. Dankie vir die aanmoediging om hierdie sprong te neem asook die geleentheid te bied en die ondersteuning om oorsee te kan studeer. Dit sou nie moontlik gewees het sonder Pappa se hulp nie. Ek weet dat opvoeding vir Pappa baie belangrik is en ek waardeer dit dat Pappa my ondersteun het om so ver te kon kom.

Karien, Gideon, Bailey en baba Leah, baie dankie vir die steun en amper daaglikse fotos van Leah (veral in my laaste jaar of twee) en ook die video chats – dit het my baie gehelp

om haar glimlaggie en vrolike persoonlikheid te sien en ook hoe sy so mooi opgroei. Dit het ook gehelp om my aan te moedig om klaar te maak so dat Christiaan en ek ook 'n familie kan begin. Karien ook vreeslik baie dankie vir die mooi "cover" en "inside pages" van my boek, jy het so `n onsetend mooi kreatiwiteit en talent wat ek dink jou baie ver gaan bring in die lewe. Ek kry baie komplemente oor my oorbelle van jou wat ek daagliks dra!

Oupa en Ouma Visser, elke keer as ek met Ouma praat dan vra Ouma of ons al 'n terapie onwikkel het om MS te stop, ongelukkig het ons nog nie so ver gekom nie. Maar ons het darem klein stappies al geneem om meer te verstaan wat gebeur in die siekte aktiwiteit van MS. Oupa ek onthou nog hoe ons baie aande op die stoep gesit het en gepraat het oor my belangstelling en wat ek een dag wil doen, een ding was virseker ek wou nie physics doen nie (maar dit het terug gekom op 'n klein manier tydens my studie). Van Oupa het ek geleer om vas te byt en enige ding kan n werklikheid word as jy net wil. Altwee baie dankie vir julle ondersteuning van al die pad uit Moorreesburg!

Oupa James, eendag het Oupa vir my iets gesê wat in my gedagtes gebly het "mense versamel altyd iets, dit kan geld wees, dit kan familie wees, dit kan materialistiese goed wees of kennis" in my geval is dit kennis en dit het Oupa ook goed geweet (alhoewel Oupa gedink het ek moes n "lawyer" geword het)! Ek het ook altyd vir Oupa gesê as ek ooit my PhD klaar maak sal dit met die van Langelaar naam wees omdat ons dit nie kan aandra in ons familie nie. Baie dankie vir al Oupa se ondersteuning en wyse woorde deurgaans!

My schoonfamilie and Nuggy, without your support and willingness to take me on as your extra daughter, I would never have been able to come to the Netherlands to join Christiaan. I am so happy we met you in Taiwan. I will also never forget how small the world is when we returned to South Africa and my dad came home to say he works in the same building as Rene without even knowing where he was working at that time. Hellen, you were always ready for a chat when I returned home during the days we were still living with you. Thank you so much for your encouragement and long chats!! Rene, you are always ready with some wise words, we even went through a couple of coaching sessions while you were in Australia. Thank you both for always listening and being there when I needed it the most and Rene for helping me with my Dutch summary (although it has changed alot now). My sister in-law, Meylin, quite a challenging name when parts of my thesis involves the word myelin, every time I typed this word, I would type your name first! Thank you for your continued support and the sometimes random conversations we have had to take my mind off of the PhD. Although that was difficult, I always wanting to vent about various things, but you were always there ready with a listening ear or a glass of red wine! The moments I enjoyed the most were when we started jogging and then after a couple of minutes we decide naahh and just walk the rest of the way while dad runs ahead of us (I am now sad that while living in Delft these moments are very rare and only happen from time

to time in Markelo….). You are a very positive person by nature and I am very happy that you managed to find your own passion by now working at the World Animal Protection agency – I wish you continued success in this career path! Ook bedankt aan oma Hulleman voor de interesse en aanmoediging.

To my husband Christiaan, thank you for listening to my joyous moments, frustrations and long stories (with one ear open and the other covered by headphones while playing X-box)! Your enormous amount of support and wanting to come up with solutions to my problems has helped me through this journey. When the day came that you started your PhD, I thought finally I have a companion that understands all the ups and downs of doing a PhD. But so wrong was I, because from day one I could see the passion and joy you have for your own project. Your positivity during your project has inspired me and helped me to put a positive spin on difficult situations. You are almost there with completing your thesis – hang in there and I know you will do great at your defence! I could go on and write a whole book about our journey starting from way back when we met in Taiwan, surviving a time of long distance and then finally me joining you in The Netherlands. Hopefully from here on we can hop from country to country together as a family ☺. Thank you again for all your patience and understanding with early mornings and long nights throughout this time. Ek is onsettend lief vir jou!

To end with I would like to dedicate this thesis to Oupa James van Langelaar (94) and Prof. Rogier Hintzen.

CURRICULUM VITAE

Jamie van Langelaar was born in East London, South Africa on the 3rd of January 1991. When she was younger she moved with her family around South Africa. Her education started at a farm school in the Free-State and soon after that she attended a small international school situated in Lesotho. Thereafter, in 2002 she and her family moved to Taiwan and in 2009 she completed the International Baccalaureate (IB; high school) at the Taipei European School. From 2010 until 2012, she studied Molecular Biology and Biotechnology at Stellenbosch University, South Africa. Her love for traveling and interacting with different cultures encouraged her to further expand her education abroad. After obtaining her Bachelor's degree she moved to the Netherlands in 2013 where she studied a Master's degree in Brain and Cognitive Science at the University of Amsterdam. During her studies she became especially interested in neuroscience and immunology. Perfectly combining these two interests as part of her master's degree she did an internship entitled "Correlation between T-cell phenotypes and clinical parameters in the disease course of multiple sclerosis" at the Department of Immunology (Erasmus MC, Rotterdam), under the supervision of Dr. Marvin M. van Luijn (Neuroimmmunology Brain Group; NIB). In 2016 she started her PhD project, focusing on unraveling the different B and T cell subsets that play a role in multiple sclerosis, under the supervision of Dr. Marvin M. van Luijn, Prof.dr. Rogier Q. Hintzen (who passed away in May 2019), Prof.dr. Peter D. Katsikis and Prof.dr. Peter A.E. Sillevis Smitt at the Department of Immunology (Erasmus MC, Rotterdam). After the completion of her PhD project, she will continue her work as a postdoctoral researcher in the NIB group.

PHD PORTFOLIO

Name PhD student	Jamie van Langelaar
Erasmus MC department	Immunology
Research school	Molecular medicine (MolMed)
PhD period	2016 – 2020
Promotors	Prof. Dr. P.D. Katsikis
	Prof. Dr. P.A.E. Sillevis Smitt
Copromotor	Dr. M.M. van Luijn

PhD training

Courses and workshops

2015	Basic and Advanced Flow Cytometry course (BD)
2016	Workshop Presenting Skills for Junior Researchers
2016	Annual course on Molecular Medicine
2016	Basic Introduction course on SPSS
2016	Course on Molecular Diagnostics X
2016	Course in Virology
2016	Research Integrity
2017	Advanced Immunology
2017	Introduction to Data Analysis (NIHES)
2017	Workshop Photoshop and Illustrator CS6
2018	Research Management for PhD students
2018	Biomedical English Writing Course
2019	Biobusiness Summer School

(Inter)national Scientific meetings and presentations

2016	10th International Congress on Autoimmunity, Leipzig, Germany (**Oral presentation**)
2016	Annual MolMed meeting (**Poster**)
2016	Joint BSI/NVVI congress, Liverpool, United Kingdom (**Poster**)
2017	MS research days, Leiden, The Netherlands (**Poster**)
2017	Annual MolMed meeting (**Poster**)
2017	EMBO B cell research meeting, Barcelona, Spain (**Poster**)
2017	MS research days, Tiel, The Netherlands (**Oral presentation**)
2017	NVVI, Noordwijk, The Netherlands (**Oral presentation**)
2018	Annual MolMed meeting (**Oral presentation** and **Poster**)
2018	Keystone B cell research meeting, Dresden, Germany (**Oral presentation**)

2018	European Congress Immunology (ECI), Amsterdam, The Netherlands (**Oral presentation**)
2019	Annual MolMed meeting (**Elevator pitch**)
2019	MS research days, Groningen, The Netherlands (**Oral presentation** and **Poster**)
2019	NVVI, Noordwijk, The Netherlands (**Oral presentation**)

Grants and Awards

2016	Travel grant from Erasmus MC Trustfund (Joint BSI/NVVI congress, Liverpool, United Kingdom)
2018	EMBO travel grant (EMBO B cell research meeting, Barcelona, Spain)
2018	EFIS-EJI travel grant (ECI, Amsterdam, The Netherlands)
2018	Bright Sparks award (ECI, Amsterdam, The Netherlands)

Teaching

2016-2019	Histology teaching (1st year medical students)
2017-2019	Supervision of HLO and master students (research internships)

Other activities

2016-2017	PhD committee member
2016-2019	Journal club at the Department of Immunology
2016-2020	Seminars and minisymposia at the Department of Immunology

LIST OF PUBLICATIONS

Jamie van Langelaar, Annet F. Wierenga-Wolf, Johnny P.A. Samijn, Caroline J.M. Luijks, Theodora A. Siepman, Pieter A. van Doorn, Andrew Bell, Menno C. van Zelm, Joost Smolders and Marvin M. van Luijn. *The association of Epstein-Barr virus infection with CXCR3$^+$ B-cell development in multiple sclerosis: impact of immunotherapies*. European Journal of Immunology, 2020; Epub ahead of print.

Malou Janssen, Arlette L. Bruijstens*, **Jamie van Langelaar***, Yu Yi M. Wong, Annet F. Wierenga-Wolf, Marie-José Melief, Liza Rijvers, Daniëlle E. van Pelt ED, Joost Smolders, Beatrijs H. Wokke and Marvin van Luijn. *Naive B cells in neuromyelitis optica spectrum disorders: impact of steroid use and relapses*. Brain Communication, 2020; Epub ahead of print.

Steven C. Koetzier, **Jamie van Langelaar***, Katelijn M. Blok*, Thierry P.P. van den Bosch, Annet F. Wierenga-Wolf, Marie-José Melief, Kim Pol, Theodora A. Siepman, Georges M. Verjans, Joost Smolders, Erik Lubberts, Helga E. de Vries and Marvin M. van Luijn. *Brain-homing CD4$^+$ T cells display glucocorticoid-resistant features in multiple sclerosis*. Neurology, Neuroimmunology and Neuroinflammation, 2020; 7.

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; 11.

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-related gene CLEC16A in the B cell receptor-mediated HLA class II pathway*. Journal of Immunology, 2020; 205:945-956.

Jamie van Langelaar*, Liza Rijvers*, Malou Janssen, Annet F. Wierenga-Wolf, Marie-José Melief, Theodora A. Siepman, Helga E. de Vries, Peter-Paul 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; 86:264-278.

Liza Rijvers, Marie-José Melief, Roos M. van der Vuurst de Vries, Maeva R. Stéphant, **Jamie van Langelaar**, Annet Wierenga-Wolf, Jeanet M. Hogervorst, Anneke J. Geurts-Moespot, Fred C. Sweep, Rogier Q. Hintzen and Marvin M. van Luijn. *The macrophage inhibitory factor pathway in human B cells is tightly controlled and dysregulated in multiple sclerosis*. European Journal of Immunology, 2018; 48:1861-1871.

Jamie van Langelaar*, Roos M. van der Vuurst de Vries*, Malou Janssen*, Annet F. Wierenga-Wolf, Isis M. Spilt, Theodora A. Siepman, Wendy Dankers, Georges M. Verjans, Helga E. de Vries, Erik Lubberts, Rogier Q. Hintzen^{+} and Marvin M. van Luijn^{+}. *T helper 17.1 cells associate with multiple sclerosis disease activity: perspectives for early intervention*. Brain, 2018; 141:1334-1349.

* or $^{+}$ denotes shared authors