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Immunomonitoring Lymphocyte Subpopulations in Multiple Sclerosis Patients

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Abstract: Advances in the understanding of pathogenic mechanisms of diseases have led to the defining of new biomarkers for diagnosis, prognosis, and therapy response. In this context, flow cytometry has been positioned as one of the most useful technologies for monitoring immune-mediated diseases, such as multiple sclerosis (MS), allowing a detailed analysis of lymphocyte subpopulations in peripheral blood. The autoimmune inflammatory response in MS results in changes in lymphocyte subpopulations that might be useful as surrogate markers for the evaluation of disease activity, progression, and monitoring of therapy response. This chapter discusses the role of T-lymphocyte and B-lymphocyte subpopulations in MS pathogenesis, the effect of MS treatments on these subsets, and their potential usefulness as biomarkers of treatment response.

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Introduction

There is evidence of patients with the same disease responding differently to the same treatment. Thus, it is necessary to define biomarkers to stratify patients, monitor the course of the disease, and predict response to treatment. Peripheral blood leukocytes play an important role in the pathogenesis of autoimmune diseases. It has been demonstrated that immunomodulatory treatments decrease the percentage of these cell populations, alter the expression of their surface markers, and modify their functionality (i.e., cytokine production, proliferation, and induction of apoptosis). For these reasons, it has been hypothesized that systematic analyses of peripheral blood immune cells could serve as surrogate biomarkers of activity of the disease and/or response to therapy, leading to the development of personalized medicine (1–4).

FLOW CYTOMETRY, A TOOL FOR IMMUNE-MONITORING

Flow cytometry enables the analysis of a panel of surface molecules at single-cell level that not only determines the percentages of peripheral lymphocytes but also their differentiation stage. In addition, the activation state of peripheral lymphocytes and their memory or effector functions can be measured. Recent advances in the development of multiparametric flow cytometry have made detailed characterization of lymphocyte subsets possible in whole blood or isolated peripheral blood mononuclear cells (PBMC) of healthy donors and patients, and it has been presented as a powerful tool for immunomonitoring of response to treatment (5, 6). Concurrent to this development, several international consortia have been created to standardize immune-monitoring using flow cytometry for immune-mediated diseases, transplantation, and hematological diseases, for potential use in clinical settings (7–9).

Pathogenic Mechanisms of Multiple Sclerosis

Multiple sclerosis (MS) is a chronic, inflammatory demyelinating disease of the CNS, characterized by infiltration of T-lymphocytes, B-lymphocytes, macrophages, NK cells, demyelination, and axonal damage (10–12). The etiology of MS remains unknown; however, it has been proposed that there is a selective autoimmune response against myelin autoantigens causing damage to the CNS. However, like the majority of autoimmune diseases, the triggers of this response are unknown. Both environmental and genetic factors have been postulated. A 40% concordance in monozygotic twins as well as association with HLA-DRB1*1501 and DQB1*0602 alleles have been described (11, 13). GWAS studies in MS patients have shown the involvement of several loci related to the immune system, of which the HLA locus presents the highest association (14–16).

The existing evidence on the induction and perpetuation of the disease points to an important role of autoreactive CD4+ T-cells (2). Studies in the animal model of MS, experimental autoimmune encephalomyelitis (EAE), have shown that the effector CD4+ T-subpopulations, Th1 and Th17, play an important role in the pathogenesis of the disease. These subpopulations have been found increased in the CNS of patients with MS, mainly in CSF and the perivascular space (3, 4). In addition, oligoclonal expansions of activated CD8+ T-cells in CNS lesions of MS patients have been described, indicating their participation in CNS damage (5, 6). The involvement of B-lymphocytes in the pathogenesis of MS is better understood: they produce autoantibodies; induce, maintain, and reactivate CD4+ T-cells; act as antigen-presenting cells; and produce pro-inflammatory cytokines (7). Impairment in the immunoregulatory function of NK cells in MS patients has also been decribed (12). A schematic overview of the roles of immune cells in MS pathogenesis is represented in Figure 1.

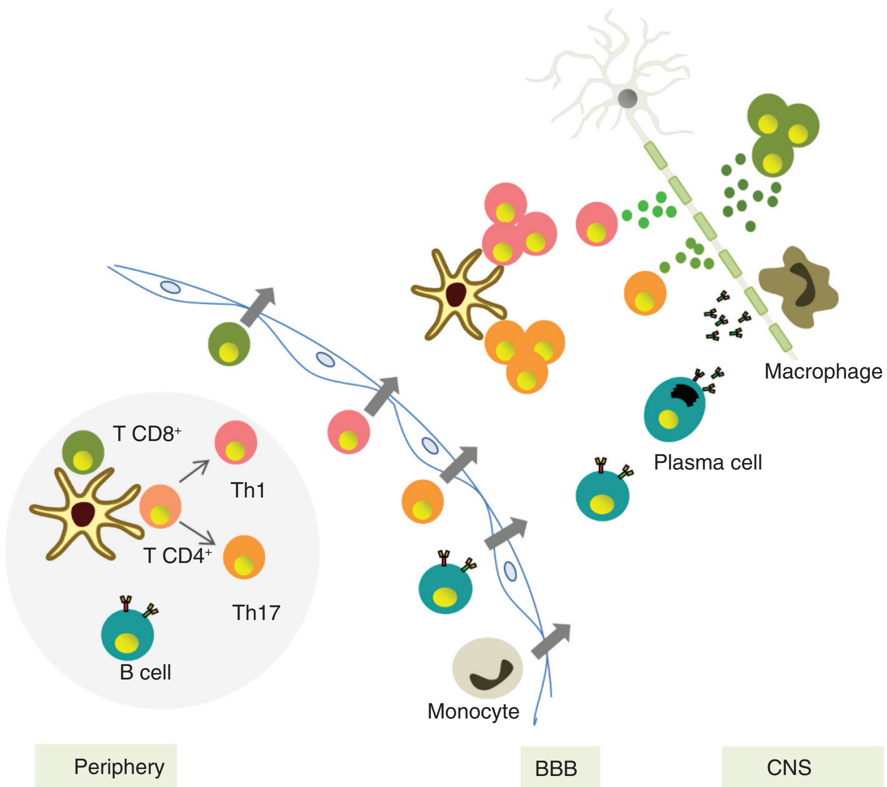

Figure 1 Pathogenic mechanisms of multiple sclerosis. Autoreactive T-cells and B-cells are activated in peripheral lymph nodes where they are differentiated into effector cells, CD8+ T-cells, and CD4+ T-cells (Th1 and/or Th17). Activated cells migrate through the blood–brain barrier (BBB) where they are further activated by local antigen-presenting cells. These processes induce cytokine and chemokine production, facilitating the entry of other cell types from peripheral blood. At the central nervous system (CNS), macrophages and activated T-cells attack myelin components and release cytokines that activate B-cells which mature to antibody-producing plasma cells. This increases the inflammatory response and causes demyelination and axonal damage.

Lymphocyte Subpopulations in MS

The autoimmune inflammatory response in MS results in changes in lymphocyte subpopulations of peripheral blood (17–20). These changes might be useful surrogate markers for the evaluation of disease activity, progression, and monitoring of therapy response.

T-CELL SUBPOPULATIONS

T-cell subpopulations can be divided into naïve, central memory, effector memory, and other minor effector subsets such as terminally differentiated effector cells (T_{EMRA}), based on the expression of CD45RA, CCR7, and CD27 (7, 21). Studies published until now regarding T-cell subpopulations in MS patients are discrepant. Differences among studies might be due to different genetic backgrounds, stages of the disease, analysis of small groups of patients, and also different monoclonal antibodies used to define T-cell subpopulations. These discrepancies are particularly relevant in studies regarding $CD8^+$ T-subpopulations. Whereas some authors report an increase of effector $CD8^+$ T-cells (22, 23), other authors describe a decrease in effector memory and T_{EMRA} $CD8^+$ T-cells in peripheral blood (24). Analysis of the cellularity of the CNS infiltrates show enrichment in the number of effector memory and T_{EMRA} $CD8^+$ T-cells in patients with MS and other inflammatory neurological diseases (25, 26). In these studies, the increase in central memory and effector memory $CD8^+$ T-cells in peripheral blood, and in CSF, were related to active disease or early-stage disease. In contrast, in patients with less active disease, no changes in central memory $CD8^+$ T-cells or the percentages of $CD8^+$ early effector memory in peripheral blood were found, although a decrease in absolute counts of $CD8^+$ early effector memory T-cells could be observed, which would suggest that in MS patients these cells migrate to the CNS (17).

TH17 AND TREG SUBPOPULATIONS

The increased percentage of Th17 in the peripheral blood of RRMS patients has been widely reported and a pathogenic role for these cells postulated (27, 28). Moreover, Th17Th1 cells, a subpopulation which secretes both IL-17 and IFN-γ, have also been related to MS pathogenesis (29). Regarding Treg subpopulations, most of the reports found a similar percentage of Tregs in MS patients compared with healthy donors, although a functional impairment has been found in *in vitro* assays (30–32). In this context, an increase of the Th17/Treg balance has been associated with higher disease activity and severity (20, 33).

B-CELL SUBPOPULATIONS

Although the involvement of B-lymphocytes in the pathogenesis of MS has been a focus in recent years, a full characterization of B-cell subpopulations in peripheral blood of MS patients is still lacking (34, 35). Most of the studies on B-cells are focused on their changes in response to treatments (36, 37).

Current Therapies for MS and Their Effect on Lymphocyte Subpopulations

Even though a number of new drugs have been developed to treat MS, a treatment that can cure the disease has not been developed as yet. Approved treatments reduce the frequency of relapses and decrease inflammation but fall short of stopping CNS degeneration. Current treatments can be divided basically into two groups: those that treat acute relapses (megadoses of metilprednisolone) and disease-modifying therapies (DMTs). DMTs include classic injectable drugs (interferon-β and glatiramer acetate (GA)), oral substances (fingolimod, terifunamide, and dimethyl fumarate (DMF)), and monoclonal antibodies—anti-CD49d (natalizumab) and anti-CD52 (alemtuzumab). Other monoclonal antibodies such as anti-CD25 (daclizumab) and anti-CD20 (ocrelizumab) that cause depletion of B-cells are expected to be in the clinics soon. DMT treatments have broad immune-modulatory/immunosuppressive effects affecting peripheral blood subpopulations (38–41). The major changes in lymphocyte subpopulations in response to DMT treatments are summarized in Table 1.

INTERFERON β (1A AND 1B)

Interferon β (IFN-β) was the first treatment approved for MS. It decreases the number of relapses, progression of disability, and disease activity (measured by MRI). The mechanism of action of IFN-β, although extensively studied, is not fully understood. The known mechanisms include a decrease in lymphocytes activation and proliferation, a reduction in pro-inflammatory cytokines production, and an increase in anti-inflammatory cytokines. IFN-β has a nonspecific immunomodulatory effect on various immune cells, and it has been demonstrated that it interferes with the transmigration of leukocytes through the blood–brain barrier (BBB). This treatment induces a weak leukopenia, an increase of IL-10 that has been associated with an increase of both $CD4^{+}$ and $CD8^{+}$ T regulatory cells, and $CD56^{bright}$ NK cells (42–44). Moreover, some studies described a decrease of IL-17 production, and Th17 cells, in peripheral blood in MS patients under IFN-β treatment (45, 46). It has also been described that the effect of IFN-β causes a decrease of activated and memory T-cells (44, 47); on the other hand, it induces an increase of B-cells production—an increase in transitional (immature) Bccells and k-deleting recombination excision circles (KRECs), thereby supporting its use for increasing B-cell release from bone marrow (17, 48). Its effect on thymic egress of recent thymic emigrants (RTEs) is still unclear, but it seems that IFN-β may induce a decrease of RTEs and TCR recombination excision circles (TRECs) in peripheral blood (48, 49).

GA OR COPOLYMER-1

It is a polymer composed of the most frequent aminoacids in the myelin basic protein (L-tyrosine, L-glutamate, L-alanine, and L-lysine) (13). Its mechanism of action is poorly understood, but it is postulated that GA acts by binding the major histocompatibility complex class II molecules, competing with other antigens as

TABLE 1 Main changes in lymphocyte subpopulations induced by DMT treatments

Drug	ACL	T-cells	CD4+ T-cells	CD8+ T-cells	T-Cells Memory Subsets	RTEs	Th17	Tregs	B-Cells	B-Cells Memory Subsets	B Transitional/ Immature	Bregs
Interferon	↓	↓			↓ memory and activated	NC/↓	↓	↑			↑	
Glatiramer acetate					↑ Th2			↑				
Natalizumab	↑	↑	↑	↑	NC	↑	=	NC	↑	↓ naive ↓ memory	↑	
Fingolimod	↓	↓	↓	↑	↓ naive/CM ↑ EM/TEMRA	↑	NC	↑	↓	NC	↑	↑
Dimethyl fumarate	↓				↑naive ↓EM ↑Th2			=/↑			↑	↑
Alemtuzumab	↓	↓	↓	↓	↑ EM ↑ TEMRA		=	↑	↑			
Teriflunomide	↓				↓ activated*				↓	↓ activated*		

ACL = absolute count lymphocytes, CM = central memory, EM = effector memory, TEMRA = terminally differentiated effector cells, RTEs = recent thymic emigrants; NC = nonconclusive.
*Inhibition synthesis of rapidly dividing lymphocytes.

myelin basic protein, and inhibiting the activation of myelin basic protein-specific T-cells (50, 51). GA has a nonspecific effect on the immune system because no specific changes have been described in peripheral blood of patients under treatment. Some studies describe that GA induces a shift in the CD4 T-cells' response to a Th2 profile. Moreover, it has been proposed that it induces an increase in Treg subpopulation (50, 52).

DIMETHYL FUMARATE

DMF is an oral drug of the fumaric acid ester. It induces activation of the transcription factors Nfr2 (decreasing inflammation) and NF-κB (modifying cytokines production), and diminishes neuroinflammation by promoting the cytoprotection of CNS cells against oxidative stress (41). DMF induces a pronounced lymphopenia that has been associated with the occurrence of rare and fatal cases of progressive multifocal leukoencephalopathy (PML) associated with JC virus infection (53, 54). DMF reduces the number of lymphocytes with a decrease of B-cells and $CD4^+$ and $CD8^+$ T-cells. A decrease of central and effector memory T-cells with a concomitant expansion of naïve T-cells in peripheral blood of patients under treatment with DMF have been reported. Moreover, a shift in T helper (Th) subpopulations (a decrease in Th1 and Th17, and an increase in Th2 and regulatory T-cells) has been reported (55–58). Regarding B-cell subpopulations, an increase of a subset of B-cells with regulatory capacity has been described (59).

TERIFLUNOMIDE

Teriflunomide is an active metabolite of leflunomide, an approved treatment for other autoimmune diseases. It inhibits dihydroorotate dehydrogenase, blocking the *de novo* pyrimidine synthesis that is required by rapidly dividing lymphocytes, resulting in a reversible cytostatic effect that limits the expansion of stimulated T-cells and B-cells. It is administered orally (60–62). Teriflunomide impairs the production of activated lymphocytes (inhibiting their proliferation). Specific changes in lymphocyte subpopulations have not been reported.

FINGOLIMOD

Fingolimod is the first oral drug approved for MS treatment. It is a structural analogue of sphingosine and its phosphorylated metabolite, sphingosine 1-phosphate (S1P). S1P and its receptor ($S1P_1$) mediate the circulation of T-cells and B-cells between blood and lymph nodes (LNs). In physiological conditions, the interaction between S1P and $S1P_1$ promotes their egress from LNs by overcoming retention signals as the chemokine receptor CCR7. Naive and central memory T-cells as well as B-cells express CCR7. In contrast, effector memory T-cells and terminally differentiated effector T-cells (T_{EMRA}) are $CCR7^-$ and may egress from LNs independently of $S1P_1$ receptor. Fingolimod binds to four of the five subtypes of S1P receptors, causing the internalization and degradation of these receptors, and consequently blocking the egress of $CCR7^+$ lymphocytes from LNs (21, 63, 64). The main effect of fingolimod is a decrease of $CCR7^+$ cells in peripheral blood, specifically of naïve and central memory T-cells (65–68). In contrast to T-cells,

B-cell subsets have not been extensively studied in patients under fingolimod treatment. Literature on the effect of fingolimod in naïve and memory subset subpopulations is scarce and equivocal (69–71). An increase in immature and transitional B-cells (71, 72) and Treg cells has been reported in peripheral blood of MS patients under fingolimod treatment (67, 70, 73–76), supporting the conclusion that fingolimod can exert an alternative immunomodulatory mechanism inducing the production of Treg cells, as previously suggested by *in vitro* and *ex vivo* experiments (77–79). Results regarding the effect of fingolimod on Th17 cells are inconclusive and contradictory (67, 72, 75, 80). This is probably a consequence of the diversity in surface markers used to define this T-cell subset. Specifically, CCR7 (a clue marker for cells homing to LNs) can differentiate effector Th17 cells ($CCR7^-$) from central memory or pre-Th17 cells ($CCR7^+$). In a longitudinal study (72), we detected an increase in the percentages of effector Th17 cells, defined as $CD4^+CCR7^-CCR6^+CCR4^+$ following the international consensus of 2008 (21), in accordance with other studies (67). In contrast, Mehling et al. observed, in a cross-sectional study, that Th17 lymphocytes of MS patients were predominantly central memory Th17 and that their percentages were decreased in patients under fingolimod treatment compared with untreated MS patients and healthy donors. These authors did not analyze the effector Th17 subpopulation (80).

ALEMTUZUMAB

It is a humanized monoclonal antibody against CD52, recently approved for MS treatment (previously approved and widely used in the treatment of leukemia). It is administered via intravenous route (13, 41). As CD52 is a panleucocitary molecule, it promotes a rapid, marked, and sustained depletion of T-lymphocytes and B-lymphocytes, NK cells, monocytes, and some granulocytes. Studies performed in a transgenic mouse model postulated that the mechanism of lymphocyte depletion is predominantly antibody-dependent cytolysis (81). A decrease in the percentage of T-cell subpopulations at day 7 posttreatment with the onset of reconstitution 1 month after treatment has been described (82). Although $CD4^+$ and $CD8^+$ T-cell depletion lasts for months after treatment, there is a selective delayed reconstitution of some $CD4^+$ T-cells subsets that remain decreased for up to 24 months after treatment (82, 83). In contrast, there is an increase in the percentages of Tregs with an increase of suppressive activity. No differences in Th1 and Th17 percentages have been reported after reconstitution of the $CD4^+$ T-cell pool (83).

$CD8^+$ T-cell pool reconstitution is faster than $CD4^+$, normalized at the third month after treatment with the dominance of effector subsets (T_{EMRA}) for at least 24 months (82, 84). These results indicate that T-cell recovery is due to homeostatic expansion. In contrast to T-cells, the repopulation of $CD19^+$ B-cells reaches percentages above baseline in the first 12 months of treatment (85). Interestingly, in B-cell reconstitution, there is an output from bone marrow reflected in a significant frequency of immature B-cells in the first months after treatment. The B-cell pool is dominated by memory B-cells at 12 months after treatment; however, they remain below the baseline levels (86). The efficacy of alemtuzumab has been found to last longer than the lymphocyte depletion, probably due to the fact that after treatment there is a reconstitution with a different lymphocyte repertoire (87).

Furthermore, the selectively delayed $CD4^+$ T-cell repopulation can contribute to the suppression of the disease activity (82). The main adverse effect of alemtuzumab is autoimmunity, the most frequent being thyroid autoimmunity, that appears in 30% of patients after treatment (84, 85, 87). The development of autoimmunity could be explained by the homeostatic expansion that occurs in the T-cell pool reconstitution (84).

NATALIZUMAB

Natalizumab is a humanized monoclonal antibody against CD49d (subunit α4 of VLA-4 integrin). The strong adhesion between VLA-4 of lymphocytes and VCAM-1 of the endothelium is very important for the migration of leucocytes through the BBB and entry to the CNS. Natalizumab is administered intravenously, and it binds to CD49d, blocking the transmigration of leucocytes through the BBB. This treatment decreases the occurrence of relapses by up to 90%, inducing a decrease of disease progression and MRI activity. The main side effect of natalizumab is the risk of developing PML caused by JC virus infection, which is associated with high mortality. As natalizumab blocks the transmigration of leukocytes through the BBB, in the peripheral blood of MS patients under treatment with natalizumab, there is an increase in the absolute numbers of B, T $CD4^+$, T $CD8^+$ (without alterations in CD4/CD8 ratio), and NK cells (88–90). The effect of natalizumab on lymphocyte subpopulations is not fully defined, although it has been described that memory T-cells would be increased in peripheral blood and would induce changes in memory B-cells (90–92). Moreover, natalizumab treatment interferes with the mechanisms of bone marrow egress of hematopoietic stem cells, inducing an increase of $CD34^+$ cells in peripheral blood, specifically lymphoid progenitors, transitional B-cells, and RTEs (17, 91, 93–97).

Changes in Lymphocyte Subpopulations as Biomarkers of Therapy Response

Immunomonitoring of peripheral lymphocyte subpopulations may be useful to assess treatment response. In DMF treatment, patients with stable disease had lower numbers of $CD4^+$, $CD8^+$ T, and B-cells than those with active disease (98). Moreover, percentages of $CD8^+$ T-cells and B-cells at 6 months after treatment could predict response to treatment (98). Regarding response to fingolimod treatment, Song et al proposed that percentages of central memory $CD4^+$ T-cells could predict relapse (76). In a pilot study, our group described that the baseline percentage of RTEs and transitional B-cells are lower in responder patients. Therefore, immunomonitoring their percentages could be a tool for predicting which patients would be good candidates to receive fingolimod treatment. Moreover, the percentage of late effector memory $CD4^+$ T-cells and RTEs could provide information on the response to therapy as early as 1 month after starting this therapy (72). Using quantitative flow cytometry as a tool for immune-monitoring, a method for immunomonitoring CD49d receptor occupancy in MS patients under natalizumab therapy has been reported. Using this method, it is possible to determine

the percentage of CD49d molecules bound to natalizumab and identify those patients with low receptor occupancy (suboptimal doses), which in a long-term sustained therapy context would show a decrease in treatment efficacy (99).

Conclusion

DMTs induce changes in lymphocyte subpopulations that can be detected in peripheral blood using flow cytometry. Treatment with monoclonal antibodies (natalizumab and alemtuzumab), fingolimod, and DMF induces a clear effect on different peripheral blood lymphocyte subpopulations. In contrast, IFN-β, GA, and teriflunomide produce nonspecific changes. Immunomonitoring lymphocyte subpopulations allows to define biomarkers of therapy response and opens up the opportunity to initiate a personalized therapy in MS treatments, enabling clinicians to choose the best treatment for each patient and predict which patients are the most suitable for receiving a specific therapy.

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