Circulating immune cells in multiple sclerosis

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Summary

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. Utilizing 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 phenotypical descriptions to assist in experimental design and highlights areas of future research.

Keywords: B cells, flow cytometry, multiple sclerosis, T cells

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 characterized 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 (Ig)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. Immunological 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 T helper 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 understand more clearly the immunopathology of MS. In this review

we provide discussion and phenotypical descriptions of human T and B cell subsets associated with MS pathogenesis, highlighting the importance of multi-parameter analyses in elucidating subset heterogeneity and identifying pathogenic subsets.

CD4$^+$ T helper cells

Background

Historically, autoimmune diseases such as MS were viewed as interferon (IFN)-γ, T helper type 1 (Th1)-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-γ$^+$IL-17$^+$ double-positive Th17.1 cells emerge from the Th17 population in response to cytokines, including transforming growth factor (TGF)-β 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-γ$^+$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.

Table 1. T helper (Th) subset phenotypical markers

	Th1	Th17	Th17.1
CD161	–	+	+
CCR6	–	++	+
CXCR3	++	–	++
CCR4	+	++	+
T-bet	+	–	+
ROR-γt	–	+	+

ROR-γt = retinoic acid receptor-related orphan receptor gamma t.

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 upon GM-CSF [14]. In humans, GM-CSF production is associated with IFN-γ-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-γ- and GM-CSF$^+$IFN-γ$^+$CD4$^+$ T cells in samples from RRMS patients was reported recently 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 phenotypical markers summarized 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 retinoid-related orphan receptor gamma t (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 signal transducer and activator of transcription 5 (STAT-5) signalling; however, STAT-5 is non-specific for GM-CSF production and also plays a role in regulatory T cell (T$_{reg}$) development and Th17 differentiation [24].

Using ICS, Th1, Th17 and Th17.1 subsets can be identified as CD3$^+$CD4$^+$ T cells with an IFN-γ$^+$IL-17-, IFN-γ-IL-17$^+$ or IFN-γ$^+$IL-17$^+$ phenotype, respectively (Fig. 1). The proinflammatory profile of T cells can be elucidated by further staining simultaneously 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

Fig. 1. Intracellular cytokine staining to identify T helper type 1 (Th1), Th17 and Th17.1 cells. In comparison to a healthy control sample (a), the proportion of Th17 and Th17.1 cells can be increased substantially in samples from patients with clinically isolated syndrome (CIS), particularly in the setting of active disease (b). Plots represent $CD3^+CD4^+$ T cells gated on live, single-cell peripheral blood mononuclear cells (PBMC), following a 4-h stimulation with phorbol myristate acetate (PMA)/ ionomycin in the presence of brefeldin A (BD leucocyte activation cocktail with GolgiPlug).
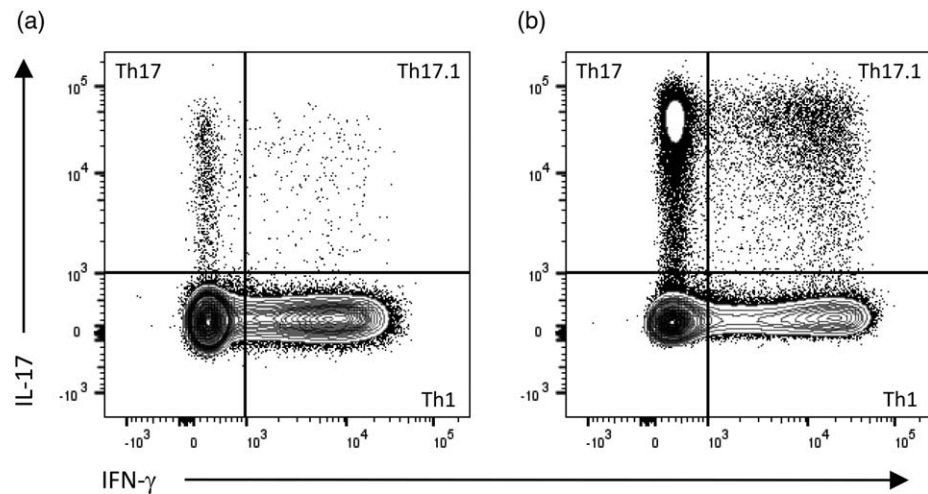
suggest that Th1, Th17 and Th17.1 cells obtained following cell culture are predominantly of a memory ($CD45RO^+$) phenotype [6], whereas $CD4^+GM-CSF^+$ T cells are approximately equally naive ($CD45RA^+$) and memory cells [20]. For the purposes of interstudy comparison, it is important to consider 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 expressed constitutively by epithelial cells of the choroid plexus, and CCR6 expression is essential for Th17 cell migration into the CNS [28]. Th17 cells can thus be described as $CD3^+CD4^+CD161^+CCR6^{hi}CXCR3^-CCR4^{hi}$ T cells.

In accordance with their mixed Th1/Th17 profile, Th17.1 cells have a $CD3^+CD4^+CD161^+CCR6^{lo}CXCR3^+CCR4^{lo}$ phenotype. The expression of CXCR3 by these cells may contribute to their pathogenic potential, as its ligand, C-X-C motif chemokine 10 (CXCL10), is increased significantly in the CSF of patients with MS [29].

GM-CSF single-positive cells have been distinguished from $GM-CSF^+$ cells co-expressing IFN-γ 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-γ^+IL-17^+ double-positive cells [30]. Although not expressed exclusively 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 in 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 studied regularly 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 (T_{reg})

Background

T_{reg} 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 T_{reg} in preventing neuroinflammatory demyelinating disease. Depletion or inactivation of T_{reg} increases the susceptibility of mice to the development of EAE [32], whereas adoptive transfer of T_{reg} in T_{reg}-deficient animals can prevent EAE development [33]. The frequency of PB T_{reg} in patients with MS is not significantly different to healthy controls [4,34]; however, T_{reg} from MS patients are less competent at suppressing $CD4^+$ T cell proliferation [4,34,35]. Accordingly, any examination of T_{reg} in the context of MS should incorporate markers of suppressive function.
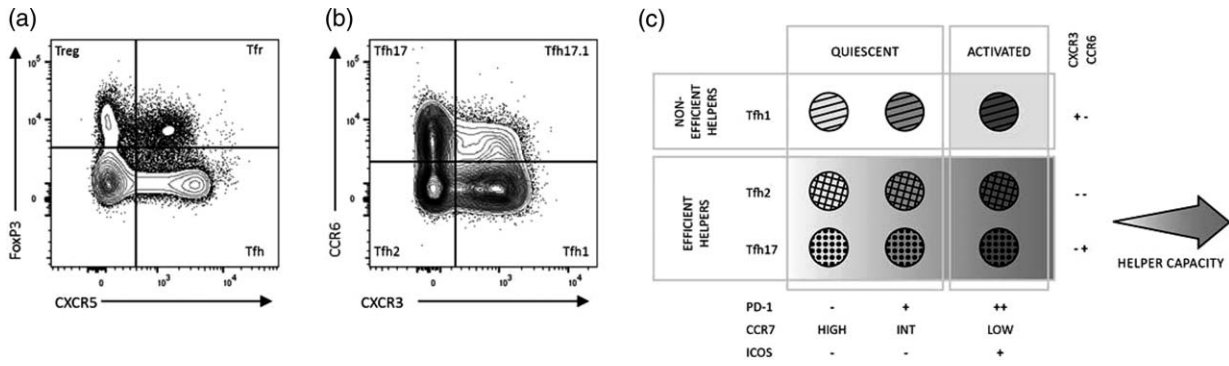
Fig. 2. Peripheral blood follicular T cells. These cells are distinguished from conventional CD4$^+$ T cells by the expression of CXCR5 and helper subsets categorized based on chemokine receptor, programmed death 1 (PD)-1 and inducible T cell co-stimulator (ICOS) expression. (a) Examining forkhead box protein 3 (FoxP3) and CXCR5 expression on live, single-cell, CD3$^+$CD4$^+$ T cells reveals four distinct subsets (clockwise from top left quadrant): CCXR5$^-$FoxP3$^+$ regulatory T cells (T$_{reg}$); CXCR5$^+$FoxP3$^+$ follicular T helper (Tfr); CXCR5$^+$FoxP3$^-$ Tfh; and CXCR5$^-$FoxP3$^-$ conventional T cells. The absence of CXCR5 fluorochrome-conjugated antibody in T$_{reg}$ analysis would result in Tfr being analysed simultaneously with conventional T$_{reg}$. Simultaneously, FoxP3 fluorochrome-conjugated antibodies (or other regulatory markers) are required to distinguish between Tfh and follicular regulatory T (Tfr) cells. (b) CXCR5$^+$FoxP3$^-$CD45RA$^-$ Tfh cells can be categorized as Tfh17 (CCR6$^+$CXCR3$^-$), Tfh17.1 (CCR6$^+$CXCR3$^+$), Tfh1 (CCR6$^-$CXCR3$^+$) and Tfh2 (CCR6$^-$CXCR3$^-$). CCR4 and CD161 expression may be used to refine Tfh subsets further (refer to Table 1). (c) While helper capacity of Tfh1 cells is restricted to the activated ICOS$^+$PD-1^{++}CCR7^{lo} 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.

Phenotyping

The ability to phenotype T$_{reg}$ accurately was enhanced by the discovery of forkhead box protein 3 (FoxP3), the transcription factor required for the development of regulatory CD4$^+$CD25$^+$ T cells in the thymus [36]. As FoxP3 expression correlates inversely with cell surface CD127 expression [37,38], T$_{reg}$ may be phenotyped as CD4$^+$CD25$^+$FoxP3$^+$ T cells, CD4$^+$CD25$^+$CD127^{lo}/$^-$FoxP3$^+$ T cells, or to circumvent cell permeabilization and conduct functional analyses, as CD4$^+$CD25$^+$CD127$^{lo/-}$ 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 T$_{reg}$ in CIS, but the frequency of T$_{reg}$ and levels of FoxP3 mRNA are similar to those reported for RRMS [41,42]. FoxP3 expression and T$_{reg}$ function is restored in response to a number of DMTs, including glucocorticoids, IFN-β and glatiramer acetate [43]. Due to its essential role in T$_{reg}$ 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 T$_{reg}$ that express CD39 is of relevance in MS due to the ability to suppress Th17 cells effectively [44]. Deficits in the number and function of CD4$^+$CD25$^+$CD127^{lo}FoxP3$^+$CD39$^+$ T$_{reg}$ have been reported in patients with RRMS [44,45], but not SPMS [44]. As CD39$^+$ T$_{reg}$ are predominantly CD45RO$^+$ memory cells [45], this finding matches an earlier report that functionally suppressive CD45RO$^+$ T$_{reg}$ 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 T$_{reg}$ 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$^+$ T$_{reg}$ 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

Also found within the CD4$^+$ T cell pool are follicular T helper (Tfh) and follicular T regulatory (Tfr) cells. 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]. Characterized primarily as $CD4^+$ T cells expressing CXCR5, the receptor for CXCL13 (Fig. 2a), Tfh cells bearing a memory phenotype ($CD45RO^+/CD45RA^-$) may be categorized further as IFN-γ-producing Tfh1 cells ($CXCR3^+CCR6^-$), IL-4-producing Tfh2 cells ($CXCR3^-CCR6^-$) or IL-17-producing Tfh17 cells ($CXCR3^-CCR6^+$) (Fig. 2b) [51].

Inducible T cell co-stimulator (ICOS) and programmed cell death protein 1 (PD-1) are up-regulated in active PB Tfh cells, differentiating them from their quiescent counterparts (Fig. 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 $ICOS^+PD-1^{2+}$ 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 Tf 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 $CD4^+CD25^+CD127^-$ T_{reg}, are reduced in patients with MS, and skewed compositionally towards the Th17-like subset [52]. This study reported a lower percentage of Tfr in samples from MS patients, without a significant difference in Tfh populations [52]. The recognition of Tfr cells has implications for Tfh and T_{reg} analysis. An inability to discriminate between $CXCR5^+$ follicular T cells and conventional $CD4^+$ populations would result in misclassification of cells, as can be visualized in Fig. 2a. Thus, the inclusion of regulatory and follicular markers in future Tf and T_{reg} analyses, respectively, is advised.

$CD8^+$ cytotoxic and T_{reg}

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 a hypothesized 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 allow development of MS.

In addition to their role in viral defence, $CD8^+$ T cells are implicated in the autoreactivity and immune activation associated with MS, as well as exhibiting impaired regulatory function. CNS autoantigen-targeted $CD8^+$ T cells can injure neuronal cells directly, 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 $CD8^+$ T_{reg} in MS vary, significant reductions in the frequency and suppressive function of PB and CSF $CD8^+$ T_{reg} have been noted during periods of relapse [61–63]. These disturbances may contribute directly to MS onset or relapse, as $CD8^+$ T_{reg} 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 naive ($CD45RA^+CCR7^+$), 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 the involvement of CD161-expressing $CD8^+$ T cells in MS [65–67]. These cells fit closely the description of MAIT cells, which are predominantly $CD3^+CD4^-CD8^+$ or $CD3^+CD4^-CD8^-$ T cells expressing high levels of CD161, and identified more specifically by the Vα7.2 T cell receptor chain [68]. The associations found between MAIT cells and MS,
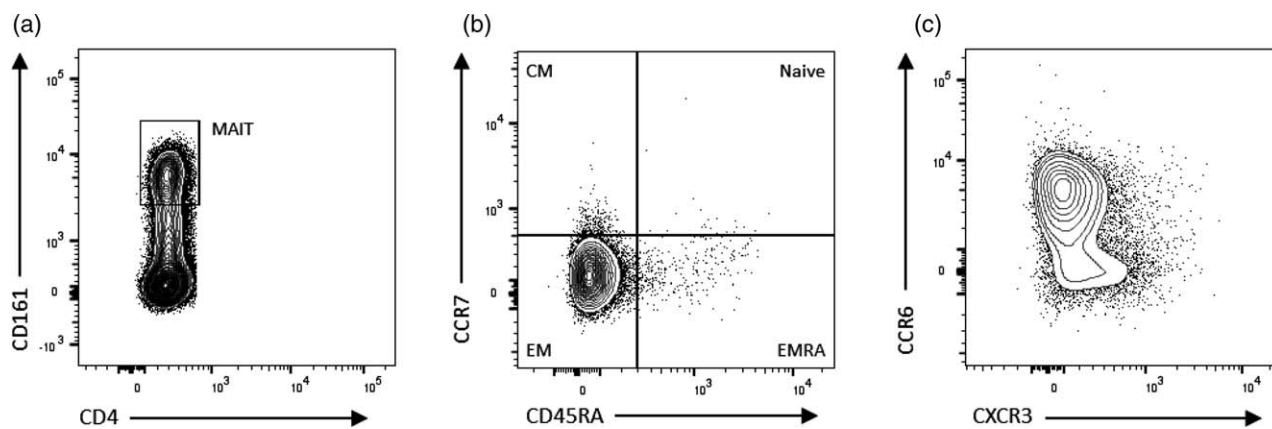
Fig. 3. Mucosal-associated invariant T (MAIT) cells possess a phenotype consistent with central nervous system (CNS) infiltration and proinflammatory cytokine production. (a) $CD3^+CD4^-$ MAIT cells (predominantly $CD8^+$, not shown) express high levels of CD161, and are predominantly effector memory (EM) ($CCR7^-CD45RA^-$) (b) and $CCR6^+$ cells (c). This phenotype is consistent with migration to inflamed tissue, the capacity to cross the blood–brain barrier (BBB) and exert effector function, including interleukin (IL)-17 production.

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 αβT cells [66]). The percentage of MAIT cells correlated inversely with disease activity, being lowest during relapse [66]. The $CCR6^+$ and EM phenotype of MAIT cells (Fig. 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 cell population [30], 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 markers of IL-17 production in $CD4^+$ T cells, only relatively minor percentages of $CD8^+CD146^+$ T cells ($\sim$8·4%) [69] and MAIT cells ($\sim$0·2% [65] to $\sim$1·5% [66]) are IL-17$^+$. Thus, for the purposes of examining IL-17 production specifically in $CD8^+$ T cells, ICS represents the most reliable method.

Studies utilizing ICS to examine PB Tc17 ($CD3^+CD8^+IL-17^+$) [70,71] and Tc17 co-expressing IFN-γ ($CD3^+CD8^+IL-17^+IFN-\gamma^+$) [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 $CD8^+CD25^+CD28^-$ T_{reg} [62], the percentage of $CD8^+FoxP3^+$ T_{reg} and the median fluorescence intensity (MFI) of FoxP3 were reduced significantly, 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 act instead by reducing B cell cytokine production and antigen presentation [73,74]. While certain B cell subsets contribute to neuroinflammation, regulatory B cells (B_{reg}) inhibit Th1 and Th17 cell differentiation and induce $CD4^+$ T_{reg} [75,76], but are reduced numerically and functionally 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.

Table 2. Phenotypical description of core B cell subsets

Transitional	$CD19^+CD20^+IgD^+CD27^-CD24^{hi}CD38^{hi}$
Naïve	$CD19^+CD20^+IgD^+CD27^-CD24^{lo}CD38^{lo}$
Double-negative memory	$CD19^+CD20^+IgD^-CD27^-$
Non-switched memory	$CD19^+CD20^+IgD^+CD27^+$
Switched memory	$CD19^+CD20^+IgD^-CD27^+$
Plasmablasts	$CD19^+CD20^-IgD^-CD27^{hi}CD38^{hi}CD138^-$
Plasma cells	$CD19^+CD20^-IgD^-CD27^{hi}CD38^{hi}CD138^+$

Ig = immunoglobulin.

Phenotyping B cells by cell surface markers

Circulating B cells exist in several different stages of maturation and activation. Identified primarily 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 to 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 decreases significantly 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 co-stimulatory molecule expression is the small number of parameters assessed simultaneously, prohibiting subset categorization and patterns of co-expression. Current generation multi-colour flow cytometry is better equipped to contextualize chemokine receptor and co-stimulatory 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 B_{reg} 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 B_{reg} in samples from MS patients has yielded conflicting results. Transitional B_{reg}, 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 associated both inversely [92] and positively [93] 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 B_{reg} not being a specific lineage, but rather a phenotype induced by inflammation [76]. Thus, different B_{reg} may emerge in different disease settings. Furthermore, the studies mentioned are limited by a lack of in-depth phenotyping. Expression patterns of B_{reg} are highly complex, and attempts to simplify phenotyping to a few markers may result in misclassification [94]. Only through ascertaining their function can B_{reg} be identified confidently [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 TLR-9 ligation, whereas CD40 ligation stimulates $CD27^-$ B cells specifically [95]. Cell culture experiments utilizing CD40 ligation with or without the addition of a TLR-9 ligand find consistently 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 TLR-9 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 TLR-9 ligand and B cell receptor (BCR) [97]. However,

Table 3. Reported changes in peripheral blood (PB) immune cell subsets in different stages of multiple sclerosis (MS) in comparison to healthy controls

	CD4$^+$ Th cells	CD4$^+$ T$_{reg}$	Tf cells	CD8$^+$ T cells	B cells
CIS	↑ Th17 (% CD4) [16] ↑ (↑↑ in active disease) CD146$^+$ (% CD4) [30]	No significant differences reported in the cited literature	↔ Tfh (% CD4) ↓ Tfr (% T$_{reg}$) [52]	↓ EM/RA (% PBMC) [56] ↔ Tc17 (% CD8) [72] ↔ FoxP3$^+$ (% CD8) [61]	↓ CD27$^+$ (% B cells) in active disease [80]
RRMS	↑ Th17 (% CD4) [17] ↔ Th17 (% memory CD4) [6,18] ↑ CD146$^+$ (% CD4) [30] ↑ GM-CSF$^+$ (% CD4) [20]	↓ CD39$^+$ (% T$_{reg}$) [44] ↓ CD39$^+$ (% PBMC) [45] ↓ memory T$_{reg}$ (% CD4) [35] ↓ FoxP3 MFI [39,40]	↔ Tfh (% CD4) [52] ↑ Tfh (number) [55] ↑ ICOS$^+$ (% Tfh) [7] ↓ Tfh1 (% Tfh) [7] ↓ Tfr (% T$_{reg}$) [52] ↑ Tfr17 (% T$_{reg}$) [52]	↓ EM/RA (% PBMC) [56] ↑ Tc17/IFN-γ$^+$ Tc17 (% CD8) [70] ↑ MAIT (% CD8) [65] ↓ MAIT (% memory CD8 [67] and αβT cells [66]) ↑ GM-CSF$^+$ (% CD8) [20]	↔ B cells (% PBMC) [80] ↔ core subsets (% B cells) [80,81] ↓ IL-10$^+$ (% B cells) [77]
RRMS - active	↑↑ Th17 and Th17.1 (% memory CD4) [6,18] ↑↑ CD146$^+$ (% CD4) [30]	↓ CD39$^+$ (% T$_{reg}$) [44] ↓ memory T$_{reg}$ (% CD4) [35] ↓ FoxP3 MFI [39,40]	↑↑ number of Tfh and ICOS$^+$ Tfh [55]	↓ EM/RA (% PBMC) [56] ↑ Tc17 [70,71]/IFN-γ$^+$ Tc17 (% CD8) [70] ↓ FoxP3$^+$ (% CD8) and FoxP3 MFI [61] ↓↓ MAIT (% αβT cells) [66]	↓ CD27$^+$ (% B cells) [80,82] ↓ CCR5$^+$ (% total, CD27$^+$ and CD27$^-$ B cells) [82] ↓ IL-10$^+$ (% B cells) [77]
SPMS and PPMS	↑ Th17 (% CD4) [7] ↑ CD146$^+$ (% CD4) [30]	No significant differences reported in the cited literature	↑ Tfh17 (% Tfh) [7] ↓ Tfr (% T$_{reg}$) [52]	↓ EM/RA (% PBMC) [56]	No significant differences reported in the cited literature

CIS = clinically isolated syndrome; RRMS = relapsing–remitting multiple sclerosis (MS); SPMS = secondary progressive MS; PPMS = primary progressive MS; T$_{reg}$ = regulatory T cell; Tfh = follicular T helper; EM/RA = effector memory re-expressing RA T cell; ICOS = inducible T cell co-stimulator; MAIT = mucosal-associated invariant T; PBMC = peripheral blood mononuclear cells; GM-CSF = granulocyte–macrophage colony-stimulatory factor; FoxP3 = forkhead box protein 3; MFI = mean fluorescence intensity; IFN = interferon; Th = T helper.

whereas PMA/ionomycin stimulates B cells to mature into IL-10$^+$ cells, BCR inhibits the production of IL-10 that is induced otherwise by TLR-9 ligation [98]. Ireland and colleagues [97] detected 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, B$_{reg}$ 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 co-stimulatory molecule expression. As B cell compartment 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 characterized by elevated proportions of a range of inflammatory T and B lymphocytes, coupled with impairments in regulatory populations (summarized 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 multi-parameter analysis capable of examining patterns of co-expression in identifying novel pathological markers or profiles. Such analyses will facilitate more efficient monitoring of disease activity and the effects of DMTs, as well as aid the development of new, or 'personalized', 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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