Development, Specificity and Local Recruitment of Lymphocytes Contributing to Central Nervous System Demyelinating Disease

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Development, Specificity and Local Recruitment of Lymphocytes Contributing to Central Nervous System Demyelinating Disease

Ontwikkeling, specificiteit en lokale rekrutering van lymfocyten betrokken bij demyeliniserende ziekten van het centraal zenuwstelsel

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Chapter 1

General introduction

GENERAL INTRODUCTION

Multiple sclerosis (MS), neuromyelitis optica spectrum disorders (NMOSD) and myelin oligodendrocyte glycoprotein (MOG) IgG associated disorders (MOGAD) are immune-mediated demyelinating diseases of the central nervous system (CNS)[1-5]. For all three entities, disability accumulates due to ongoing brain volume loss and incomplete recovery from relapses[6]. These diseases are presumed to be mediated by pro-inflammatory B and T cells and/or antibodies that cross the endothelial cell layer between peripheral blood and the CNS (in simplified form called the blood brain barrier, BBB) and target specific parts of the CNS. This is most likely the myelin-sheath in MS and MOGAD and the astrocyte in AQP4 IgG-positive NMOSD. Local inflammation, demyelination and CNS cell death (neurons, oligodendrocytes and astrocytes) are seen as typical and probably sequential pathological events[2, 7-10]. In contrast to MS, the transfer of peripherally produced autoantibodies into the CNS is probably the main pathogenic event in both NMOSD and MOGAD[11]. In NMOSD, MOGAD and MS, (auto)antigen-specific targeting of the CNS is likely the result of pathogenic lymphocytes that are inadequately controlled in the periphery.

Increased understanding of the lymphocyte subsets that drive MS, NMOSD and MOGAD may help to better predict the disease course and optimize current treatment regimens in a more personalized manner. New insights into their pathogenicity will even provide a rationale for developing more targeted therapeutic strategies. In the current thesis, the development, antigen specificity and local recruitment of disease-relevant lymphocyte subsets will be explored in patients with MS, NMOSD and MOGAD.

Epidemiology and clinical features of MS

The prevalence of MS is 127 per 100,000 in Western Europe and the global number of patients exceeds 2 million[12]. MS affects mostly young people (disease onset most frequently between 20 and 40 years of age), resulting in a significant loss of quality-adjusted life years[12]. Although the cause of MS remains elusive, factors that are known to increase MS risk include genetic predisposition, infections, female sex and smoking[13-15]. These factors all can impact the immune system as will be discussed later in this chapter.

A schematic representation of the MS course is displayed in figure 1. For most MS patients, the disease starts with an episode of clinical symptoms reflecting subacute demyelination in optic nerves, spinal cord or brain stem5, 16. This first episode is called clinically isolated syndrome (CIS, Table 1)17. During this episode, lymphocyte subsets infiltrate the central nervous system (CNS), predominantly white matter, due to profound leakage of the BBB resulting in typical demyelinated plaques18, 19. Most patients with CIS also have oligoclonal bands (OCB) composed of immunoglobulins (Ig) in their cerebrospinal fluid (CSF). These CSF OCB (identified after correction for bands in peripheral blood) are probably the result of

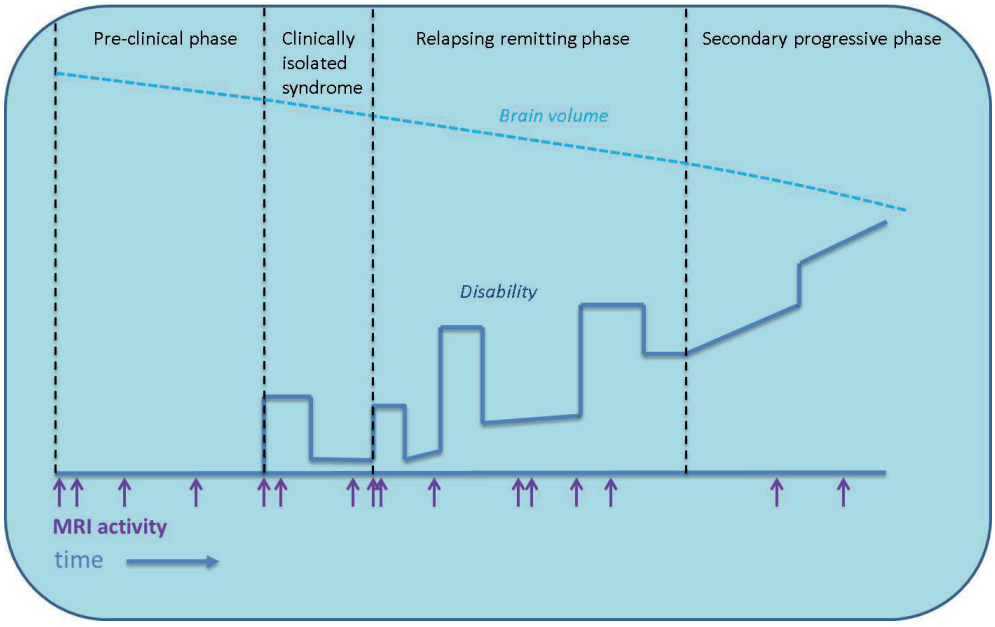

Figure 1. The typical clinical course in an individual MS patient

In the pre-clinical phase, first typical MRI lesions may be present without any clinical signs (radiologically isolated syndrome). In general, MRI-based disease activity is more frequent than clinical disease activity and sometimes goes unnoticed. The occurrence and duration of first symptoms caused by inflammation or demyelination defines a clinically isolated syndrome. When dissemination in space and in time is detected (via a second relapse or proven disease activity on MRI), the diagnosis of MS is clinically definite (relapsing-remitting MS). After a variable period (typically between 10-15 years), approximately 60% of the patients progress during which brain volume further declines due to neurodegeneration. This phase is called secondary progressive MS (SPMS).

intrathecal Ig production[20-22]. After CIS, a significant proportion of patients (~60%) will experience a second demyelinating attack. This most often occurs within the next months or years, but intervals of 10-20 years have been reported[17, 23]. A second clinical relapse can define relapsing remitting MS (RRMS). In some patients, MS can be diagnosed without or even before second clinical relapse by showing dissemination in lesion location and in time using MRI[24, 25]. It is challenging to foresee whether individual patients will develop RRMS, although some risk factors are known including clinical factors (disease dissemination and first lesion location[26]) and exposure to environmental factors (discussed later on)[27]. Next to patients presenting with CIS and RRMS, some patients (~10-15%) show a gradual increase of neurological disability from disease onset onwards. This disease type is referred to as primary progressive MS (PPMS)[28-30] and is characterized by slow accumulation of lymphocytes in the perivascular space without significant BBB leakage[19].

Table 1. Clinical phenotype, patient distribution and pathological hallmarks of various MS stages, NMOSD and MOGAD. s

		Clinical phenotype	Patients	Pathology
	CIS	First episode of demyelination	Female predominance (60-70%)	BBB leakage. CNS infiltration of mononuclear cells
Multiple sclerosis	RRMS	Lesions have occurred at multiple locations and various time points	Female predominance (60-70%)	BBB leakage. CNS infiltration of mononuclear cells
	SPMS	Gradual progression, relapses in some patients can still occur but are less prominent	Female predominance (60-70%)	Slow accumulation of mononuclear cells in PVS
	PPMS	From initiation phase onwards progression dominates disease course	Equal sex ratio	Slow accumulation of mononuclear cells in PVS
NMOSD		Fierce attacks of demyelination in optic nerves and/or spinal cord	Female predominance (90%)	AQP-4 Ig produced in periphery infiltrates and damages CNS (astrocytes)
MOGAD		More often monophasic demyelination of optic nerves and/or spinal cord	Equal sex ratio	MOG-Ig induced pathology

RRMS patients will experience relapses of which the timing, duration and subsequent complaints are hard to predict[9, 28, 31]. Neuronal loss that occurs gradually from the start of disease can further increase due to incomplete relapse recovery. Around 60% of RRMS patients[32] will develop secondary progressive multiple sclerosis (SPMS)[28, 29, 33], who show more gradual and progressive deterioration. The pathogenesis of SPMS is more similar to PPMS than to RRMS[19]. Risk factors for developing secondary progressive MS are older age, male sex, longer disease duration, high disability score, rapid initial disease progression and increased number of relapses in the year prior to SPMS conversion[34, 35].

Risk factors for MS

Early detection of individuals who are prone for developing MS would advance prognosis and treatment. Furthermore, it would aid in understanding the disease process and provide possible options to intervene. Many risk factors have been described for MS, of which the most have an impact on the immune system.

Sex

MS is much more common among women compared to men (3:1 ratio)[3, 36]. In general, females live longer and healthier lives compared to males[37] and female immune responses are more intense[38]. This is a double edged sword since females are more prone for most autoimmune diseases (AID)[39, 40]. The sex-based differences may originate from genes located at the X-chromosome[41-43], harboring the largest number of immune-related genes of all chromosomes[44]. X-inactivation is not successful for about 15% of genes[45], resulting in a cumulative higher expression of those genes in women. Furthermore, DNA-methylation patterns of the X-chromosome are parent-of-origin specific, resulting in reduced expression in paternally inherited X-chromosomes. This is female-specific as males always inherit their X-chromosome maternally[42, 46]. Overactivation of the female immune system via these pathways might contribute to the shifted sex-ratio in autoimmune diseases, including MS. Also sex-associated hormones are known to affect the immune system[47-50]. Compared to healthy males, male MS patients have lower levels of testosterone and these levels correlate with increased disability[51]. Restoring testosterone levels in male patients induces neuroprotection and reduces brain atrophy[52, 53]. *In vitro* testosterone exposure impairs differentiation of pathogenic lymphocytes[54]. In contrast, normal levels of female hormones induce pro-inflammatory responses whereas high levels, for instance during pregnancy, reduce the pro-inflammatory response[40].

Pregnancy

MS disease activity is significantly lowered in the third trimester of pregnancy and MS relapse risk rises again after parturition[55-58]. These differences in MS risk coincide with pregnancy-induced shifts in hormone levels such as estrogens that show a peak in the third trimester and are reduced postpartum[59]. During pregnancy, there is need for a delicate balance between an active immune system providing protection to both mother and fetus and simultaneously maintaining a state of immune tolerance towards partly allogenic fetal HLA. Previously, it has been shown that the default T cell response in pregnant women switches from pro- to anti-inflammatory[48]. In pregnant healthy women, it has been shown that certain lymphocyte subsets of the adaptive part of the immune system alter in frequency during pregnancy and recuperate post-partum[48, 60]. Understanding which exact disease-relevant lymphocyte subsets change during the peri-pregnancy period might aid in predicting which patients are at increased risk for a post-partum relapse and deserve frequent monitoring or prophylactic treatment.

Smoking

Smoking increases individual MS risk with approximately 50%[61, 62] and in individual patients correlates with a more aggressive disease course[63]. Tobacco smoke induces production of

pro-inflammatory cytokines like interleukin (IL)-17[64-66]. Smoking cessation can reduce MS risk and reduce disability progression in confirmed cases[67]. In individuals with genetic susceptibility for MS, smoking increased MS risk much more compared to patients without genetic predisposition[63, 68, 69].

Sunlight

The incidence of MS is much lower in countries around the equator. The increased sun exposure in these countries results in higher circulating levels of active vitamin D, which is likely to protect people from developing MS[70-72]. Vitamin D is a lipid-soluble precursor of active $1,25(OH)_2D$ and can be recognized by the vitamin D receptor (VDR). VDR, a nuclear receptor transcription factor, is expressed in a variety of immune cells and binds to VDR responsive elements (VDRE) of gene promoter regions, which are enriched in MS risk loci[73, 74]. Dendritic cells, involved in both the innate and adaptive parts of the immune system, are crucial in mediating the immune modulatory function of $1,25(OH)_2D$ by transporting vitamin D metabolites to the draining lymph nodes[75]. $1,25(OH)_2D$ reduces the differentiation of pro-inflammatory $CD4^+$ T cells[71]. Altogether, $1,25(OH)_2D$ shifts the human immune system to a more anti-inflammatory profile, which might explain the protective effect of appropriate vitamin D levels on MS risk[71, 76].

Epstein Barr Virus infection

One of the strongest environmental risk factors for MS development is a history of symptomatic infection with Epstein Barr virus (EBV)[77, 78]. EBV is a B- and plasma cell-tropic virus infecting the majority of individuals (~90%). MS patients are almost universally seropositive for EBV[78]. Primary EBV infection occur most often during childhood and is asymptomatic or with minor complaints[79]. However, with increasing age, primary EBV infection is more frequently associated with infectious mononucleosis (IM)[80], a symptomatic infection with sometimes long lasting complaints of fatigue. It has been reported that IM increases permeability of the blood-brain barrier with subsequent infiltration of lymphocytes into the CNS[81]. MS patients more often show a history of IM than healthy individuals[82]. In light of the association between age of primary infection and IM, it is interesting to see that high titers of EBV antibodies in mothers during pregnancy increase the risk for MS in the offspring[83]. Here, it might be possible that these Igs delay the child's own EBV-specific immune response due to long lasting maternal protection. Individuals with genetic MS predisposition have higher titers of EBV-specific antibodies[84, 85].

Genetics

MS is partly heritable as shown by the increased likelihood of developing MS when a family member has been diagnosed[86, 87]. Concordance in monozygotic twins is around 25-30%[88-90]

and in discordant monozygotic twins, the healthy twins immune system often shows signs of aberrant activation[91]. A significant part of the heritability has been explained almost entirely via genes acting on the adaptive immune response. The largest genetic risk allele is found in the protein complexes involved in presenting foreign antigens to T cells from the adaptive immune system: the human leukocyte antigens (HLA, explained later in more detail). The presence of HLA-DRB1*15:01 increases MS risk approximately three times[92-95]. This haplotype increases HLA class II expression on various antigen-presenting cells[96, 97], making these cells more likely to be involved in immune responses. In HLA-DRB1*15:01-positive MS patients, brain homing of cells involved in adaptive immunity (i.e. B and T lymphocytes) is induced[98]. In contrast, certain HLA class I haplotypes are associated with relative protection against MS development[99, 100]. Next to the major HLA haplotypes, minor genetic variants are associated with increased MS risk as well. Genome wide association studies (GWAS) with large numbers of patients and controls identified 200 additional risk alleles[43], of which the majority is active in B and T lymphocytes[101].

The role of the immune system in MS

It has long been considered that the immune system plays an important role in the initiation and progression of MS. The presence of oligoclonal immunoglobulin bands in CSF has been known for over 60 years[102] and MS is generally recognized as an autoimmune disease although this has not been fully proven. In patients with autoimmune diseases (AID), lymphocytes specifically recognize and respond to an autoantigen or epitope, eventually resulting in tissue damage. The originally defined criteria for AID in Witebksy's postulates[103, 104] include the presence of circulating antibodies directed against an autoantigen, development of comparable clinical phenotype after immunization with this particular antigen and/or disease induction after transfer of autoantigen-specific immune cells or serum containing autoantibodies[22]. In MS, however, the target antigen(s) of lymphocytes still has to be uncovered and therefore does not fulfil these criteria. Multiple studies have been trying to identify the antigenic targets of B cells and T cells in MS immunopathology, until now without satisfying results.

Further evidence of the role of the immune system in MS comes from the fact that most of the various drugs that exist for reducing MS disease activity and preventing relapses, target the adaptive arm of the immune system[105]. For instance, anti-CD20 and -CD52 therapy is highly effective in MS by reducing both B- and T-cell responses[106, 98]. Interferon-β, the oldest approved drug for MS treatment, is a cytokine likely to suppress interferon-γ activity and by doing so shifts the T cell response to a more anti-inflammatory one[107]. Congruently, glatiramer acetate[108], a compound containing four amino acids found in myelin basic protein (MBP), reduces T cell pro-inflammatory cytokine responses. Furthermore, natalizumab, an antibody preventing CNS-infiltration of lymphocytes (discussed later on in more detail)

is an effective treatment in MS, preventing relapses and disease progression[109, 110]. Finally, fingolimod, an agent blocking lymphocyte egress from lymph nodes, reduces frequencies of circulating immune cells, including B and T cells[111, 112].

Elucidating the role the immune system plays in MS, therefore, remains an important aspect of unravelling the complex pathophysiology of MS.

Adaptive immunity: specific protection against infections

The human immune system protects the body against pathogens via innate and adaptive responses. The innate response is initiated after the detection of pathogen-associated molecular patterns (PAMPs) which are recognized by pattern recognition receptors (PRR) expressed on macrophages and dendritic cells (DC)[113, 114]. PRR-mediated signaling triggers microbicidal and pro-inflammatory responses that are required to eliminate infectious agents[115, 116]. Furthermore, PRR enable detection cellular debris[116]. DC are professional antigen-presenting cells (APC) that internalize, process and present antigens on the cell surface via human leukocyte antigen (HLA) molecules[75]. HLA-molecules are encoded by the highly polymorphic major histocompatibility complex (MHC) gene complex[117]. Two HLA classes exist, both with three different HLA loci: HLA-A, HLA-B and HLA-C for class I[118, 119] and HLA-DR, HLA-DP and HLA-DQ for class II[120-122]. DC, like other antigen-presenting cells (macrophages, thymocytes, B cells), express HLA class II molecules enabling the presentation of exogenously derived and phagocytosed antigens (e.g. bacteria or fungi) to $CD4^+$ T helper (Th) cells[121]. HLA class I molecules are expressed on every nucleated cell in the human body and classically present peptides generated within a cell, for example viral antigens[123], to $CD8^+$ cytotoxic T cells (CTLs). DCs also have the ability to present exogenously derived antigens on HLA class I molecules, a process termed cross presentation[124].

Development of T and B cells

All T and B cells develop from the common lymphocyte progenitor cell (fig 2). The antigen-specific receptors are generated via a process of relatively random DNA rearrangements of variable, joining and in some cells also diversity gene segments, a process called V(D)J recombination[125-127], resulting in a broad repertoire of antigen-specific T and B cell receptors (TCR and BCR respectively).

After the development in the bone marrow, T lineage cells transmigrate into to the thymus and mature into pro-thymocytes. Via TCR rearrangements, cells expressing a functional TCR differentiate into common thymocytes. Double-negative thymocytes evolve in double positive thymocytes, expressing both CD4 and CD8 receptors. These undergo positive selection in the thymic cortex, eliminating cells with non-responsive TCRs. Cells become either $CD8^+$ or $CD4^+$ T cells based on the affinity of the TCR for HLA class I and II molecules, respectively[128]. After positive selection, thymocytes are directed into the medulla. where cells that show

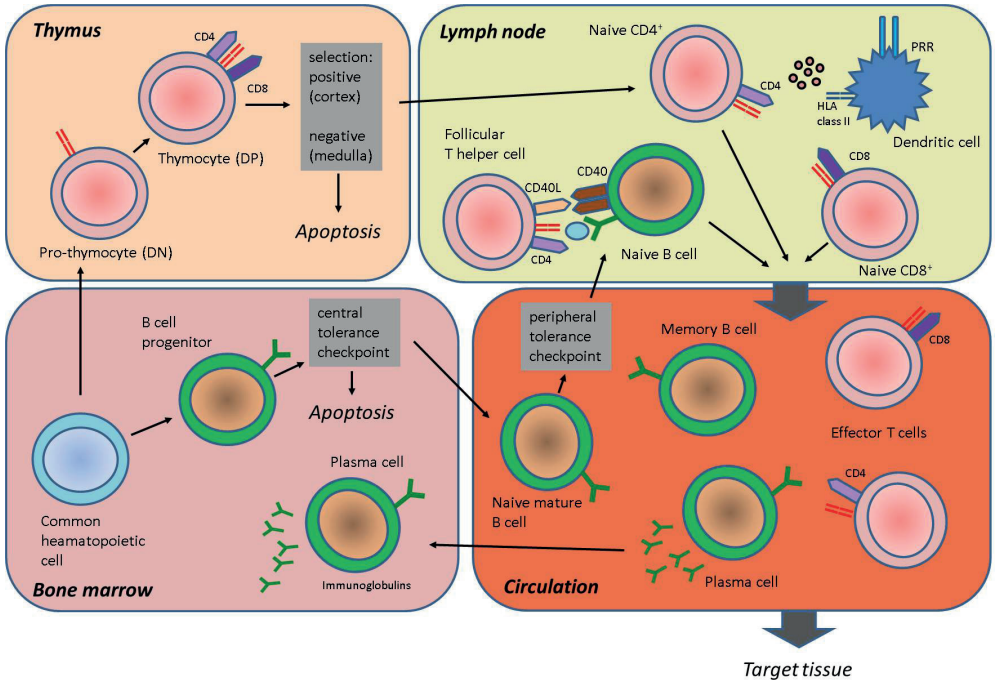

Figure 2. Lymphocyte development and maturation within lymphoid organs.
Common hematopoietic cells reside in the bone marrow and can give rise to various types of lymphocytes in the human blood. Pro-thymocytes enter the thymus and, after positive and negative selection, leave the thymus as a naive T cell. In secondary lymphoid organs, they are activated by dendritic cells and develop into effector and memory cells. B cells develop in the bone marrow and, after passing central tolerance checkpoints, enter the circulation as transitional B cells. At the follicular border within secondary lymphoid organs, naive mature B cells interact with $CD4^+$ T cells and subsequently become short lived plasmablasts (not shown) or enter the germinal center (GC). After GC-dependent interaction with T cells, B cells can become effector memory cells or plasma cells. For responses to T-cell independent antigens, B cells can become natural effector B cells and plasmablasts without any T cell help, which occurs for example in the marginal zone of the spleen (not shown).

high affinity to autologous peptides presented by thymic antigen-presenting cells are deleted (negative selection)[129]. Alternatively, autoreactive cells can become regulatory T cells[130]. Naive $CD4^+$ and $CD8^+$ T cells eventually leave the thymus and enter the circulation.

B cells emerge from common hematopoietic cells in the bone marrow. BCR repertoire diversities are further increased via random nucleotide deletions, which can result in reading frame shifts[131]. Comparable to TCR, BCR selection occurs, first via the central tolerance checkpoint[132-134]. B cells with strong responses to autologous peptides undergo apoptosis. Alternatively, BCR can be edited via secondary recombination to alter their specificity[134]. After passing this checkpoint, immature B cells undergo positive selection and become transitional IgM^+ B cells that can no longer edit their BCR[134]. These cells enter the circulation. In

the circulation, autoreactive B cells will go through peripheral tolerance checkpoints in the secondary lymphoid organs[135-137]. These checkpoints prevent the activation of naive mature B cells recognizing peptides derived from self-antigens in the absence of T cell help[135-138].

Generation of a T-cell response

After DC have engulfed and processed an exogenous antigen, they migrate into secondary lymphoid organs. Here, the DC present peptides on HLA molecules to naive T cells, a process called priming[139]. Primed $CD4^+$ T cells will proliferate, produce cytokines, express activation markers and become effector $CD4^+$ T cells. These cells can in turn can help macrophages in killing engulfed pathogens by producing cytokines and provide B cell help for proliferation and antibody production[140, 141]. Different effector $CD4^+$ T-cell subsets can be distinguished based on their cytokine secretion and receptor expression, as will be discussed later in this chapter. Like $CD4^+$ T cells, primed cytotoxic $CD8^+$ T cells undergo clonal expansion[142]. When effector $CD8^+$ T cells encounter their HLA-peptide complex, they release substances that induce cell lysis (perforin)[143] or apoptosis (granzymes)[144]. If the infection is cleared, most $CD4^+$ and $CD8^+$ T cells undergo apoptosis. However, some will develop into memory cells, providing protection when the same pathogen is encountered later in life.

Generation of a B-cell response

BCR can recognize soluble and membrane bound peptides. Soluble forms of the BCR are known as antibodies[8, 145, 146]. Antibody binding to target cells induces cellular cytotoxicity or apoptosis (activation of death receptors, antigen-crosslinking or blockade of cell survival pathways)[147]. Naive B cells can be activated in a T cell dependent and independent manner. A T cell-independent (TI) antigen can activate B cells via BCR crosslinking or triggering of Toll-like receptors (TLRs), a subtype of PRRs recognizing structures that are common on micro-organisms[148]. In response to TI antigen, B cells can become natural effector cells or short lived plasmablasts. TI immune responses are generally characterized by low avidity and the absence of memory formation[149, 150]. In contrast, a T cell-dependent B-cell response is initiated in the follicular border within secondary lymphoid organs. After antigen processing and presentation by HLA class II molecules, naive mature B cells are activated by T follicular helper (T_{FH}) cells via costimulatory receptors and cytokine production. B cells can then either leave the follicle and differentiate into short-lived plasmablasts or further mature within so-called germinal centers (GC)[151]. Increased strength and duration of the interaction between B and T_{FH} cells facilitates their entry into the GC[151]. The GC is composed of two zones: the light zone (affinity selection via interaction with T_{FH} cells) and the dark zone (B cell receptor diversification and clonal expansion)[145]. Cells circulate between these zones until they leave the GC as switched memory (IgG^+ or IgA^+), unswitched memory (IgM^+) or long-lived plasma cells[145] (fig 2).

In MS, antigenic targets of pathogenic B and T cells have not yet been elucidated. It is a matter of debate whether their pathogenicity is induced via infection with common viruses or bacteria, leading to bystander inflammatory demyelination[152-154]. In general, adaptive immune responses will give rise to tissue-resident memory lymphocytes that can respond quickly upon recognition of their target antigen. These cells are also found in the CNS of both healthy individuals and MS patients[155-157].

The concept of CNS immune privilege

Historically, the CNS was considered an immune privileged site with the BBB preventing entrance of infectious agents and potential harmful lymphocytes. However, during CNS infections, lymphocyte migration is necessitated and can therefore still occur[158-161]. More recently, the concept of immune privilege has been re-evaluated. It is now generally accepted that immune cells in meninges and parenchymal perivascular space (PVS) provide CNS immune surveillance[159]. The PVS has been shown to be an important location for T and B cell interaction and tissue resident lymphocytes found here are phenotypically distinct from populations in the peripheral blood[81]. PVS tissue resident cells show reduced expression of differentiation markers and transcription factors as well as increased tissue homing markers[156]. Within the CNS, T cell populations differ greatly as well, as indicated by the predominance of $CD4^+$ T cells in CSF, whereas brain parenchyma harbors significantly more $CD8^+$ T cells[155]. Immune cells and large particles drain via CSF and interstitial fluid to deep cervical lymph nodes, using a functional lymphatic system originating from meninges[162].

Peripheral lymphocytes can enter the CNS using different routes: via the choroid plexus, meningeal vessels or the PVS[163]. Lymphocytes expressing certain chemokine receptors migrate towards their cognate chemoattractant in the CNS[164, 165]. For instance, C-X-C chemokine receptor 3 (CXCR3) facilitates migration towards C-X-C motif chemokine ligand 10 (CXCL10)[166] whereas C-C chemokine receptor 6 (CCR6) induces attraction towards CCL20[167]. Interestingly, increased proportions of CSF T cells expressing CXCR3[164] as well as CCR6[168] have been shown. Other proteins like integrins[169, 170], and adhesion molecules[171] can facilitate cell migration as well. For instance, very-late antigen 4 (VLA-4) is an integrin involved in lymphocyte homing to various site of inflammation, including the gut, skin and the brain[172-174]. VLA-4 on lymphocyte cell surface interacts with vascular cell adhesion molecule 1 (VCAM-1), expressed by endothelial cells composing blood vessels[175]. Blocking this interaction is the rationale behind natalizumab treatment: an antibody targeting VLA-4, shown to be effective in reducing MS disease activity[109, 170, 176, 177]. Lymphocytes can recirculate between CNS and periphery[178, 179]. Due to selective migration it seems worthwhile to compare relative lymphocyte subset distribution in periphery and CNS in MS. Furthermore, it would be favorable to study antigen specificity of pathogenic lymphocytes using cells derived from the actual

disease compartment, since antigen-specific cells are likely to be enriched in the disease compartment.

Altered adaptive immunity and MS

A frequently used animal model to study neuroinflammation in MS is experimental autoimmune encephalomyelitis (EAE)[180, 181]. In this mouse model, CNS antigens (most often myelin-derived) are intrathecally injected, with or without an adjuvant to boost the immune system. This procedure gives rise to $CD4^+$ T cells that carry TCR specific for myelin epitopes, which induce CNS lesions that are comparable to those found in MS patients[182, 183]. Adoptive transfer of these antigen-specific $CD4^+$ T-cells to naive animals causes similar lesions[184]. Although EAE provides a valuable model studying neuroinflammatory processes, this model has its limitations. For example, the absence of genetic diversity due to use of inbred mouse stains[185] and the known target of the antigen-specific response due to active immunization[186] differ with respect to MS. Since there are also differences in the immune system between men and mice[187], one should be reserved when interpreting results in patients with MS.

$CD4^+$ T cells

From historical perspective, $CD4^+$ T cells have been considered the main drivers behind MS pathology[9, 188, 189], which is supported by the major risk locus HLA-DRB1*15:01[190, 191] and observations in EAE[93-95]. Multiple Th subsets exist that can be distinguished based on cytokine production and corresponding chemokine receptor expression[128] (fig 3). Th1 (mainly producing interferon (IFN)-γ) and Th17 (mainly producing IL-17A and -F) cells are involved in pro-inflammatory responses such as in EAE and MS[192-194]. IFN-γ has been shown to induce oligodendrocyte cell death[195] and is abundant in MS white matter lesions[196]. IL-17A has been reported to be associated with breakdown of the BBB in RRMS[197]. Th1 cells express transcription factor T-bet, which triggers surface expression of CXCR3[198]. Th17 cells express transcription factor RORγT[199] and chemokine receptor CCR6[200]. In contrast, Th2 cells classically help B-cells to differentiate and proliferate and thereby facilitate antibody production[194]. This subset provides protection to parasitic infections and, via typical adaptive responses in atopic individuals, can contribute to asthma pathology[201]. Th2 cells express transcription factor GATA-3[202] and can be identified by surface expression of CCR3, CCR4 and CCR8[203]. In MS, Th2 cells were found in type II brain lesions, characterized by antibody and complement disposition[204]. Tregs highly express FoxP3 and CD25[205] and are critical for maintaining peripheral tolerance, limiting chronic inflammation and preventing autoimmune responses. Their most important cytokines are IL-10, TGF-β and IL-35[206, 207]. In MS, it has been shown that Tregs have reduced immune suppressive capacities[208, 209] and adoptive transfer of this subset has been proposed as a therapeutic option[210]. Follicular T helper cells (T_{FH}) convey a separate subset mainly helping B cells to produce antibodies and can be identified by the expression

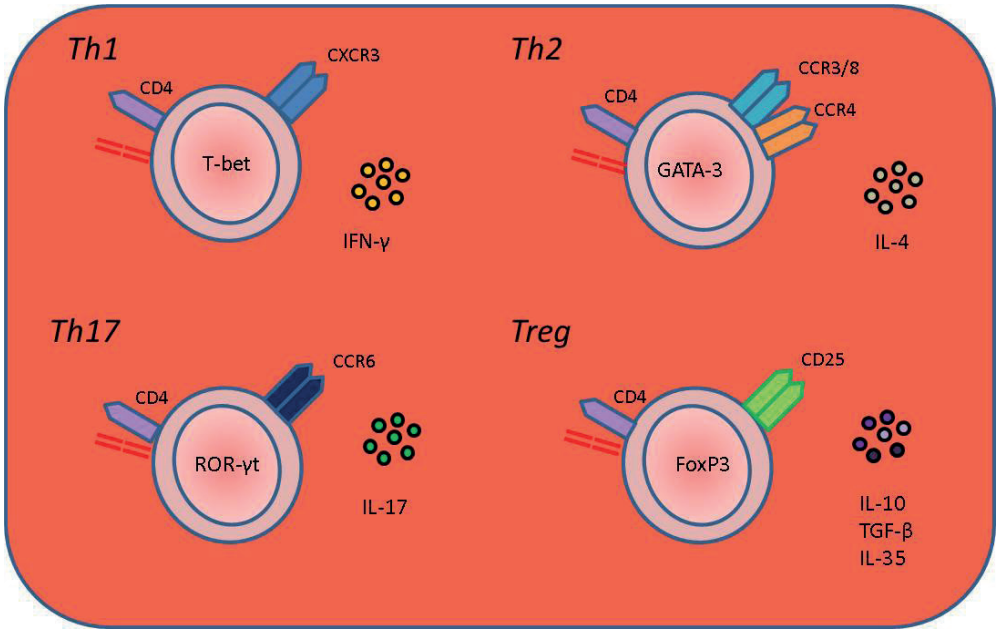

Figure 3. Classical T helper subsets with associated transcription factors, cytokines and chemokine receptors. Tfh cells, all expressing CXCR5 and producing IL-21, can be divided in comparable subsets.

of CXCR5[211-213]. Follicular T helper cells are subdivided into T_{FH1}, T_{FH2}, T_{FH17} and T_{FHregs} based on similar cytokine and transcription factor expression profiles as other Th subsets[214].

Multiple studies have been focused on addressing antigen specificity of $CD4^+$ T cells in MS. Myelin peptides were most frequently assessed, but antigens derived from other cells in the CNS (i.e. neuronal or glial proteins) have been proposed as well[215-218]. Myelin-reactive $CD4^+$ T cells have been shown to be present in MS patients and healthy individuals[219-221]. However, such T cells in healthy individuals were much less frequent[222]. Furthermore, in healthy individuals these cells showed a reduced activation status[221] and produced anti-inflammatory IL-10 in contrast to pro-inflammatory IFN-γ, IL-17 and GM-CSF[223]. Recently, $CD4^+$ T cells recognizing self-peptides and EBV antigens have been identified in MS patients carrying HLA-DRB1*15:01[98, 224]. Other reports indicate that EBV-reactive $CD4^+$ T cells can cross react with myelin peptides[78, 225].

$CD8^+$ T cells

Next to $CD4^+$ T cells, cytotoxic $CD8^+$ T cells are involved in MS immunopathology and activated $CD8^+$ T cells are found in MS brain lesions[155, 157, 226]. $CD8^+$ T cells could damage CNS tissue directly via cytotoxic responses since HLA class I is upregulated in the MS brain, most profound in severe and active lesions[185, 227-229]. Apoptotic-epitope specific $CD8^+$ T cells with

strong inflammatory potential were found in MS CSF[230], possibly aggravating newly formed lesions. Furthermore, oligoclonal expansion of CSF-derived memory CD8$^+$ T cells has been observed[231, 232] and granzyme B levels produced by these cells correlate to disease activity[233], providing evidence for a local immune response. Certain proteins are associated with increased pathogenicity and CNS migration of CD8$^+$ T cells in MS, like CCR6[234] and melanoma cell adhesion molecule (MCAM)[235]. Expression of VLA-4, another protein enabling CNS migration discussed earlier, is upregulated on effector CD8$^+$ T cells[236, 237]. The exact target of CNS-infiltrating CD8$^+$ T cells in MS patients remains unknown. Antigen-specific CD8$^+$ reactivity towards myelin basic protein (MBP) was shown in a small cohort using CSF-derived T cells[238]. Myelin reactive CD8$^+$ T cells expressing low levels of CD20 have been described recently[239]. These cells disappeared after anti-CD20 therapy and it cannot be ruled out that this contributes to success of this therapy. More recently, EBV-responsive CD8$^+$ T cells were found in higher frequencies in MS patients with active disease compared to patients with stable disease or healthy controls[155, 240-242].

B cells

In MS, in contrast to other AID like NMOSD[133], only peripheral and not central B-cell tolerance checkpoints are impaired, which suggests MS-specific alternations in the selection of autoreactive naive B cells. As a result, not transitional but naive mature B cells show increased numbers of autoreactive clones and have an activated phenotype in the blood of MS patients[136]. These autoreactive B cells can contribute to MS immunopathology in distinct ways.

Antibody-dependent function

B cells are abundant in MS cerebrospinal fluid[243], often harboring OCB[244], and clonally expanded memory B and plasma cells are present in white matter demyelinated lesions[245, 246]. Since short lived plasmablasts are the most predominant lymphocytes in MS CSF and abundance of these cells correlates with disease severity[247, 248], it seems likely that these cells produce the OCB-IgG. Successful natalizumab treatment frequently omits OCB after two years[244]. However, although successful in reducing MS disease activity[106, 249, 250], B cell depletion therapy via anti-CD20 therapy fails to omit the OCB[106], even when applied intrathecally[251]. Until now, no clear universal target for MS OCB-IgG has been shown. Previously, OCB-IgG specificity for myelin epitopes has been reported[252, 253]. More recently, reactivity was shown to be rather patient specific[254], targeting ubiquitous intracellular proteins[255] or debris as result of cellular damage[256]. However, due to the high prevalance of OCB in MS and the correlation with different clinical characteristics, intrathecal antibody production by antibody secreting cells (ASC) can not be ruled out as pathogenic factor in MS.

Antibody-independent functions

In addition to antibody production, B cells can alter T-cell responses via antigen presentation, costimulation and cytokine production[122, 257-259]. After CD20-directed antibody therapy, pro-inflammatory and autoproliferative T cells were reduced in MS patients[260]. Furthermore, successful treatment of MS patients with glatiramer acetate affects B cell antigen-presentation resulting in reduced differentiation of pro-inflammatory T cells and simultaneous induction of regulatory T cell differentiation[261]. Ectopic B cell follicle-like structures, enabling local B cell and T cell interaction, have been described in MS leptomeninges[262-264]. The presence of these structures correlates with subpial demyelination, advocating their pathological relevance[263-265]. Together these observations imply an important role for the antibody-independent B cell functions in MS immunopathology. An interesting hypothesis on B cell involvement in MS immunopathology has been put forward recently[266]. B-cell activation in peripheral lymph nodes enables CNS migration via expression of chemokine receptors and integrins. In the CNS, memory B cells can activate local T cells to mediate local inflammation and demyelination. Furthermore, memory B cells in the CNS can be re-activated and differentiate into professional ASC after interaction with pathogenic Th cells, producing antibodies directed against yet unknown antigens[112, 266].

Neuromyelitis optica spectrum disorder and myelin oligodendrocyte glycoprotein antibody associated disease

Epidemiology and clinical features of NMOSD and MOGAD

Compared to MS, NMOSD are rare, with a disease prevalence of about 1 in a million[267]. The spectrum conveys a broad range of severe clinical presentations that are caused by (relapsing) inflammation of the optic nerve(s) and/or myelum[1, 2, 268]. Disease exacerbations are generally accompanied with greater disability compared to MS[269, 270]. Complaints of optic nerve involvement can be visual field defects, loss of visual acuity or loss of color vision. Spinal cord involvement can induce a relative acute paralysis of legs and sometimes arms together with disturbances in the sensory pathways, quite often accompanied by bladder dysfunction[1, 2]. An important difference between NMOSD and MS is the undisputed role of pathogenic antibodies with known specificity in most NMOSD patients and NMOSD's complete fulfillment of the Witebsky criteria for AID[103, 104]. In ~75% of patients with NMOSD-like symptoms, antibodies are found that target aquaporin-4 (AQP4), a water channel protein expressed on astrocytes within the CNS[271]. Transfer of AQP4 antibodies to animal models yields NMOSD-like disease[272], demonstrating their pathogenicity and confirming the Witebsky criteria[103, 104]. About 10% of NMOSD patients are seropositive for antibodies targeting myelin oligodendrocyte glycoprotein (MOG). These antibodies have been shown to cause brain injury as well[273]. This subgroup of patients is diagnosed with MOG IgG associated disorders

(MOGAD) with an NMOSD phenotype[274]. In 60% of patients with MOGAD, MOG-specific B cells are detected in the peripheral blood[275]. Clinical features of AQP4-IgG-positive NMOSD and MOGAD patients are significantly different[276-278], including a different sex distribution, more relapses and increased need for immune modulatory medication for AQP4 seropositive patients[279]. The frequency of other AID in patients with NMOSD is 20-30%[280], whereas MOGAD patients showed no elevated risk for these diseases[281]. Due to this difference in clinical characteristics, it is tempting to speculate that the immunopathogenesis is different between NMOSD and MOGAD.

Immunopathogenesis

Recently, it has been reported that in patients with AQP4-IgG-positive NMOSD, both central and peripheral B tolerance checkpoints are defective[133, 282]. This enables autoreactive B cells to differentiate into ASC in a germinal center-dependent but also an antigen-independent manner[10, 133, 282]. Whether similar checkpoint deficits are involved in MOGAD pathology remains unknown. Plasmablast frequencies in the blood have been shown to increase during a NMOSD relapse, whereas it remains stable during an exacerbation in patients with MOGAD. This suggests the peripheral B-cell development is differentially regulated in these two diseases[283]. Some reports provide evidence for Th17 cell involvement in NMOSD and MOGAD immunopathology[284-289]. High IL-17 levels are associated with severe disability[289]. T cell involvement is further indicated by the IgG1 subclass of most AQP4- and MOG-specific antibodies[285, 288]. It is also not surprising that T_{FH} subsets favoring B-cell differentiation and antibody production are more prominent in NMOSD[290]. In brain biopsies and post-mortem tissues, activated $CD4^+$ cells were found in NMOSD[286] and MOGAD[287] patients.

Adaptive immunity in NMOSD and MOGAD, the unknowns

Individual patient based fine-tuning of current treatment is warranted. At present, steroids are given to treat acute disease relapses in both NMOSD and MOGAD, but their use is also accompanied by serious side effects[291]. Furthermore, due to these effects they can only be prescribed for a limited time frame, and thus they are not suitable for preventing relapses in NMOSD patients[292]. Adequate prediction of which patients will benefit from steroid treatment and at which specific moment in time is currently not possible. Since most previous research has been performed using patients receiving immunomodulatory treatment, the determination of essential B cell subsets involved in NMOSD/MOGAD immunopathology can be hampered. Development of a prediction model defining optimal starting and cessation points for steroid treatment would benefit patients to a great extent. Furthermore, if pathogenic lymphocyte subsets can be identified in NMOSD and MOGAD, it might be possible to develop compounds that selectively interfere with their development in the periphery.

SCOPE OF THIS THESIS

This thesis focusses on the development, specificity and local recruitment of lymphocytes in different central nervous system autoimmune diseases (AID): multiple sclerosis (MS), neuromyelitis optica spectrum disorders (NMOSD) and myelin oligodendrocyte glycoprotein (MOG)-antibody associated disease (MOGAD) with a clinical presentation of NMOSD. **Chapter 1** will provide an introduction based on relevant current understanding of both diseases. MS is most the most prevalent AID of the CNS whilst NMOSD is relatively rare. Both disorders however can provide distinct knowledge about protective immunity and alterations that can yield pathogenic immunity contributing to neurological disease. In this thesis, we aimed to uncover the identity, specificity and effector function of lymphocytes involved in CNS inflammatory demyelinating disease. Furthermore, we tried to find markers predicting individual clinical courses. The first part focusses on T-lymphocytes in MS, historically thought as most important in its disease pathology; **Chapter 2** studies antigen-specificity of intrathecal $CD4^+$ and $CD8^+$ T cells derived from the cerebrospinal fluid against a panel of candidate auto-antigens. In **chapter 3** we examine a newly discovered type of $CD4^+$ T cell: the Th17.1 cell, and compare its presence and activation status in MS blood, cerebrospinal fluid and brain tissue. In **chapter 4**, we address a functionally distinct B cell subset known to be involved in autoimmunity: the T-bet+ B cell. We compare its presence in early MS (CSF and blood) as well as different post-mortem CNS compartments from end-stage MS donors, and perform in vitro assays to study its development and CNS infiltration capacity. **Chapter 5** focusses on the impact of pregnancy, a naturally occurring modifier of MS disease activity, on GC-dependent B-cell differentiation both ex vivo and in vitro. Finally, in **chapter 6**, we study how B-cell differentiation can be linked to relapses and steroid treatment in patients with NMSOD and MOGAD. The composition of the peripheral B-cell compartment is compared to patients with MS. In **chapter 7**, the main findings of this thesis are brought together and discussed in detail.

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Chapter 2

Intrathecal CD4+ and CD8+ T cell responses to endogenously synthesized candidate disease-associated human autoantigens in multiple sclerosis patients

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ABSTRACT

MS pathology is potentially orchestrated by autoreactive T-cells, but the antigens recognized remain unknown. A novel APC/T-cell platform was developed to determine intrathecal CD4+ and CD8+ T-cell responses to candidate MS-associated autoantigens (cMSAg) in clinically isolated syndrome (CIS, n=7) and MS (n=6) patients. Human cMSAg encoding open reading frames (n=8) were cloned into an Epstein–Barr virus (EBV)-based vector to express cMSAg at high levels in EBV-transformed B-cells (BLCLs). Human cMSAg cloned were myelin-associated MAG) and -oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), proteolipid protein (PLP), ATP-dependent potassium channel ATP-dependent inwards rectifying potassium channel 4.1 (KIR4.1), S100 calcium-binding protein B (S100B), contactin-2 (CNTN2), and neurofascin (NFASC). Transduced BLCLs were used as autologous APC in functional T-cell assays to determine cMSAg-specific T-cell frequencies in cerebrospinal fluid derived T-cell lines (CSF-TCLs) by intracellular IFN-γ flow cytometry. Whereas all CSF-TCL responded strongly to mitogenic stimulation, no substantial T-cell reactivity to cMSAg was observed. Contrastingly, measles virus fusion protein-specific CD4+ and CD8+ T-cell clones, used as control of the APC/T-cell platform, efficiently recognized transduced BLCL expressing their cognate antigen. The inability to detect substantial T-cell reactivity to eight human endogenously synthesized cMSAg in autologous APC do not support their role as prominent intrathecal T-cell target antigens in CIS and MS patients early after onset of disease.

INTRODUCTION

MS is a chronic neurological disorder characterized by inflammation, demyelination, and axonal loss leading to accumulative disability[1]. MS immunopathology has been widely studied in EAE models[2]. EAE is induced by immunization of animals using various, mainly myelin-derived CNS proteins, or by adoptive transfer of autoreactive T-cells. Autoreactive CD4+ and CD8+ T-cells are analogously assumed to orchestrate MS pathology, but candidate MS autoantigen (cMSAg) targets remain enigmatic[1,2].

Several cMSAgs have been proposed including oligodendrocyte-specific proteins like myelin-associated glycoprotein (MAG), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP), glia-specific proteins like inwards rectifying potassium channel (KIR4.1, ATP-dependent inwards rectifying potassium channel 4.1) and S100 calcium-binding protein (S100B) and neuron-specific proteins like contactin-2 (CNTN2) and neurofascin 155kD isoform (NFASC)[1,2]. The majority of studies detailing cMSAg-specific T-cells in MS patients assayed peripheral blood with conflicting outcome[1,2]. Some observed increased auto-reactive T-cell frequencies in MS, while others described equivalent numbers in patients and controls questioning their role in MS[3]. The poor correlation between blood and intrathecal T-cell phenotypes, and TCR repertoires[4] dispute extrapolation of systemic T-cell data to MS immunopathology.

Most studies assayed intrathecal cMSAg-specific T-cell responses using autologous PBMC, pulsed with synthetic peptides or recombinant (animal) cMSAg, as APC[2]. This strategy has several limitations[2,3]. First, cMSAg of different species and bacterial-produced antigens have a different amino acid composition and conformation compared with native human cMSAg, respectively, which potentially affect processing and presentation of the cognate epitope[5]. Second, peptide binding is highly HLA allele-specific. Distinct HLA alleles are dominant MS risk or even protective factors[1]. Thus, differences in cMSAg T-cell responses in MS patients and controls may be due to intercohort HLA genotype disparity[1] ensuing mandatory HLA matching of patients and controls. Third, CD8+ T-cells preferentially recognize endogenously synthesized proteins. This is only partially overcome using cMSAg-spanning synthetic peptides as corresponding epitopes may not be presented when processed intracellularly and might need length optimization[5]. Finally, previous studies commonly used superphysiological peptide concentrations, typically in the 10–250 µM range, which may lead to activation of disease-irrelevant T-cells expressing low-avidity TCR[6].

To overcome the aforementioned limitations we developed a novel APC/T-cell platform that facilitates high and stable expression of individual cMSAgs in Epstein–Barr virus (EBV)-transformed B-cells (BLCL), an easy to generate and efficient APC system to assay CD4+ and CD8+ T-cell responses simultaneously by flow cytometry. This APC system was used to determine the frequency of CD4 and CD8 T-cells directed to eight different human cMSAgs in

short-term cerebrospinal fluid (CSF)-derived T-cell lines (CSF-TCLs) obtained from MS (n=6) and clinically isolated syndrome (CIS; n=7) patients.

RESULTS AND DISCUSSION

BLCL express and present recombinant proteins to CD4 and CD8 T-cells efficiently

The advantage of using immortalized BLCL as APC is their high expression of HLA class I and II (HLA-I/II) and costimulatory molecules crucial for T-cell activation. Ectopic expression of EBV nuclear antigen 1 facilitates episomal replication of the origin of latent replication (oriP) containing EBV genomes. We optimized an eukaryotic EBV-based vector, containing the EBV OriP and a geneticin transferase expression cassette, facilitating stable plasmid maintenance in BLCL[7]. The original pNS vector was modified to clone and express human cMSAg proteins more efficiently (Fig. 1A).

To validate that stable transduction of BLCL leads to efficient processing and presentation of endogenously expressed antigen by both HLA-I and II, we cloned measles virus fusion protein (MVF) into the pNS vector (pNS.MVF). The pNS.MVF-transduced BLCL grown for several weeks under geneticin selection showed uniform MVF surface expression (Fig. 1B; left panel) (Supplementary Fig. 1A). Next, we assayed recognition of transduced HLA-matched BLCL (GR-BLCL) by well-defined MVF-specific CD4+ (4-F99) and CD8+ T-cell clones (2-F40; TCCs)[8,9]. Both TCC showed a dose-dependent response to BLCL pulsed with their cognate peptides (Fig. 1C). TCCs cultured alone or cocultured with mock-transduced GR-BLCL secreted negligible IFN-γ amounts, whereas both TCCs stimulated with phorbolmyristate-acetate and ionomycin (P/I) secreted high IFN-γ levels. Notably, both TCCs efficiently recognized pNS. MVF-tranduced GR-BLCL, but not empty vector (pNS.empty)-transduced BLCL (Fig. 1C). Thus, the pNS/BLCL-system facilitates high expression and efficient presentation of CD4+ and CD8+ T-cell epitopes of an endogenously expressed antigen.

No evidence for intrathecal T-cell responses to cMSAg in CIS and MS patients

No consensus exists on autoantigens specifically recognized by systemic and intrathecal T-cells in MS. Limited studies have addressed cMSAg-specificity of CSF-derived T-cells in MS[10–12]. Ideally, antigen specificity of CSF-derived T-cells is determined ex vivo. However, limited cell numbers in surplus CSF samples, particularly in CIS cases, prohibited functional T-cell assays for multiple cMSAgs directly (Table 1). Therefore, we generated short-term CSF-TCLs by stimulating CSF-derived cells from CIS and MS patients in a single round of mitogenic stimulation to characterize intrathecal human cMSAg-specific CD4+ and CD8+ T-cell responses simultaneously and to perform repetitive analysis to determine interassay variability.

The coding sequences of human cMSAg for oligodendrocyte (i.e. MAG, MBP1, MOG, and PLP), glia (i.e. KIR4.1 and S100B) and neuronal origin (i.e. CNTN2 and NFASC) were cloned in the pNS vector and subsequently used to nucleofect autologous BLCL (autoBLCL). Flow cytometry showed that all viable transduced BLCLs uniformly expressed the respective cMSAg intracellularly after 2–3 weeks of geneticin selection (Fig. 1B). Whereas all assayed cMSAg,

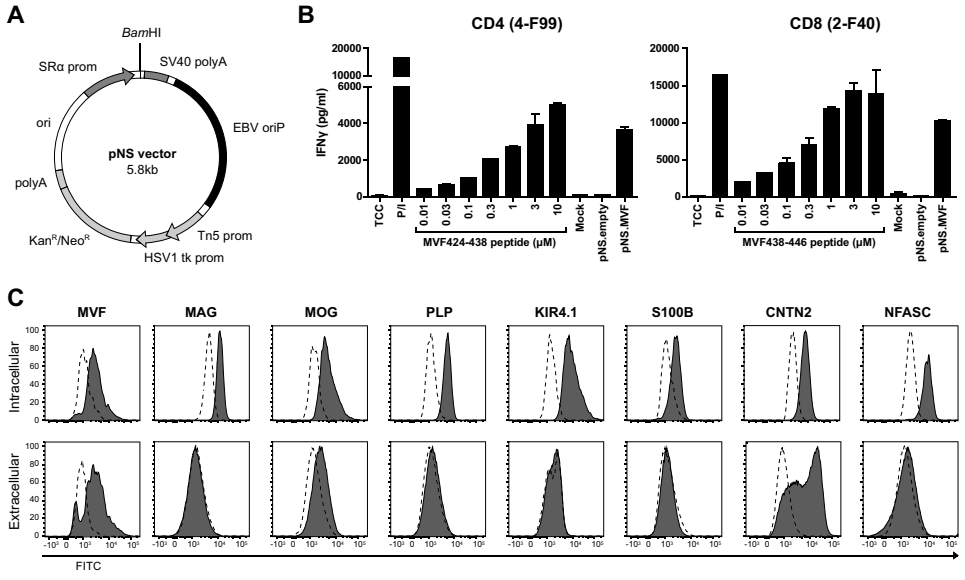

Figure 1.

EBV-based episomal pNS vector enables stable expression and efficient presentation of a cloned gene in BLCL to CD4+ and CD8+ T cells. (**A**) Schematic representation of pNS expression vector containing EBV origin of latent replication P (EBV oriP) for episomal replication in EBV-transformed B-cell lines (BLCLs). Combined kanamycin/neomycin transferase under control of the prokaryotic transposon Tn5 and eukaryotic herpes simplex type 1 (HSV-1) promoter enabled positive geneticin selection of transduced BLCL. Antigens of interest were cloned in *Bam*HI site and expressed under control of SRα promoter and SV40 polyadenylation signal (poly A). (**B**) Histograms of flow cytometry analyses of cells gatedfor lymphocytes, based on forward and side scatter and viability using fluorescent viability staining of representative BLCL transduced with pNS encoding measles virus fusion protein (MVF) and seven individual candidate human MS-associated autoantigens (cMSAg) cultured under gentamycin selection for >2 weeks. Dark area represents antigen-specific staining and the dotted line represents the respective isotype control staining. Vertical axis shows cell counts normalized to mode. Histograms are representative of two independent experiments. (**C**) HLA-matched BLCL pulsed with increasing synthetic peptide concentrations encompassing epitopes of MVF-specific $CD4^+$ (4-F99) and $CD8^+$ (2-F40) T-cell clones (TCCs) and stably pNS.MVF-transduced BLCL expressing MVF protein were cocultured for 24 h with respective TCC. IFN-γ levelswere determined by ELISA. As controls, TCC were non-stimulated (TCC), stimulated with phorbolmyristate-acetate and ionomycin (P/I) or cocultured with untransfected BLCL (mock) and BLCL transduced with empty pNS vector (pNS.empty). Data are shown as mean + SD (n = 4) and are pooled from two independent in-duplo experiments.

Table 1. Patient demographics and diagnosis

Patient ID	Age; Sex[a]	Disease entity[b]	Disease duration[c]	CSF WBC (x10^3/mL)[d]	CSF OCB[e]	IgG index[f]	Follow-up (months)	Progression to MS[g]
1	37; F	CIS	10	5	no	0.5	50	no
2	34; F	CIS	22	5	no	0.5	3	no
3	21; F	CIS	8	5	multiple	0.7	46	yes, to RRMS
4	25; M	CIS	1	12	multiple	0.9	42	yes, to RRMS
5	37; M	CIS	4	3	no	0.5	25	no
6	37; M	CIS	2	2	no	0.5	24	no
7	42; M	CIS	4	3	multiple	0.7	30	no
8	51; F	PPMS	54	11	multiple	0.9	2	n.a.
9	36; F	RRMS	102	4	multiple	1.4	29	n.a.
10	34; F	RRMS	9	14	multiple	1.0	26	n.a.
11	29; F	RRMS	30	25	multiple	1.0	32	n.a.
12	41; F	RRMS	36	7	multiple	2.1	51	n.a.
13	38; F	RRMS	18	5	n.d.	0.5	51	n.a.

a) Age in years; F, female; M, male.

b) CIS, clinical isolated syndrome and PP, primary progressive MS; RRMS, relapsing remitting MS.

c) Disease duration in months at time of lumbar puncture.

d) White blood cell count (WBC) per mL cerebrospinal fluid (CSF).

e) Presence and number of oligoclonal bands (OCB) in CSF only; n.d. not determined

f) IgG index was determined by the following formula: (CSF [IgG] / CSF [albumin]) / (serum [IgG] / serum [albumin]).

g) No, no progression to MS during follow-up; n.a. not applicable

except MBP1 and S100B, are transmembrane proteins only MOG- and CNTN2-specific mAb target extracellular domains of the protein. Transmembrane localization of MOG and CNTN2 was confirmed by extracellular staining. Unfortunately, no MBP1-specific mAb was available.

To determine the lower level of detection of antigen-specific CD4 and CD8 T-cells in T-cell lines using the APC/T-cell platform developed, we spiked a T-cell mixture consisting of two irrelevant varicella zoster virus-specific CD4+ (TCC 146) and CD8+ (TCC 38) with increasing numbers of the MVF-specific CD4+ and CD8+ TCCs and measured the response to pNS.MVF-transduced GR-BLCL by intracellular IFN-γ flow cytometry[8,9]. The estimated detection limit of CD4+ and CD8+ T-cells directed to endogenously expressed protein, here represented by MVF, in transduced GR-BLCL was 1 and 3%, respectively (Supplementary Figs. 1B and 2). Next, the frequency of cMSAg-specific CSF-derived CD4+ and CD8+ T-cells was determined by coculturing CSF-TCL with human cMSAg-transduced autoBLCL. P/I stimulation of all CSF-TCL showed that the majority of CD4 and CD8 T-cells produced high levels of IFN-γ indicating that the CSF-derived T-cells cultured were immunecompetent and not exhausted (Fig. 2A). To correct for background T-cell reactivity (e.g. intrathecal EBV-specific T-cells recognizing BLCL)[13], cMSAg-specific CD4+ and CD8+ T-cell responses were normalized for reactivity toward pNS.empty-transduced autoBLCL (Supplementary Fig. 3). Interassay variation for CD4+ and CD8+ T-cell responses was determined by calculating the SD of all mean netto cMSAg-specific responses. The variation was lower for CD4+ T-cells (0.33%) as compared with CD8+ T-cells (0.86%). Consequently, the threshold for positive calls was set at 1.2% for

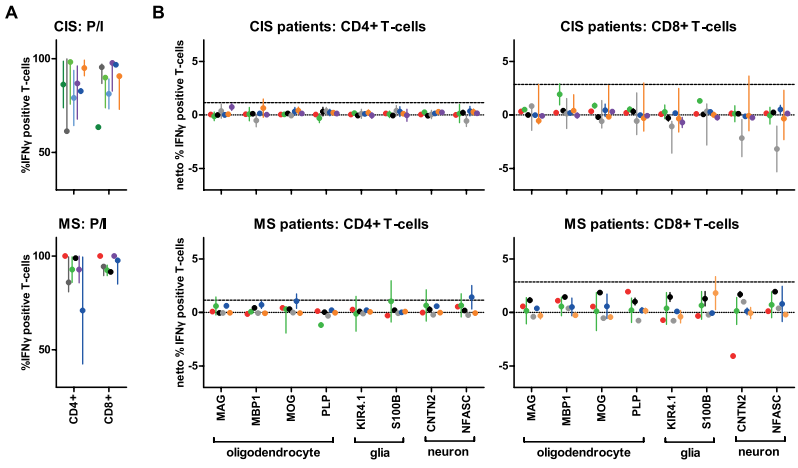

Figure 2.

CD4+ and CD8+ T cell reactivity to candidate MS-associated human autoantigens in CSF-derived T-cell lines from CIS and MS patients. (**A**) CSF-derived T-cell lines (CSF-TCLs) generated from patients diagnosed for clinically isolated syndrome (CIS; n = 7) and MS (n = 6) were stimulated with phorbolmyristate-acetate and ionomycin (P/I). Phenotype and frequency of responding T cells was determined by flow cytometry. Cells were gated on viable lymphocytes using fluorescent viability staining, expression of CD3, subdivided on CD4 and CD8 and subsequently intracellular IFN-γ expression. Colored dots and vertical bars represent median reactivity and range of individual CSF-TCL. (**B**) Autologous BLCL (autoBLCL) were nucleofected with pNS vector encoding candidate MS-associated antigens (cMSAgs; n = 8) and positively selected in culture for >2 weeks with geneticin to generate stable cMSAg expressing BLCLs. CSF-TCLs were cocultured with cMSAg-transduced autologous BLCLs. The netto frequency of cMSAg-specific T cells, corrected for reactivity toward pNS.empty-transduced BLCL, is shown as percentage of IFN-γ$^+$ CD4 (left panels) or CD8 T cells (right panels). Threshold for positive cMSAg T-cell reactivity, indicated by a horizontal dashed line, was calculated as 3.09-times SD of all mean netto cMSAg-specific CD4$^+$ and CD8$^+$ T-cell responses, allowing a 0.1% one-tailed false discovery rate. Significance of variation in T-cell reactivity per cMSAg was determined by one-way analysis of variance for CD4$^+$ and CD8$^+$ T cells independently. CSF-TCL T-cell reactivity to cMSAg-transduced autoBLCLs was performed in monoplo and determined at least twice, except for RRMS patient #9 (red dots) and #12 (gray dots), which were measured only once.

CD4+ T-cells and 2.9% for CD8+ T-cells allowing a one-tailed 0.1% false discovery rate, respectively[14]. This closely resembles the detection limit determined using titrated MVF-specific TCCs and MVF-transduced GR-BLCL as APC (Supplementary Fig. 2). Notably, the only positive call for cMSAg reactivity was the CSF-TCL of patient #13 containing a median 1.4% (range 0.3–2.5%) frequency of NFASC-specific CD4 T-cells. However, no significant variation was observed between pMSAg for CD4+ (p = 0.74) and CD8+ T-cells (p = 0.30, one-way analysis of variance) (Fig. 2B).

CONCLUDING REMARKS

Intrathecal autoreactive T-cells are anticipated to be involved in MS pathology, but we were unable to detect substantial CD4+ and CD8+ T-cell reactivity to a broad panel of cMSAgs (n=8) in short-term cultures of CSF-derived T-cells of seven MS and six CIS patients. Few studies enumerated intrathecal antigen-specific CD4+ and CD8+ T-cells responses simultaneously using endogenously processed antigen[2,3]. Whereas antigen processing and presentation may differ between BLCL and the currently undefined local APC in MS lesions, the use of cMSAg-transduced autoBLCL as artificial APC represents a reasonable compromise to detect intrathecal cMSAg-specific T-cells. Congruent to our findings, Wuest and colleagues also did not observe significant reactivity of CSF-derived T-cells toward self-antigens using cell-, myelin-, and brain-derived lysate pulsed autologous dendritic cells used as APC[12].

Still, a cautious interpretation of our data is warranted, as there are a few limitations. First, the assay format is not well equipped to detect low frequencies of cMSAg-specific T-cells, as the overall background was 1.1±0.3% and 2.6±0.9% (mean±SD) reactive CD4+ and CD8+ T-cells, respectively. Nevertheless, local frequencies of Ag-specific T-cells observed by others typically exceeded our detection limit in compartmentalized T-cell mediated diseases like Chronic Chagas' Cardiomyopathy[15] and autoimmune thyroiditis[16,17]. Moreover, using BLCL as APC we have previously detected profound frequencies (>5%) of intralesional T-cells. T-cells directed to the triggering herpes virus in patients with herpetic ocular diseases[18,19]. Second, a limited number of 13 patients were included in this study, which warrants further studies on larger cohorts of patients and disease controls. However, we included patients early in disease development, particularly seven CIS patients. Notably, two of them progressed to RRMS during clinical follow-up, suggesting that intrathecal reactivity against the explored antigens did not contribute to their disease evolution.

In conclusion, the inability to detect substantial intrathecal T-cell reactivity to eight human cMSAgs in CIS and MS patients do not support their role as prominent target antigen for intrathecal T-cell responses in CIS and MS patients.

MATERIALS AND METHODS

Patients and clinical specimens

Paired blood and CSF samples from CIS (n=7) and MS (n=6) patients were obtained as part of diagnostic workup at the Erasmus MC (Rotterdam, the Netherlands) (Table 1). No patient received immunomodulatory therapy at time of lumbar puncture, except one CIS patient (patient #2) who received Avonex treatment. CSF was always obtained >1 month after start of clinical symptoms. The study was performed according to the tenets of the

Helsinki declaration, approved by the local medical ethical committee and written informed consent was obtained from all participants.

Generation of BLCL and CSF-TCL

Isolation of PBMC and generation of autoBLCL were performed as described previously[8,13]. CSF-TCL were generated from surplus CSF cells by stimulation with 1 µg/mL phytohemagglutinin-L (Roche, Branfort, CT) in the presence of 30 Gray γ-irradiated allogeneic PBMC used as feeder cells[13]. Cells were grown in RPMI-1640 supplemented with antibiotics and 10% heat-inactivated bovine (B-cells) or pooled human (T-cells) serum. T-cells received recombinant human IL-2 (25 U/mL) and IL-15 (25 ng/mL; Myltenyi biotec, Bergisch Gladbach, Germany) every 3–4 days and were harvested around day 14, aliquoted and stored frozen[13]. Generation, characterization, and maintenance of MVF-specific CD4+ and CD8+ TCC was described[8].

Cloning of human cMSAg in the EBV-based episomal pNS vector

The eukaryotic expression vector pNS.CD8α described by Mazda et al.[7] was modified to the current pNS vector by removing murine CD8α by PvuII/HindIII digestion, generation of blunt ends using T4 DNA polymerase and subsequent self-ligation (all from New England Biolabs, Ipswich, MA) (Fig. 1A). ORFs encoding human MAG, MBP1, MOG, PLP1, S100B, CNTN2, and NFASC were PCR amplified from cDNA clones obtained from Darmacon (Lafayette, CO) and cloned in pCR4.1-TOPO (Invitrogen). Restriction endonuclease target sequences engineered into amplification primers were used to shuttle ORFs to pNS vector's BamHI site (Supplementary Table 1). ORFs encoding MVF were cloned from pEC12.MVF[20] and human KIR4.1 (Damacon) from a cDNA clone by SacI or SacII/SmaI digestion into pNS vectors's BamHI site after generating blunt ends using T4 DNA polymerase, respectively. DNA sequences of all cloned vectors were verified by Sanger sequencing using an ABI prism 3130XL genetic Analyzer (Applied Biosystems, Foster City, CA) with pNS vector specific 5'- and 3'-end cMSAg flanking primers and if no overlapping sequences were obtained internal cMSAg-specific primers (data not shown).

Generation of BLCL expressing MVF and candidate MS-associated neuroantigens

BLCL were nucleofected using Amaxa Cell line nucleofection kit V (Lonza; Bazel, Switzerland) per manufacturer's instructions and transduced cells positively selected using 500 µg/mL geneticin (Life Technologies; Carlsbad, CA) for >2 weeks. Expression of cMSAgs and MVF were determined by extracellular and intracellular flow cytometry on a FACSCanto II (BD Biosciences; San Jose, CA) using specific antibodies. Intracellular staining was performed using Cytofix/Cytoperm Kit (BD Biosciences) per manufacturer's instructions. The

following mAbs were used at predefined concentrations: Mouse-anti-MVF (clone F3-5), mouse-anti-MAG (clone 513) (Millipore; Darmstadt, Germany), mouse-anti-MOG (clone 8–18C5; Millipore), mouse-anti-PLP1 (clone sc-73336) (Santa Cruz; Dallas, TX), mouse-anti-KIR4.1 (clone 1C11) (Sigma-Aldrich; St. Louis, MO), mouse-anti-S100B (clone 19/S100B; BD Biosciences), mouse-anti-CNTN2 (clone 372913) (R&D systems; Minneapolis, MN), and rabbit-anti-NFASC (ab31457) (AbCAM; Cambridge, UK). Secondary fluorescein-conjugated goat-anti-mouse IgG (BD Pharmingen; San Diego, CA) or swine-anti-rabbit IgG (Dako; Glostrup, Denmark) were used. Respective mouse IgG1, IgG2a, IgG2b, or Rabbit IgG isotypes were used as negative controls. Data analysis on viable stained cells was performed using FlowJo software version 10 (Ashland, OR). Gating strategy for BLCL is shown in Supplementary Fig. 1A.

T-cell reactivity to endogenously expressed MVF in BLCL

CD4+ and CD8+ MVF-specific TCCs were incubated with pNS.MVF-transduced HLA-matched BLCL (GR-BLCL). Negative controls included TCC alone, TCC cocultured with BLCL or BLCL nucleofected with the pNS vector without insert. Positive control for T-cell stimulation included stimulation with phorbolmyristate-acetate (50 ng/mL) and ionomycin (500 ng/mL) (Sigma-Aldrich). Additionally, TCC were incubated with GR-BLCL pulsed overnight with increasing concentrations of synthetic peptide, range 0.01–10 μM, representing minimal T-cell epitopes[8]. In all assays, 5 × 104 BLCL were cocultured with either TCC at a 1:1 ratio for 24 h. Secreted IFN-γ was quantified by ELISA in conditioned culture medium (eBioscience; San Diego, CA). Experiments were performed twice.

The detection limit of T-cell responses toward endogenously expressed antigen was determined by titrating the MVF-specific CD4+ (4-F99) and CD8+ TCC (2-F40) into a T-cell mixture, consisting of VZV-specific CD4+ (TCC 146; patient 4) and CD8+ TCC (TCC 38; patient 3)[9], ranging from 0.1 to 10% of either MVF-specific TCC in the T-cell mixture. T-cell mixtures (total 105 cells) were cocultured with pNS.MVF-transduced GR-BLCL in a 1:1 ratio for 6–8 h in the presence of Golgistop per manufacturer's instructions (BD Biosciences). GR-BLCL transduced with pNS.empty and GR-BLCL pulsed overnight with 3 μM of aforementioned synthetic peptides were used as negative and positive control APC, respectively. T-cells were phenotyped with fluorochrome-conjugated mAbs directed to CD3 (clone SP34-2) (BD Pharmingen; San Diego, CA), CD4 (clone SK3; BD Biosciences), and CD8α (clone RPA-T8; eBioscience) stained for viability (violet live/dead stain; Invitrogen), fixed, permeabilized, and subsequently stained for intracellular IFN-γ (clone B27; BD Pharmingen) followed by flow cytometry. Lymphocytes were first gated based on forward and side scatter characteristics and viability using violet live/dead staining and subsequently on differential expression of surface markers CD3, CD4, and CD8 (Supplementary Fig. 1B). Finally, intracellular IFN-γ

expression was defined for both gated T-cell subsets (Supplementary Fig. 2). Experiment was performed twice.

Intrathecal T-cell reactivity to endogenously expressed human cMSAg expressed in autologous BLCL

CSF-TCL (105 cells) were cocultured with autoBLCL stably transduced with aforementioned cMSAg (n=8) in a 1:1 ratio for 6–8 h in the presence of Golgistop per manufacturer's instructions (BD Biosciences). Negative and positive controls included CSF-TCL incubated with pNS.empty-transduced autoBLCL and CSF-TCL solely stimulated with P/I, respectively. T-cells were stained and analyzed using flow cytometry as described above (Supplementary Fig. 3). Experiments were performed at least twice, except for RRMS patient #9 and #12 where limited numbers of CSF-TCL cells were available.

Statistical analysis

Netto T-cell reactivity toward cMSAg-transduced BLCL was calculated by subtracting reactivity toward pNS.empty-transduced BLCL. Threshold for positive cMSAg T-cell reactivity was calculated as 3.09-times SD of all mean netto cMSAg-specific CD4+ and CD8+T-cell responses, allowing a 0.1% one-tailed false discovery rate[14]. Significance of variation in T-cell reactivity per cMSAg was determined by one-way analysis of variance for CD4+ and CD8+ T-cells independently.

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CONFLICT OF INTEREST

The authors declare no commercial or financial conflict of interest.

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SUPPLEMENTAL DATA

Supplementary Table 1. Cloning of putative MS-associated autoantigens in pNS vectormeasles virus fusion protein

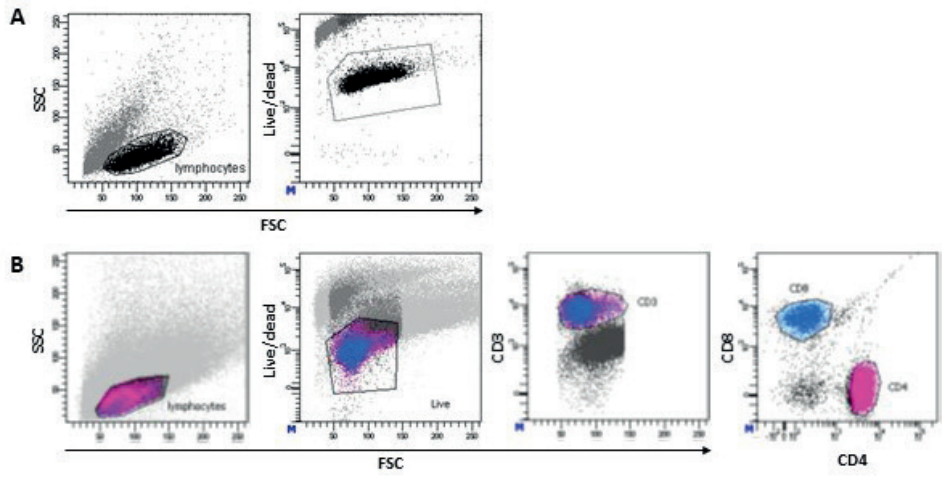
Gene[a]	GenBank Accession No	Length (aa)[b]	Primer sequence[c] 5'-end forward (5'-3')	Primer sequence[c] 3'-end reverse (5'-3')	Restriction site ORF[d] 5'-end	Restriction site ORF[d] 3'-end
MAG	BC053347	626	CT*CTTAAG*TACAGA**ATG**ATATTCCTCAC	CT*ATGCAT*ACTTGACCCGGATTTCAG	*Afl*II (T4)	*Nsi*I (T4)
MBP1	BC065248	197	CT*CTTAAG*ATTCAGG**ATG**GGAAACCAC	CT*ATGCAT*GCTACGTGCCAGTTCTTCC	*Afl*II (T4)	*Nsi*I (T4)
MOG	BC035938	295	CT*CTTAAG*TAGAG**ATG**GCAAGCTTATC	CT*ATGCAT*TCTAACAGCTGGCTTCTTTGC	*Afl*II (T4)	*Nsi*I (T4)
PLP	NM_000533	277	CT*GGATCC*CAAAGAC**ATG**GGCTTGTTAG	CT*GGATCC*GAACTTGGTGCCTCGGC	*Bam*HI	*Bam*HI
KIR4.1	BC131627	379	n.a.	n.a.	*Sac*II (T4)	*Sma*I
S100B	BC001766	92	CT*GGATCC*GAGG**ATG**TCTGAGCTGGAGAAGGCC	CT*GGATCC*TCAAAGAACTCGTGGCAGGCAG	*Bam*HI	*Bam*HI
CNTN2	BC129986	1040	CT*GGATCC*ATCCACC**ATG**GGGACAGCCAC	CT*GGATCC*GAAGGGGCTGGCTGTGTCCG	*Bam*HI	*Bam*HI
NFASC	BC137013	1169	GA*GGATCC*GGGCCAGGTGCCGAGG**ATG**	GA*GGATCC*GTGGTTTTGTCTCCCCTTCTC	*Bam*HI	*Bam*HI
MVF	AM237414	553	n.a.	n.a.	*Sac*I (T4)	*Sac*I (T4)

a. MAG; myelin associated glycoprotein, MBP1; myelin basic protein isoform 1, MOG; myelin oligodendrocyte protein, PLP; proteolipid protein, KIR4.1; inward rectifying potassium channel 4.1, S100B; S100 calcium binding protein B, CNTN2; contactin-2, NFASC; neurofascin, and MVF; measles virus fusion protein.

b. aa, amino acid.

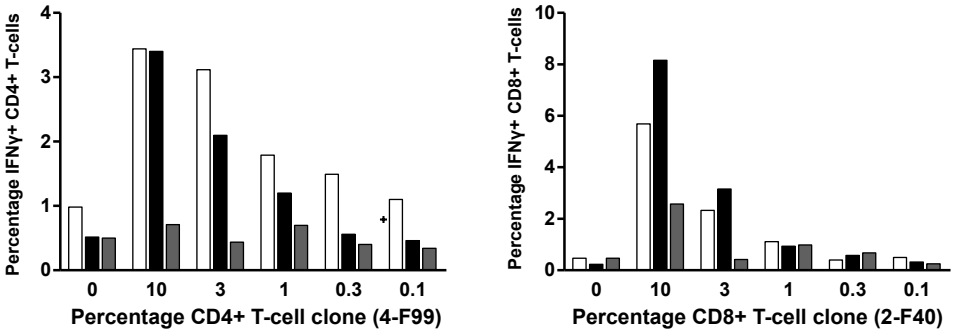
c. Primer sequence annotation: restriction site, start codon; n.a. not applicable.

d. ORF, open reading frame; T4, restriction site blunted using T4 DNA polymerase.



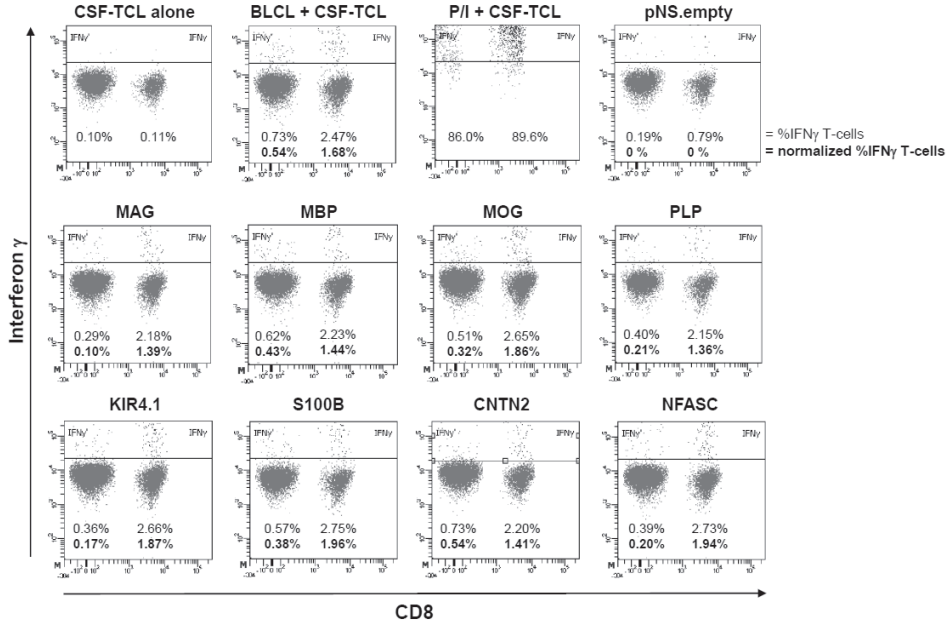
Supplementary Figure 1. Gating strategy for EBV-transformed B-cells and T-cell subsets.

(**A**) Cultures of EBV-transformed B-cells (BLCL) were stained for viability with the violet live/dead buffer and subsequently for intra-cellular or surface expression of candidate multiple sclerosis-associated antigens as depicted in Figure 1B. Viable BLCL were identified based on lymphocyte forward and side scatter characteristics and cell viability. (**B**) Cell cultures, consisting of T-cells and BLCL, were stained for surface markers CD3, CD4 and CD8 and viability using violet live/dead buffer. Viable T-cells were identified based on lymphocyte forward and side scatter characteristics, live cells and subsequently CD3 expression. Finally, viable CD3+ cells were separated into CD4+ (pink) and CD8+ (blue) viable T-cells.



Supplementary Figure 2. Estimation of CD4+ and CD8+ T-cell detection limit recognizing recombinant protein expressing EBV- transformed B-cells used as antigen presenting cells.

EBV-transformed B-cells (BLCL) were nucleofected with a eukaryotic expression vector (pNS) encoding measles virus F (MVF) protein to generate stable high MVF protein expressing BLCL. Indicated percentages of two different MVF-specific T-cell clones (TCC), one CD4+ (clone 4-F99) and one CD8+ (clone 2-F40) TCC, were spiked into a T-cell population consisting of two irrelevant varicella zoster virus-specific CD4+ and CD8+ TCC. T-cell mixtures were co-cultured with MVF-expressing BLCL in a 1:1 ratio for 6 to 8 hrs at 37°C in the presence of Golgistop and subsequently stained for intracellular IFNg and surface CD4 and CD8 followed by flow cytometry analysis (black bars). Cells were gated for viable lymphocytes, based on forward and side scatter and cell viability using violet live/dead buffer, expressing CD3 and subsequently subgated for viable CD4+ and CD8+ T-cells expressing IFNg. Frequencies of reactive T-cells are shown as percentage of IFNg+ CD4+ or CD8+ T-cells. T-cell reactivity towards non-transduced BLCL pulsed overnight with 3 µM synthetic peptide encompassing the MVF- specific TCC cognate MVF epitopes were included as positive control (white bars). BLCL transduced with the empty pNS vector were used as negative control (grey bars). Data of an experiment, performed in monoplo, are shown. The experiment was performed twice.



Supplementary Figure 3. T-cell reactivity towards endogenously synthesised cMSAg of a cerebrospinal fluid-derived T-cell line (CSF-TCL) of multiple sclerosis (MS) patient #11.

Autologous EBV-transformed B-cells (BLCL) were nucleofected with the eukaryotic expression vector pNS encoding candidate human MS-associated antigens (cMSAg; n=8) and positively selected in vitro with geneticin for >2 weeks at 37°C to generate stable cMSAg expressing autologous BLCL. CSF-TCL generated from surplus CSF of patient #11 were co-cultured with cMSAg-transduced autologous BLCL in a 1:1 ratio for 6 to 8 hrs at at 37°C in the presence of Golgistop and subsequently stained for intracellular IFNg and surface CD3, CD4 and CD8 followed by flow cytometry analysis. Cells were gated for lymphocytes forward and side scatter characteristics, viability using violet live/dead buffer and subsequently CD3 expression. Finally, viable CD3+ cells were subgated for CD4+ (pink) and CD8+ (blue) T-cells and subsequently intracellular IFNg expression. Frequency of reactive T-cells are shown as percentage of IFNg+ CD4+ (pink) or CD8+ (blue) T-cells. The normalized frequency of cMSAg-specific T-cells, corrected for reactivity towards autologous BLCL transduced with the empty pNS vector (pNS.empty, is shown as netto percentage of IFNg+ CD4+ or CD8+ T-cells (bold). CSF-TCL alone and co-cultured with non- transduced autologous BLCL served as negative controls. CSF-TCL stimulated solely with phorbolmyristate-acetate and ionomycin (P/I) served as positive control for T-cell activation. Data of an experiment, performed in monoplo, are shown. The experiment was performed twice.

Chapter 3

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

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

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.

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 RPMI1640 (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 RPMI1640 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), $MgCl_2$ (0.2M; Sigma-Aldrich), DTT (100mM; Invitrogen), random hexamers (50μM; 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, 0.2μM 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 50ºC and 10 min at 95ºC followed by 40 cycles of 15 s at 95ºC and 1 min at 60ºC. 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 (900ng/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.

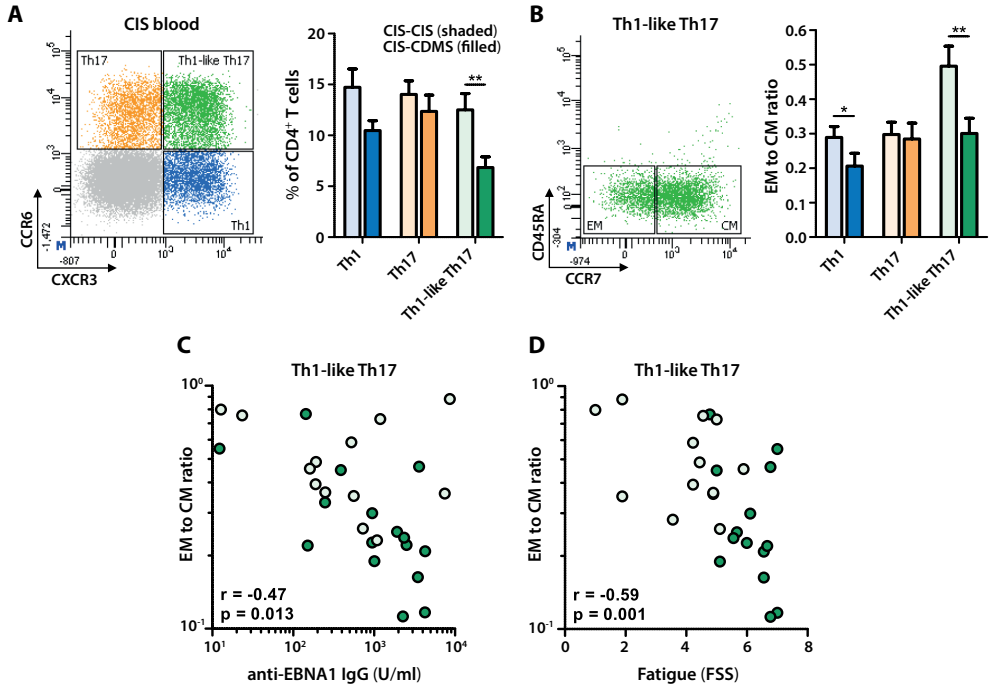

Figure 1. Reduction of Th1-like Th17 effector cells in the blood of CIS patients with short time to CDMS.

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**). * $p < 0.05$; ** $p < 0.01$.

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].

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.

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)

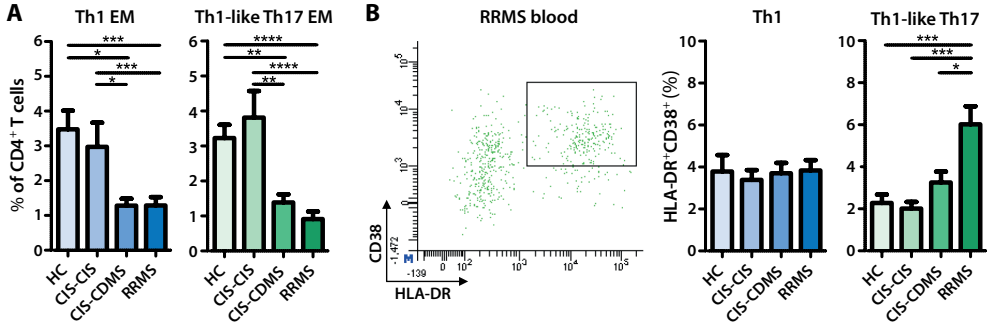

Figure 2. Th1-like Th17 effector cells are highly activated and less present in the blood after MS diagnosis.
(**A**) Th1 and Th1-like Th17 effector memory (EM) frequencies in CD4$^+$ T cells from CIS-CIS (n = 14) and CIS-CDMS (n = 16) as well as RRMS (n = 18) and both age- and gender-matched healthy 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. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

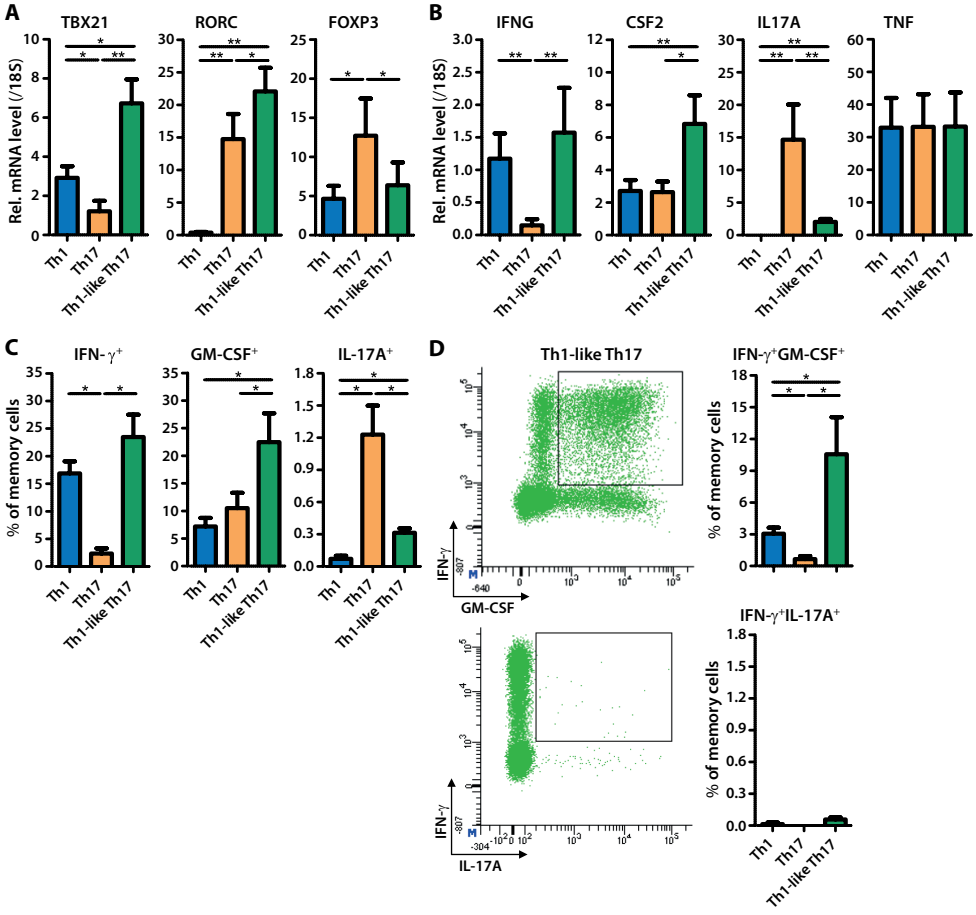

Figure 3. Blood Th1-like Th17 cells predominantly express T-bet and RORγt, and are high IFN-γ and GM-CSF, but low IL-17A producers.

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

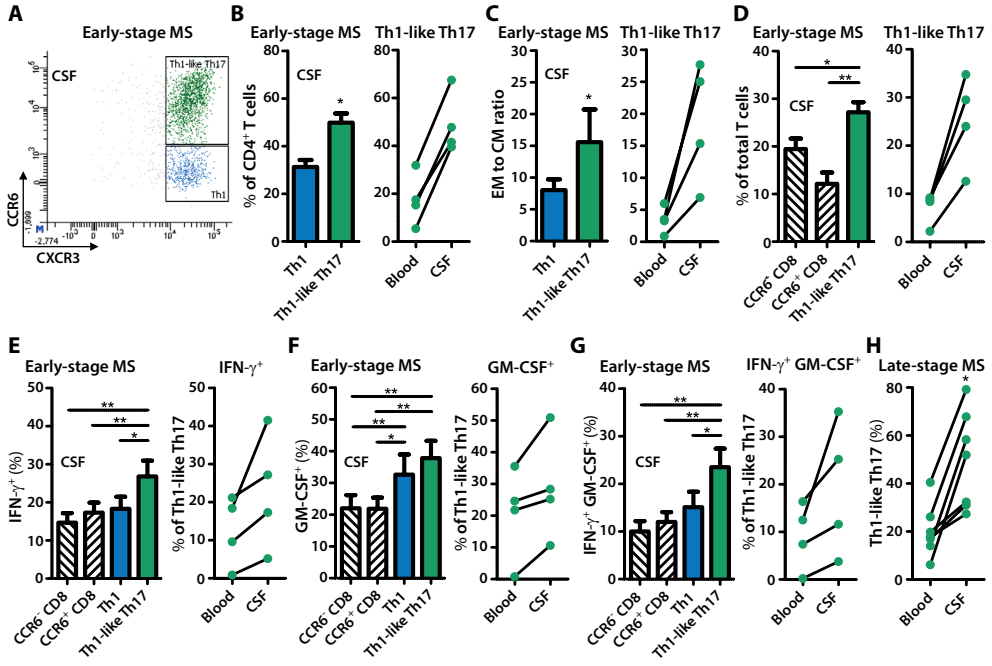

Figure 4. Prevalence of pro-inflammatory Th1-like Th17 cells in CIS and MS CSF compared to blood T cell cultures. (**A-D**) Presence of Th1-like Th17 ($CCR6^+CXCR3^+$) subsets in short-term CSF T cell cultures from 10 early-stage MS patients (CIS, n = 7; RRMS, n = 3). CSF Th1-like Th17 were compared 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). * $p < 0.05$; ** $p < 0.01$.

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 (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

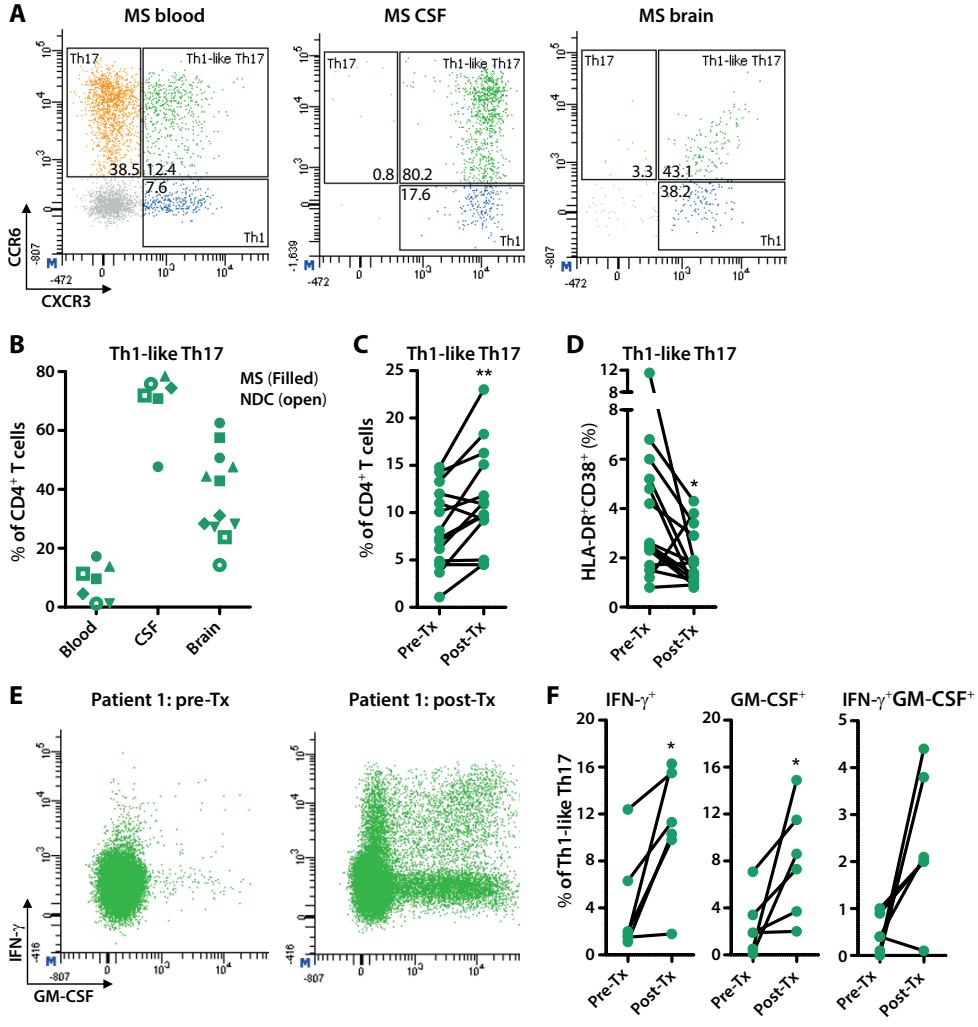

Figure 5. Th1-like Th17 recruitment to the CNS and targeting by natalizumab in MS patients.

(**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. * $p < 0.05$; ** $p < 0.01$.

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.

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).

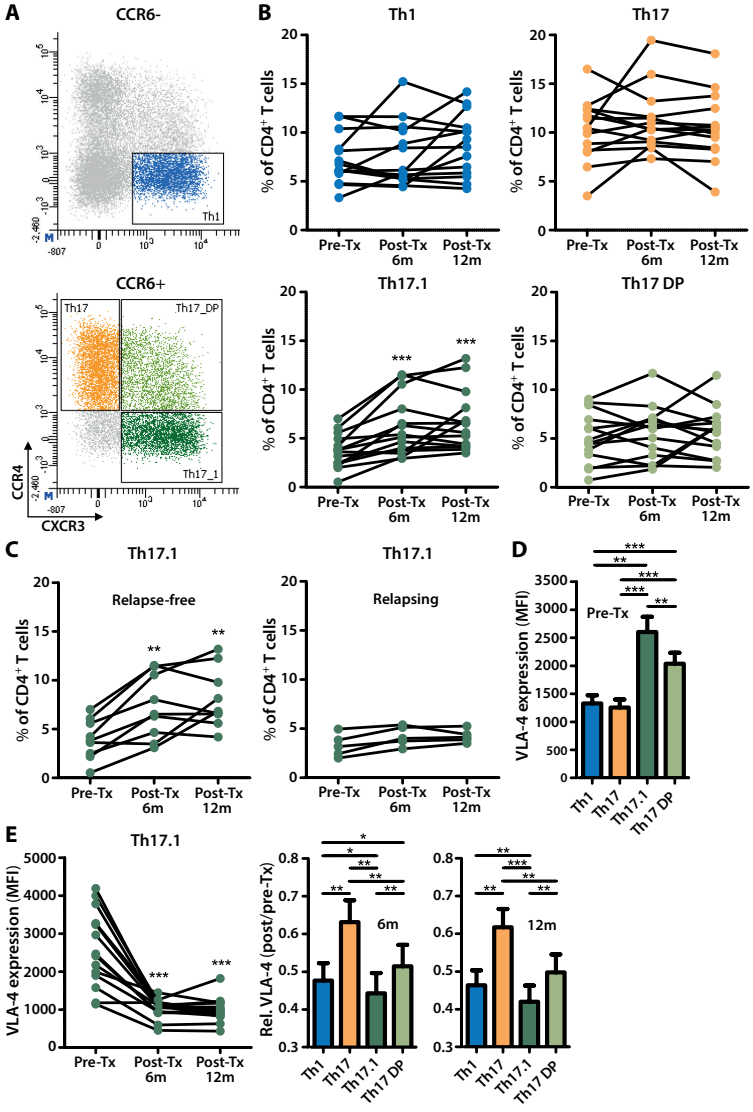

Figure 6. Selective accumulation of Th17.1 cells in natalizumab-treated MS patients who do not experience clinical relapses.

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.

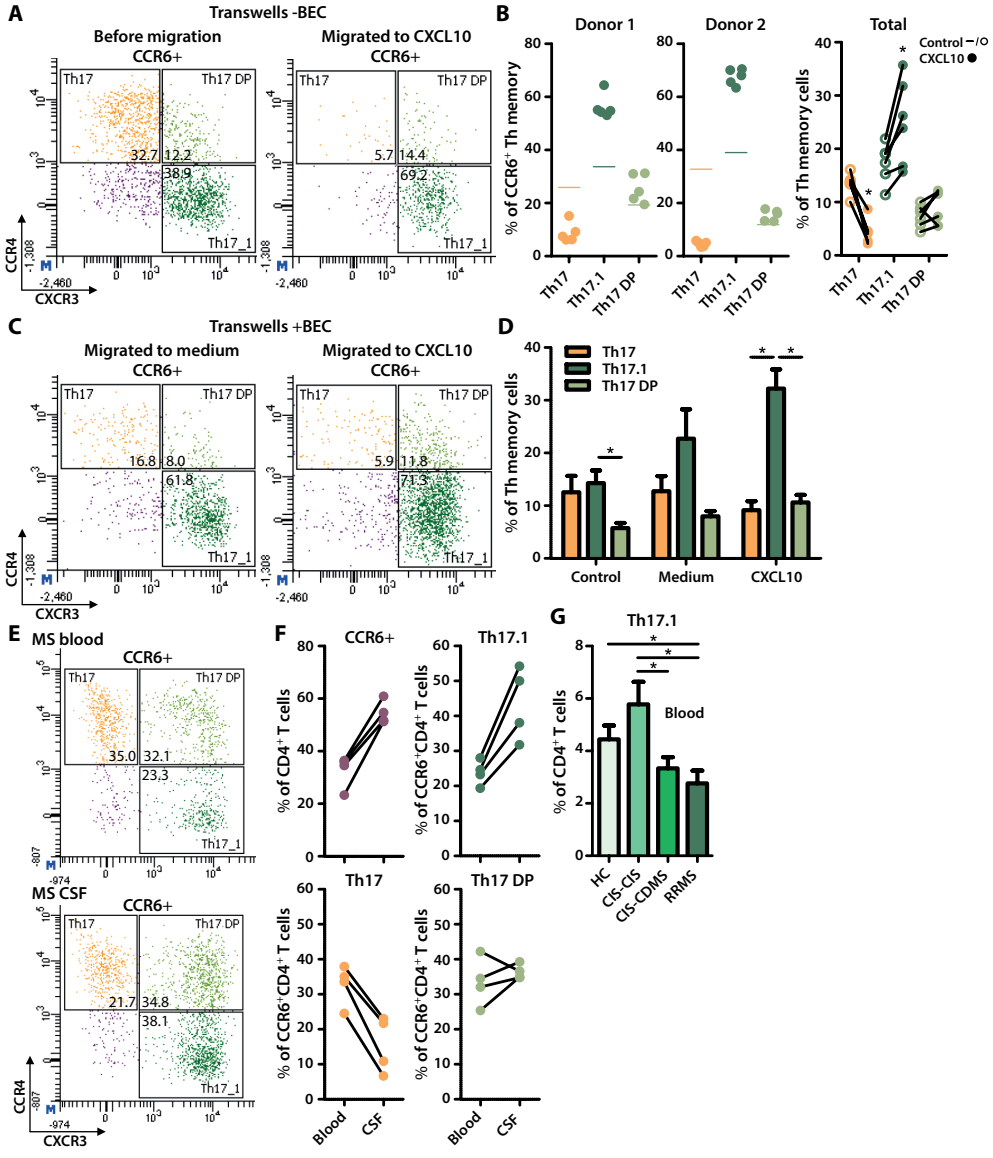

Figure 7. Enhanced CNS transmigration potential of Th17.1 cells and their recruitment to CSF in early MS.

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. * $p < 0.05$.

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).

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-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] human. 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-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].

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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.

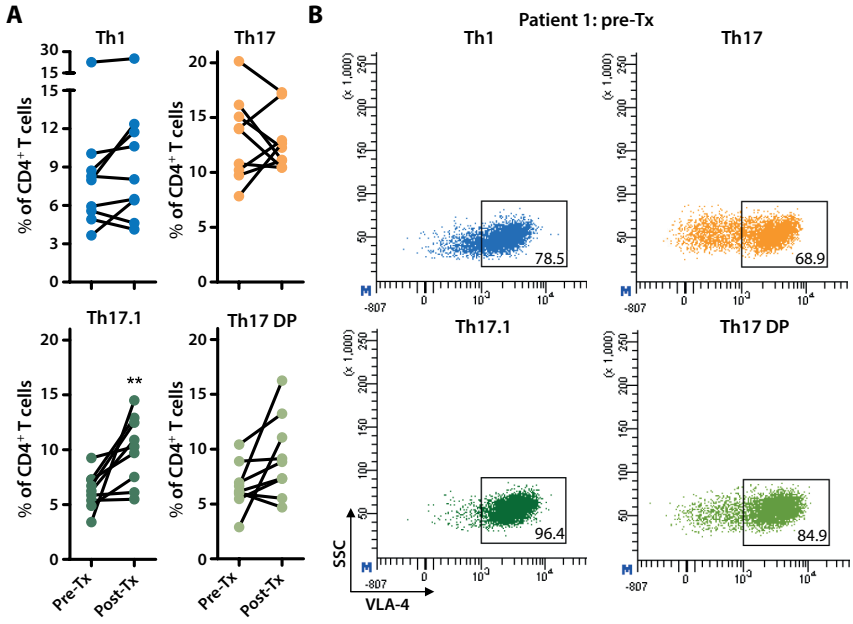
Supplementary Table 2. qPCR primer sequences

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

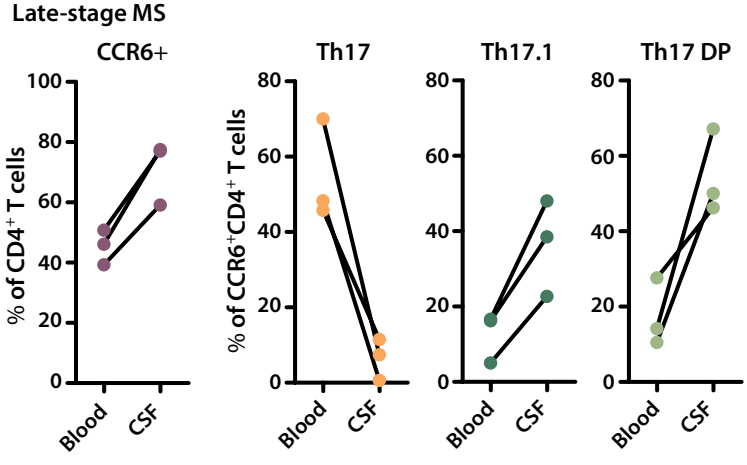
Surface marker	Cohort	CD4+ Th subpopulations						
CD25	n=10	6-3+	6+3-	6+3+	6-3+4-	6+3-4+	6+3+4-	6+3+4+
% positive cells	HC	9.6 ± 0.8	28.1 ± 3.0	19.8 ± 1.7	6.9 ± 0.7	33.9 ± 3.1	13.9 ± 1.8	24.8 ± 2.0
	RRMS	10.3 ± 0.7	31.1 ± 1.7	20.3 ± 1.7	7.9 ± 1.0	36.6 ± 1.3	11.9 ± 1.0	26.1 ± 1.9
MFI positive	HC	1303 ± 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.

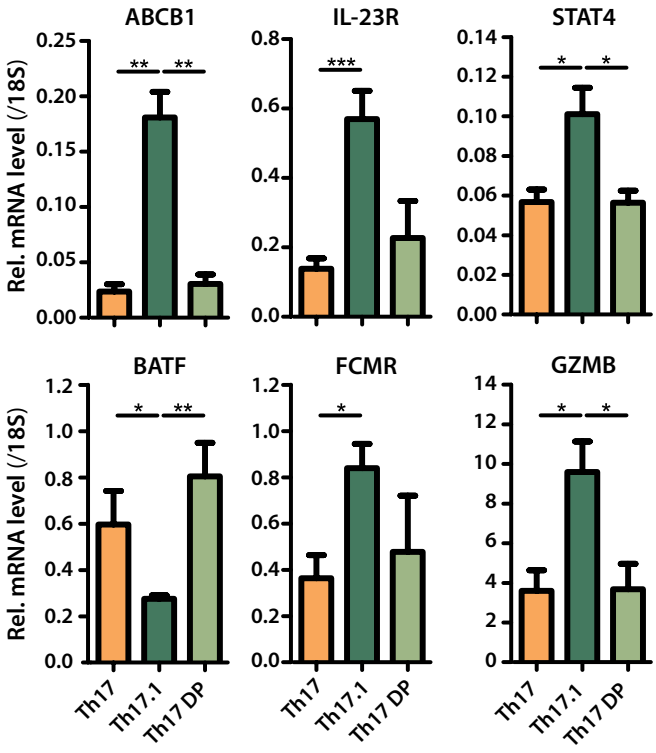


Supplementary Figure 1.

(**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-4$^+$ cells within Th1, Th17, Th17.1 and Th17 DP subpopulations in pre-treatment MS blood.

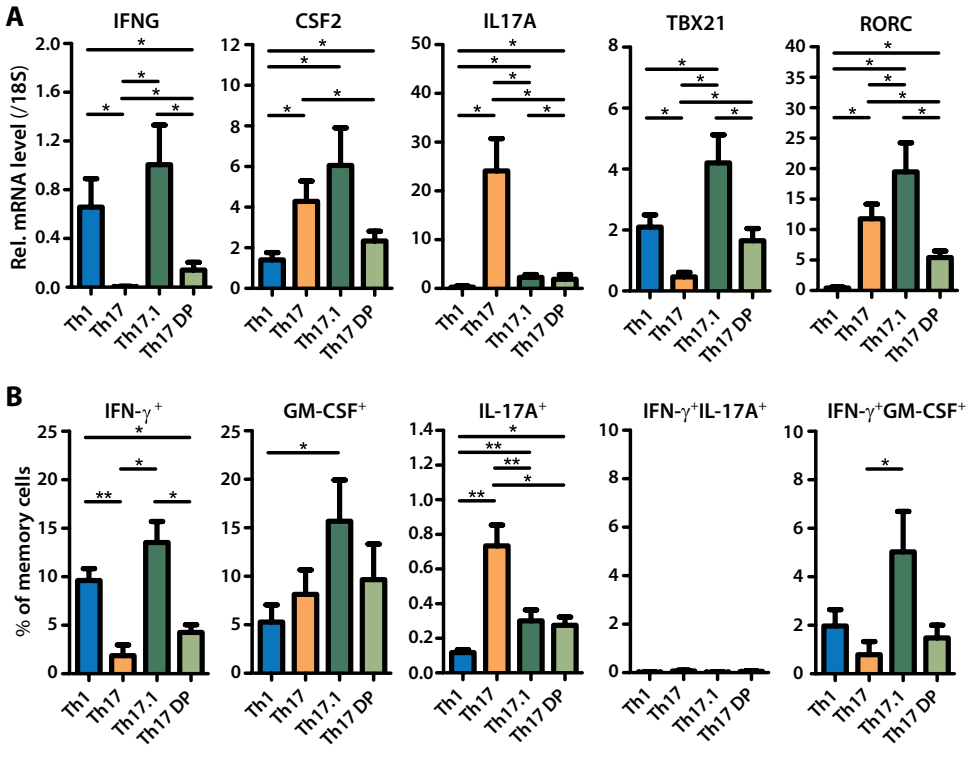


Supplementary Figure 2. Flow cytometric analysis of Th17 subpopulations in paired *ex vivo* CSF and blood samples of three late-stage MS patients.



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 4

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

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ABSTRACT

Objective: Results from anti-CD20 therapies demonstrate that B- and T-cell interaction is a major driver of multiple sclerosis (MS). The local presence of B-cell follicle-like structures and oligoclonal bands in MS patients 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 CXCR3high 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-bethigh 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 approved by the medical ethics committee of the Erasmus Medical Center (Rotterdam) and VU University Medical Center (Amsterdam, The Netherlands).

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.

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].

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 0.3 µm pore size (2×10^5 cells/well; Corning, Amsterdam, the Netherlands). B-cell migration towards medium or CXC chemokine ligand (CXCL)10 (900 ng/ml; R&D Systems, Abingdon, UK) was

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

analyzed after 3h in 37°C. In addition, 2,5-5×10^5 memory B cells were placed on confluent monolayers of human brain endothelial cells (hCMEC/D3) on 5 µm pore size transwell plates (Corning) coated with collagen, and migration was analyzed after 5 hours[22]. Percentages of memory B-cell subsets were compared before and after transmigration using flow cytometry.

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

BCR-mediated uptake and presentation of *Salmonella typhimurium (S. typhimurium)* SL1344 was used as a model for antigen presentation, as previously demonstrated[23]. mAb anti-human IgG (MH16-1, Sanquin, Amsterdam, The Netherlands) was mixed with mAb against *S. typhimurium* lipopolysaccharide (LPS; 1E6, Invitrogen, Paisley, UK) and rat anti-mouse IgG1 antibody (RM161.1, Sanquin) to generate BCR-LPS tetrameric antibody complexes. Exponentially grown bacteria were washed twice with PBS, incubated with BCR-LPS tetrameric antibody complexes for 30 min at room temperature, and washed twice to remove

unbound antibodies. B cells were incubated with viable anti-IgG coated *S. typhimurium*[23] at 20 bacteria per cell for 45 min at 37°C without antibiotics. Next, cells were washed 3 times and cultured for 60 min in 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].

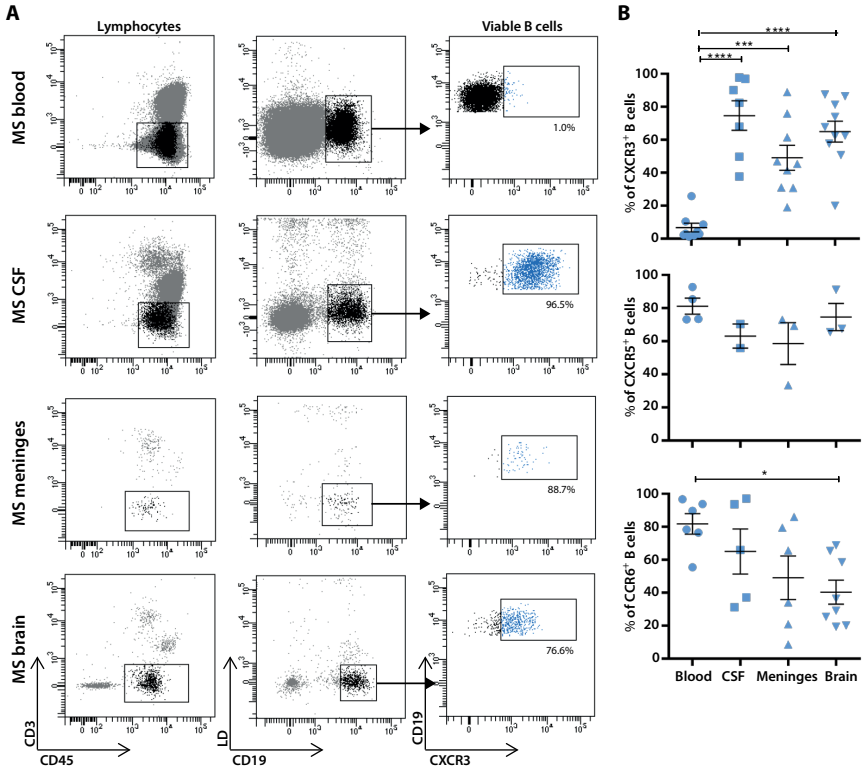


Figure 1. CXCR3⁺ B cells are abundant in the CNS compared to blood of MS patients.

(**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

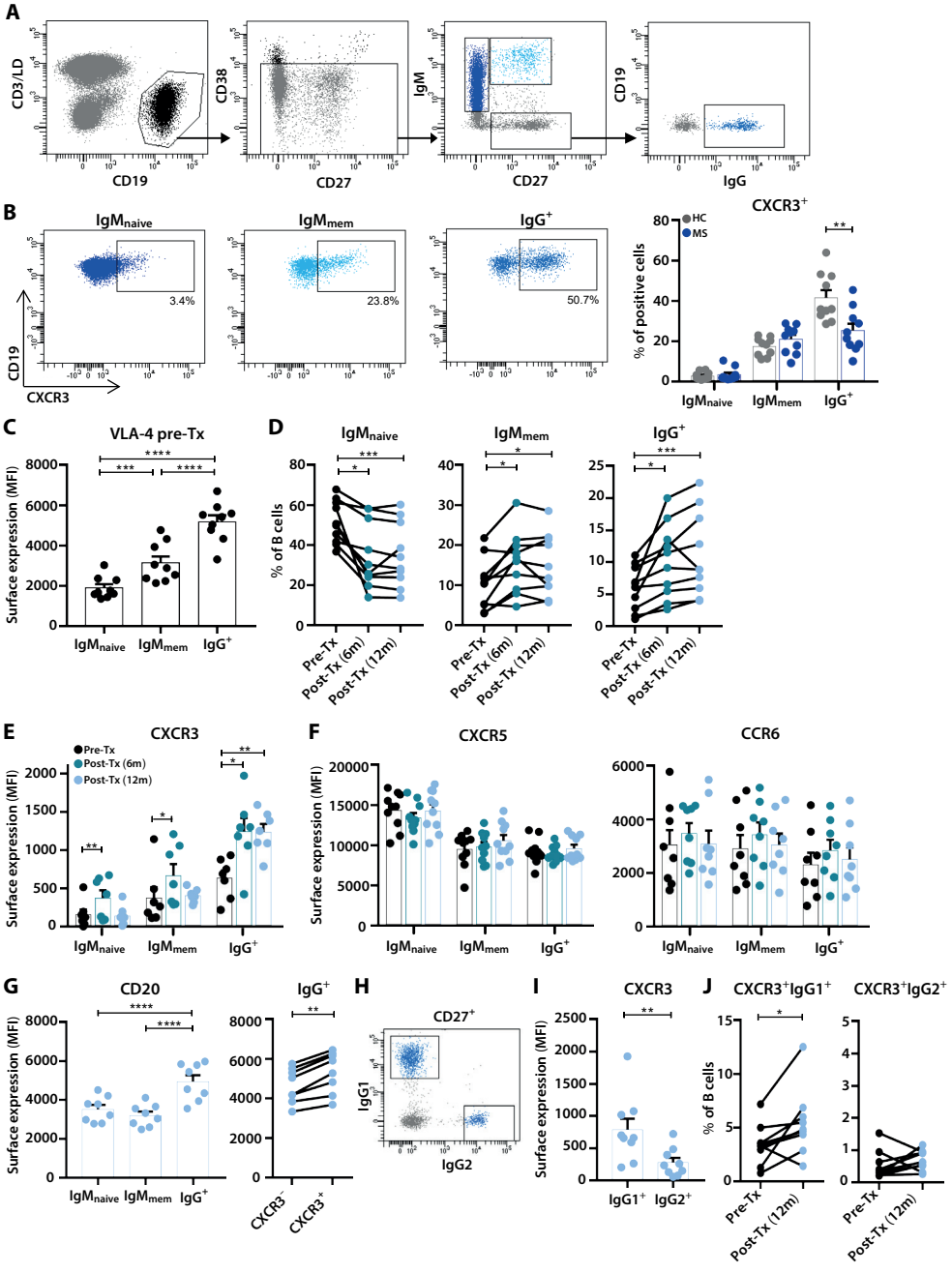
To determine how CXCR3 is involved in the local attraction of different B-cell populations in MS, we assessed the proportions of CXCR3-expressing naive ($CD27^-IgM^+$; IgM_{naive}) and both IgM^+ memory ($CD27^+IgM^+$; IgM_{mem}) and IgG^+ memory ($CD27^+IgG^+$) B cells in the peripheral blood of untreated 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).

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.

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
IgMnaive
IgMmem
IgG+
C
VLA-4 pre-Tx
Surface expression (MFI)
IgMnaive
IgMmem
IgG+
D
IgMnaive
IgMmem
IgG+
% of B cells
Pre-Tx
Post-Tx (6m)
Post-Tx (12m)
E
CXCR3
Pre-Tx
Post-Tx (6m)
Post-Tx (12m)
Surface expression (MFI)
IgMnaive
IgMmem
IgG+
F
CXCR5
CCR6
Surface expression (MFI)
IgMnaive
IgMmem
IgG+
G
CD20
IgG+
Surface expression (MFI)
IgMnaive
IgMmem
IgG+
CXCR3-
CXCR3+
H
CD27+
IgG1
IgG2
I
CXCR3
Surface expression (MFI)
IgG1+
IgG2+
J
CXCR3+IgG1+
CXCR3+IgG2+
% of B cells
Pre-Tx
Post-Tx (12m)
Pre-Tx
Post-Tx (12m)

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 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

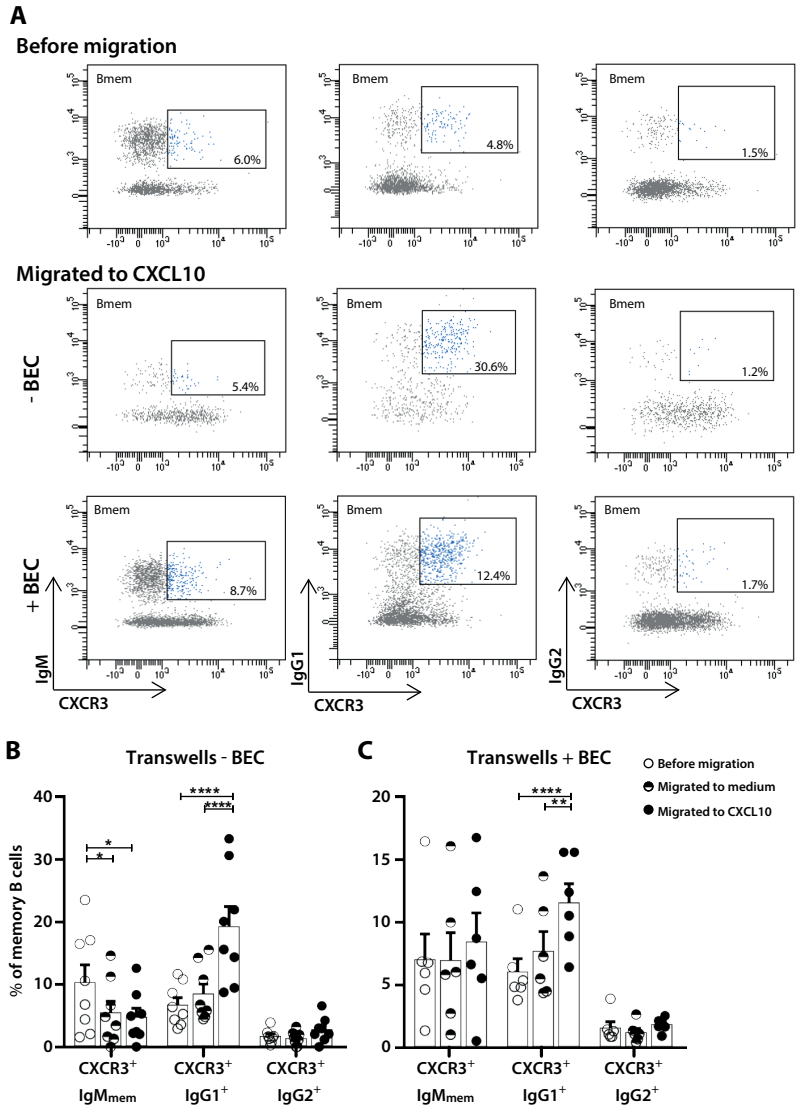


Figure 3. Enhanced migration of CXCR3⁺IgG1⁺ B cells across transwell filters and human brain endothelial monolayers *in vitro*.

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**).

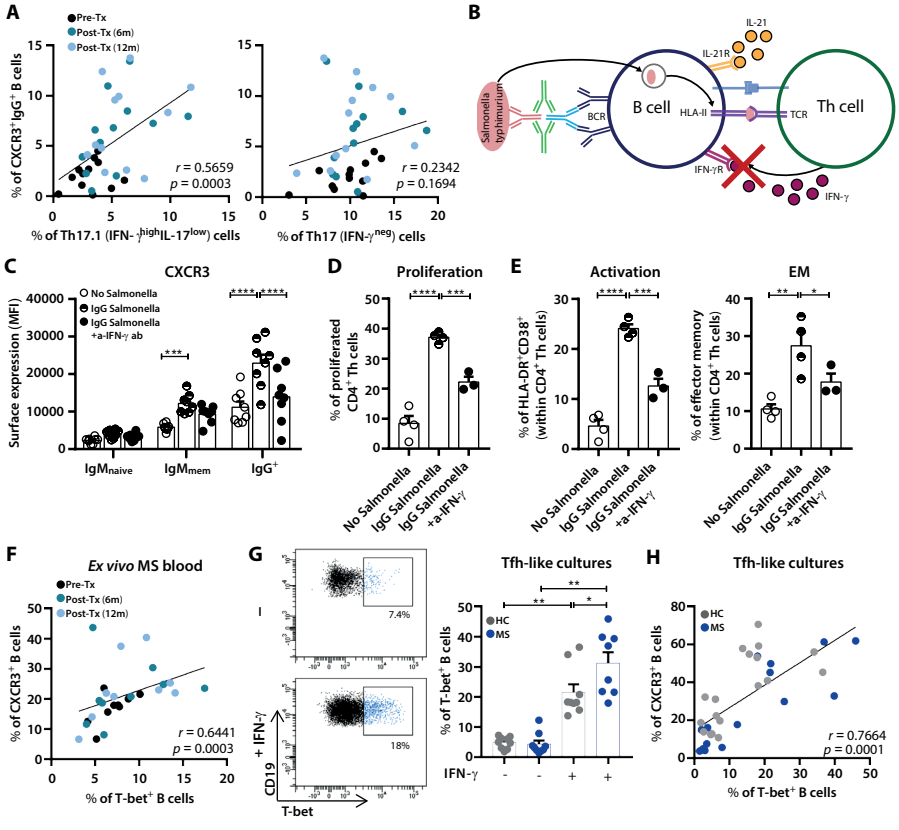


Figure 4. T helper 1 cytokine IFN-γ is a major trigger of CXCR3⁺(T-bet⁺) B-cell differentiation in MS.

(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.

(r = 0.644, p = 0.0003) and showed a similar association with Th17.1 (IFN-γ^{high}IL-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-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-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-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 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).

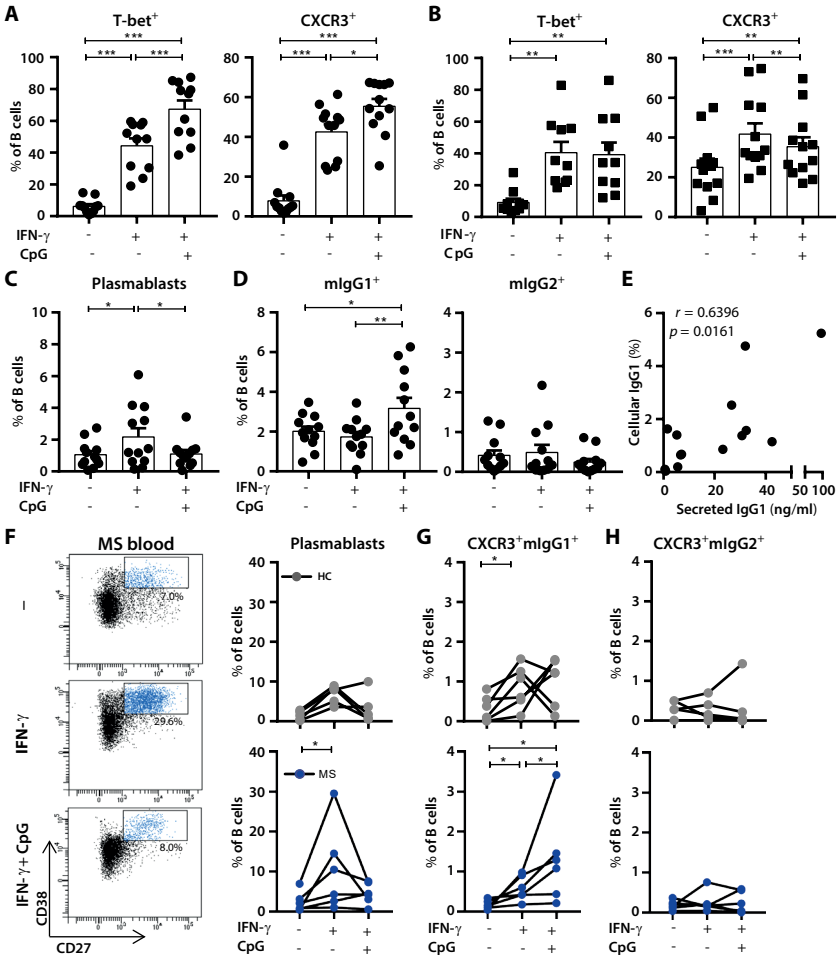


Figure 5. IFN-γ induces plasmablast differentiation, whereas both IFN-γ and CpG-DNA further upregulate T-bet and trigger IgG1 switching in B cells of MS patients.

(**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 $T\text{-}bet^+$ 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.

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 $T\text{-}bet^+$ 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

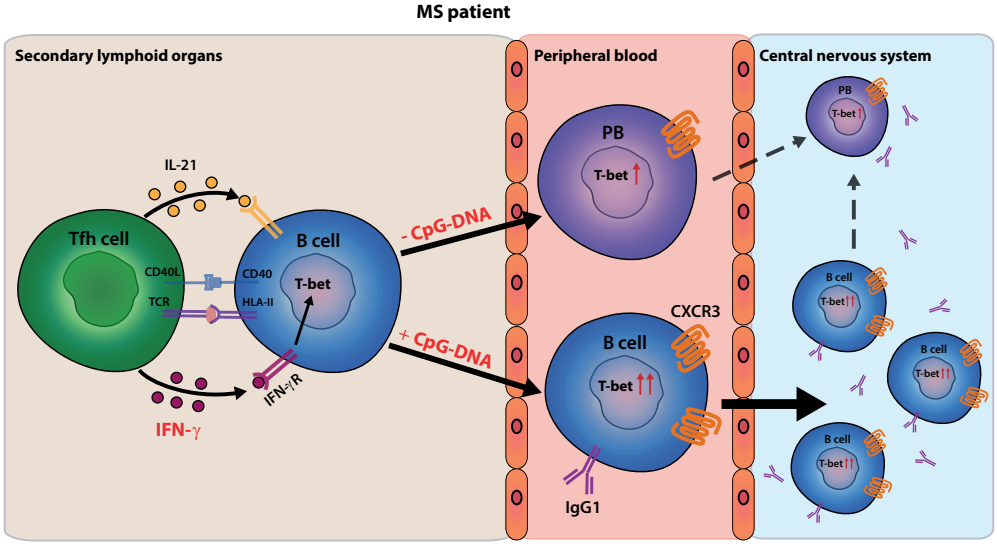


Figure 6. IFN-γ and TLR9 signaling upregulate T-bet in peripheral B cells, likely driving CXCR3-mediated recruitment and IgG1 production in the CNS of MS patients.

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.

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 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.

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Chapter 5

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

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Submitted

ABSTRACT

Background: In MS, pathogenic memory B cells are likely triggered by T follicular helper (T_{FH}) cells to infiltrate the brain and develop into antibody-secreting cells. Chemokine receptors not only define their brain-homing capacity, but also their maturation within germinal centers. How this corresponds to MS relapse risk is underexplored.

Objective: To study the impact of pregnancy, as a model for relapse risk, on B-cell differentiation in MS.

Methods: Germinal center-like B-cell phenotypes and outgrowth were compared between 19 MS patients and 12 healthy controls during pregnancy (low risk) and early after delivery (high risk).

Results: Transitional, and not naive mature, B-cell frequencies dropped in the third trimester, which was strongest in patients who experienced a pre-pregnancy relapse. In the postpartum period, these frequencies raised again, while those of memory B cells showed a modest reduction. CXCR4 was downregulated and CXCR5, CXCR3 and CCR6 were upregulated on postpartum memory B cells, implying enhanced recruitment into germinal center light zones for interaction with T_{FH} cells. Postpartum memory B cells of MS patients expressed higher CCR6 levels and preferentially developed into plasma cells during T_{FH}-like cultures.

Conclusion: These data provide indications how memory B-cell outgrowth may contribute to increased postpartum relapse risk in MS.

INTRODUCTION

MS is a chronic inflammatory and demyelinating disease of the CNS, for which a key role for B cells in the pathogenesis has been shown in recent years[1]. In the relapsing phase of MS, naive B cells escape peripheral tolerance checkpoints[2] and develop into memory populations that activate brain-homing, IFN-γ-producing $CD4^+$ T cells[3]. The latter is supported by the reduction in T-cell activation after B cell-depleting therapy in relapsing MS[4]. This interaction of B cells with so-called T follicular helper (T_{FH}) cells occurs within germinal centers (GCs) of secondary lymphoid organs. Our group recently showed that B cells, in turn, use T_H cell-derived IFN-γ to develop into $CXCR3^+$ populations, which infiltrate the CNS tissue of MS patients[5]. After reaching the CNS, memory B cells are likely reactivated in perivascular spaces and ectopic GC-like structures found in the cerebral meninges[6] to further mature into plasmablasts/plasma cells and contribute to focal inflammation[7].

Besides the homing into inflamed tissue, IFN-γ-mediated induction of B cells results in the formation of autoimmune ectopic GCs, as reported in mice. Furthermore, CXCR3 upregulation on B cells contributes to aberrant plasma cell differentiation within GCs[8]. Other chemokine receptors such as CXCR4 and CXCR5 also orchestrate the GC response by guiding B cells into the dark and light zone, respectively. Whereas centroblasts undergo several rounds of proliferation and somatic hypermutation in the dark zone, centrocytes interact with follicular dendritic cells and T_{FH} cells to undergo antigen-specific selection in the light zone[9]. The presence of CCR6 on B cells has been reported to promote their development and antigen responsiveness within the light zone[10, 11]. Therefore, aberrancies in the expression of these chemokine receptors may affect GC B-cell outgrowth into memory and plasmablasts/plasma cells, thereby increasing the chance of causing autoimmunity[12, 13]. Currently, it remains to be determined whether and how this associates with relapse risk in MS.

In the natural course of untreated MS patients who are pregnant, relapse risk is reduced by approximately 70% in the third trimester[14]. Although current MS diagnostic and treatment strategies likely contribute to an attenuation of this fluctuation[15], third trimester and postpartum period remain a period of relative low and high relapse risk, respectively. Pregnancy induces a shift from T_H1 to T_H2 responses[16] and promotes the expansion of circulating T_{FH} cells[17], likely affecting the development of GC B cells. Hence, by comparing their development in context of chemokine receptor expression between periods of low and high relapse risk within patients, insights will be gained into how functional B-cell outgrowth is associated with the unpredictable, relapsing MS course.

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

into plasmablasts or plasma cells differs between periods of relatively low (third trimester) and high (early postpartum) relapse risk. Patients with relapses the year preceding pregnancy were analyzed separately to include a sensitivity analysis on patients with a very high relapse risk.

METHODS

Participants

We included 19 pregnant woman with RRMS according to the McDonald criteria[18]. Patient characteristics are displayed in Table 1[19]. Patients did not use any immune modulatory medication during the study period (pre-pregnancy, during pregnancy as well as early after delivery, except for one who received methylprednisolone prior to pregnancy and IVIg treatment during the first trimester postpartum. One patient conceived pregnancy using *in vitro* fertilization and one via intracytoplasmic sperm injection. One patient conceived while using leuprolide medication. Three patients gave birth via a cesarean section and all others had vaginal deliveries. Nine patients breastfed their child. During the early postpartum high risk period, 6 patients developed a clinically manifest relapse. Since we have measured these 6 patients separate from the rest of the group, expression levels were not compared between these 6 patients and the other non-relapse MS patients or HC. No MRI evaluations for disease activity were performed.

Additionally, 12 age-matched pregnant women were included as healthy controls, who did not have central nervous system or inflammatory disease. None of them used immunomodulatory medication before or during the study. For 4/13 women in this group, no information about lactation was available. Three used *in vitro* fertilization and one used intracytoplasmic sperm injection to become pregnant. One woman gave cesarean birth and one woman developed preeclampsia. All participants were seen at the outpatient clinic of Erasmus MC (Rotterdam, The Netherlands) and gave written informed consent. This study was approved by the medical ethics committee of Erasmus MC.

PBMC isolation, flow cytometry and antibodies

PBMCs from patients and controls were collected in the first and third trimester as well as 4-8 weeks after delivery. For sample collection, we used Vacutainer CPT® tubes containing sodium heparin according to manufacturer's instructions (BD Biosciences, Erembodegem, Belgium). After isolation, cells were taken up in RPMI 1640 (Lonza, Basel, Switzerland) containing 20% fetal calf serum (Lonza) and 10% dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO) and stored in liquid nitrogen until further use. Cells were incubated with Fixable Viability Stain 700 (BD Biosciences) for 15 min, after which the following monoclonal antibodies were

Table 1.

Subject	Age	Pre-preg relapse°	Post-preg relapse	*In vitro*	*Ex vivo*	BF°°	Med	PG°°°	Special
MS1	33	Yes	No	No	Yes	1	MP (pre-pregnancy)/ IVIg (postpartum)	0	
MS2	26	Yes	No	Yes	Yes	0	none	0	
MS3	23	Yes	No	No	Yes	1	none	0	
MS4	26	Yes	No	No	Yes	1	none	1	
MS5	33	Yes	No	Yes	Yes	1	none	0	
MS6	37	No	No	Yes	Yes	1	none	1	
MS7	37	No	No	Yes	Yes	0	none	1	IVF* + sectio
MS8	33	No	No	Yes	Yes	1	none	0	
MS9	26	No	No	Yes	Yes	1	none	1	
MS10	29	Yes	No	Yes	Yes	0	none	1	sectio
MS11	30	Yes	No	Yes	Yes	0	none	1	
MS12	37	No	No	No	Yes	0	none	0	
MS13	33	Yes	No	No	Yes	0	none	0	
MS14	22	Yes	Yes	Yes	Yes	1	none	1	
MS15	34	No	Yes	No	Yes	1	none	0	Leucrin use
MS16	32	No	Yes	No	Yes	1	none	0	
MS17	35	No	Yes	Yes	Yes	0	none	1	ICSI**
MS18	31	No	Yes	No	Yes	0	none	1	
MS19	29	No	Yes	Yes	Yes	0	none	1	sectio
HC1	33	n.a.	n.a.	No	Yes	1	n.a.	0	
HC2	22	n.a.	n.a.	No	Yes	?	n.a.	0	
HC3	27	n.a.	n.a.	Yes	Yes	0	n.a.	0	
HC4	27	n.a.	n.a.	Yes	Yes	1	n.a.	1	IVF
HC5	33	n.a.	n.a.	No	Yes	?	n.a.	0	Pre-eclampsia
HC6	32	n.a.	n.a.	No	Yes	0	n.a.	0	IVF
HC7	34	n.a.	n.a.	Yes	Yes	?	n.a.	0	
HC8	36	n.a.	n.a.	No	Yes	0	n.a.	0	ICSI
HC9	34	n.a.	n.a.	Yes	Yes	0	n.a.	0	sectio
HC10	25	n.a.	n.a.	Yes	Yes	1	n.a.	0	
HC11	33	n.a.	n.a.	No	Yes	?	n.a.	0	
HC12	41	n.a.	n.a.	No	Yes	1	n.a.	0	
HC13	34	n.a.	n.a.	Yes	No	0	n.a.	0	IVF

° = year prior to conception, °° BF = breast feeding, °°° PG = primi gravida (first child), * = in vitro vertilization, ** ICSI = intra cytoplasmatic sperm injection. N.a. = not applicable

added for 30 min at 4°C: CD27 (BV421, M-T271), CD138 (BV605 and PE-CF594, MI15), CXCR4 (PE-CF594, 12G5), CXCR5 (PercCP, RF8B2), IgD (PE-CF594, IA6), IgG (APC-H7, G18-145; all BD Biosciences), CCR6 (PE, G034E3), CD19 (BV785, HIB19), CD38 (BV605 and PE-Cy7, HIT2), CXCR3 (APC and PE-Cy7, G025H7), CXCR4 (APC-Cy7, 12G5) and IgM (BV510, MHM-88; all Biolegend, London, UK). For ex vivo phenotyping, cells were measured using an LSRII-Fortessa flow cytometer. For in vitro cultures, memory ($CD19^+CD3^-CD27^+$) B cells were purified using a FACSAria III sorter. Data were analyzed using FACS Diva software, version 8.0.1 (all BD Biosciences).

Germinal center-like B-cell differentiation assay

In vitro B-cell differentiation assays were performed as recently described[5, 20]. In short, irradiated murine 3T3 fibroblasts expressing human CD40L were co-cultured with sorted memory B cells in the presence of IL-21 (50 ng/ml; Thermo Fisher Scientific, Landsmeer, The Netherlands). After 6 days of culturing, viable $CD19^+$ cells were analyzed using flow cytometry and supernatants were collected and stored at -80°C until further use.

IgM and IgG ELISA

IgM and IgG levels were determined in supernatants of memory B cells cultured for 6 days using ELISA. After overnight coating with goat anti-human Ig (1 mg/ml; Southern Biotech, Birmingham, USA) at 4°C, flat-bottom 96-well plates (Corning, Tewksbury, USA) were washed with PBS/0.05%Tween-20 and subsequently blocked with PBS/5%FCS for 2 h at RT. Samples were added for 1.5 h at RT. After washing, peroxidase-conjugated goat anti-human IgG (Thermo Fisher Scientific) or rabbit anti-human IgM (Jackson, Uden, The Netherlands) were used to detect bound antibody. TMB substrate (Thermo Fisher Scientific) was used to reveal peroxidase activity. Reactions were stopped with sulfuric acid and optical densities were measured at 450 nm using a BioTek Synergy 2 reader (Winooski, USA). Concentrations were calculated using standard curves for IgM and IgG.

Statistical analysis

Graphpad Prism Software, version 8 was used for statistical analysis. Both percentages and MFI are displayed as individual data points together with the mean. Wilcoxon signed-rank test was performed to compare the different gestational periods, Two-way ANOVA was performed to compare HC with MS patients, unless stated otherwise. P values < 0.05 were considered statistically significant.

Data availability

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

RESULTS

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

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

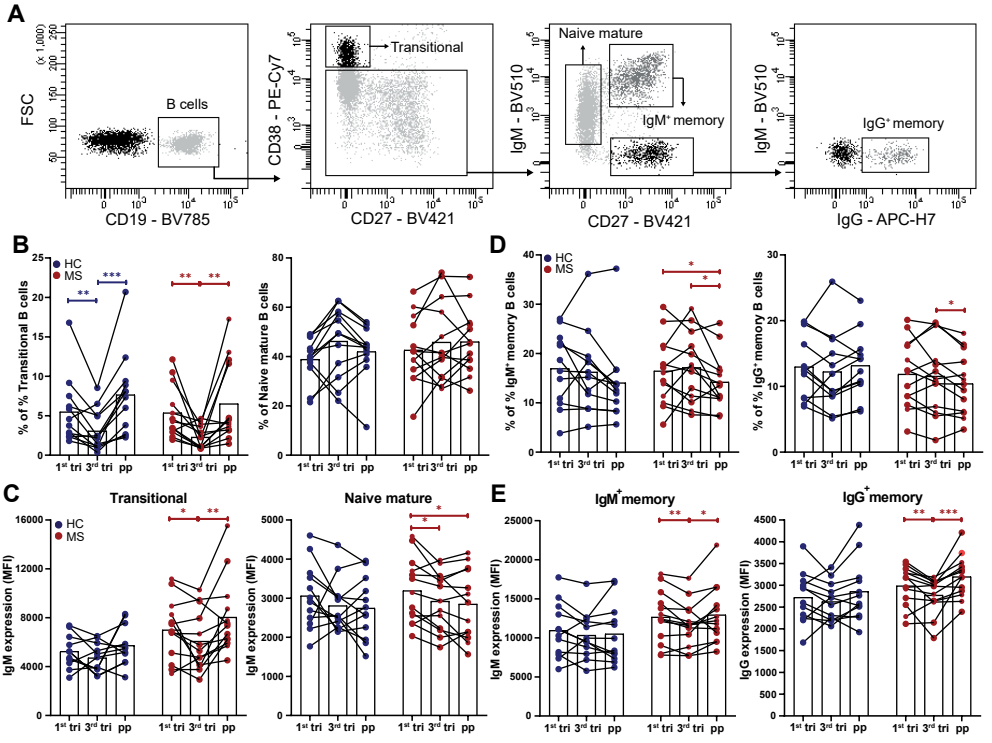

Figure 1. Frequency of circulating naive and memory B-cell subsets during and early after pregnancy in MS patients and healthy controls.

(A) Representative gating of viable CD19^{+} B-cell subsets: transitional (CD38^{high}CD27^{-}), naive mature (CD38$^{-/dim}$CD27^{-}IgM^{+}), IgM memory (IgM^{+}CD27^{+}) and IgG memory (IgG^{+}CD27^{+}) B cells. The percentages of transitional B cells, naive mature B cells **(B)** as well as IgM and IgD expression (MFI) on these subsets **(C)** were compared between paired first trimester (1st tri), third trimester (3rd tri) and early postpartum (pp) samples of 13 MS patients (red) and 12 HC (blue). Similar analyses were performed for IgM^{+} and IgG^{+} memory B cells (**D** and **E**). Wilcoxon signed-rank test was performed to compared the different gestational periods. * p<0.05, ** p<0.01, *** p<0.001

In MS patients without a postpartum relapse (n = 13) and healthy controls, the proportion of transitional B cells declined from first to third trimester and recuperated after delivery (5.4%, 2.3.% and 6.5% for MS and 5.5% vs 3.1% vs 7.7% for HC, (Figure 1B), as reported earlier[21]. Naive mature B-cell frequencies were not different between periods, resulting in elevated naive mature/transitional B-cell ratios per subject (Supplementary Figure 1A). In the third trimester, these ratios were even more raised in patients with a relapse one year before pregnancy (Supplementary Figure 1B) and were lower in MS patients with an early postpartum relapse (n = 6; Table 1 and Supplementary Figure 1C). IgM and not IgD expression was significantly increased on postpartum transitional B cells, which was not seen for naive mature B cells and the most pronounced in MS patients ($p = 0.0056$, Figure 1C and Supplementary Figure 1D). Both IgM^+ and IgG^+ memory B cells showed a moderate decline in frequencies, but a significant increase in Ig surface expression in the postpartum period ($p = 0.0473$ for IgM^+ and $p = 0.0010$ for IgG^+; Figure 1D). These differences in expression level and memory fractions were not influenced by an early pre-pregnancy or postpartum relapse (data not shown and Supplementary Figure 1E and F) or differences in third trimester estradiol, progesterone and cortisol levels (data not shown).

The pregnancy-induced disturbances in naive B-cell development and formation of IgM^{high} memory B cells *ex vivo* may imply favored differentiation in GCs during the high risk postpartum period[22].

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

Chemokine receptors selectively regulate GC organization and maturation of B cells (Figure 2A). First trimester, third trimester and postpartum B cells were analyzed for the expression of CXCR4 and CXCR5, which mediate dark and light zone localization, respectively[9], as well as CCR6 and CXCR3, which contribute to memory recall and antibody responses[8, 23], respectively.

CXCR4 was downregulated, while CXCR5 showed an upregulation on postpartum versus third trimester B cells. This was seen for both memory (IgM^+ and IgG^+; Figure 2B and C) and naive mature (Supplementary Figure 1G) B cells. The postpartum rise in CXCR5 was significant in the MS but not the control group. CXCR4 and CXCR5 levels were higher on naive mature than memory B cells (IgM^+CD27^- > IgM^+CD27^+ > IgG^+CD27^+; Supplementary Figure 2A). In addition to this, CCR6 and CXCR3 were upregulated on postpartum B cells in both groups (Figure 2D and E; Supplementary Figure 1H). CCR6 levels were more increased and significantly different compared to first trimester B cells in the MS group ($p = 0.0126$ for IgM^+ memory and $p = 0.0081$ for IgG^+ memory; Figure 2D and E). For all groups and periods, CCR6 was highest expressed on naive mature B cells (IgM^+CD27^- > IgM^+CD27^+ > IgG^+CD27^+), while CXCR3 was most predominant on memory B cells (IgM^+CD27^- < IgM^+CD27^+ < IgG^+CD27^+;

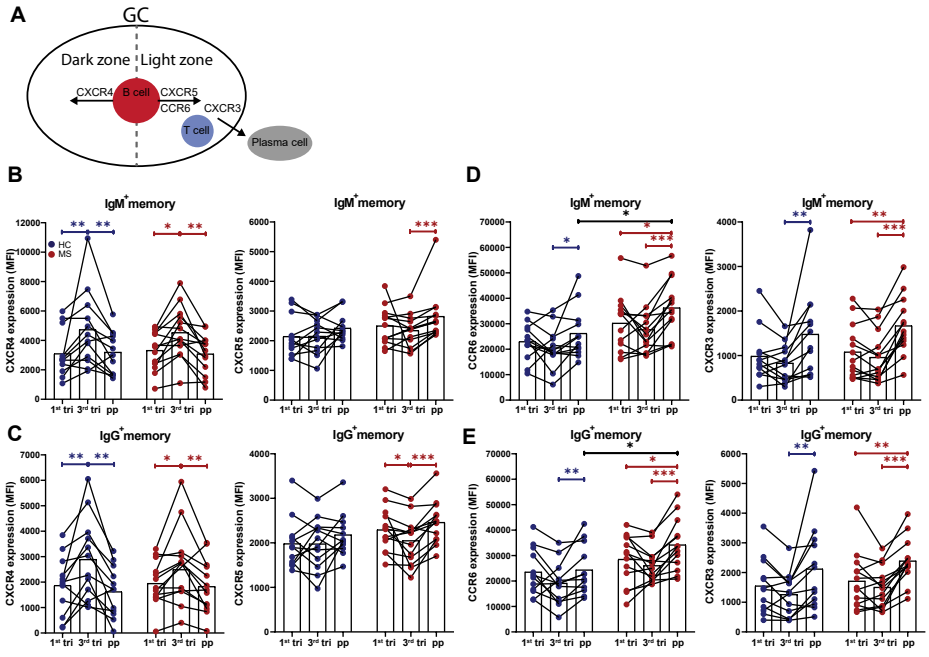

Figure 2. Chemokine receptor expression on circulating memory B cells during and early after pregnancy in MS patients and healthy controls.

(**A**) Schematic display of chemokine receptors involved in GC-dependent organization and maturation of B cells. Expression of dark zone-associated CXCR4 and light zone-associated CXCR5, CCR6 and CXCR3 was compared between IgM^+ (**B, D**) and IgG^+ (**C, E**) memory B cells from first trimester (1^{st} tri), third trimester (3^{rd} tri) and early postpartum (pp) samples from 13 MS patients (red) and 12 HC (blue). Wilcoxon signed-rank test was performed to compare the different gestational periods. Two-way ANOVA was performed to compare HC with MS patients. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Supplementary Figure 2B). We found no significant impact of a pre-pregnancy or postpartum relapse on chemokine receptor expression (data not shown and Supplementary Figure 3).

Together with the increased Ig surface levels (Figure 1C and D), the chemokine receptor expression profile especially found for postpartum B cells of MS patients ($CXCR4^{low}CXCR5^{high}CCR6^{high}CXCR3^{high}$) implies an increased potential of these cells to enter and further develop in GC light zones.

Early postpartum memory B cells from MS patients preferentially develop into Ig-secreting plasma cells under T_{FH}-like conditions *in vitro*

Effective B cell memory is generated through the help of T_{FH} cells within the GC light zone. After reaching the CNS, memory B cells are likely reactivated to develop into potent antibody-secreting cells in MS patients. To assess whether B cells are prone for such recall

responses in the high-risk postpartum period in MS, memory ($CD27^+$) B cells were purified from paired third trimester and postpartum samples of 11 MS patients and compared for their outgrowth into plasmablasts/plasma cells *in vitro*. Under IL-21- and CD40L-inducing conditions (mimicking a T_{FH} cell response), more antibody-secreting cells ($CD38^{high}CD27^{high}$) were formed in cultures with postpartum versus third trimester memory B cells ($p = 0.0234$, Figure 3A and B). This was similar to paired memory B-cell samples from healthy controls ($n = 5$, 0.1250). When we analyzed plasma cell ($CD138^+CD38^{high}CD27^{high}$) frequencies, these were mainly increased for postpartum memory B cells from MS patients, which were significantly higher than those from controls ($p = 0.001$, Figure 3C). For the MS group, IgM^+ and not IgG^+ plasmablasts ($CD38^{high}CD27^{high}$) seemed to be more induced in both third trimester and postpartum memory B-cell cultures Figure 3D). Especially IgM levels were elevated in supernatants of postpartum memory B-cell cultures, which were not different between patients and controls (Figure 3E).

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

DISCUSSION

Pregnancy causes a transient period of immune suppression, which results in an increased relapse risk early after delivery in MS[24]. Here, we studied how peripheral B-cell differentiation is regulated during pregnancy-associated low and high relapse risk periods in patients with MS. The observed differences in IgM, CXCR3, CXCR4, CXCR5 and CCR6 expression levels point towards a GC light zone phenotype of postpartum versus third trimester B cells *ex vivo*. This is reflected by the increased capacity of memory B cells to develop into plasma cells under T_{FH}-like conditions *in vitro*.

Regarding naive B cells, Lima et al[21] showed previously that transitional B cell frequencies are reduced in the third trimester. We replicated this observation and additionally showed kinetics of this observation during pregnancy. Reduced proportions of transitional B cells were seen from the first towards the third trimester, resulting in increased naive mature/transitional B-cell ratios. The pregnancy-induced alterations in B lymphopoiesis are probably the result of increased hormone levels in the third trimester[17], keeping transitional B cells in check due to a lack of multidrug resistance receptor 1 (MDR1)[25], a glycoprotein which pumps steroids out of cells. We found a similar impact of steroid treatment in patients with MS, AQP4-IgG^+ neuromyelitis optica spectrum disorder and MOG-IgG-associated disease[26]. Because of the sharp rise in IgM^{high} transitional B cells early after delivery, one could speculate that this leads, via increased entrance of autoantigen specific naive mature B cells to

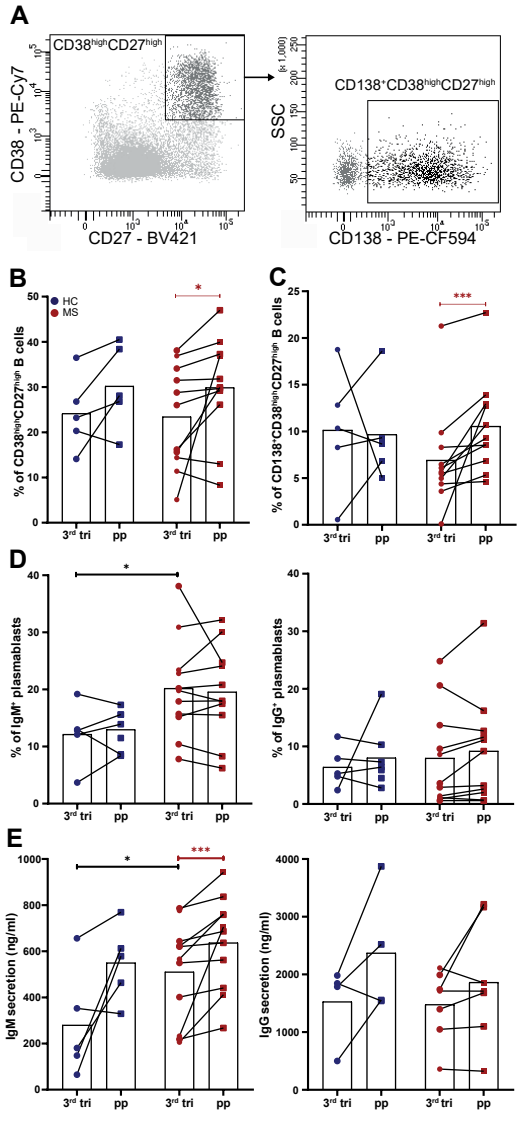

Figure 3. Germinal center-dependent antibody-secreting cell outgrowth using memory B cells from paired third trimester and early postpartum blood samples.

(**A**) Representative gating of $CD38^{high}CD27^{high}$ (plasmablasts/plasma cells) and $CD138^{+}$ (plasma cells) cells after culturing of memory B cells under T_{FH}-like conditions using IL-21 and 3T3-CD40L for 6 days. Percentages of $CD38^{high}CD27^{high}$ (**B**), $CD138^{+}CD38^{high}CD27^{high}$ (**C**) and IgM^{+} and IgG^{+} plasmablasts (**D**; FACS), as well as IgM and IgG secretion (**E**; ELISA) were compared for cultures with third trimester (3rd tri) and early postpartum (pp) memory B cells from 11 MS patients (red) and 5 HC (blue). Wilcoxon signed-rank test was performed to compare third trimester and postpartum samples. Two-way ANOVA was performed to compare HC with MS patients. * $p<0.05$, *** $p<0.001$.

the GC reaction, to more GC-experienced pathogenic subsets[22] that are destined to enter the CNS in MS patients. Although we did not touch upon their CNS transmigration capacity in this study, we can at least assume that the postpartum increase in CXCR3 expression contributes to the local B-cell enrichment in MS.5 This may be further induced by the elevated levels of CCR6 found for postpartum B cells of MS patients versus healthy controls.

The postpartum upregulation of CXCR5 especially seen in MS patients is likely involved in B-cell organization rather than recruitment in the CNS[9, 27]. Our group reported earlier an upregulation of CXCR4 during MS onset in non-pregnant patients[28]. The here found reduction in CXCR4 expression during the high relapse risk postpartum period is therefore counterintuitive. It may be that autoreactive CXCR4^{high} naive B cells escape from T_{FH}-mediated selection in the GC light zone in peripheral lymphoid organs during a primary response[13], while CXCR4^{low} memory B cells are more prone to interact with T_{FH} cells and develop into long-lived plasma cells during a secondary response in the CNS. CCR6 can facilitate such recall responses[23] and, together with CXCR3, is associated with the production of high affinity antibodies[29].

Until now, not much was known about the potential of memory B cells to locally develop into plasma cells in MS. Despite the current focus on antibody-independent B-cell functions, this is of high relevance as long-lived plasma cells reside within the chronically inflamed MS CNS[30]. The clinical relevance of local Ig production has become apparent from the increased risk of CIS to MS conversion in patients with CSF oligoclonal bands[31],which are present in more than 95% of MS patients and indicates ongoing IgG production in the CNS. Consistently, the absence of B cells in brain lesions of MS patients is associated with a lack of CSF oligoclonal bands, a lower intrathecal IgG production, and a more favorable outcome[32]. The observation that (short term) anti-CD20 treatment reduces CSF B cell numbers while oligoclonal bands persist[33] suggests that intrathecal IgG are mainly produced by (CD20^{-}) long-lived plasma cells in the CNS. Recently, it has been shown that MS myelin is bound by IgG and that IgG immune complexes trigger human microglia, resulting in enhanced production of pro-inflammatory cytokines[34].

Our study has some limitations. The relatively low numbers of included subjects hampered a subdivision in MS risk groups based on disease activity before and after pregnancy. Sequential data collection of such patients is difficult, but the accumulating evidence for the safe continuation during pregnancy of various disease modifying therapies may increase options[35]. Furthermore, we did not perform *in vitro* B-cell cultures with sera or female hormones. The recent described effects of estradiol and progesterone on preferred outgrowth after antigenic triggering of memory B cells and plasma cells in healthy pregnant individuals[36] raises the question whether this is differentially regulated in MS patients. Finally, B-cell EBV load could play an additional role in the impact of CXCR3^{high} plasma cell formation[20] as seen early after delivery, which was not studied here.

Together, this work provides new insights into how B-cell development is affected during pregnancy and may contribute to an increased postpartum relapse risk in MS. With these findings, we reveal a link between chemokine receptor expression profiles and the ability of brain-homing memory B cells to differentiate into plasma cells, which should be further studied in the near future. This may not only help to decipher the underlying mechanisms of local B-cell accumulation and antibody production, but also offer new tools to better predict disease activity in patients with MS.

DECLARATION OF CONFLICTING INTERESTS

JS: consultancy and/or lecture fee of Biogen, Merck, Novartis and Sanofi Genzyme. All the other authors declare that there is no conflict of interest.

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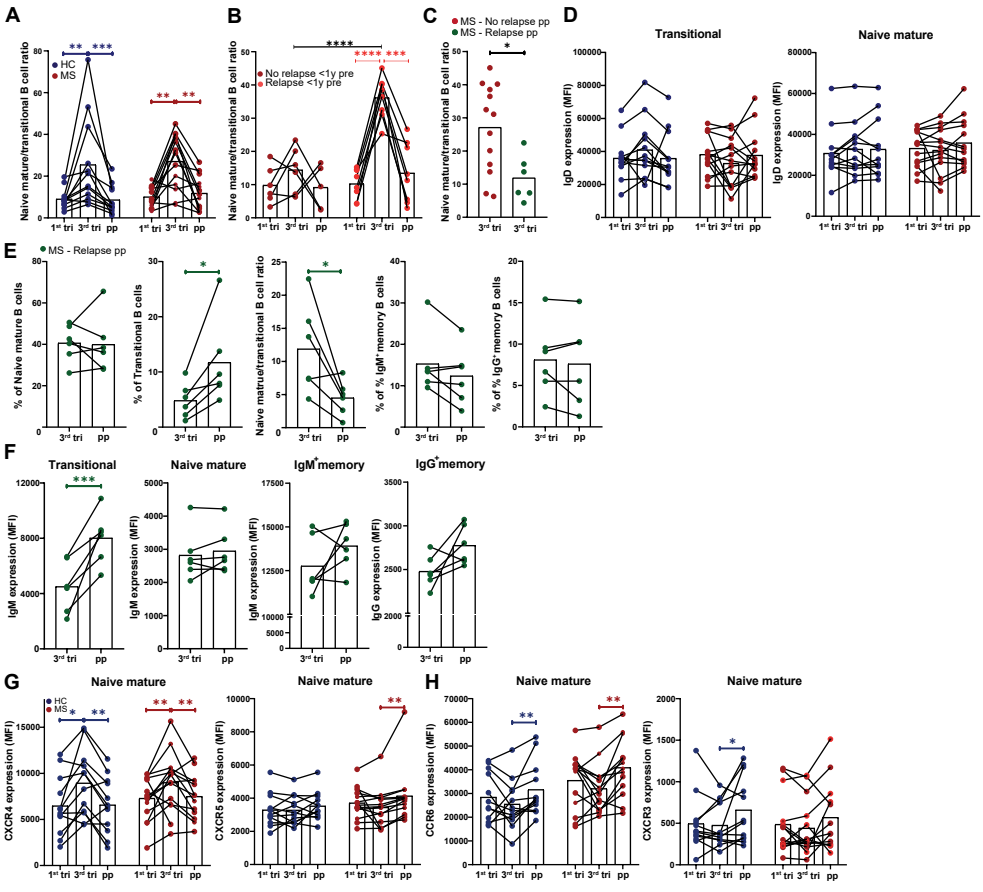
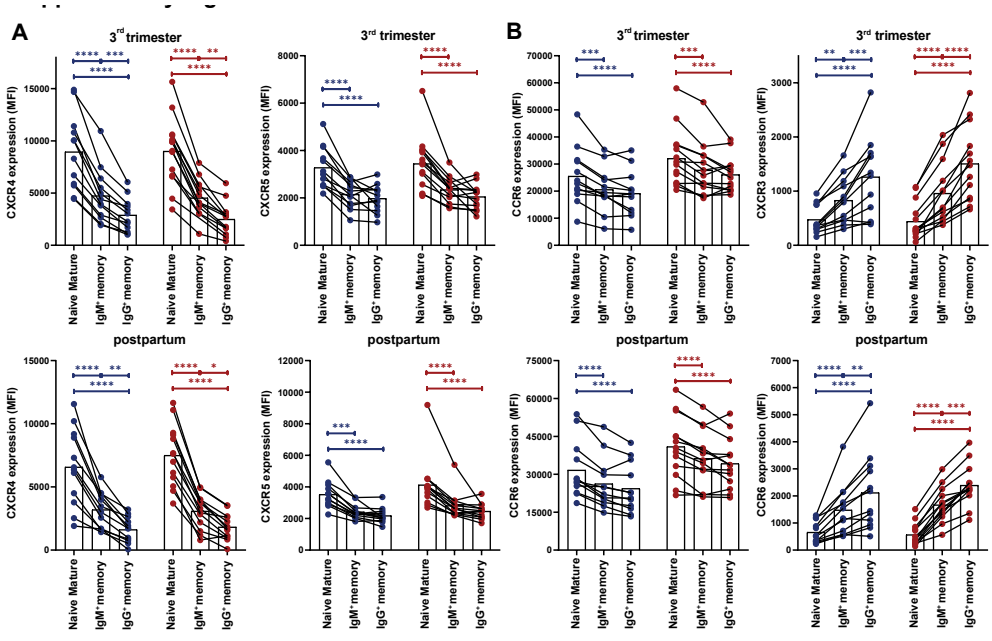
SUPPLEMENTAL DATA



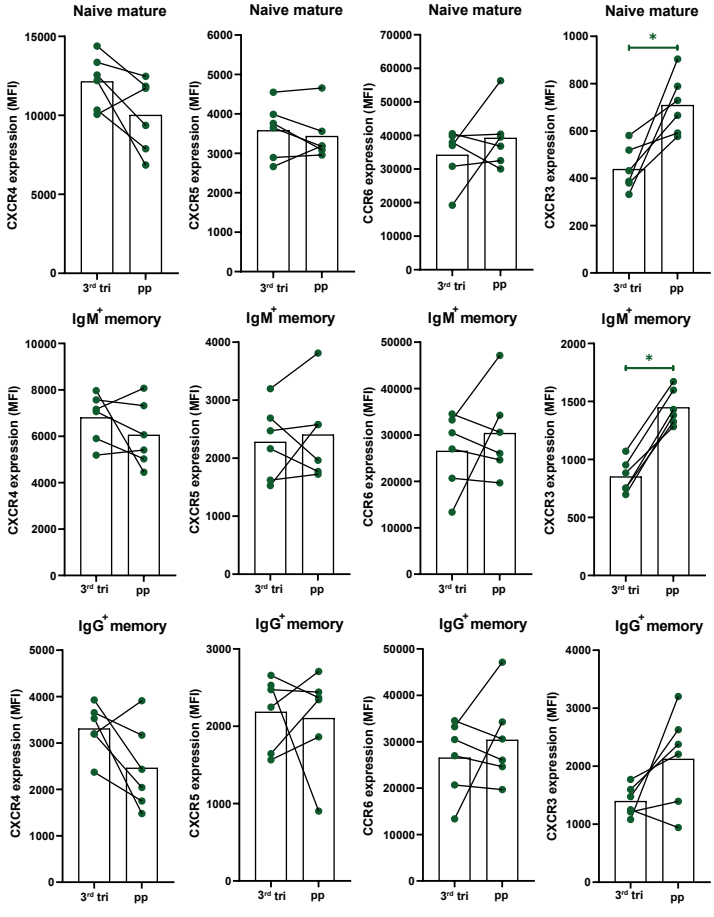
Supplementary Figure 1. The naive mature/transitional B cell ratio, frequencies of B cell subsets in MS patients with a postpartum relapse and chemokine expression levels on naive mature B cells of HC and MS patients

(**A**) The naive mature/transitional B cell ratio in the different gestational periods of 12 HC (blue) and 13 MS patients (red), which in **(B)** were separated into MS patients with (n = 8) or without (n = 6) a relapse within 1 year before pregnancy. **(C)** The naive mature/transitional B cell ratio in the 3rd trimester of MS patients with a postpartum relapse (n=6) or without (n=13). **(D)** IgD expression on transitional and naive mature B cells was compared between paired first trimester, third trimester and early postpartum samples of 12 HC (blue) and 13 MS patients (red). **(E)** The percentage of transitional and naive mature B cells in MS patients with a post-pregnancy relapse (n = 6), as well as their ratio and the frequency of IgM^+ and IgG^+ memory B cells. (**F**) Immunoglobulin expression on memory B cells of post-pregnancy relapsing patients (n = 6). (**G-H**) Expression of dark zone associated CXCR4 and light zone associated CXCR5, CCR6 and CXCR3 on naive mature B cells in the first trimester, third trimester and early postpartum period for 12 HC (blue) and 13 MS patients (red). Wilcoxon signed-rank test was performed to compare the different gestational periods. Mann-Whitney U test was performed in **C**. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.



Supplementary Figure 2. Chemokine expression levels on naive mature, IgM⁺ and IgG⁺ memory B cells in the third trimester and early after delivery.

Expression of dark zone-associated CXCR4 and light zone-associated CXCR5, CCR6 and CXCR3 was compared between naive mature, IgM⁺ and IgG⁺ memory B cells from third trimester and early postpartum samples from 12 HC (blue) and 13 MS patients (red). Two-way ANOVA was performed to compare the different B cell subsets within a group. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.



Supplementary Figure 3. Chemokine expression levels on naive mature, IgM⁺ and IgG⁺ memory B cells in the third trimester and early after delivery of post-partum relapsing MS patients

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

Chapter 6

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

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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 in a separate group of 9 NMOSD and MOGAD patients who received corticosteroid treatment. These findings need to be confirmed in longitudinal studies. 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 was only found for relapsing cases. These data indicate that naive B-cell homeostasis is different and selectively targeted by corticosteroids in NMOSD patients. This also supports further exploration of naive B cells for their 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 (Wingerchuk *et al.*, 2007). Although the exact cause of these divergent presentations remains poorly understood, the dominant role of the B-cell lineage is undisputed (Sabatino *et al.*, 2019). In approximately 75% of NMOSD patients, IgG antibodies are found that target the neuronal water channel protein aquaporin-4 (AQP4; Saadoun and Papadopoulos, 2010). Furthermore, 30-40% of AQP4-IgG-negative NMOSD patients test positive for antibodies against myelin oligodendrocyte glycoprotein (MOG; Pelt van *et al.*, 2016a; Hamid *et al.*, 2017), 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 (Kitley *et al*., 2012; Pelt van *et al.*, 2016b; Jurynczyk *et al.*, 2017), 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 (Wilson *et al.*, 2018; Cotzomi *et al*., 2019). 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 (Kowarik *et al*., 2017; Wilson e*t al.*, 2018; Cotzomi *et al*., 2019). In systemic autoimmune disease, which coexists in ~20% of AQP4-IgG-positive NMOSD patients (Shahmohammadi *et al.*, 2019), 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 (Sindhava *et al*., 2017; Wang *et al*., 2018).

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 (Wingerchuk *et al.*, 2015) 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 RT. Sample and a human IgG standard (Sigma-Aldrich/Merck, Darmstadt, Germany) were added for 1.5 h at RT. 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 H2SO4 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	**41/F**	ON + ON	None	7	Yes	7644	NMOSD	Yes
Patient 2	**39/F**	TM + 2xTM	None	78	Yes	13365	NMOSD	Yes
Patient 3	**56/F**	ON bilateral + ON/BS	None	6	Yes	13736	NMOSD	Yes
Patient 4	**61/F**	TM	None	5	No	7469	NMOSD	Yes
Patient 5	**46/M**	ON bilateral	None	15	No	545	NMOSD	Yes
Patient 6	**47/F**	ON	None	2	No	15437	NMOSD	Yes
Patient 7	**27/F**	TM	None	46	No	7874	NMOSD	Yes
Patient 8	**26/F**	BS	None	122	No	7262	NMOSD	Yes
Patient 9	**36/F**	ON	None	0	No	ND	NMOSD	Yes
Patient 10	**46/F**	TM	None	4	No	20533	NMOSD	No
Patient 11	**34/F**	TM + TM	P	5	Yes	82	CS-treated	No
Patient 12	**50/F**	TM + 2xTM	MP	13	Yes	17061	CS-treated	No
Patient 13	**19/F**	BS + TM	MP + P	6	Yes	14055	CS-treated	No
Patient 14	**58/F**	TM/ON bilateral	MP + P	2	No	15618	CS-treated	No
Patient 15	**37/F**	BS/ON bilateral	MP + P	4	No	16701	CS-treated	No
MOG-IgG								
Patient 1	**38/F**	ON + 5xON	None	95	Yes	24237	MOGAD	Yes
Patient 2	**26/M**	ON + ON	None	25	Yes	5099	MOGAD	Yes
Patient 3	**25/F**	TM + 2xON	None	17	Yes	424	MOGAD	Yes
Patient 4	**32/M**	ADEM + TM	MP	326	Yes	1681	CS-treated	Yes
Patient 5	**52/M**	ON	None	10	No	136	MOGAD	Yes
Patient 6	**40/F**	ON bilateral	None	10	No	1234	MOGAD	Yes
Patient 7	**56/F**	TM	None	18	No	696	MOGAD	Yes
Patient 8	**37/M**	TM/ON	MP	1	No	6495	CS-treated	Yes
Patient 9	**26/F**	ON bilateral	MP + P	1	No	2058	CS-treated	Yes
Patient 10	**25/F**	ON bilateral + 3xON	None	201	Yes	6617	MOGAD	No
Patient 11	**32/M**	E + E	MP	181	Yes	21	CS-treated	No
Patient 12	**29/M**	TM	None	51	No	1200	MOGAD	No

[#] 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 (Ketelslegers *et al.*, 2011; Pelt van *et al.*, 2016a; Pelt van *et al.*, 2016b). 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 i*n 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 (van Langelaar *et al.,* 2019). 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.

Data availability

The raw data that support the here described findings are available from the corresponding author upon reasonable request.

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; (Pelt van *et al.*, 2016a), 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 (Timmermans *et al.*, 2016; Heeringa *et al.*, 2018) was elevated in NMOSD versus healthy controls (mean 16.2% vs 5.6% for transitional B cells and 47.3% vs 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 (Berkowska *et al.*, 2011) 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 (Berkowska *et al.*, 2011) (Supplementary Fig. 2B) or plasmablasts ($CD38^{++}CD27^{++}$; Supplementary Fig. 3A), did not differ between groups. None of the 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

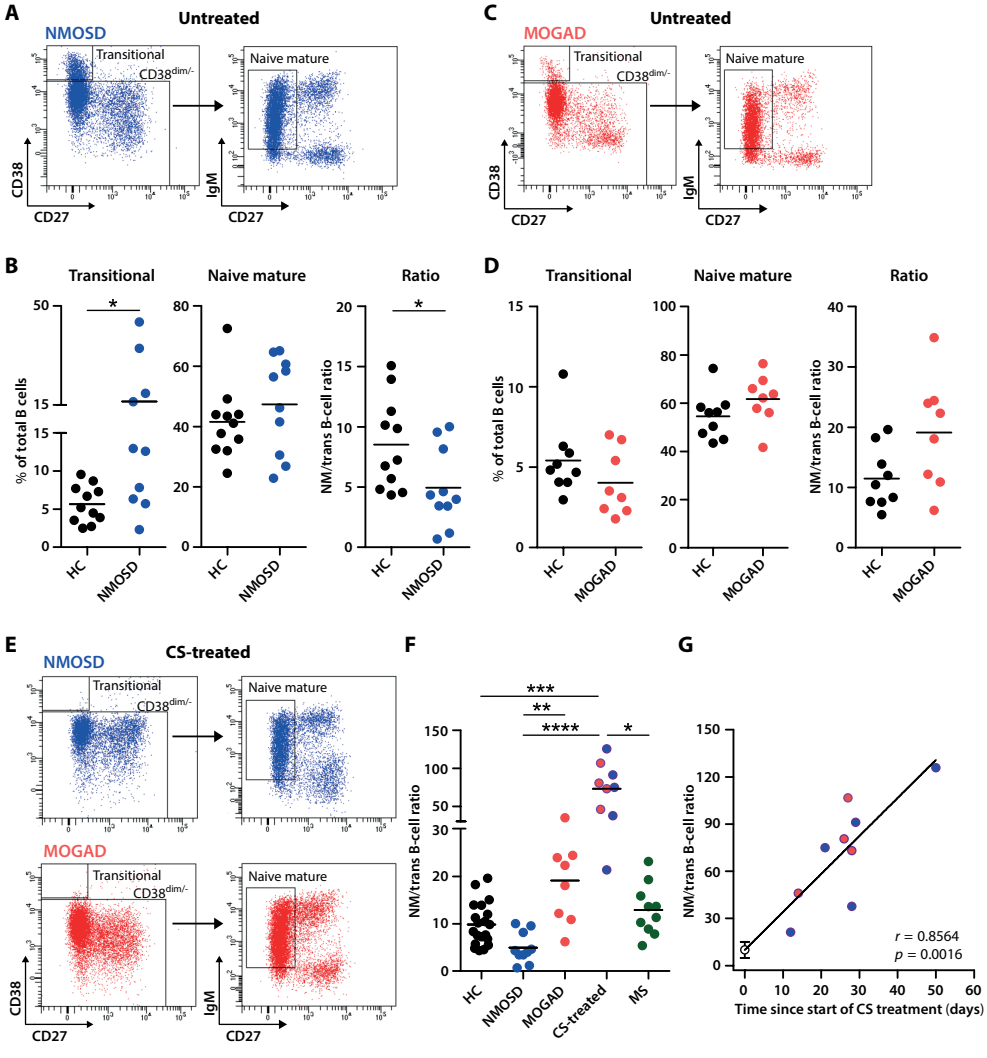

Figure 1. Transitional and naive mature B-cell frequencies in the blood of different NMOSD, MOGAD, MS and healthy control groups.

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

treatment. In this group, naive mature/transitional B-cell ratios were significantly elevated (fold change vs 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 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*

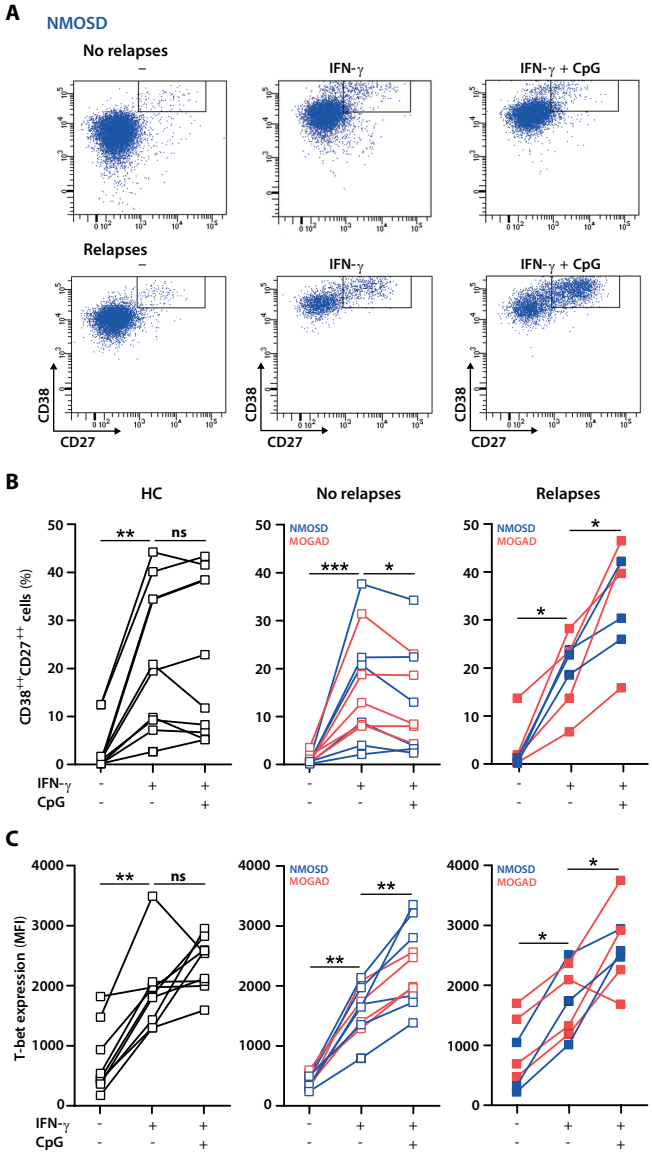

Figure 2. ***In vitro*** **plasmablast outgrowth for naive mature B cells from subgroups with and without relapses under different germinal center-like conditions.**

(**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.

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, i*n 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

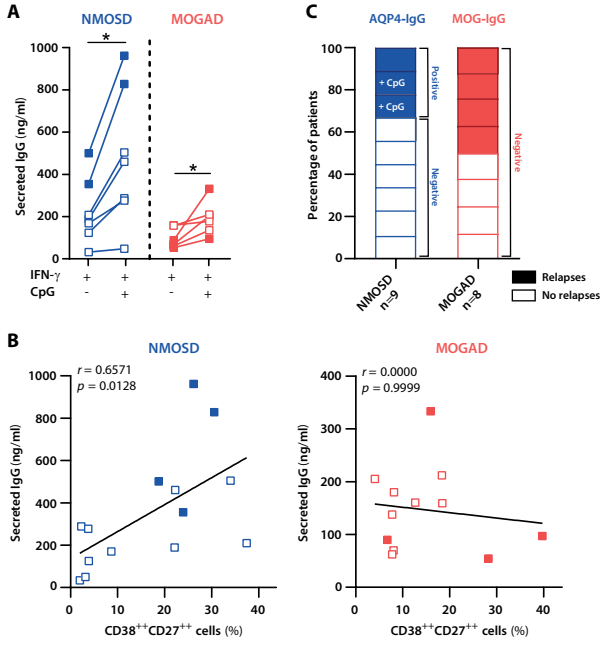

Figure 3. IgG secretion by *in vitro*-generated plasmablasts using naive mature B cells from subgroups with and without relapses.

(**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.

(Fig. 3C; ΔMFI, positive control: 7322). In this group, total IgG levels did not correlate with i*n vitro* plasmablast formation (Fig. 3B).

DISCUSSION

AQP4-IgG production by peripheral B cells is an important driver of NMOSD (Hauser *et al.*, 2008; Bar-Or *et al.*, 2010; Kim *et al.*, 2011). Since central B-cell tolerance mechanisms are defective in NMOSD (Kinnunen *et al.*, 2013; Cotzomi *et al.*, 2019), 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 (Cotzomi *et al.*, 2019). 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 effect of steroids on transitional B cells is probably explained by their lack of multidrug resistance receptor 1 (Wirths and Lanzavecchia, 2005), a glycoprotein that pumps a wide range of substances including corticosteroids out of the cell (Brinkmann and Eichelbaum, 2001). 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 (Kowarik *et al.*, 2017). 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 (Wilson *et al.*, 2018), and somatic hypermutation was shown to be essential for generating AQP4-specific antibodies (Cotzomi *et al.*, 2019). We did not find any increase in circulating memory B-cell subsets, including those lacking CD27 expression (Kowarik *et al.*, 2017). 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 (Chihara *et al.*, 2011; Kowarik *et al.*, 2017). 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 (Kyu *et al.*, 2009) 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 (Bennett *et al.*, 2015; Wilson *et al.*, 2018).

Whereas the ability of *ex vivo* plasmablasts to produce AQP4-IgG is highly controversial (Chihara *et a*l., 2011; Wilson *et al*., 2018), 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 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 (van Langelaar *et al.*, 2019). The same is true for naive mature B cells of NMOSD patients (this study). However, in contrast to MS patients (van Langelaar *et al.*, 2019), 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 (Cotzomi *et al*., 2019). 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 (Jackson *et al.*, 2014), but synergizes with IFN-γ to potentiate autoreactive $T\text{-}bet^{+}$ B cells (Rivera-Correa *et al.*, 2017). 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 (Arkatkar *et al*., 2017), leading to TLR9 upregulation (Carvalho *et al*., 2011). TLR ligation has been previously associated with the activation of transcription factor X-box binding protein 1 (XBP-1; Martinon *et al*., 2010), which enhances IgG secretion (Iwakoshi *et al.*, 2003)2003 and links to the IL-6-driven survival of AQP4-IgG-secreting plasmablasts (Chihara *et al*., 2011).

A limitation of this study is the relatively low sample size. The results obtained here need to be confirmed in longitudinal studies and in additional cohorts. Nonetheless, to our knowledge, this is the first study that assessed the phenotype and responsiveness of naive B cells of NMOSD patients without immunomodulatory treatment and compared these to MOGAD, MS and healthy control groups. The impact of steroids on transitional but not on

naive mature B cells could be a mechanistic explanation why NMOSD relapses recur after discontinuation of steroid treatment. Our findings also provide a rationale for exploring the underlying mechanisms of naive B-cell development in patients with active or stable disease. Further assessment of CpG-ODN-mediated outgrowth of naive B cells under germinal center-like, IFN-γ-containing *in vitro* conditions may be a new approach to predict NMOSD relapses.

ACKNOWLEDGMENTS

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COMPETING INTERESTS

The authors declare no competing interests.

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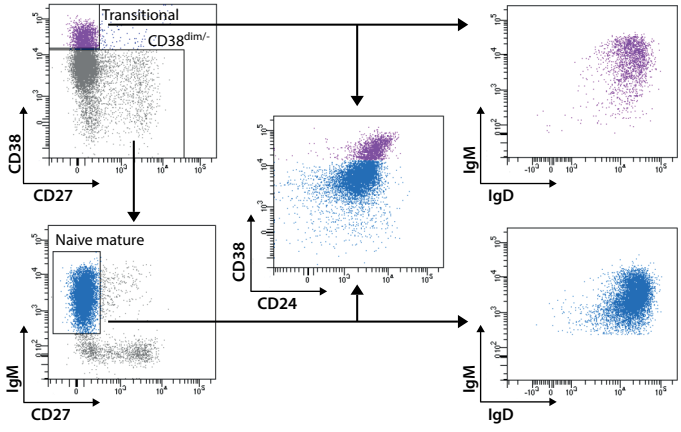
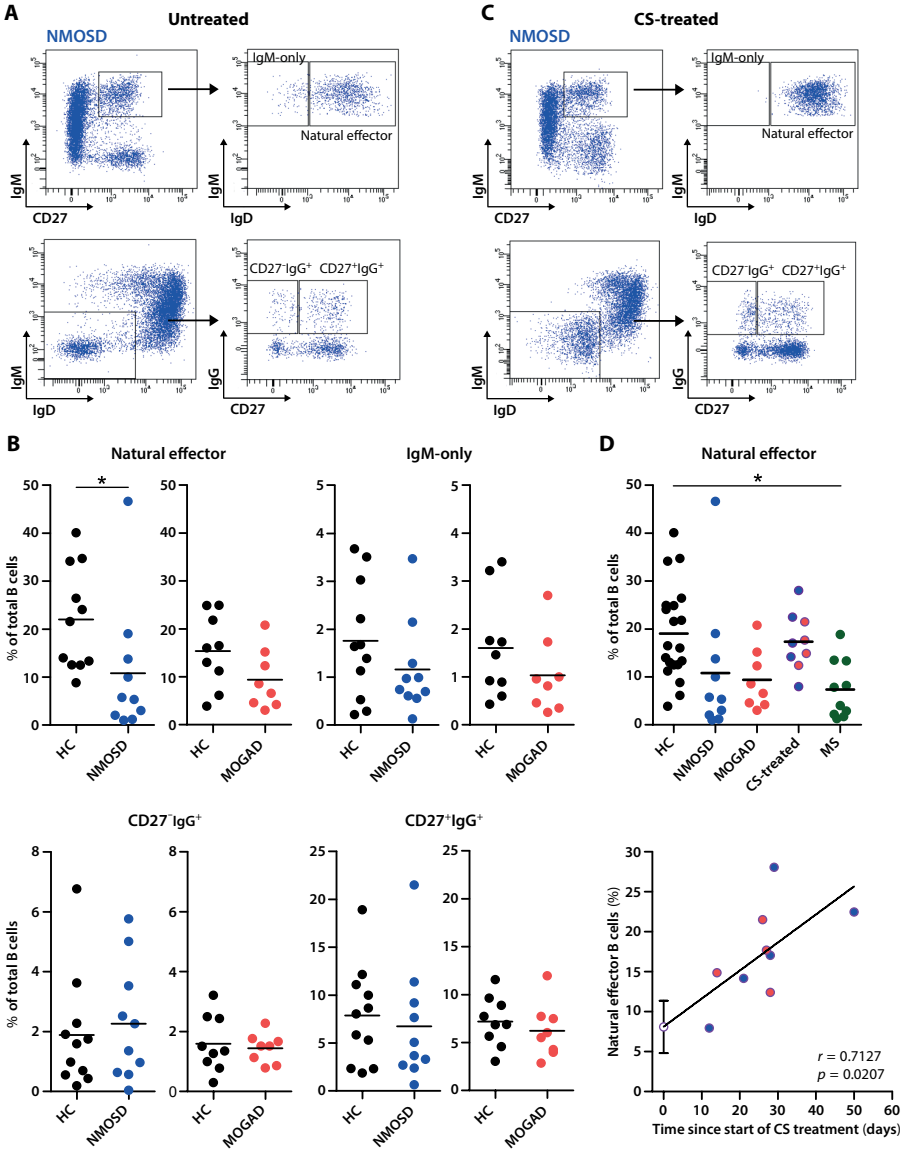
SUPPLEMENTAL DATA

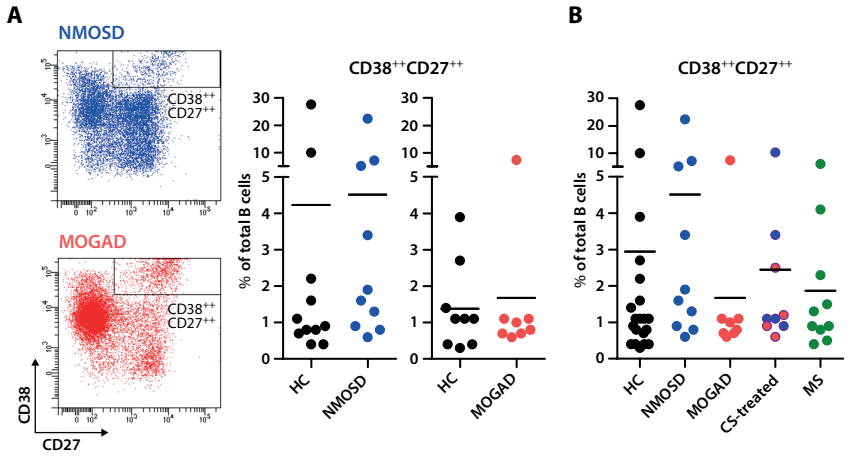


Supplementary Figure 1. Expression of CD24, IgD and IgM on transitional and naive mature B cells in the blood.



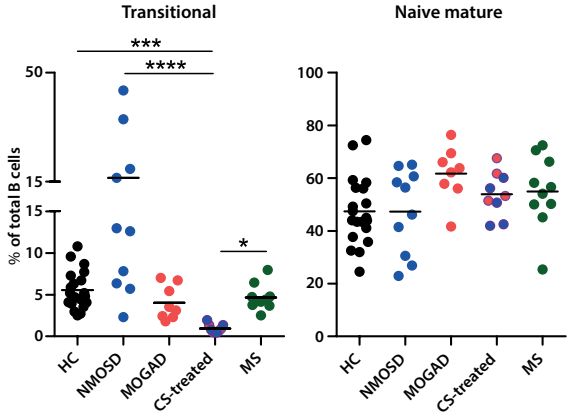
Supplementary Figure 2. Presence of *ex vivo* memory B-cell subsets in the blood of untreated and CS-treated NMOSD, MOGAD, MS and HC subgroups.

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.



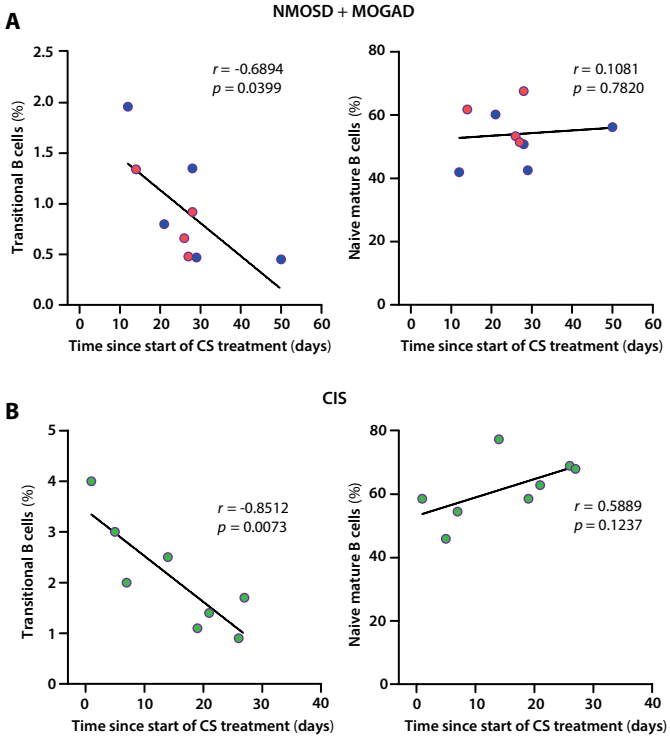
Supplementary Figure 3. Frequencies of *ex vivo* plasmablasts in the blood of untreated and CS-treated NMOSD, MOGAD, MS and HC subgroups.

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.



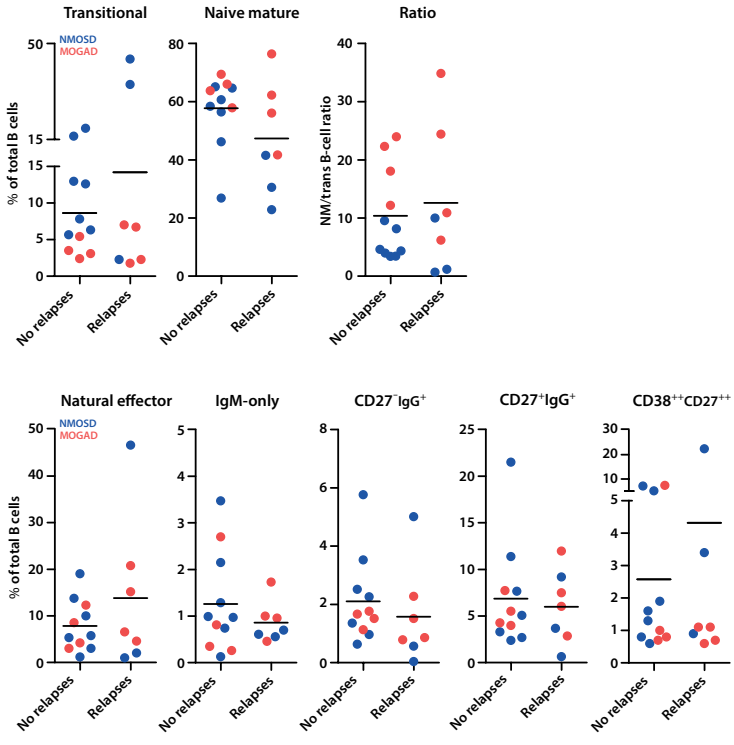
Supplementary Figure 4. Composition of the naive B-cell pool in the blood of untreated and CS-treated NMOSD, MOGAD, MS and HC subgroups.

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.



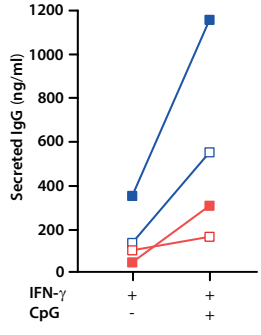
Supplementary Figure 5. The impact of CS treatment on the presence of transitional and naive mature B cells in the blood.

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

Discussion

DISCUSSION

Multiple sclerosis (MS), neuromyelitis optica spectrum disorders (NMOSD) and myelin oligodendrocyte glycoprotein Ig associated disease (MOGAD) are autoinflammatory diseases of the central nervous system (CNS) differing in incidence, pathogenesis and clinical features[1-5]. These diseases are mediated by pathogenic lymphocytes, which are triggered in the periphery and contribute to local inflammation and demyelination. This was summarized in **chapter 1**.

In this thesis, we assessed the antigen specificity, local recruitment and development of such lymphocytes to increase our understanding of the immunopathology of these diseases and find new ways to improve disease prediction and current treatment regimes. In this final chapter, the most important findings will be discussed and directions for future studies will be proposed.

Antigen-specific T-cell responses in MS

In **chapter 2**, we assessed antigen-specific $CD4^+$ and $CD8^+$ T-cell responses using cell lines generated from cerebrospinal fluid (CSF) of early-stage MS patients. We used autologous Epstein Barr virus (EBV)-immortalized B-lymphoblastoid cell lines (BLCL) as antigen presenting cell (APC)[6, 7]. BLCL were transfected using a plasmid vector containing open reading frames[8] of one of eight MS candidate autoantigens (MSAg), selected based on previous reports[9-19]. With use of this system, we showed that none of these $CD4^+$ or $CD8^+$ CSF T cell lines (TCL) showed substantial reactivity towards any of the selected oligodendrocyte (myelin associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP) and myelin basic protein(MBP)), glial (Kir4.1 (inwards rectifying potassium channel) and S100 calcium binding protein (S100B)) or neuronal (contactin-2 (CNTN2), neurofascin 155-kD isoform (NF155)) antigens. These results are in sharp contrast to studies on peripheral blood derived T-cell studies[9-19]. This inconsistency can be explained by methodological differences, amongst others mononuclear cell (MNC) source, culture characteristics, used autoantigens and read out systems. These confounders will be discussed hereafter.

Historically, the majority of research on T-cell antigen specificity in MS has been performed using peripheral blood mononuclear cells (PBMC). CSF contains immune cells specifically trafficking between brain parenchyma and the cervical lymph nodes[20-23]. Therefore, CSF MNC and PBMC differ in terms of phenotype and T cell receptor repertoire[24] and since MS pathology is confined to the CNS, antigen-specific T cells are likely to be enriched here[25]. The lack of antigen specific responses found in our study is in line with the report by Wuest et al, who also used CSF derived T cells and autologous dendritic cells pulsed with CNS antigens as APC[26]. In contrast to Wuest et al, we generated short lived TCL[27] in order to perform repeated analysis and validation experiments. Since in subjects with chronic immune responses like

MS, T cell exhaustion may occur[28, 29] possibly causing selective outgrowth of our TCL and obliterating relevant responses. In contrast, techniques have been developed to facilitate prediction of T cell antigen-specificity on single cell level. For instance, by sequencing individual TCR bound to labeled major histocompatibility complex (MHC)-tetramers with known epitopes. Combining these TCR sequences and correlating them to their specificity will eventually enable prediction of T cell antigen-specificities based on TCR sequence[30]. Even more recently, an algorithm has been developed also including mRNA and protein levels in combination with CDR3 sequence[31]. Future studies might use these techniques to determine antigen specificities for individual brain infiltrating T cells in MS directly *ex vivo* to circumvent culture induced alterations.

An important issue to consider when attempting to prevent culture induced alterations is the presence of post-translational modifications[32]. In our study, BLCL expressed full length proteins, enabling post-translational modification by autologous APC. Alternatively, post-translational modifications are present in studies using brain derived proteins for antigenic stimulation[10, 11]. However, the use of brain-derived proteins as antigen requires highly pure protein fractions to prevent xenoreactivity against contaminants, leaving the possibility that these observed responses were false positive. When using peptides, post-translational modifications are absent, possibly skewing T cell responses. In order to compare T cell responses to different peptide levels with our MSAg BLCL-APC system, we used a measles virus fusion (MVF) protein specific TCL and MVF-nucleofected BLCL. Our MVF-BLCL induced similar levels of T cell responses compared to MVF antigen concentrations of 1 µM. This is in contrast to most studies using often high, non-physiological concentrations (25-250 µM)[33] of peptides to stimulate T cells, increasing the likelihood low-avidity TCR responses[15, 25, 33-35]. Cells carrying these TCR are likely not to be activated in physiological circumstances. Different types of peptides can be used as well; some studies used synthetic overlapping peptides[9, 12-17] or *in silico* predicted human leukocyte antigen (HLA) class I or class II binding peptides[14, 36, 37]. Selecting peptides based on prediction models inevitably introduces a bias for the selected HLA haplotypes. The same is true for tetramers[38, 39]. This can result in HLA-haplotype-associated skewing of antigen-specific responses[40-44]. In our study, we used autologous EBV-BLCLs circumventing HLA-haplotype selection. The capacity of BLCL-APC system to detect autoantigen-specific responses, if present, has been shown in type 1 diabetes[45-47]. In light of the limitations associated with protein or peptide usage for detecting antigen specific responses, the use of our autologous APC system with endogenous expression of MSAg to trigger both antigen-specific $CD4^+$ and $CD8^+$ T cell responses is preferable.

To detect antigen specific responses, we used intracellular cytokine staining. In our CSF $CD4^+$ and $CD8^+$ TCL, we only stained for IFN-γ. This cytokine was chosen since both $CD4^+$ (Th1) and $CD8^+$ T cells have been reported to produce this in high levels[48, 49]. Furthermore, most studies described earlier used this cytokine for readout, so validating the BLCL-APC

system using this cytokine was preferable. However, single staining for IFN-γ omits detection of other cytokines. For instance, granulocyte-macrophage colony-stimulating factor (GM-CSF) has been reported to be an important contributor to MS immunopathology[50, 51] and IFN- γ coproduction has been shown to be important in MS in **chapter 3**. Future studies are recommended to include antibodies targeting multiple cytokines when studying MS antigen specific T cell responses.

Undeniably, the candidate antigens included in our screening panel affect outcome. Since publication of our article, some antigens have been shown to be the target of humoral responses (which necessitates $CD4^+$ T cell help) in MS patients, including Kir4.1[52] , neurofascin[53] and PLP[54]. In contrast, S100B has recently been shown to be a more relevant biomarker in NMOSD compared to MS[55] and a recent report states that MOG is not associated with adult MS[56]. New candidate antigens have been proposed since our paper has been published. For instance, $CD4^+$ autoreactive T cells towards self-peptides (mainly HLA-DR2a and DR2b derived)[43] have been described in patients carrying MS risk haplotype HLA-DR15. Interestingly, these $CD4^+$ cells can cross react with EBV and bacterium *Akkermansia*, found in gut microbiome previously shown to be enriched in MS patients[57] and a target of MS oligoclonal band (OCB) IgG[58]. Also in patients without the HLA-DR15-haplotype, autoreactive $CD4^+$ T cells with cross reactivity towards gut microbiome peptides have been shown to infiltrate the CNS[59]. These results provide the possibility that cross reactivity contributes to previously described autoimmune T cell responses in MS. In a follow up study using the BLCL-APC system and MS associated antigen panel, T cells obtained from white matter lesions end-stage MS donors did not show CNS antigen-specific responses[60]. Interestingly, a strong reactivity of $CD8^+$ T cells towards non-transfected BLCL was detected, suggesting a local EBV-specific response in MS lesions.

For future studies after antigen specificity of T cells in MS, it would be of interest to include T cells obtain from leptomeningeal follicles, structures often present in MS (and discussed later in this chapter)[61, 62]. Furthermore, TCR derived from brain infiltrating T cells can be analyzed and cloned into TCR-null T cells to perform antigen-discovery experiments. In conclusion, auto-reactive T cells are likely to be involved in MS pathology, although the exact target of these cells remains enigmatic.

Highlights

- Autologous BLCL are suitable as antigen presenting cell for studying reactivity of both $CD4^+$ and $CD8^+$ T cells to pre-defined candidate antigens
- No significant antigen-specific T cell response was detected against a panel of eight candidate MS-associated auto-antigens

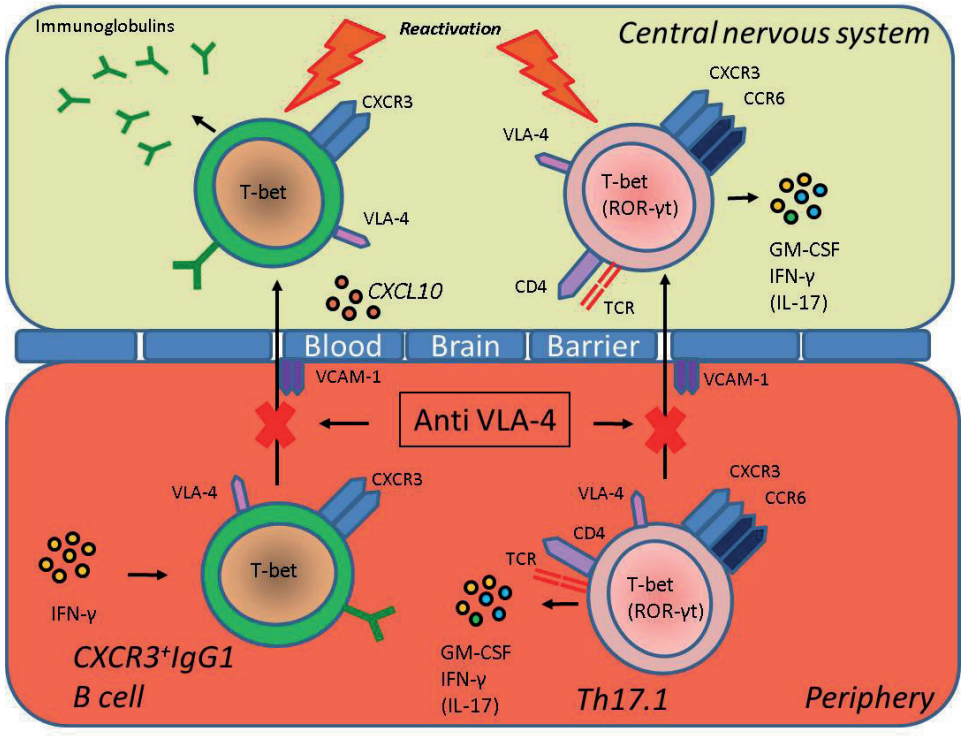


Figure 1. Model of distinct CNS-homing, T-bet-expressing B and T cell subsets in early MS.

CXCR3+IgG(1)$^+$ B cells and Th17.1 cells migrate across the blood brain barrier with use of adhesion molecules, including VLA-4/VCAM-1 interaction among others. VCAM-1 is enriched on endothelial cells of brain vessels under inflammatory conditions. CXCL10 (cognate ligand for CXCR3) is upregulated in the inflamed CNS, which mediates the local attraction of these types of pathogenic lymphocytes. Classical Th17 do not express CXCR3 and are therefore not recruited by MS specific upregulation of this chemokine. In MS patients treated with natalizumab, we found that both subsets selectively accumulate in the peripheral blood. Their accumulation, re-activation and proliferation within the CNS likely contributes to local inflammation and demyelination.

Recruitment of pathogenic T and B-cell subsets to the MS central nervous system

Th1-like Th17 cells

In **chapter 3**, we investigated the frequency and distribution of IFN-γ, IL-17A and GM-CSF producing CD4$^+$ T cell subsets in patients with CIS and MS. IL-17 producing CD4$^+$ T cells (Th17) are traditionally characterized by expression of the ROR-γt transcription factor in combination with C-C motif chemokine receptor CCR6, whereas Th1 cells express C-X-C chemokine receptor CXCR3 via transcription factor T-bet[63, 64]. In peripheral blood of early onset CIS and RRMS patients, we observed lower frequencies of highly activated (HLA-DR$^+$CD38$^+$) CD4$^+$ T cells expressing both CXCR3 and CCR6 as well as T-bet and ROR-γt. CXCR3 and CCR6 facilitate

different migration routes into the CNS[65]. CCR6 expression primarily enables migration across the choroid plexus[66], whereas CXCR3 migration is initiated towards its ligand C-X-C motif ligand CXCL10, that is highly produced in the MS brain parenchyma and CSF[67-69]. T-bet knock-out animals were shown to be resistant for EAE induction due to downregulation of IFN-γ and upregulation of regulatory IL-10[70]. EAE was aggravated in mice deficient for STAT1, a signaling molecule upstream of T-bet[71].

In our study, IFN-γ was co-expressed with GM-CSF and less so with IL-17 in $CCR6^{+}CXCR3^{+}$ Th cells, which is in sharp contrast to mice[72]. GM-CSF can infiltrate the CNS via diffusion and active transport[73] and is able to attract lymphocytes from the circulation[74]. Furthermore, both GM-CSF and IFN-γ contribute to breakdown of the blood brain barrier (BBB) by inducing local immune responses[75, 76] and modifying BBB tight junctions[77] respectively. BBB disruption is important in the early phase of MS since it facilitates infiltration of pathogenic immune subsets[78, 79]. Consistent with this, blocking of GM-CSF has been shown to prevent EAE disease onset in mice[80].

In line with their reduced proportions in the peripheral blood, short-term T cell cultures of CSF mononuclear cells from patients with early MS contained significantly more Th1-like Th17 cells (**chapter 3**). Furthermore, in these cultures, effector to central memory (EM/CM) ratios were significantly higher for Th1-like Th17 compared to Th1 and Th17 cells. After PMA/ionomycin stimulation, CSF Th1-like Th17 cells produced more IFN-γ and GM-CSF compared to their counterparts in the peripheral blood, confirming the high pro-inflammatory capacity of this brain-homing subset. Their local enrichment was confirmed by *ex vivo* analysis of late-stage MS CSF and brain tissues. The proportion of Th1-like Th17 cells was also increased in brain tissues from controls but to a much lower extent. Under inflammatory conditions, all memory $CD4^{+}$ T-cell subsets are able to migrate across the BBB[81]. In contrast, in non-inflammatory conditions, $CXCR3^{+}$ T cells (including Th1-like Th17 cells) show an increased migratory potential[81]. Of all the memory $CD4^{+}$ T cells analyzed in our study, Th1-like Th17 cells and in particular the $CXCR4^{-}$ Th17.1 (discussed later in more detail) preferentially crossed non-inflamed hBEC monolayers *in vitro* even without the addition of CXCL10. Since CXCL10 is not expressed in MS normal appearing white matter or healthy control brain tissue[69], it is tempting to speculate that this subset triggers MS disease onset.

Based on our analyses of *ex vivo* blood from natalizumab-treated patients, in which leukocyte transmigration into the CNS is impaired, and paired blood and CSF samples of treatment-naive patients, we confirmed that mainly Th17.1 cells are locally enriched in the early phase of MS. For these analyses, we included CCR4 as a third marker to subdivide Th1-like Th17 cells into Th17.1 ($CCR4^{-/dim}$; IFN-γ^{high}GM-CSF^{high}IL-17^{low}) and Th17 DP ($CCR4^{+}$; IFN-γ^{low}GM-CSF^{low}IL-17^{int}) subsets. In contrast to non-responders to natalizumab (demonstrated by the ongoing clinical relapses), Th17.1 and not Th17 or Th17 DP cells selectively accumulated in the blood of patients who responded to natalizumab treatment. This suggests that the

blockade of this brain-homing subset contributes to the beneficial therapeutic effects. Due to the presence of anti-natalizumab antibodies in unresponsive patients[82], it would be worthwhile identifying mechanisms by which migration of Th17.1 cells into the CNS can be blocked in other ways. Recent work postulates that natalizumab resistance may be caused by certain single nucleotide polymorphisms[83], but further research providing immunological support for this statement is required.

The selective increase of Th17.1 cells in the blood of clinical responders could explain partly results of a recent study, in which Th17.1 cells were moderately enriched in the twins with MS compared to non-affected twins[84]. Of the 43 patients included in this study, 21 used immune modulatory drugs, including natalizumab. However, the details on which twins where using which particular drugs were not available. A follow-up study including treatment-naive monozygotic MS discordant twins will provide more direct insights in the effects of genetic and epigenetic factors on Th17.1 and other T-cell subsets. The pathogenicity of Th17.1 cells has been shown in other autoimmune and neurological diseases as well, including myasthenia gravis[85], sarcoidosis[86], systemic lupus erythematosus[87] and Graves' disease[88]. In patients with neurodegenerative Huntington's disease, Th17.1 cells seemed to be associated with the initiation and not progression of the disease, since this subset was enriched in the CSF of patients with preceding onset of clinical disease[89]. More insights into the induction and activation of Th17.1 cells are warranted to understand how this pathogenic subset contributes to CNS immunopathology. One important aspect may be the fraction of follicular T helper cells, which have recently been reported to be enriched in MS CSF[90]. Follicular T cells not only get signals from B cells, but also trigger germinal center (GC) B cells to develop into memory and antibody-secreting cells. In MS patients, pathogenic T- and B-cell subsets likely interact both in the periphery and the CNS to mediate the pathogenesis.

CXCR3$^+$ memory B cells

In **chapter 4,** the frequencies and brain-infiltrating capacity of CXCR3$^+$ B cells in MS patients were assessed. Particularly in mice, this B-cell population is known to be highly pathogenic and involved in other models of CNS inflammation and autoimmunity[91]. In accordance with Th17.1 cells in **chapter 3**, the proportion of IgG$^+$CXCR3$^+$ B cells in peripheral blood was significantly reduced in treatment-naive MS patients compared to age-and-gender matched HC. When looking in deceased end-stage MS patients, CXCR3$^+$ B cells were reduced in the peripheral blood and enriched in CSF, leptomeninges and brain parenchyma. CXCR3$^+$ IgG$^+$ B cells accumulated in the blood of MS patients treated with natalizumab, supporting the fact that memory subsets enter the CNS in a VLA-4-dependent manner[92]. Interestingly, the proportions of IgG$^+$CXCR3$^+$ B cells positively correlated to those of Th17.1 and not Th1 or Th17 cells. The accumulating CXCR3$^+$ memory B-cell populations in natalizumab-treated patients mainly expressed IgG1, a T cell- dependent Ig subclass that is predominantly found in CSF

oligoclonal bands (OCB)[93, 94]. This corresponded to the expression of T-bet, which induces IgG2a (the murine equivalent of human IgG1) switching in murine B cells[95-97]. When assessed *in vitro*, CXCR3^{high} IgG1^{+}B cells preferentially crossed confluent monolayers of human brain endothelial cells towards CXCL10 as compared to IgM^{+} and IgG2^{+} memory B cells. The disease-contributing role of IgG1 and T-bet expressing B cells has been shown in other autoimmune diseases, for instance in Sjogren's syndrome and systemic lupus erythematosus[98, 99]. The selective recruitment of CXCR3^{+} B cells or plasmablasts towards the diseases sites has been shown previously in NMOSD patients[100] and in a viral encephalomyelitis model[91].

IFN-γ has been shown earlier to induce CXCR3 expression on B cells[101]. We assessed the effect of IFN-γ on T-bet and CXCR3 expression by peripheral blood B cells of MS patients and healthy controls *in vitro* in T_{FH}-like cultures to assess GC responses i*n vitro*. IFN-γ upregulated T-bet and consecutive T-bet mediated CXCR3 expression in MS B cells to a larger extent compared to HC B cells. No enhanced T-bet or CXCR3 expression was seen in absence of IFN-γ. To analyze the impact of IFN-γ on the antigen-presenting function of B cells, we performed autologous T-cell co-cultures with B cells taking up Th1-associated Salmonella typhimurium bacteria via surface IgG. This crosslinking-based method was reported earlier to induce BCR-mediated uptake and antigen presentation[102]. During co-cultures with and without IFN-γ blocking antibody, we showed that IFN-γ production by autologous Th cells upregulates CXCR3 on IgG^{+} B cells. Proliferation and activation of co-cultured autologous T cells was reduced after IFN-γ blockade. These in vitro findings are in line with the described crucial role of T-bet^{+} B cells in the animal model autoimmune responses in systemic lupus erythematosus[103], facilitating production of autoantibodies and GC generation. Another study reported that IFN-γ mediates GC-dependent development of autoreactive B cells in mice[104]. We observed an increase in plasmablast differentiation *in vitro* after addition of IFN-γ to culture medium.

The effects of IFN-γ on B cells can be influenced by Toll-like receptor (TLR) stimulation[105, 106]. TLR9 recognizes unmethylated CpG repeats in DNA sequences of pathogens and TLR9 triggering has been shown to induce T-bet expression in B cells[107] and enhance regulatory functions in the absence of IFN-γ[108, 109]. In naive, and not memory, MS B cells CpG-ODN synergized with IFN-γ to further enhance T-bet expression under T_{FH}-like culture conditions (Fig. 4). In addition, this TLR9 triggering blocked plasmablast development, but induced IgG1^{+} memory formation. This implies that both stimuli contribute to the formation of IgG1^{+} memory B cells. The high sensitivity of B cells from MS patients to IFN-γ and CpG110, probably facilitates migration of CXCR3^{high}IgG^{+} subsets into the CNS. CXCR3^{high}IgG^{+} B cell possibly migrate into the leptomeningeal follicle-like structures that have been reported frequently in MS patients[61, 111, 112]. Recent reports have shown that their presence correlates with demyelination, both subpial[113, 114] and in the spinal cord[115]. Furthermore, our meningeal samples of end-stage MS patients contained significant numbers of CXCR3^{+} B cells. T-bet^{+} B cells have

recently been described to be present in follicle like structures in kidney of SLE mice[103] and patients[116]. Once IgG1$^+$CXCR3$^+$ memory B cells have crossed the BBB, local re-activation can be sufficient to induce development of tissue-resident antibody secreting cells (ASC) in MS chronically inflamed CNS[117]. Future research is warranted to assess the antigenic target of these ASC. A possible target of local immune responses in the MS CNS might be EBV suggested by a recent report on EBNA1- and EBNA2-specific IgG derived from MS brain[118] and the observation that memory B cells with high EBV load show increased expression of CXCR3, facilitating CNS homing[119].

CXCL10 in MS

Both CXCR3$^+$ cell types discussed here, Th17.1 cells and IgG1-T-bet$^+$ B cells, preferentially migrate towards CXCL10 in our *in vitro* experiments. It is tempting to speculate this, at least partly, contributes to the correlation of CXCL10 levels with MS disease course. An alternative name for CXCL10 is interferon gamma-induced protein 10[67, 120]. However, it can also be produced upon TNF-α stimulation or after exposure to proteins from bacteria or virusses[121, 122]. CXCL10 can be produced by various cell types, including lymphocytes, fibroblasts and endothelial cells. In MS patients, various cell types have been shown to produce higher levels of CXCL10 after IFN-γ and LPS stimulation compared to controls[123]. Like mentioned before, CXCL10 is abundant in MS CNS[67-69]. CXCL10 has been shown to be important for microglial activation in an EAE model[124] and oligodendrocytes in MS brain express more CXCL10-receptor CXCR3 compared to oligodendrocytes from control brain[125]. In viral models, IFN-γ produced in the CNS induces CXCL10, attracting CXCR3$^+$ T cells and damaging tight-junctions of the BBB, creating a feedforward loop with ongoing infiltration of pathogenic lymphocytes[126, 127]. In MS, similar mechanisms could occur, further supported by the significant decrease of CSF CXCL10 levels in patients that have been successfully treated with natalizumab[128], potentially hampering this cascade. GM-CSF production, for instance by IFN-γ and GM-CSF co-producing Th17.1 cells, might further damage the BBB[74]. In light of correlations of clinical MS course with CXCL10 levels, preventing CXCR3$^+$ lymphocytes from entering the CNS by hampering CXCL10 mediated migration is therefore an interesting target for MS therapy. It has been reported that interferon-β increases peripheral levels of CXCL10[129], thereby likely suppressing the gradient-mediated migration of CXCR3$^+$ cells. Furthermore, nano-antibodies have been developed recently to selectively block CXCL10[130]. Future studies analyzing effect of these treatments on selective migration of Th17.1 and IgG1CXCR3$^+$ B cells are warranted to investigate whether this can reduce disease activity, for instance in patients not responding to natalizumab treatment.

Main findings

- Out of all memory CD4+ T- and B-cell subsets analyzed, Th17.1 and CXCR3^{+} B cells are selectively recruited to the CNS of MS patients
- The increased brain-homing capacity of both subsets is confirmed in patients treated with natalizumab and *in vitro* using human brain endothelial monolayers
- IFN-γ is highly produced by Th17.1 cells and synergizes with TLR9 ligand CpG-ODN to induce CXCR3 expression and class switching in differentiating human B cells

GC-like differentiation of B cells in MS: impact of pregnancy and comparison to AQP4-IgG^{+} NMOSD and MOGAD

The final part of this thesis was devoted to development of pathogenic B cells and plasmablasts in neuro immunological disease. For this, we studied differentiation of naive B cells after entrance of the germinal center reaction in high- and low relapse risk MS as well as in the other demyelinating neuro immunological disorders AQP4-IgG^{+} NMOSD and MOGAD.

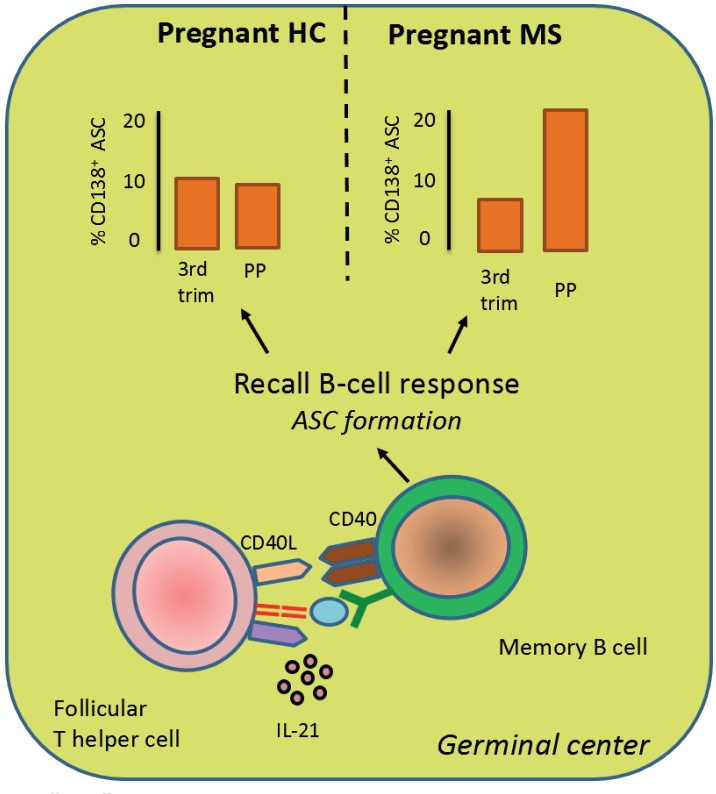


Figure 2. ***In vitro*** **recall B cell response.**

T_{FH}-like conditions with CD40L and IL-21 using sorted memory B cells from 3rd trimester and early postpartum from MS patients and HC.

MS relapse risk is reduced during pregnancy and increases again early after delivery[131, 132], resulting in a low-relapse risk 3rd trimester and a high relapse risk post-partum. In **chapter 5,** we find clues that the capacity of third trimester memory B cells to infiltrate the CNS and differentiate into ASC is attenuated, thereby potentially contributing to the low relapse risk during this period.

First, we assessed *ex vivo* B cell subset distribution in different pregnancy stages. We observed reduced proportions of transitional B cells during pregnancy, most pronounced in 3rd trimester, replicating earlier reports[133]. This reduction was seen in both MS patients and matched controls, rendering this a generic effect of pregnancy for instance mediated by a generic pregnancy-associated factor, such as increased circulating hormone levels[117]. This reduction might result in reduced numbers of autoreactive naive mature B cells. In normal situations in MS, primary B cell responses give rise to memory B cells, that can infiltrate brain parenchyma with use of the right receptors or integrins. It has been hypothesized that after local reactivation and recall B cell response, these cells develop into tissue resident ASC[79]. However, chemokine receptors also contribute to B cell localization in the GC[134]. In our *ex vivo* analysis, B cells that were obtained from 3rd trimester MS patients were likely to express chemokine receptors that retained cells into the GC-dark zones, thereby preventing development of GC-dependent memory B cells. In contrast, in postpartum B cells we observed reduced expression of CXCR4, enhanced expression of CXCR5[134] on IgM and IgG memory B cells as well as naive mature B cells facilitating migration to GC-light zones. The upregulation of CXCR5 expression was only significant in MS. Postpartum B cells expressed more CCR6, associated with memory recall response[135] and CXCR3, contributing to antibody responses[136] respectively. CCR6 expression was enhanced more strongly on MS B cells. Together, chemokine receptor expression of postpartum B cells was $CXCR3^{high}CXCR4^{low}CXCR5^{high}CCR6^{high}$, facilitating T_{FH} interaction in GC-light zones and memory B cell development. Immunoglobulin expression was altered by pregnancy as well. IgM was significantly higher on postpartum translational B cells, most pronounced in MS. *Ex vivo* expression of surface Ig was increased postpartum for both IgM and IgG memory cells. IgM^{high} memory B cells primarily originate from GC[137], again suggesting preferential GC-dependent memory cell formation postpartum.

In our in T_{FH}-like cultures, we found that memory B cells from postpartum blood samples of MS patients were more prone to differentiate into plasma cells. This resulted in increased production of IgM and IgG, which was not different between patients and controls. This similarity seems counterintuitive due to increased numbers of ASC in MS. However, it might be possible that affinity of MS antibodies was higher, due to altered chemokine receptor expression profile and increased CD138 expression. Future studies confirming this are warranted. Local antibody deposition is a pathological characteristic of MS white matter lesions[138], and a recent report showed that intrathecal IgG complexes may break human microglial tolerance[139] causing production of pro-inflammatory cytokines and subsequent lymphocyte

CNS recruitment. The relevance of IgGs for MS is further highlighted by the high proportion (95%) of patients with oligoclonal bands at diagnosis[140], which associates with lesional B cell infiltration and activity in autopsy studies[141]. Patients without OCB have in general less brain atrophy[142] and disability[143]. Hence, the here described alterations in chemokine receptor expression on memory B cells may prevent local ASC formation and therefore contribute to the reduced relapse occurrence during pregnancy.

Before entering the GC for further maturation, autoreactive B-cell clones are negatively selected during central and peripheral tolerance checkpoints. In MS, only the peripheral checkpoints are defective[144], whereas in NMOSD both central and peripheral tolerance checkpoints are affected[145, 146]. Whether these checkpoints are impaired and contribute to the pathogenesis in patients with MOGAD is currently unknown. In **chapter 6** we studied the naïve B cells and germinal center responses in NMOSD and MOGAD patients (Fig. 3). In treatment-naive NMOSD patients, transitional B cells were significantly upregulated.

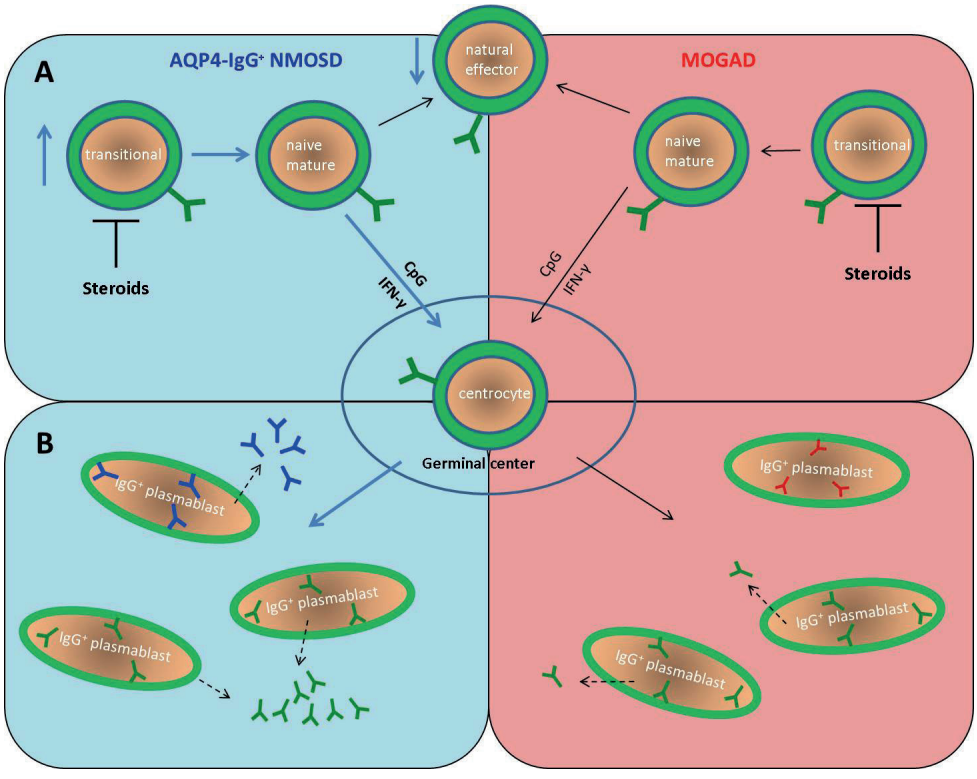


Figure 3. Representation of naive B cell to plasmablast differentiation in AQP4-IgG+ NMOSD and MOGAD patients with relapsing disease.

A) In the blood of AQP4-IgG+ NMOSD patients, transitional B cells show increased frequencies and are targeted by steroid treatment. **B)** TFH-like cultures, mimicking in vivo GC-reactions, induce plasmablast outgrowth and Ig production.

Interestingly, this upregulation seemed associated with disease activity, since it was most pronounced in patients experiencing a relapse close to cell sampling. It is very likely that the central checkpoint deficits in NMOSD results in increased release of autoreactive transitional B cells into the circulation[146]. Since B cell depleting CD20 antibody rituximab was recently shown to be effective in preventing NMOSD relapses[147, 148] and transitional B cells are the first to recover[149], studies should focus on analyzing the long term effect of this treatment in NMOSD. In patients with MOGAD, the proportion of transitional B cells was not increased, which may imply that central tolerance checkpoints are not affected. Of note, while pregnancy reduces MS disease activity (as discussed earlier), relapse risk has been reported to be aggravated in pregnant NMOSD patients[150, 151], although this more recently has been questioned due to lack of sufficient data[152]. This implies differential regulation of transitional B cells in the NMOSD peri-partum period. In NMOSD and MOGAD as well as CIS patients, steroid treatment reduced transitional B cell levels to a large extent. The lack of multi-drug resistant gene 1 (MDR1) in this subset probably renders cells more vulnerable for steroid-induced cell death[153, 154], explaining the treatment success of steroids in dampening acute disease activity as well as the frequent and quick relapses occurring after this treatment in NMOSD and MOGAD patients without maintenance therapy [155-157]. Interestingly, in the high-level steroid hormone carrying pregnant woman (**chapter 5**), low levels of transitional B cells were observed as well. In treatment-naive NMOSD patients, proportions of circulating GC-independent $CD27^+IgM^+IgD^+$ memory (natural effector) B cells were reduced compared to healthy controls and MOGAD patients, suggesting preferential GC-entrance of B cells. After steroid treatment, the frequencies of natural effector B cells returned to baseline levels. No differences were observed in ASC frequencies *ex vivo*, which may be explained by ASC homing to inflamed sites or bone marrow[2, 145]. Another reason may be the fact that we used frozen PBMC for our study. Future studies using non-frozen cells would be valuable, since freeze-thaw cycles affect plasmablasts[158] and, in other models like viral infections[159], such freezing effects can be different between patients and healthy individuals.

The earlier described T_{FH}-like culture system was used in NMOSD and MOGAD (**chapter 6**) and MS (**chapter 4**) patients to investigate primary B cell responses (Fig. 4). For inducing primary B cell responses, naive mature B cells were cultured in the presence of IL-21 and CD-40-3T3 cells. Additionally, 1/3 of cultures received IFN-γ and 1/3 received IFN-γ together with TLR9 triggering CpG-ODN. In HC and treatment-naive MS patients included in **chapter 4**, plasmablast outgrowth was induced upon IFN-γ stimulation under these T_{FH}-like conditions. In cultures with both IFN-γ and CpG-ODN, more IgG^+-memory B cells and less plasmablasts were induced. For naive mature B cells from NMOSD and MOGAD patients (**chapter 6**), the addition of CpG-ODN to IFN-γ-containing cultures yielded alternative effects; only in patients with relapsing disease, irrespective of serotype (MOG-/AQP4-IgG), IFN-γ and CpG synergized to enhance plasmablast formation outgrowth compared to those with IFN-γ

only. Plasmablast outgrowth was reduced upon CpG addition in patients with stable disease, which was in line with effects on HC and MS patient B cells as shown in **chapter 4** (Fig. 4). T-bet was upregulated by both IFN-γ and CpG-ODN in both relapsing and non-relapsing groups. In NMOSD, IFN-γ possibly induces IL-6 production by B cells[160], with consequent TLR9 upregulation[161]. IL-6 facilitates survival of plasmablasts[162, 163] and induces the expansion of circulating TFH cells in NMOSD[164], possibly creating a feedforward loop by further enabling B-cell differentiation into ASC. In this particular aspect, MOGAD seems to be more similar to NMOSD compared to MS, since increased ASC outgrowth upon CpG stimulation in patients with active disease is seen in this group as well. However, in our studies using MS B cells, samples were not separated based on disease activity. It might be interesting to separate MS patients with active and more stable disease and observe whether this synergistic effect is NMOSD/MOGAD specific or more associated with disease activity. Furthermore, it might be of additional value to compare sample B cells from NMOSD patients with and without

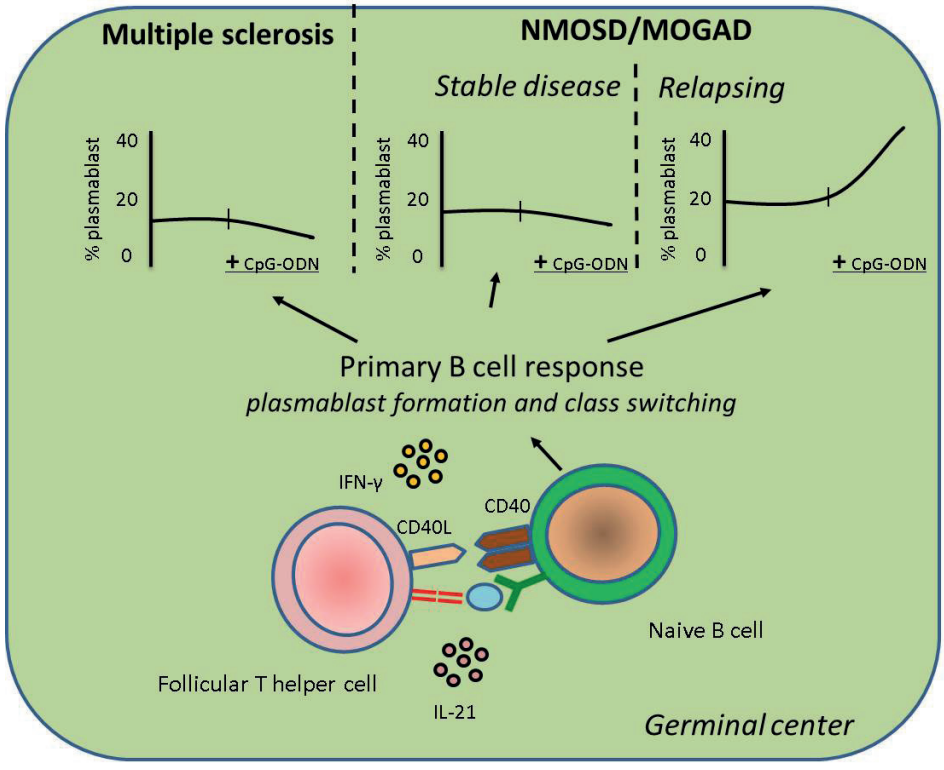


Figure 4. Investigating *in vitro* primary B cell responses in demyelinating disease.

T_{FH}-like conditions were simulated with CD40L and IL-21. IFN-γ and CpG was added to some cultures using sorted memory B cells from MS patients and NMOSD and MOGAD patients with stable and relapsing disease.

additional autoimmune disease, present in 20% of all patients[165], since this can affect cell characteristics, for instance T-bet induction[116], as well.

Antibody production by peripheral B cells is presumed to be an important driver of NMOSD[166-168]. The importance of IgG in NMOSD pathology is demonstrated by the correlation between antibody titers and disability[169] and the reduced AQP4-IgG serum levels after successful anti-CD20 therapy[170, 171]. Naive mature B cells from NMOSD patients gave rise to AQP4-IgG producing B cells from relapsing NMOSD in the most basic culture condition (+ CD40L and IL-21) even in absence of antigen (Fig. 3B). This supports the idea that AQP4 specific B cells can produce AQP4-IgG in the periphery that eventually cause pathology in the CNS[100, 162, 172, 173]. Cotzomi et al[146] reported somatic hypermutation, and therefore GC-reactions, are indispensable to generate functional (i.e. detected by the cell based assays in our study) AQP4 antibodies. In contrast, exposing naive mature B cells from patients with MOGAD to T_{FH}-like culture conditions did not result in disease-specific (i.e. MOG) IgGs.

Main findings

- In the early postpartum (high relapse risk) period, memory B cells of MS patients show a chemokine receptor expression pattern that reflects localization in the GC light zones and an increased capacity to develop into ASC
- Naive B-cell composition and outgrowth into ASC is associated with disease activity and AQP4-IgG production in patients with NMOSD

This thesis was devoted to development, antigen specificity and local recruitment of lymphocytes in CNS autoimmunity. Although many questions remain and need to be investigated, here we identified relevant T- and B-cell subsets and triggers that are involved in MS, NMOSD and MOGAD. Since we used primarily patient material it is hard to prove causation. Combining research in human samples with studies using animal models in which many factors can be controlled, will be valuable. New techniques based on information technology and mathematical models, might add even more. We argue that future research into the development and reactivity of the here discussed pathogenic subsets will result in valuable tools for improved prognostication and more targeted and personalized therapeutic approaches.

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Addendum

Abbreviations

Summary

Samenvatting

Dankwoord

Curriculum Vitae

PhD Portfolio

Publications

LIST OF ABBREVIATIONS

AID	autoimmune disease
APC	antigen presenting cell
APRIL	a proliferation inducing ligand
AQP4	aquaporin 4
ASC	antibody secreting cell
BBB	blood brain barrier
BCR	B cell receptor
BLCL	EBV transformed B cell
CCR/L	c-c motif chemokine receptor/ligand
CD	cluster of differentiation
CD40L	CD40 ligand
CDMS	clinically definite multiple sclerosis
CIS	clinically isolated syndrome
CM	central memory
cMSAg	candidate MS associated antigen
CNS	central nervous system
CpG-ODN	CpG oligodeoxynucleotides
CSF	cerebrospinal fluid
CTN2	contactin 2
CXCR/-L	CXC chemokine receptor/ligand
EAE	experimental autoimmune encephalomyelitis
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
ELISA	enzyme-linked immune sorbent assay
EM	effector memory
FACS	Fluorescence activated cell sorting
GC	germinal center
GM-CSF	granulocyte macrophage colony-stimulating factor
HC	healthy control
HEK	Human embryonic kidney cell
HLA	human leukocyte antigen
IFN	interferon
Ig	Immunoglobulin
IL	interleukin
KIR4.1	potassium inwardly-rectifying channel
MAG	myelin associated glycoprotein

MBP	myelin basic protein
MFI	mean/median fluorescence intensity
MHC	major histocompatibility complex
MNC	mononuclear cell
MOG	myelin oligodendrocyte glycoprotein
MOGAD	myelin oligodendrocyte glycoprotein IgG associated disease
MS	multiple sclerosis
MVF	measles virus fusion protein
NFASC	155 kd isoform of neurofascin
NMOSD	neuromyelitis optica spectrum disorders
OCB	oligoclonal bands
ORF	open reading frame
PB	plasmablast
PBMC	peripheral blood mononuclear cell
PC	plasma cell
PLP	proteolipid protein
PP	post-partum
PPMS	primary progressive MS
PRR	pattern recognition receptor
RRMS	relapsing remitting multiple sclerosis
S100B	S100 calcium binding protein B
SLE	systemic lupus erythematosus
T-bet	T-box transcription factor
TCC	T cell clone
TCL	T cell line
TCR	T cell receptor
Tfh	follicular T helper cell
Th	T helper cell
TLR	toll like receptor
VLA-4	very late antigen 4 (= integrin 4α1β; = CD49a)

SUMMARY

Multiple sclerosis (MS), neuromyelitis optica spectrum disorders (NMOSD) and myelin oligodendrocyte glycoprotein (MOG) antibody disease (MOGAD) are demyelinating diseases of the central nervous system (CNS). Although these diseases differ in clinical features, an aberrant response of lymphocytes is thought to be driving the pathogenesis of all three disorders. In this thesis, specificity, local recruitment and development of lymphocyte subsets involved in the pathogenesis of these diseases have been studied.

Chapter 1 outlined the current state of knowledge of MS, NMOSD and MOGAD epidemiology and risk factors. The similarities and differences between diseases are discussed as are the different views on pathogenesis which have changed significantly over the years.

In **Chapter 2**, we studied the antigen specificity of cerebrospinal fluid (CSF) derived T cell lines in MS. As antigen-presenting cells, we used autologous B cells immortalized with EBV and transfected with a selection of MS-associated antigens (MSAg; contactin-2, inward rectifying potassium channel protein, myelin associated glycoprotein, myelin basic protein, myelin oligodendrocyte glycoprotein, neurofascin, proteolipid protein, protein S100B). This physiologically advantageous model was shown to be useful for triggering both antigen-specific $CD4^+$ and $CD8^+$ T cells. However, no significant responses were detected against the MSAg of choice.

In **chapter 3,** we found that a functionally distinct Th1-like Th17 subset termed Th17.1 was selectively associated with early disease activity in MS patients. This IFN-γ^{high}GM-CSF^{high}IL-17^{low} CD4+ T cell subset was less present in the peripheral blood of patients who rapidly develop MS, enriched in MS CSF and brain tissues and highly capable of migrating across confluent human brain endothelial monolayers *in vitro*. Interestingly, in RRMS patients treated with natalizumab, only Th17.1 cells accumulated in the blood of clinical responders. This was not seen in non-responders, supporting the role of this brain-homing T-cell subset in inducing MS disease activity.

Regarding B cells, we showed in **Chapter 4** that IFN-γ-related $CXCR3^+$ populations were significantly enriched in MS CSF, meninges and brain compared to paired blood. The brain-infiltrating capacity of $CXCR3^+$ B cells was underlined both *in vitro* and *ex vivo* using the same transmigration systems and blood samples from natalizumab-treated patients as in Chapter 3. During T_{FH}-like cultures with naive B-cells IFN-γ mainly triggered plasmablast formation, while the addition of both IFN-γ and Toll like receptor (TLR) 9 ligand (CpG-ODN) resulted in switching to IgG1.

Chapter 5 addressed B-cell differentiation in pregnant MS patients and healthy controls in the context of relapse risk. When comparing B cells between the high relapse risk period (4-8 weeks after delivery) and low relapse risk period (third trimester of pregnancy), memory B cells showed an altered chemokine receptor expression profile related to increased

migration into the CNS and preferential localization in the T_{FH} cell-containing germinal center light zone. In addition, we found that postpartum memory B cells are more able to differentiate into plasma cells in a T_{FH}-dependent manner *in vitro*, which may contribute to their final maturation in the CNS.

In **Chapter 6** we observed high frequencies of transitional B cells in treatment naive patients with NMOSD compared to patients with MOGAD, MS and healthy controls. These high frequencies were most prominent in patient samples obtained close to relapse. Steroid treatment reduced proportions of transitional B cells in NMOSD, MOGAD and MS. *In vitro*, germinal center-like cultures containing IFN-γ induced plasmablast outgrowth. However, only in relapsing NMOSD and MOGAD patients, CpG-ODN synergized with IFN-γ to further enhance plasmablast outgrowth. In patients with stable disease, addition of CpG-ODN to culture medium reduced plasmablast proportions. AQP4 specific IgG could be found in the supernatant of patients with relapsing NMOSD. MOG specific IgG was not found. In conclusion, naive B cell homeostasis is altered in NMOSD and affected by corticosteroids.

Chapter 7 summarized results, compared outcomes between different studies and provided suggestions for future studies.

SAMENVATTING

Multiple sclerosis (MS), neuromyelitis optica spectrum disorders (NMOSD) en myeline oligodendrocyt glycoproteïne (MOG) antistof geassocieerde ziekte (MOGAD) zijn demyeliniserende ziekten van het centrale zenuwstelsel (CZS). Hoewel deze ziekte verschillen in pathologie, incidentie en patiëntkarakteristieken, hebben ze gemeenschappelijk dat er een afwijkende reactie van lymfocyten ten grondslag ligt aan de immunopathologie van de ziekten. In dit proefschrift is gekeken naar specificiteit, lokale verrijking en ontwikkeling van verschillende groepen lymfocyten die zijn betrokken bij de pathogenese van deze drie ziekten.

Hoofdstuk 1 introduceert de huidige stand van zaken van de wetenschap omtrent MS, NMOSD en MOGAD en de betrokken lymfocyten. Gelijkenissen en verschillen tussen deze ziekten worden behandeld alsook de veranderende kijk op de pathogenese door de jaren heen, aangezien daar de nodige wijzigingen in zijn opgetreden.

Hoofdstuk 2 kijkt naar de antigeen specificiteit van T cel lijnen die zijn gehaald uit hersenvocht van patiënten met MS. Om dit goed te kunnen bestuderen, is er gebruik gemaakt van autologe B cellen die onsterfelijk gemaakt zijn middels EBV infectie en via transductie één van een geselecteerd panel van kandidaat antigeen tot expressie brengen (contactin-2, kaliumkanaaleiwit, myeline geassocieerd glycoproteïne, myeline basiseiwit, myeline oligodendrocyt glycoproteïne, neurofascine, proteolipide eiwit en S100B eiwit). Deze manier van antigeen presentatie maakte het uitlokken van antigeen specifieke responsen bij zowel $CD4^+$ als $CD8^+$ T cellen mogelijk. Er werd geen significante antigeen specifieke reactiviteit van de T cel lijnen gevonden tegen dit panel van kandidaat MS antigenen.

In **hoofdstuk 3** vonden we dat aanwezigheid van de specifieke Th17.1 subset, die zowel op Th1 als Th17 lijkt, gecorreleerd was met vroege ziekteactiviteit in MS. De subset produceert significante hoeveelheden IFN-γ en GM-CSF en een kleine hoeveelheid IL-17. Th17.1 waren verlaagd aanwezig in het bloed van patiënten die snel MS ontwikkelden, verrijkt in MS hersenvocht en breinweefsel en hadden een groot migrerend vermogen over een cellaag met brein endotheelcellen *in vitro.* In het bloed van patiënten die werden behandeld met natalizumab, een VLA4 antistof, werd een selectieve ophoping van dit celtype gezien waarschijnlijk door verminderde migratie naar het CZS. In patiënten die ondanks natalizumab behandeling toch een nieuwe aanval kregen, vond geen ophoping van Th17.1 plaats, suggererend dat niet effectieve blokkade van de Th17.1 in deze patiënten bijdraagt aan de ontwikkeling van MS schubs.

Kijkend naar B cellen in **hoofdstuk 4,** ontdekten we dat een $CXCR3^+$ celtype selectief verrijkt was in het CZS (hersenvocht, meningen en hersenweefsel) vergeleken met bloed. Het migrerend vermogen van deze cellen naar het brein werd ondersteund door door vergelijkbare *in vitro* en *ex vivo* bevindingen als in hoofdstuk 3. Tijdens *in vitro* kweken met T_{FH}-condities zorgde toevoeging van IFN-γ vooral voor het ontstaan van plasmablasten. Als

daarbovenop nog Toll like receptor (TLR) 9 ligand CpG-ODN werd toegevoegd, trad voornamelijk isotype verandering naar IgG1 op.

Hoofdstuk 5 bestudeerde B cellen in zwangere MS patiënten en gezonde controles als model van ziekte activiteit. Bij het vergelijken van B cellen tijdens periodes van hoge ziekte activiteit (4-8 weken na bevalling) en lage ziekte activiteit (derde trimester van de zwangerschap) viel een verschil op tussen de chemokine receptoren die de geheugen B cellen tot expressie brachten. Postpartum faciliteerden deze receptoren migratie naar het brein alsook lokalisatie in de lichte zone van de kiemcelcentrumreactie. Daarnaast vonden we dat B cellen die verkregen waren van postpartum MS patiënten makkelijker differentieerden tot plasma cellen *in vitro* in kweken die de aanwezigheid van folliculaire T-helpercellen nabootsen. Dit draagt mogelijk bij tot hun differentiatie tot mature cellen in het CZS.

In **hoofdstuk 6** ontdekten we dat in onbehandelde patiënten met NMOSD hoge percentages transitionele B cellen aanwezig zijn in vergelijking met onbehandelde MS en MOGAD patiënten en gezonde controles. Deze waren het meest uitgesproken aanwezig bij patiënten die kort voor of na bloedafname een ziekteaanval kregen. Deze B cell subset werd specifiek verlaagd in patiënten die behandeld waren met steroïden. *In vitro* ontstonden er in aanwezigheid van IFN-γ in kweken van alle patiënten plasmablasten. Echter, alleen in patiënten met een recente ziekteaanval werd het aantal plasmablasten verder verhoogd na toevoeging van een TLR9 ligand. In patiënten met stabiele ziekte werd de plasmablast differentiatie hierdoor juist verminderd. In het supernatant van deze kweken van sommige NMOSD patiënten met recidiverende ziekteaanvallen konden AQP4 specifieke antistoffen worden aangetoond. De aanwezigheid en ontwikkeling van naïeve B cellen in NMOSD patiënten verschilt ten opzichte van controles en patiënten met andere neuroimmunologische ziekten. Dit wordt beïnvloed door corticosteroïden.

In **hoofdstuk 7** werden de resultaten samengevat, de uitkomsten vergeleken en aanbevelingen gedaan voor toekomstig onderzoek.

DANKWOORD

Het is een cliché, maar die zijn meestal waar: een promotie doe je niet alleen. Daarom zou ik graag de volgende mensen willen bedanken voor hun bijdrage.

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CURRICULUM VITAE

Malou Janssen was born, April 14th 1989, and raised in Purmerend. She graduated in 2007 from the Jan van Egmond College and proceeded to the technical university of Delft, where she studied technology, policy and management for one year. After that, she started medical school at the Erasmus University in Rotterdam. After finishing her BSc, she started two MSc programs simultaneously: medicine and research master neuroscience. The latter one she finished in 2013 and subsequently she started her PhD, described in this thesis, under supervision of prof. Rogier Hintzen and prof. Jon Laman. After finishing a 4 year PhD period, she continued with her medical internships, finishing as MD in 2019. Her first job as a MD was at the intensive care unit of the Fransciscus Gasthuis in Rotterdam. In January 2020 she transferred to the ICU of the Erasmus MC. In 2021 she started her residency in Internal Medicine. Malou is living in Delft and enjoys social activities with friends and participating in local politics via the city council.

PHD PORTFOLIO

Name PhD student:	Malou Janssen
Erasmus MC Department:	Immunology and Neurology
PhD period:	1 sept 2013 – 31 aug 2017
Promotors:	P. D. Katsikis, P.A.E. Sillevis Smitt
Co-promotor:	M.M. van Luijn

General Courses

Endnote course	*Erasmus MC Rotterdam*	2013
Management for promovendi and post-docs	*NIBI Utrecht*	2014
Writing grants	*Erasmus MC Rotterdam*	2014
English biomedical writing and communication	*Erasmus MC Rotterdam*	2015-2016
Workshop presenting skills for PhD students and Post Docs (MolMed postgraduate school)	*Erasmus MC, Rotterdam*	2016

Specific courses

Annual MolMed course (MolMed postgraduate school)	*Erasmus MC, Rotterdam*	2014
Advanced Immunology (MolMed postgraduate school)	*Erasmus MC, Rotterdam*	2014
European School of Neuro Immunology (ESNI) course	*Prague (travel grant)*	2015
Biostatistical Methods	*NIHES Rotterdam*	2015

Seminars and workshops

FACS course, beginner, advanced and BD	*Erasmus MC, Rotterdam*	2013-2014
Comparative pathology	*Erasmus MC, Rotterdam*	2014
Genetics for dummies	*Erasmus MC, Rotterdam*	2014
Scientific integrity	*Erasmus MC, Rotterdam*	2014

Conferences and presentations

MS research days	*(poster 2015, poster + oral 2016)*	2013-2017
ISNI, Mainz	*(royal academy of science travel grant)*	2014
ISNI, Jerusalem	*(oral + poster, travel grant)*	2016
NVvI/BSI, Liverpool	*(oral + poster, travel grant)*	2016

Other meetings and presentations

NVVI course Noordwijkerhout (’13), Kaatsheuvel (’14)	2013, 2014
NVVI wintermeetings Lunteren (*poster* 2014)	2013, 2014
MolMed day Rotterdam	2013-2015

Other

Departments PhD-committee	2013-2014
Instructor Weekendschool	2014-2017
Seminars Department Immunology	2013-2017
Journal Clubs Department Immunology	2013-2017
BROK course and exam	2014
SMBWO exam	2014

Teaching

Immunology cases with medical students	2015-2017
Supervision of MSc student	2015
Supervision of student of applied sciences	2017

PUBLICATIONS

This thesis

Janssen M*, Rijvers L*, Koetzier SC, Wierenga-Wolf AF, Melief MJ, van Langelaar J, Runia TF, de Groot CJ, Smolders J, Neuteboom R , van Luijn MM. *Pregnancy-induced effects on memory B-cell development in multiple sclerosis.*
Submitted.

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