Circulating immune cells in multiple sclerosis

Short title: Circulating immune cells in multiple sclerosis

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Abbreviations: BCR = B cell receptor; Breg = regulatory B cell; CCL/R = C-C motif chemokine ligand/receptor; CXCL/R = C-X-C motif chemokine ligand/receptor; CD = cluster of differentiation; CIS = clinically isolated syndrome; CNS = central nervous system; CSF = cerebrospinal fluid; DMT = disease modifying therapy; EAE = experimental autoimmune encephalomyelitis; EBV = Epstein-Barr virus; ELF = ectopic lymphoid follicle-
like structures; EM = effector memory T cell; EMRA = effector memory re-expressing RA T cell; FoxP3 = forkhead box P3; GC = germinal centre; GM-CSF = granulocyte-macrophage colony stimulating factor; ICOS = inducible T cell costimulator; ICS = intracellular cytokine staining; IFN = interferon; Ig = immunoglobulin; IL = interleukin; MFI = median fluorescence intensity; MS = multiple sclerosis; PB = peripheral blood; PD-1 = programmed cell death protein 1; PMA = phorbol 12-myristate 13-acetate; PPMS = primary progressive multiple sclerosis; ROR-γt = retinoic acid receptor-related orphan receptor gamma; RRMS = relapsing remitting multiple sclerosis; SPMS = secondary progressive multiple sclerosis; T-bet = T-box expressed in T cells; Tc = cytotoxic T cell; Tfh/r = follicular T helper/regulatory cell; Th = T helper cell; TLR = toll-like receptor; Treg = regulatory T cell.
Abstract

Circulating T and B lymphocytes contribute to the pathogenesis of the neuroinflammatory autoimmune disease, multiple sclerosis (MS). Further progress in the development of MS treatments is dependent upon a greater understanding of the immunological disturbances that underlie the disease. Analyses of circulating immune cells by flow cytometry have revealed MS-associated alterations in the composition and function of T and B cell subsets, including temporal changes associated with disease activity. Disturbances in circulating immune populations reflect those observed in the central nervous system and include skewing towards proinflammatory CD4+ and CD8+ T cells and B cells, greater proportions of follicular T helper cells, and functional defects in the corresponding T and B regulatory subsets. Utilising the analytical power of modern flow cytometers, researchers are now well positioned to monitor immunological changes associated with disease activity or intervention, describe immunological signatures with predictive value, and identify targets for therapeutic drug development. This review discusses the contribution of various T and B lymphocyte subsets to MS pathogenesis, provides current and relevant phenotypic descriptions to assist in experimental design, and highlights areas of future research.

Introduction

Multiple sclerosis (MS) is a chronic, progressive, neuroinflammatory disease, and the leading cause of neurological disability in young and middle-aged adults in the developed world (1). While the disease course and symptoms are heterogeneous, the disorder is characterised pathologically by immune-mediated inflammation, demyelination and axonal damage in the brain and spinal cord (collectively the central nervous system [CNS]). Despite decades of
research there remains only a small set of reliable markers for diagnosing and monitoring MS. In clinical practice these include magnetic resonance imaging to assess the dissemination of lesions in time and space, and the measurement of immunoglobulin G, oligoclonal bands and neurofilament in the cerebrospinal fluid (CSF) (2, 3). However, obtaining these data involves significant expertise and patient burden, and they provide limited information regarding the underlying immunological disturbances, on which to base the development of new therapeutic agents.

Peripheral blood (PB), meanwhile, represents an accessible biological sample and provides a “window” into the immunopathogenesis of MS. Immunologic characteristics of MS lesions, including infiltration of proinflammatory immune cells and defects in immunoregulation, are reflected in PB immune cells of patients with MS (4-6), and there is early evidence that certain PB immune disturbances correlate with the severity of disease progression (7). Experience with B cell-depleting disease modifying therapies (DMTs) demonstrate the significant role B cells play in MS pathology (8). However, non-B cell targeted DMTs also display efficacy (9), and an increased risk of MS is associated with polymorphisms in genes related to helper T cells (10). Collectively, these data indicate that the immunological disturbances that underlie MS span a range of immune cell subsets.

Technological advances in flow cytometry have greatly increased the depth of analysis achievable at the single-cell level, and these developments can be applied to better understand the immunopathology of MS. In this review we provide discussion and phenotypic descriptions of human T and B cell subsets associated with MS pathogenesis, highlighting the importance of multiparameter analyses in elucidating subset heterogeneity and identifying pathogenic subsets.
**CD4+ T helper cells**

*Background*

Historically, autoimmune diseases like MS were viewed as interferon-γ (IFN-γ), T helper (Th)1-mediated conditions, following the Th1/Th2 model first described by Mosmann and colleagues (11, 12). This model was challenged when later studies revealed a protective role of IFN-γ in the murine model of MS (experimental autoimmune encephalomyelitis [EAE]) (13), and, following the discovery of interleukin (IL)-17-producing Th17 cells, the concept of MS as a combined IFN-γ− and IL-17-driven condition was developed (13). Substantial evidence implicating Th17 cells, as well as IFN-γ+IL-17+ double-positive Th17.1 cells, in MS pathogenesis has accrued, however, there is now also interest in a third, granulocyte-macrophage colony stimulating factor (GM-CSF)-producing “ThPath” subset, which may be critical to the disease process (10, 14).

Th17 cells contribute to CNS demyelination via their effects on the protective brain epithelial cells and activation of inflammatory immune cells. IL-17 impairs the integrity of the blood-brain barrier, permitting entry of circulating immune cells into the CNS, while also stimulating astrocytes and microglia to produce inflammatory mediators (15).

Elevated proportions of PB Th17 cells have been reported in various stages of the disease, including clinically isolated syndrome (CIS, the earliest symptomatic presentation of demyelinating disease) (16), relapsing remitting MS (RRMS) (6, 17, 18), as well as in the primary progressive (PPMS) and secondary progressive (SPMS) manifestations (7). PB Th17 cells may also be indicative of relapse, as in the active disease phase (the definition of which
varies between studies), the percentage of Th17 cells is several fold greater than that observed in healthy controls (6, 18).

IFN-\(\gamma\)+IL-17+ double-positive Th17.1 cells are evidence of Th17 developmental plasticity, emerging from the Th17 population in response to cytokines including TGF-\(\beta\) and IL-23 in the microenvironment (19). Although there is currently little published on Th17.1 cells in MS, two studies reported a significant increase in IFN-\(\gamma\)+IL-17+ double-positive CD4+ T cells in samples from patients with RRMS, the greatest proportions occurring in conjunction with relapse (6, 18). These studies show that, collectively, elevated Th17 cells are detectable across the spectrum of MS phenotypes, and may particularly indicate active disease processes.

Despite evidence for the pathogenic role of IL-17 in MS and EAE, emerging lines of evidence implicate GM-CSF-producing T helper cells in the initiation and maintenance of autoimmune neuroinflammation (10, 14). GM-CSF promotes the maturation and activation of myeloid cells (monocytes, dendritic cells), which are the predominant immune cells in MS CNS lesions, increasing their antigen presentation and cytokine production (10, 14). In mice, Th17 cells are the predominant source of GM-CSF, and whereas IL-17 alone is not mandatory for EAE development, the disease is contingent on GM-CSF (14). In humans, GM-CSF production is associated with IFN-\(\gamma\)-producing Th1, rather than Th17, cells, although distinct GM-CSF single-positive cells have also been identified (14, 20). Higher proportions of total GM-CSF+, GM-CSF+IFN-\(\gamma\)- and GM-CSF+IFN-\(\gamma\)+ CD4+ T cells in samples from RRMS patients was recently reported by Rasouli and colleagues (20).

**Phenotyping**
Th cells can be identified through a variety of means, usually involving the use of cell surface markers in combination with lineage-specific transcription factors (Th1, Th17 and Th17.1 phenotypic markers summarised in Table 1) or intracellular cytokine staining (ICS). The transcription factor T-bet controls the expression of IFN-γ (21), and thus serves as a marker of Th1 cells when combined with surface expression of CD3 and CD4, while ROR-γt is required for the differentiation of Th17 cells (22). IFN-γ+IL-17+ double-positive Th17.1 cells co-express T-bet and ROR-γt (23). Production of GM-CSF is enhanced through STAT5 signalling, however, STAT5 is non-specific for GM-CSF production and also plays a role in regulatory T cell (Treg) development and Th17 differentiation (24).

Using ICS, Th1, Th17 and Th17.1 subsets can be identified as CD3+CD4+ T cells with a IFN-γ+IL-17-, IFN-γ-IL-17+, or IFN-γ+IL-17+ phenotype, respectively (Figure 1). The pro-inflammatory profile of T cells can be further elucidated by simultaneously staining for GM-CSF to determine the proportion of GM-CSF single-positive cells, as well as cells co-expressing GM-CSF, IFN-γ and/or IL-17. Reports suggest that Th1, Th17 and Th17.1 cells obtained following cell culture are predominantly of a memory (CD45RO+) phenotype (6), whereas CD4+GM-CSF+ T cells are approximately equally naïve (CD45RA+) and memory (20). For the purposes of inter-study comparison, it is important to consider the plasticity of T cells in response to cytokines, and that cytokine production is influenced by the method of T cell activation (19, 25).

Production of IL-17 is limited to cells that express CD161 (26). In addition to CD3, CD4, and CD161, Th17 cells express high levels of the chemokine receptors CCR6 and CCR4, but not the Th1-identifying CXC chemokine receptor CXCR3 (27). The ligand of CCR6, CCL20, is constitutively expressed by epithelial cells of the choroid plexus, and CCR6 expression is
essential for Th17 cells migration into the CNS (28). Th17 cells can thus be described as CD3+CD4+CD161+CCR6hiCXCR3-CCR4hi T cells.

In accordance with their mixed Th1/Th17 profile, Th17.1 cells have a CD3+CD4+CD161+CCR6loCXCR3+CCR4lo phenotype. The expression of CXCR3 by these cells may contribute to their pathogenic potential, as its ligand, CXCL10, is significantly increased in the CSF of patients with MS (29).

GM-CSF single-positive cells have been distinguished from GM-CSF+ cells co-expressing IFN-\(\gamma\) or IL-17 by their CCR10+CCR4+CCR6- CXCR3- phenotype (14). However, due to conflicting data (20) ICS represents the most reliable method for identifying GM-CSF+ Th cells at the current time.

An additional marker, CD146 (melanoma cell adhesion molecule), has been associated with greater IL-17 and GM-CSF mRNA expression in CD4+CD161+CCR6+ cells, and is found on \(~78\%\) of IFN-\(\gamma\)+IL-17+ double-positive cells (30). Although not exclusively expressed on Th17 or GM-CSF+ cells, CD146 may be a significant indicator of pathogenic potential due to the preferential migration of CD146+ lymphocytes across the blood brain barrier (30). CD4+CD146+ cells were found at significantly increased proportions in the PB of patients with CIS, RRMS, SPMS and PPMS, the highest levels being detected during active disease (30).

While Th17 cells are regularly studied in MS, there remains scope to refine our understanding of subset heterogeneity, and the relative temporal changes with disease activity and progression. Such knowledge may assist in identifying new drug targets, or guide treatment with existing therapies known to alter the Th17 population (31).
**CD4+ Regulatory T cells**

*Background*

Treg are crucial players in the maintenance of immune tolerance due to their ability to regulate the number and function of autoreactive T cells. A key component in the pathogenesis of MS is a disturbed balance between regulatory and effector compartments of the immune system, resulting in T cell-driven autoreactive inflammation.

Murine models support a role of Treg in preventing neuroinflammatory demyelinating disease. Depletion or inactivation of Treg increases the susceptibility of mice to the development of EAE (32), whereas adoptive transfer of Treg in Treg-deficient animals can prevent EAE development (33). The frequency of PB Treg in patients with MS is not significantly different to healthy controls (4, 34); however, Treg from MS patients are less competent at suppressing CD4+ T cell proliferation (4, 34, 35). Accordingly, any examination of Treg in the context of MS should incorporate markers of suppressive function.

*Phenotyping*

The ability to accurately phenotype Treg was enhanced by the discovery of forkhead box P3 (FoxP3), the transcription factor required for the development of regulatory CD4+CD25+ T cells in the thymus (36). As FoxP3 expression inversely correlates with cell surface CD127 expression (37, 38), Treg may be phenotyped as CD4+CD25+FoxP3+ T cells, CD4+CD25+CD127lo/-FoxP3+ T cells, or to circumvent cell permeabilisation and conduct functional analyses, as CD4+CD25+CD127lo/- T cells.
In MS, FoxP3 expression appears to change according to the disease stage and activity. It is lowest in RRMS, but is recovered in the later SPMS phase (39, 40). Few data are available regarding Treg in CIS, but the frequency of Treg and levels of FoxP3 mRNA are similar to those reported for RRMS (41, 42). FoxP3 expression and Treg function is restored in response to a number of DMTs including glucocorticoids, IFN-β and glatiramer acetate (43). Due to its essential role in Treg function, consistent findings of reduced expression in CIS and RRMS, and modulation by DMTs, FoxP3 should be considered an essential marker in MS research.

A subset of Treg that express CD39 is of relevance in MS due to the ability to effectively suppress Th17 cells (44). Deficits in the number and function of CD4+CD25+CD127lowFoxP3+CD39+ Treg have been reported in patients with RRMS (44, 45), but not SPMS (44). As CD39+ Treg are predominantly CD45RO+ memory cells (45), this finding matches an earlier report that functionally suppressive CD45RO+ Treg are reduced in early RRMS, but return to normal by the SPMS stage (35).

Another intracellular marker of interest is the Ikaros zinc finger transcription factor, Helios, which is thought to be a marker of activated Treg with increased suppressive function (46, 47). To our knowledge the only examination of Helios in patients with MS was conducted without comparison to a healthy control group (48). However, treatments that expand the Helios+ Treg pool have shown clinical benefit in EAE studies (48, 49), supporting further investigation of Helios in MS.

**CD4+ follicular T helper and regulatory cells**

*Background*
Follicular T (Tf) cells are a subset of CD4+ memory T cells, consisting of helper (Tfh) and regulatory cells (Tfr). Tfh cells are involved in the formation of germinal centres (GC), and the activation, expansion and differentiation of B cells into antibody-producing cells (50, 51). Tfr cells, meanwhile, regulate GC responses and the elimination of autoreactive B cells (52).

It is proposed that Tf cells are recruited to sites of CNS inflammation in response to the chemokine receptor ligand, CXCL13 (53), whereupon they contribute to the development of ectopic lymphoid follicle-like structures (ELF), and antibody-mediated demyelination (50). ELF in the CNS have been associated with a younger age of MS onset, more pronounced demyelination and more rapid progression to physical disability (54). Potentially reflecting sustained immune activity, the number and proportion of PB Tfh cells are elevated in samples from RRMS and SPMS patients, being most pronounced in active disease (7, 55). Although certain DMTs reduce the frequency of circulating Tfh cells, the levels remain above those in healthy controls (55).

The propensity towards CNS inflammation may be exacerbated by a disturbance in the number and function of Tfr cells (52). In comparison to healthy controls, a significantly decreased frequency of Tfr cells, in conjunction with an increased Tfh:Tfr ratio, was found in RRMS patients in one study (52). Tf cells are currently under-investigated in MS, although the existing evidence encourages further research.

**Phenotyping**

Peripheral Tfh cells are heterogeneous and display differing expression of surface markers depending on their activation status and Th-like phenotype (51). Primarily characterised as CD4+ T cells expressing CXCR5, the receptor for CXCL13 (Figure 2a), Tfh cells bearing a
memory phenotype (CD45RO+/CD45RA-) may be further categorised as IFN-γ-producing Tfh1 cells (CXCR3+CCR6-), IL-4-producing Tfh2 cells (CXCR3-CCR6-), or IL-17-producing Tfh17 cells (CXCR3-CCR6+) (Figure 2b) (51).

Inducible T cell costimulator (ICOS) and programmed cell death protein 1 (PD-1) are upregulated in active PB Tfh cells, differentiating them from their quiescent counterparts (Figure 2c) (51). The majority of quiescent PB Tfh cells are ICOS-PD-1-, although a proportion express low levels of PD-1. Tfh subsets have distinct B cell helper capacities, the most efficient helpers being ICOS-PD-1+ and ICOS+PD-1++ Tfh2 and Tfh17 (51). A disturbance in the balance of Tfh subsets, specifically lower proportions of non-efficient Tfh1 cells, has been noted in the PB of RRMS, SPMS and PPMS patients (7).

Regulatory Tfh cells retain the activated CD4+CXCR5+PD-1+ profile and Th-like subsets, but are differentiated by their expression of regulatory markers such as CD25, FoxP3 and Helios (52). One study has found that Tfr cells, as a percentage of CD4+CD25+CD127- Treg, are reduced in patients with MS, and compositionally skewed towards the Th17-like subset (52). This study reported a lower percentage of Tfr in samples from MS patients, without significant difference in Tfh populations (52). The recognition of Tfr cells has implications for Tfh and Treg analysis. An inability to discriminate between CXCR5+ follicular T cells and conventional CD4+ populations would result in misclassification of cells, as can be visualised in Figure 2a. Thus, the inclusion of regulatory and follicular markers in future Tfh and Treg analyses, respectively, is advised.

**CD8+ cytotoxic and regulatory T cells**

*Background*
While CD4+ T cells have largely been at the centre of T cell research in MS, CD8+ T cells may also be involved in the immunopathogenesis of the disease. Patients with MS have lower frequencies of circulating CD8+ T cells in at least some studies, providing an hypothesised link between Epstein-Barr virus (EBV) reactivation and MS development (56). As EBV infection is normally kept under tight control by CD8+ cytotoxic T cells (Tc) (57), the observed reduction in the frequency of CD8+ T cells potentially impairs defence against EBV to allows development of MS.

In addition to their role in viral defence, CD8+ T cells are implicated in the auto-reactivity and immune activation associated with MS, as well as exhibiting impaired regulatory function. CNS autoantigen-targeted CD8+ T cells can directly injure neuronal cells, and enhance CNS inflammation through cytokine production (58). This includes production of IL-17 by Tc17 and mucosal-associated invariant T (MAIT) cells which have been identified in CNS lesions (30, 59). CD8+ T cells can also damage CNS cells through the production of cytotoxic mediators such as perforin (Tc) and granzyme B (MAIT cells) (60).

Although reports on the frequency of CD8+ Treg in MS vary, significant reductions in the frequency and suppressive function of PB and CSF CD8+ Treg have been noted during periods of relapse (61-63). These disturbances may directly contribute to MS onset or relapse as CD8+ Treg can inhibit Tfh cells (64), expression of co-stimulatory molecules on antigen presenting cells, and proliferation of antigen-specific CD4+ T cells (63).

**Phenotyping**

Further to their CD3+CD8+ phenotype, CD8+ T cells can be divided into naïve (CD45RA+CCR7+), central memory (CD45RA-CCR7+), effector memory (EM) (CD45RA-
CCR7-) and effector memory re-expressing RA (EMRA) (CD45RA+CCR7-) subsets. In one study the EM and EMRA subsets in particular were reduced as a proportion of PB mononuclear cells (PBMC), but not of CD8+ T cells, in MS patients (56). The consistency of this finding throughout the disease course (CIS, RRMS and SPMS), without alteration with disease severity or duration, suggests that this disturbance reflects a primary defect, rather than being secondary to the disease process.

A number of reports suggest involvement of CD161-expressing CD8+ T cells in MS (65-67). These cells closely fit the description of MAIT cells which are predominantly CD3+CD4-CD8+ or CD3+CD4-CD8- T cells expressing high levels of CD161, and more specifically identified by the Va7.2 T cell receptor chain (68). The associations found between MAIT cells and MS, however, have been inconsistent (65-67). In contradictory studies, Annibali and colleagues (65) found a significantly higher percentage of PB MAIT cells (as a percentage of CD8+ T cells) in samples from patients with RRMS, while two other groups observed that RRMS was associated with lower levels of these cells in circulation (as a percentage of CD8+CD45RA- memory cells (67) or αβ T cells (66)). The percentage of MAIT cells inversely correlated with disease activity, being lowest during relapse (66). The CCR6+ and EM phenotype of MAIT cells (Figure 3) is consistent with migration to the CNS, as well with the reduction in CD8+ EM cells observed by Pender and colleagues (56).

CD146, which is associated with IL-17 production within the CD4+ T cells population (ref), is also present on a proportion of CD8+IL-17+ T cells, and found at increased frequencies on CD8+ T cells in other autoimmune diseases (69). However, we did not identify any research reporting on the frequency of such cells in the circulation of patients with MS. Although both CD161 and CD146 are marker of IL-17 production in CD4+ T cells, only relatively minor
percentages of CD8+CD146+ T cells (~8.4%) (69) and MAIT (~0.2% (65) to ~1.5% (66)) are IL-17+. Thus, for the purposes of specifically examining IL-17 production in CD8+ T cells ICS represents the most reliable method.

Studies utilising ICS to examine PB Tc17 (CD3+CD8+IL-17+) (70, 71) and Tc17 co-expressing IFN-γ (CD3+CD8+IL-17+IFN-γ+) (70) have found that the frequency of these cells is significantly greater in RRMS patients in comparison to healthy controls, but is stable between relapse and remission. The presence of Tc17 cells in the PB may provide information on disease progression, as it has been reported that Tc17 are not increased in the circulation of patients with CIS or early MS, despite being elevated in the CNS (72).

Regulatory function has been described in CD8+ T cells with the CD8+CD25+CD28- and CD8+FoxP3+ phenotype (61, 62). During relapse, the number of CD8+CD25+CD28- Treg (62), percentage of CD8+FoxP3+ Treg, and the median fluorescence intensity (MFI) of FoxP3, were significantly reduced, while in remission these parameters were not statistically different to healthy controls (61).

**B cells**

Although often viewed as a T cell-mediated disease, there is substantial evidence of B cell involvement in MS. Mechanisms by which B cells contribute to MS pathogenesis include the production of proinflammatory cytokines and autoantibodies to myelin proteins, and activation of autoreactive T cells (8). Strongly supporting a pathogenic role of B cells in MS is the efficacy of anti-CD20 monoclonal antibody therapies (e.g. rituximab and ocrelizumab) in reducing the number of relapses and new gadolinium-enhancing lesions (73). The specificity of these DMTs for CD20-expressing pre- and mature-B cells excludes a direct effect on antibody production,
and they may instead act by reducing B cell cytokine production and antigen presentation (73, 74). While certain B cell subsets contribute to neuroinflammation, regulatory B cells (Breg) inhibit Th1 and Th17 cell differentiation and induce CD4+ Treg (75, 76), but are numerically and functionally reduced in the PB of patients with MS (77-79).

A large proportion of B cell research in demyelinating disease examines the CNS, CSF, or lymphoid tissues, and is not necessarily generalizable to PB. Here we discuss findings relating to PB, and how changes in circulating B cell populations reflect disease activity in the CNS.

Phenotyping B cells by cell surface markers

Circulating B cells exist in several different stages of maturation and activation. Primarily identified by the pan-B cell marker CD19, the core B cell subsets are defined by variable expression of CD20, IgD, CD27, CD24, CD38 and CD138 (Table 2). The expression of chemokine receptors and T cell co-stimulatory molecules reveals further information regarding the propensity for CNS migration and T cell engagement.

Untreated remitting RRMS patients have similar proportions of total B cells and B cell subsets as healthy controls (80-82), although a higher proportion of CD27+ memory B cells has also been reported (83). Some studies have observed a proportional reduction in CD27+ memory subsets, together with a reciprocal expansion of CD27- B cells, in the PB of patients with CIS and RRMS during active phases of disease (80, 82). This is attributed to the migration of B cells towards chemokine-producing active lesions. CSF levels of CXCL13, the receptor for which (CXCR5) is expressed by a majority of B cells, are elevated in active relapse and
correlate with the accumulation of CD27+ B cells in the CSF (80). Furthermore, the proportion of PB CD27+ B cells expressing CCR5 significantly decreases during relapse (82), possibly in response to the production of CCR5 ligands in active MS lesions (29).

Aside from an increased migratory potential, there is some evidence that the B cell pool in RRMS patients comprises an elevated proportion of CD80+ and CD86+ cells (82, 84). Although increased PB CD80/86+ B cells are not found in all studies, the high proportion of CD80+ B cells in the CSF of CIS and MS patients supports a role for B cells in the generation of CNS-antigen-specific T cells (85).

A critical limitation of the studies reporting on chemokine receptor and costimulatory molecule expression is the small number of parameters simultaneously assessed, prohibiting subset categorisation and patterns of co-expression. Current generation multicolour flow cytometry is better equipped to contextualise chemokine receptor and costimulatory molecule expression, and revisiting these markers in future projects is required. Underscoring this recommendation is evidence that different CD80/86-expressing B cell subsets have proinflammatory (78) and regulatory characteristics (86).

In the absence of a unique transcription factor, descriptions of Breg are made by examining the profile of B cells exhibiting suppressor function (76). Such descriptions include transitional B cells (87), CD1d-expressing naïve and memory cells (86, 88), as well as B cells expressing CD25, FoxP3 (89) or CD5 (76). However, analysis of these Breg in samples from MS patients has yielded conflicting results. Transitional Breg, reported to be numerically and functionally deficient in other autoimmune diseases (75, 87), were reduced in number in CIS and RRMS
patients in one study (90), whereas a second found no difference between patients and healthy controls (91). Similarly, the percentage of CD5+ B cells has been both inversely (92) and positively (93) associated with disease progression, while relapse has been associated with both a decrease in the percentage of CD25+ B cells and an increase in FoxP3+ B cells (89). These conflicting results may relate to Breg not being a specific lineage, but rather a phenotype induced by inflammation (76). Thus, different Breg may emerge in different disease settings. Furthermore, the studies mentioned are limited by a lack of in-depth phenotyping. Expression patterns of Breg are highly complex, and attempts to simplify phenotyping to a few markers may result in misclassification (94). Only through ascertaining their function can Breg be confidently identified (94), and as such ICS may be preferable.

*Phenotyping B cells by intracellular cytokine staining*

Expression of the regulatory cytokine IL-10 identifies suppressive B cells. When analysed in conjunction with inflammatory cytokines the crucial balance between pro- and anti-inflammatory B cells can be ascertained. Although both memory and naïve B cells are capable of producing IL-10, the choice of stimuli is important as their responses are ligand-dependent (95).

CD27+ B cells are stimulated to produce IL-10 in response to toll-like receptor (TLR)4- and TLR9-ligation, whereas CD40-ligation specifically stimulates CD27- B cells (95). Cell culture experiments utilising CD40-ligation with or without the addition of a TLR9 ligand consistently find that B cells from patients with MS produce significantly less IL-10, but greater amounts of lymphotoxin and IL-6 than healthy controls, indicating regulatory impairments in naïve and
memory populations (96, 97). Conflicting results from ICS experiments have been reported but are probably attributable to the method of stimulation.

In one study, there were significantly fewer IL10+ B cells following stimulation of PBMC with TLR9 ligand and phorbol 12-myristate 13-acetate (PMA)/Ionomycin in samples from patients with MS than in healthy controls (77). In contrast, Ireland and colleagues reported no difference in the number of IL-10+ B cells or intracellular levels of IL-10 between patients and controls after stimulating with TLR9 ligand and B cell receptor (BCR) (97). However, whereas PMA/Ionomycin stimulates B cells to mature into IL-10+ cells, BCR inhibits the production of IL-10 that is otherwise induced by TLR9 ligation (98). Ireland and colleagues (97) did detect significantly greater levels of intracellular IL-6 in patient samples, providing further support of an imbalanced regulatory:inflammatory B cell profile in MS. Besides IL-10, Breg that suppress inflammation via the production of IL-35 or transforming growth factor β have also been described, although their relevance to MS has not been determined (95).

Published ICS analyses suffer the same shortfall as other B cell research in MS; namely, limited subset classification or analysis of chemokine receptor and costimulatory molecule expression. As B cell compartmental shifts are associated with disease activity, an understanding of differential functional capacity would be of benefit.

**Summary**

Our growing understanding of the pathogenesis of MS has revealed a large set of immunological disturbances involving complex interactions between different subsets of the
immune system. Superseding the Th1-mediated model of MS is one characterised by elevated proportions of a range of inflammatory T and B lymphocytes, coupled with impairments in regulatory populations (summarised in Table 3). Importantly, these disturbances can be observed not only in the CNS, but also through examination of PB immune cells.

Beyond providing an overview of MS-relevant T and B cell subsets, the aim of this review is to bring attention to the need for multiparameter analysis capable of examining patterns of co-expression, in identifying novel pathological markers or profiles. Such analyses will better facilitate monitoring of disease activity and the effects of DMTs, as well as aid the development of new, or “personalised”, therapeutic interventions. To this end we have noted a number of areas requiring clarification in future research, including the expression of functionally relevant markers within lymphocyte subsets, and characteristics of disease progression. Such questions would be best addressed through thorough, multi-subset, longitudinal analysis from the earliest presentation of demyelinating disease.

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### Table 2. Phenotypic description of core B cell subsets

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<td>Double-negative memory</td>
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<td>Non-switched memory</td>
<td>CD19+CD20+IgD+CD27+</td>
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<tr>
<td>Switched memory</td>
<td>CD19+CD20+IgD-CD27+</td>
</tr>
<tr>
<td>Plasmablasts</td>
<td>CD19+CD20-IgD-CD27hiCD38hiCD138-</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>CD19+CD20-IgD-CD27hiCD38hiCD138+</td>
</tr>
</tbody>
</table>
Table 3. Reported changes in PB immune cell subsets in different stages of MS, in comparison to healthy controls

<table>
<thead>
<tr>
<th>Condition</th>
<th>CD4+ Th cells</th>
<th>CD4+ Treg</th>
<th>Tf cells</th>
<th>CD8+ T cells</th>
<th>B cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CIS</strong></td>
<td>↑ Th17 (% CD4) (16)</td>
<td></td>
<td>No significant differences reported in the cited literature</td>
<td>↓ EM/RA (% PBMC) (56)</td>
<td>↓ CD27+ (% B cells) in active disease (80)</td>
</tr>
<tr>
<td></td>
<td>↑ (↑↑ in active disease)</td>
<td></td>
<td></td>
<td>↓ Tfr (% Treg) (52)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD146+ (% CD4) (30)</td>
<td></td>
<td></td>
<td>↔ Tc17 (% CD8) (72)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No significant differences reported in the cited literature</td>
<td></td>
<td></td>
<td>↔ FoxP3+ (% CD8) (61)</td>
<td></td>
</tr>
<tr>
<td><strong>RRMS</strong></td>
<td>↑ Th17 (% CD4) (17)</td>
<td>↓ CD39+ (% Treg) (44)</td>
<td>↓ Tfh (% CD4) (52)</td>
<td>↓ EM/RA (% PBMC) (56)</td>
<td>↓ B cells (% PBMC) (80)</td>
</tr>
<tr>
<td></td>
<td>↔ Th17 (% memory CD4) (6, 18)</td>
<td>↑ Tfh (number) (55)</td>
<td>↑ Tfh1 (% Tfh) (7)</td>
<td>↑ Tc17 / IFN-γ+ Tc17 (% CD8) (70)</td>
<td>↔ core subsets (% B cells) (80, 81)</td>
</tr>
<tr>
<td></td>
<td>↑ CD146+ (% CD4) (30)</td>
<td>↓ ICOS+ (% Tfh) (7)</td>
<td>↓ Tfr (% Treg) (52)</td>
<td>↑ MAIT (% CD8) (65)</td>
<td>↓ IL-10+ (% B cells) (77)</td>
</tr>
<tr>
<td></td>
<td>↑ GM-CSF+ (% CD4) (20)</td>
<td>↓ memory Treg (% CD4) (35)</td>
<td>↑ Tfr17 (% Treg) (52)</td>
<td>↓ MAIT (% memory CD8 (67) and αβT cells (66))</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ FoxP3 MFI (39, 40)</td>
<td></td>
<td>↑ GM-CSF+ (% CD8) (20)</td>
<td></td>
</tr>
<tr>
<td><strong>RRMS - active</strong></td>
<td>↑↑ Th17 and Th17.1 (% memory CD4) (6, 18)</td>
<td>↓ CD39+ (% Treg) (44)</td>
<td>↑↑ number of Tfh and ICOS+ Tfh (55)</td>
<td>↓ EM/RA (% PBMC) (56)</td>
<td>↓ CD27+ (% B cells) (80, 82)</td>
</tr>
<tr>
<td></td>
<td>↑↑ CD146+ (% CD4) (30)</td>
<td>↓ memory Treg (% CD4) (35)</td>
<td></td>
<td>↑ Tc17 / IFN-γ+ Tc17 (% CD8) (70)</td>
<td>↓ CCR5+ (% total, CD27+ and CD27- B cells) (82)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ FoxP3 MFI (39, 40)</td>
<td></td>
<td>↓ FoxP3+ (% CD8) and FoxP3 MFI (61)</td>
<td>↓ IL-10+ (% B cells) (77)</td>
</tr>
<tr>
<td><strong>SPMS and PPMS</strong></td>
<td>↑ Th17 (% CD4) (7)</td>
<td>No significant differences reported in the cited literature</td>
<td>↑ Tfh17 (% Tfh) (7)</td>
<td>↓ EM/RA (% PBMC) (56)</td>
<td>No significant differences reported in the cited literature</td>
</tr>
<tr>
<td></td>
<td>↑ CD146+ (% CD4) (30)</td>
<td></td>
<td>↓ Tfr (% Treg) (52)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure legends

**Figure 1.** Intracellular cytokine staining to identify Th1, Th17 and Th17.1 cells. In comparison to a healthy control sample (a), the proportion of Th17 and Th17.1 cells can be substantially increased in samples from patients with CIS, particularly in the setting of active disease (b). Plots represent CD3+CD4+ T cells gated on live, single cell PBMC, following a 4 hour stimulation with PMA/Ionomycin in the presence of Brefeldin A (BD Leukocyte Activation Cocktail with GolgiPlug).

**Figure 2.** Peripheral blood follicular T cells. These cells are distinguished from conventional CD4+ T cells by the expression of CXCR5, and helper subsets categorised based on chemokine receptor, PD-1 and ICOS expression. (a) Examining FoxP3 and CXCR5 expression on live, single-cell, CD3+CD4+ T cells reveals four distinct subsets (clockwise from top left quadrant): CCXR5-FoxP3+ Treg; CXCR5+FoxP3+ Tfr; CXCR5+FoxP3- Tfh; and CXCR5-FoxP3- conventional T cells. The absence of CXCR5 fluorochrome-conjugated antibody in Treg analysis would result in Tfr being simultaneously analysed with conventional Treg. Likewise, FoxP3 fluorochrome-conjugated antibody (or other regulatory markers) are required to distinguish between Tfh and Tfr cells. (b) CXCR5+FoxP3-CD45RA- Tfh cells can be categorised as Tfh17 (CCR6+CXCR3-), Tfh17.1 (CCR6+CXCR3+), Tfh1 (CCR6-CXCR3+) and Tfh2 (CCR6-CXCR3-). CCR4 and CD161 expression may be used to further refine Tfh subsets (refer to Table 1). (c) While helper capacity of Tfh1 cells is restricted to the activated ICOS+PD-1++CCR7lo subset, all Tfh2 and Tfh17 cells are capable of helping B cells with varying capacity. The intensity of the background greyscale reflects the capacity to
provide help to B cells. This figure has been adapted from reference (51) with permission from the authors.

**Figure 3.** MAIT cells possess a phenotype consistent with CNS infiltration and proinflammatory cytokine production. (a) CD3+CD4- MAIT cells (predominantly CD8+, not shown) express high levels of CD161, and are predominantly EM (CCR7-CD45RA-) (b) and CCR6+ cells (c). This phenotype is consistent with migration to inflamed tissue, the capacity to cross the BBB, and exert effector function including IL-17 production.
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