Cell-Based Modulation of Autoimmune Responses in Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis: Therapeutic Implications

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Key Words

Multiple sclerosis · Experimental autoimmune encephalomyelitis · T regulatory cells · Myeloid-derived suppressor cells · Immunoregulation

Abstract

Multiple sclerosis (MS) is a prototypic autoimmune inflammatory disorder of the central nervous system (CNS). MS pathogenesis is a complex phenomenon that is influenced by genetic and environmental factors that lead to the dysregulation of immune homeostasis and tolerance. It has been shown that pathogenic T lymphocyte subsets, such as T helper 1 (Th1) and Th17 cells, play a crucial role in the autoimmune cascade influencing disease initiation, progression and subsequent tissue damage during MS. On the other hand, several mechanisms have been described in both patients and animal models of MS with the potential to modulate myelin-specific autoimmune responses and to facilitate amelioration of disease pathology. To this end, regulatory T cells (Tregs) are considered to be a powerful cell subset not only in the maintenance of homeostasis but also in the re-establishment of tolerance. Along these lines, other cell subsets such as dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs), $\gamma\delta$ T cells and natural killer (NK) cells have been shown to regulate the autoimmune response in the

CNS under certain circumstances. This review will attempt to summarize the relevant knowledge of the regulatory mechanisms exerted by immune cells in MS that could hold the promise for the design of novel therapeutic strategies.

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Introduction

Multiple sclerosis (MS) is a common, complex disorder of the central nervous system (CNS) characterized by immune-mediated demyelination and it is associated with a variety of symptoms that result in functional deficits and handicap. It is the leading cause of neurological disability in young adults and middle-aged people in the developed world [1], affecting 0.05–0.15% of patients of Caucasian ancestry. MS is considered to be a T-cell-mediated autoimmune disease, with pathogenic T cells being directed against antigens expressed in the CNS [2–4]. Immune cell infiltration of the brain and spinal cord and the subsequent plaque formation are the key characteristics of MS [4, 5]. However, a totally differ-

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ent view has recently emerged that challenges the traditional 'outside-in' model and, via reinterpretation of currently available research data, it proposes that MS is a primary degenerative disorder and that the excess inflammation observed is an epiphenomenon due to the host's aberrant immune response ('inside-out' model) [6]. Current knowledge of the mechanisms that are involved in MS pathogenesis and resolution of the disease has been obtained by extensive studies in animal models [4]. Although these findings have significantly advanced the number of therapeutic targets in MS, the currently used treatments [7] still display only modest efficacy in a significant proportion of patients. Thus, a better understanding of the cells subsets and the molecules that regulate the myelin-specific pathogenic response will pave the way for the design of better-tolerated novel therapeutic protocols.

Epidemiology and Genetics of MS

Epidemiology

MS is one of the most well-studied diseases, but many aspects of its epidemiology remain obscure or a matter of ongoing debate. Its uneven distribution across the world, still the most apparent epidemiological characteristic [1], gave rise to the traditional view of a positive correlation between MS prevalence (the number of patients with MS alive at a specific date per 100,000 population) and latitude. This was first proposed by Kurtzke [8, 9], who described 3 MS frequency zones: high (>30 per 100,000) in the northern parts of Europe and North America, medium (5–30 per 100,000) in southern Europe and the southern USA and low (<5 per 100,000) in Asia and South America. This theory was later challenged because reports of increasing prevalence came from many regions [10, 11] that had been considered medium frequency zones. However, this issue is still not resolved as recent meta-analyses have come to contradictory conclusions that either support [12] or discard [1, 13] the association of MS prevalence with latitude.

What has become evident in several reports evaluating temporal changes in the incidence (i.e. the number of new cases per 100,000 per year) of MS is a disproportional increase in females over the last 25–50 years [14]. Initially observed in longitudinal studies from Canada [15] and Northern Europe [16], this has recently been confirmed in Mediterranean countries [11]. The increasing female-to-male ratio was also found in a Canadian study of immigrants (Canadian Collaborative Study) and was steep-

est in those migrating from certain regions (northern Europe, USA) and at a younger age (<21 years old) [17]. These findings indicate that environmental factors are operational in MS and may be gender-specific.

Genetics

In this respect, a search for possible genetic or/and environmental factors that may be linked to susceptibility to MS would be the next step. Indeed, systematic genetic-epidemiological studies assessing the risks to relatives of MS probands found that first-degree relatives are generally at a 10–25 times greater risk of developing MS than the general population [18]. This risk correlates with degree of kinship, with monozygotic twins having the greater risk ($\times 170$) followed by offspring from a conjugal pair ($\times 150$) [18, 19]. The identification of family aggregation in MS indicated that genetic factors may have a role in disease pathogenesis and prompted research for putative genes. The association of the human leukocyte antigen (HLA) gene cluster in chromosome 6p21.3 with the risk for MS was already known in 1972 [20]. Further gene association and linkage studies identified the *HLA-DR2* (*HLA-DRB1*15*) as the strongest MS susceptibility locus with heterozygosity conferring an odds ratio (OR) of 2.7 and a homozygosity of 6.7 [21]. In addition, a recent study found a higher female-to-male ratio in affected individuals with *HLA-DRB1*15*-positive genotypes compared to those without, thus implicating an epigenetic modification at the major histocompatibility complex (MHC) region, which may explain the excess female-to-male ratio in MS in recent years, as discussed above [22]. However, in other regions several other HLA haplotypes were identified with either a risk-promoting or a protective role, thus revealing a much more complex situation than was originally conceived. Interestingly, in 2007, genome-wide association studies identified 2 new genes, namely those encoding the interleukin (IL)-7 receptor α (*IL7Ra*) and *IL2Ra*, as being strongly associated with MS, albeit with relatively modest ORs (<1.35) [23]. Subsequently, the latest screen has identified 52 non-HLA MS risk loci with modest OR in the range 1.1–1.3, almost half of them with over 50% frequency in patients of European ancestry [24]. As might be expected, the vast majority of these MS-associated loci are located close to or inside genes encoding immune-system-related molecules, strongly supporting the hypothesis that MS is primarily an immune-mediated disease. Despite this great progress in MS genetics, the associated variants so far identified still only explain about 50% of the inherited risk of MS [25].

Immunology of MS

Autoreactive T lymphocytes directed against myelin proteins are thought to play a crucial role in the autoimmune pathogenesis of MS. This notion is supported by extensive studies in animal models as well as by evidence obtained from MS patients. First, in humans, disease susceptibility is controlled by genes that are critically involved in T cell activation such as the MHC (mentioned above) [26, 27]. Furthermore, myelin-specific CD4⁺ T cells have been isolated from the peripheral blood of patients with MS [28–30] and active MS lesions are characterized by infiltrating T cells with myelin reactivity [30–32], indicating a prominent role of T cells in CNS pathology during MS.

The potent role of T lymphocytes in MS pathogenesis has been demonstrated via studies in the well-characterized mouse model, termed experimental autoimmune encephalomyelitis (EAE), which closely resembles MS. EAE is induced in susceptible strains of mice upon immunization with CNS antigens in complete Freund's adjuvant including intraperitoneal injection of pertussis toxin [33]. Clinical EAE is characterized by paralysis that starts in the tail muscles and hind legs and then progressing towards the upper limbs. Disease can either spontaneously resolve or follow a chronic phase. Importantly, EAE can be transferred to syngeneic hosts by myelin-specific T cells isolated from myelin-immunized donors. The encephalitogenic T cells are CD4⁺ lymphocytes that, upon myelin recognition, secrete an array of effector cytokines, indicating that myelin-specific CD4⁺ T cells are necessary and sufficient to induce EAE.

The susceptibility as well as the clinical course of EAE can vary depending on the sensitizing antigen and the strain of animal employed. To this end, immunization of SJL/J (H-2^s) mice with myelin basic protein (MBP), myelin proteolipid protein or their peptides leads to the development of the remitting-relapsing (RR) form of EAE, while immunization of C57BL/6 (H-2^b) mice with myelin oligodendrocyte glycoprotein (MOG) peptide induces the chronic disease phenotype. Similar to in SJL/J mice, immunization of PL/J and B10.PL (both H-2^u) with MBP peptides results in acute but relapsing EAE. Besides EAE induction in wild-type animals, various T cell receptor (TCR) transgenic strains with specificity for myelin peptides have been generated and employed in order to study the mechanisms involved in CNS autoimmunity that have been reviewed elsewhere [34]. Collectively, the EAE mouse model has proved an excellent tool for investigating the pathogenetic mechanisms and immunoregulatory

networks that operate during MS, since, apart from the ethical constraints, factors such as the inaccessibility of CNS tissue have made the investigation of such processes in humans a complicated task.

The autoimmune cascade leading to MS pathogenesis could be divided into several steps (fig. 1). Initially, myelin-specific T cells should be activated in the peripheral lymphoid compartments upon recognition of myelin antigens presented by professional antigen-presenting cells (APCs). Upon activation, T cells upregulate the expression of cell-surface molecules that facilitate their exit from the lymphoid organs and their migration to the CNS. This step is highlighted by T cell adhesion to vascular walls and extravasation across the blood-brain barrier (BBB). Once in the CNS, activated T cells encounter myelin peptides presented by local APCs (including microglial cells), thereby triggering their effector functions. Specifically, upon recognition of myelin, T cells proliferate and produce effector molecules including cytokines [tumor necrosis factor (TNF)- α , interferon (IFN)- γ , IL-2 and IL-17] and chemokines. These molecules orchestrate the autoimmune response in the CNS, by attracting and activating inflammatory cells such as macrophages, monocytes, neutrophils, B cells and CD8⁺ T lymphocytes. Once in the CNS, CD11b⁺ myeloid dendritic cells (DCs) acquire and present myelin peptides leading to the proliferation of infiltrating pathogenic Th17 cells and, in this way, amplify the autoimmune cascade [35]. The net results of this cascade are demyelination, plaque formation and axonal damage [3, 5, 36].

Pathogenic T Helper Cells in MS and EAE

There are two major pathogenic effector CD4⁺ T cell subsets in EAE and MS, the T helper type 1 (Th1) and Th17 cells. Th1 cells are the main producers of IFN- γ and were thought initially to be the major pathogenic T cell subset in EAE. To this end, increased levels of IFN- γ have been found in the cerebrospinal fluid (CSF) of MS patients and the spinal cords of mice with EAE [37]. In addition, myelin-specific Th1 cells transferred into mice induce severe EAE. However, paradoxical data have demonstrated that IFN- γ ^{-/-} mice that lack Th1 cells develop more severe EAE, indicating that Th1 are not the only pathogenic cells participating in the inflammatory cascade during disease development [38]. Through cytokine profiling, a distinct CD4⁺ T cell subset was described that was characterized by the secretion of IL-17 and was therefore labeled Th17 cells [39]. These are now known to pro-

Fig. 1. Myelin-specific T cells that have escaped central tolerance circulate harmlessly in the periphery of healthy individuals and are kept silent by peripheral mechanisms of tolerance. Breakdown of these mechanisms, leads to the activation of clonal expansion followed by extravasation through the BBB to enter the CNS. Subsequently, infiltrating T lymphocytes encounter myelin antigens presented by local APCs and exert their pathogenic function via the secretion of inflammatory cytokines/chemokines, thereby am-

plifying the inflammatory cascade. This involves the recruitment of DCs, macrophages, neutrophils, B cells and CD4/CD8 T cells that contribute significantly to the destruction of myelin. However, these responses have been shown to be counterbalanced by modulatory mechanisms involving cell subsets such as Tregs, DCs, MDSCs, $\gamma\delta$ T cells, NK and NK T cells with the ultimate goal being the suppression of the autoimmune response and disease remission.

duce IL-17, IL-21 and TNF- α and have been shown to be pathogenic in EAE [40]. In MS, the analysis of MS tissue has demonstrated the presence of Th17 cells in lesions as well as in the blood of patients with MS [41]. Overall, Th1 and Th17 cells play a crucial role in the pathogenesis of EAE and have been linked to the demyelination process during MS.

B Cells in MS and EAE

It has been proposed that B cells mainly exert a pathogenic role in CNS autoimmunity through (1) the secretion of autoantibodies that react against myelin that contribute to demyelination and axonal damage and (2) the presentation of myelin antigens to T lymphocytes, thus promoting autoreactivity. The presence of oligoclonal

bands in the CSF of >95% of MS patients is the most consistent immunologic finding that argues for an abnormal B cell activation within the CNS [42]. In addition, the presence of ectopic meningeal B cell follicles in a substantial proportion of secondary progressive MS patients was associated with a more severe disease phenotype (disease onset at an earlier age and disability) and extensive cortical demyelination [43]. More recently, histopathological analysis of tissues from MS patients demonstrated the deposition of antibodies and complement on the myelin sheath of lesions, supporting the notion that B cells play an important role in disease pathogenesis [44]. As such, in MS patients in several past and ongoing phase II and III trials, treatment with rituximab or ocrelizumab depleted B cells positive for the CD20 antigen reduced gadolinium-enhancing lesions and tended to reduce the relapse rate. However, anti-CD20 therapy does not target

plasma cells and does not influence the presence of oligoclonal bands, which indicates that autoantibody production is not the main mechanism mediating the pathogenic role of B cells in MS [42].

The potential role of B cells and antibodies in EAE development has been demonstrated in myelin-specific TCR transgenic mice crossed with mice expressing the heavy chain of a myelin-specific antibody. It was found that double transgenic mice developed an aggressive autoimmune disease affecting the optic nerves and spinal cord [45]. Although these data indicate that B cells play a pathogenic role in EAE, several reports have proposed that this subset plays an immunoregulatory role in disease pathogenesis; this will be discussed in the following section of this review.

Regulatory Subsets in MS and EAE

It is well accepted that, in healthy individuals, central tolerance mechanisms are incomplete, resulting in the production of self-reactive T cells in the periphery. Once in the periphery, these cells are kept in check by additional mechanisms of tolerance, either recessive (anergy, deletion and ignorance) or dominant (regulatory T cells) [46]. Autoimmune diseases are thought to emerge upon disruption of any of these mechanisms that leads to the activation of these self-reactive T cells. Therefore, it is of great importance to better understand how individual cell subsets and molecules participate in the maintenance of immune homeostasis and tolerance as well as to investigate the mechanisms that might be involved in the re-establishment of tolerance when disrupted. Here, we review the literature on the different cell subsets that have been shown to modulate the autoimmune responses in patients with MS and in the EAE mouse model.

Regulatory T Cells

Over the last 2 decades, strong evidence has emerged for a dominant role of regulatory T lymphocytes in the regulation of self-tolerance and the maintenance of immune homeostasis in both humans and mice [47]. Initial experiments demonstrated that the depletion of CD4⁺CD25⁺ T cells from normal animals leads to the spontaneous development of various autoimmune diseases such as gastritis, thyroiditis and type 1 diabetes whereas the reconstitution of normal CD4⁺CD25⁺ T cells prevents these disorders [48, 49]. Following the potent regulatory function of the CD4⁺CD25⁺ T cell subset, these cells were termed regulatory T cells (Tregs). Subse-

quent extensive research established that Tregs are divided into two major subsets: naturally-occurring Tregs (nTregs) and inducible Tregs (iTregs) [50]. nTregs are thymic-derived cells that constitute 5–10% of the total of peripheral CD4⁺ T cells, whereas iTregs are generated in the periphery upon exposure of naïve T cells to several tolerogenic stimulus. A breakthrough in the field came with the identification of the transcription factor forkhead box P3 (FoxP3) as a specific marker for Tregs [51–53]. Foxp3 expression was also proven to be crucial for Treg development and function. Most importantly, the absence of Tregs due to FoxP3 gene mutations led to the development of severe autoimmune disorders, known as the scurfy phenotype in mice and the IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy and X-linked syndrome) in humans [54].

Tregs in MS

Autoreactive T cells specific for myelin antigens are present in healthy individuals, suggesting that they are kept silent by regulatory mechanisms that turn out to be defective with MS. Indeed, several reports have demonstrated an impairment of Treg function in MS. To this end, CD4⁺CD25^{high} Treg cells isolated from the peripheral blood of MS patients were found to have a defective suppressive function in vitro [55–57], even though their frequencies were not altered [58, 59]. Interestingly, Treg suppressive activity was restored upon treatment with IFN- β [60, 61], glatiramer acetate (GA) [62] or steroids [63] and remission was achieved. The defective function of Tregs was also correlated with a decreased expression of Foxp3 at both the mRNA and the protein level [64]. Concerning the newer monoclonal antibodies, a recent study showed that natalizumab treatment blocked the transmigration of Foxp3⁺ Tregs, similar to nonregulatory T cells, and did not restore the impaired function of Tregs in MS patients. This occurred even though the frequency of peripheral blood Tregs was unaffected by natalizumab treatment and the suppressive capacity of D4⁺CD25^{high}CD127^{low}Foxp3⁺ Tregs under in vitro conditions was not altered [65]. Even more recently, alemtuzumab, an anti-CD52 humanized monoclonal antibody that depletes potentially circulating B and T lymphocytes in the repopulation process that follows alemtuzumab administration, a population enriched with T cells with a regulatory phenotype (i.e. anergy to stimulation with allogeneic DCs and the ability to suppress the allogeneic response of autologous T cells) is recognized. In addition, alemtuzumab's ability to suppress T cells seems to rely on the presence of CD25^{hi} T cells [66].

Whether Tregs play a role in the suppression of autoreactive T cell responses in the target organ is less clear. A greater occurrence of nTregs has been found in the CSF of MS patients, but the suppressive ability of these cells is also impaired. However, a more recent study showed that an increased proportion of Tregs in the CSF expressed the CD45RO^{hi}CD95^{hi} phenotype that is sensitive to apoptosis, hypothesizing that Tregs might die via apoptosis within the MS lesion.

Additional evidence for a potential role of Tregs in the pathogenesis of MS was obtained through genome-wide association studies, which identified single nucleotide polymorphisms associated with disease risk. Specifically, CD25, CD127 and CD58, all closely related to Treg homeostasis and function, have been identified as MS susceptibility genes.

Tregs in EAE

More definitive evidence for the potential role of Tregs in MS pathogenesis has been obtained in the EAE mouse model. Initially, it was shown that a population of CD4⁺ T cells that did not express the transgenic receptor was able to prevent the development of spontaneous EAE in immunodeficient (RAG1^{-/-}) MBP TCR transgenic mice [67,68]. Furthermore, the adoptive transfer of CD4⁺CD25⁺ T cells reduced the onset and disease severity in MOG-induced EAE [69]. The protective effects of CD4⁺CD25⁺ Treg cells appear to be mediated by IL-10 since Treg cells for IL-10^{-/-} mice failed to ameliorate disease [70]. Furthermore, Treg depletion via the administration of anti-CD25 resulted in an increased severity of EAE that was accompanied by increased mortality [71] and rendered the resistant B10 mouse strain susceptible to EAE [72].

Treg cells have also been shown to play a prominent role in the remission phase of EAE. It has been demonstrated that Tregs accumulate in the CNS at the peak of the disease, and their depletion inhibits recovery. An important question that arose from these findings was whether the increased Treg frequency was due to the expansion of pre-existing nTregs or the conversion of non-Treg cells. Korn et al. [73], using transfer of Foxp3-GFP T cells, were able to demonstrate that Tregs in the CNS were expanded from pre-existing Tregs, rather than converted. Interestingly, the same study showed that although Tregs were increased in the CNS at the peak of the disease, they were unable to suppress myelin-specific T cell responses *in vitro*, possibly due to the increased levels of inflammatory mediators in the CNS [73]. On the other hand, neurons have been shown to convert effector T cells into Tregs in a TGF- β -dependent manner [74]. Collec-

tively, these studies demonstrate that Treg cells have the potential to suppress ongoing myelin-specific autoimmune responses.

Dendritic Cells/Monocytes

DCs are characterized as professional APCs with a unique ability to initiate immune responses by priming naïve T lymphocytes. Two major subsets of DCs have been described, conventional DCs (cDCs) of myeloid origin and plasmacytoid DCs (pDCs) of lymphoid origin. Despite their prominent immunogenic role, under certain circumstances, DC subsets have been demonstrated in both mice and humans to possess a regulatory role, and are thus characterized as tolerogenic DCs [75–77].

Dendritic Cells

There are several studies that have addressed the regulatory role of DCs in MS and EAE. To this end, it was demonstrated that TNF- α -treated DCs express a semi-mature DC (smDC) phenotype; intravenous injection of MOG-pulsed smDCs significantly ameliorated EAE [78]. In another study, intraperitoneal injection of embryonic stem cell-derived DCs transfected with programmed-death ligand 1 (PD-L1) in mice with EAE prevented disease development [79]. Although the precise mechanism involved in the amelioration of EAE by tolerogenic DCs remains unclear, other lines of evidence suggest that tolerogenic DCs might promote the induction and/or expansion of Treg cells. This feature was closely attributed to pDCs where it was demonstrated that autoantigen-presentation by pDCs inhibited EAE via the induction of Treg cells, and the selective inhibition of MHC class II expression by pDCs exacerbated pathology [80]. Furthermore, the antibody-mediated depletion of pDCs during the acute phase of EAE significantly exacerbated disease, indicating a regulatory role for pDCs during the progression of the autoimmune response [81]. In contrast, the depletion of pDCs during the priming of EAE significantly reduced disease onset and severity [82], suggesting that it depends on the disease stage and the respective microenvironment as to whether DCs possess a stimulatory or a tolerogenic role. IFN- β was thought to exert its action by downregulating the expression of costimulatory molecules and thus decreasing the production of IL-12 by DCs, which is required for differentiation to Th1 [83]. More recently, it was suggested that the inhibition of the processing of Toll-like receptor 9 (TLR9) underlies the beneficial effect of IFN- β treatment in RRMS/CIS patients compared to untreated patients, since activated pDCs separated from

IFN- β -treated patients had significantly reduced levels of the processed TLR9 protein, which resulted in a decreased production of both IFN- α and the proinflammatory cytokines IL-6 and TNF- α [84]. Furthermore, IFN- β efficacy in RRMS has been linked with its ability to suppress IL-23 and IL-1 β production and increase IL-10 production in human DC treatment [85]. Natalizumab also seems to decrease the expression of MHC class II molecules and the number of CD209 DCs, as shown in a recent report [86].

Monocytes

Monocytes are circulating blood cells that constitute almost 5–10% of the total leukocytes in mice and humans. They are highly heterogeneous cells, characterized mainly by the expression of the CD14 and CD16 antigens in humans and CD11b, F4/80 and CD115 in mice. In the field of CNS autoimmunity, GA, currently used as a therapeutic modality in MS patients, has been shown to directly affect the function of monocytes, rendering them regulatory. Initial studies demonstrated that GA treatment in humans increased the IL-10 production by DCs and inhibited IL-12 secretion [87, 88]. Recently, Weber et al. [89] reported that GA promoted the development of type II monocytes that have the capacity to instruct the differentiation of Th2 cells and Tregs cells. Indeed, adoptive transfer of GA-treated type II monocytes induced Tregs in vivo and conferred protection from EAE. In addition, it was recently suggested that a lack of response to IFN- β treatment may be related to perturbations of the type I IFN signaling pathway in monocytes. This was based on the observed selective increase in phosphorylated STAT1 levels and IFN receptor 1 expression in the monocytes of IFN- β nonresponders in MS patients at baseline, which resulted from an increased monocyte type I IFN secretion upon the innate immune stimuli via TLR4 [90]. With regard to the most recent MS treatments, natalizumab was associated with decreased numbers of CD14+ monocytes, and possibly their migratory potential, since their expression levels of $\alpha 4\beta 1$ were decreased, whereas fingolimod treatment resulted in a higher fraction of monocytes in the peripheral blood and CSF [91]. Notably, macrophages and monocytes express only low levels of CD52 and, as such, undergo minimal and transient depletion after alemtuzumab treatment, which does not impair their functionality [92]. At present, it is not clear whether the new oral compounds such as laquinimod, teriflunomide or dimethylfumarate affect the components of the innate immune system.

Among the different subsets of monocytic cells, classically activated M1 macrophages and alternatively activated M2 macrophages have received considerable attention. M1 macrophages protect the host during viral or bacterial infections and also participate in antitumor responses, while M2 macrophages have anti-inflammatory properties and have been shown to regulate immune responses [93]. In EAE, CNS-infiltrating macrophages polarize T cells towards pathogenic Th1 cells [94] and it has been proposed that M1-like macrophages contribute to axonal loss [95]. In contrast, other studies have demonstrated the protective role of macrophages in CNS demyelination via the induction of apoptosis in infiltrating T effector cells as well as via the secretion of anti-inflammatory cytokines such as IL-10 and TGF- β [96].

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) consist of a heterogeneous population of the myeloid precursors of macrophages, DCs and granulocytes, that, functionally, have been shown to potently suppress the ongoing immune response [97–104]. They are characterized by the coexpression of Gr-1 and CD11b and they can be divided into cells with monocytic (CD11b⁺Ly6C⁺Ly6G[−]) or granulocytic (CD11b⁺Ly6C^{low}Ly6G⁺) morphology [97, 105–108]. The role of MDSCs in the field of autoimmunity and particularly in MS is just emerging. To this end, we were the first to demonstrate, in the blood of patients with active MS, a significant enrichment of CD15⁺CD33⁺HLA-DR^{low}CD14[−] MDSCs that potently suppress autologous T cell proliferation in vitro [108]. Additional evidence was obtained in the EAE mouse model that the adoptive transfer of granulocytic MDSCs reduces the severity and delays the onset of disease. Moreover, G-MDSCs inhibited the priming of myelin-specific Th1 and Th17 priming via the coinhibitory molecule PD-L1. In line with our mouse-model data, Dardalhon et al. [109] reported that CD11b⁺Ly6G⁺ MDSCs regulate Th1 responses and EAE development. Conflicting data have been obtained regarding the role of the monocytic MDSC subset. Thus, in 1 study, CD11b⁺Ly6C⁺ MDSCs suppressed myelin-specific T cell responses in vitro [110], whereas 2 other reports demonstrated a pathogenic role of the same cell subset during the effector phase of EAE [111, 112].

Although the investigation of the role of MDSCs in suppressing autoimmune responses is still in its infancy, the increased immunosuppressive properties of these cells make them a potential target for the design of therapeutic protocols of diseases of the CNS and other autoimmune diseases.

$\gamma\delta$ T Cells

$\gamma\delta$ T cells represent an infrequent T cell subset. They express a distinct TCR composed of two glycoprotein chains, termed the γ and δ chains. It has been demonstrated that $\gamma\delta$ T cells recognize antigen in the context of MHC class IB molecules and have a prominent role in the recognition of lipid antigens.

Although $\gamma\delta$ T cells have been shown to contribute to MS pathogenesis via a cytotoxic function, other reports indicate that they could also exert an immunoregulatory role and thus mediate amelioration of the disease. A potential immunoregulatory role of $\gamma\delta$ T cells in MS has been proposed by a study in which MBP-specific T cells were used to vaccinate MS patients in an attempt to induce immune tolerance. It was demonstrated that $\gamma\delta$ T cells expanded in response to the vaccine and produced high levels of IL-2, TNF- α and IL-10 [113], proposing a possible regulatory role, in contrast to encephalitogenic T cells. Furthermore, another study showed that treatment of MS patients with IFN- β 1 α was correlated with decreased levels of IL-12 and an increase in IL-10 secretion by $\gamma\delta$ T cells, suggesting that this cell subset plays a regulatory role [114].

The immunomodulatory potential of $\gamma\delta$ T cells is further supported by studies in EAE, where the depletion of these cells in spinal cord homogenate-immunized B10.PL mice aggravated disease and decreased antigen-specific T cell responses [115]. In a similar vein, $\gamma\delta$ T cells were shown to exert immunoregulatory properties during EAE by inducing the apoptosis of encephalitogenic $\alpha\beta$ T cells [116] in a Fas/FasL-dependent manner.

Overall, the precise role played by $\gamma\delta$ T cells in the regulation of EAE and MS has not been fully elucidated because contradictory results have been reported in studies that used different mouse strains and different strategies to address this task. The conditions under which $\gamma\delta$ T cells could exert immunomodulatory properties in MS need further investigation.

B Cells

Several lines of evidence have indicated an immunoregulatory role for B cells in the development of EAE. To this end, a subset of B cells characterized as CD18^{hi}CD5⁺ has been shown to secrete elevated levels of IL-10 during EAE development, and adoptive transfer experiments have demonstrated its participation in the amelioration of disease [117]. In addition, B cells have been shown to chemoattract Treg cells to the CNS at the peak of the disease and to influence the recovery phase [118].

NK and NK T Cells

Natural killer (NK) cells are a heterogeneous population of lymphocytes that lack antigen-specific receptors, and were originally described as playing a critical role in innate immune responses.

NK T cells are of lymphocytic origin, sharing the properties of T and NK cells. NK T cells are distinct from conventional T lymphocytes, since they express a heavily biased TCR repertoire that usually recognizes lipid antigens such as α -galactosylceramide [119], in the context of the monomorphic MHC class I-like molecule CD1d [120–122]. Upon activation, both populations secrete various cytokines, chemokines or cytotoxic enzymes, thus contributing to innate and adaptive immune responses. There are several studies suggesting that NK and NK T cells could downregulate immune responses [123–127]. In addition, accumulative data also support the active involvement of these cells in the immunoregulation of MS.

NK Cells

Initially, reduced numbers of NK cells and their impaired functional activity were reported in MS patients, indicating a possible regulatory role of this cell subset in MS pathogenesis [128–130]. Subsequently, NK cells isolated from MS patients in remission were shown to express high levels of Fas (CD95) and to secrete Th2 cytokines such as IL-5 and IL-13 [131–133]. NK cells with this phenotype were termed NK2 cells and were proposed to downregulate IFN- γ secretion by T cells from PBMCs, upon stimulation with MBP [131].

The regulatory role of NK cells is further supported in rodent models of MS. Specifically, in vivo depletion of NK cells by anti-NK-cell antibody administration exacerbated the clinical features of disease and induced fatal EAE, suggesting a protective role of NK cells in EAE development [134]. In line with this, the depletion of NK cells in MOG-induced EAE resulted in a more severe form of disease and augmentation of Th1 responses in response to myelin antigen [135]. Moreover, several studies have correlated NK activity with the suppression of proinflammatory cytokine secretion (such as IFN- γ and TNF- α) by encephalitogenic T cells [136–139]. Furthermore, bone marrow-derived NK cells exhibit potent inhibitory effects on autoreactive T cell proliferation to both Con A and MBP in dark agouti rats [140]. Although it has been postulated that NK cells might directly affect the interactions between DCs and T cells [141, 142], the precise molecular mechanisms via which they downregulate autoimmune encephalitogenic responses have still to be determined.

It is noteworthy that daclizumab, a humanized monoclonal antibody against IL-2 receptor α chain (IL2RA or CD25) that showed benefits in clinical and imaging parameters in patients with RRMS, is thought to exert its biological effect by the expansion of immunoregulatory NK cells (NK CD56^{bright}) [120]. Of the other recently introduced therapies for MS, natalizumab treatment exerts no significant effects on NK cells, whereas in fingolimod-treated patients, elevated NK cells have been observed both in the periphery and in the CNS [91]. NK cells from alemtuzumab-treated huCD52 mice also retain their ability to lyse target cells in vitro [92]. It is currently not known if laquinimod, teriflunomide or dimethylfumarate act on NK cells.

NK T Cells

Similar to observations in NK cells, the frequency of the V α 24⁺ NK T cell subset was significantly reduced in the periphery of MS patients compared to healthy controls [143]. In addition, CD4⁺ NK T cells isolated from MS patients in remission were characterized by increased levels of IL-4 secretion, but the production of IFN- γ was diminished [144]. In a recent longitudinal study, IFN- β treatment of MS patients significantly increased the percentage of NK T cells in the PBMCs that were characterized by elevated production of IFN- γ , IL-4 and IL-5 upon glycosphingolipid α -GC stimulation [145].

The results obtained from EAE studies have provided a better understanding of the role of NK T cells in the regulation of disease. To this end, a series of elegant reports has demonstrated that the activation of NK T cells by the administration of the glycosphingolipid α -galactosylceramide can result in the amelioration and even full prevention of EAE [146–148]. These results raise important implications regarding the therapeutic potency of glycosphingolipids because these ligands can also activate human NK T cells, thus providing an approach that could be promising for the treatment of MS. Finally, NK T cells have been shown to actively participate in the recovery phase of EAE, so they do possess a regulatory role [149].

Astrocytes in MS

Until recently, astrocytes, apart from their role in establishing the BBB, were considered ‘bystanders’ in acute MS plaques or as responsible merely for the formation of a gliotic scar in chronic MS lesions [150, 151]. However, in the last 10 years, the discovery that neuromyelitis op-

tica (NMO), a demyelinating condition initially considered to be an atypical, aggressive MS variant, is caused by antibodies against aquaporin-4 [(AQP4) the most abundant water channel protein in the CNS, mainly expressed on astrocytic foot processes], created an upsurge of interest and called for a reappraisal of the role of astrocytes in MS. As such, in pathologic specimens of active MS lesions, the findings of loss of perivascular astrocyte endfeet and astrocyte hypertrophy at the margin of the acute lesion were unambiguous. Notably, astrocyte damage persisted even after acute inflammation and macrophage activity had subsided [151]. Evidence is accumulating (extensively reviewed by Brosnan and Raine [151]) that astrocytes may contribute to lesion development in MS by expressing cytokines and chemokines that attract leukocytes to sites of injury in the CNS and by acting as tissue-damage mediators by producing factors toxic for oligodendrocytes and neurons. In addition, they are mechanistically involved in disruption of the BBB and may also have a role in upregulating adhesion molecules and matrix metalloproteases, thus facilitating the entry of activated T lymphocytes into the CNS parenchyma. Astrocytes possibly also contribute to lesion repair not only through scar formation but also through limiting lesion development [151]. Furthermore, it has been suggested that axonal degeneration in MS may be associated with mitochondrial energy failure. In this respect, white matter astrocyte dysfunction may play a role. It is postulated that white-matter astrocytes in MS show a reduced metabolism of adenosine triphosphate-generating phosphocreatine, which may impair the astrocytic sodium potassium pump and lead to a reduced sodium-dependent glutamate uptake, thus leading to extracellular glutamate accumulation. In addition, a deficiency in astrocytic β 2 adrenergic receptors may be responsible for a reduced glycogenolysis, resulting in a decreased formation of lactate and glutamine, which are energy sources for axons, and for increased levels of nitric oxide. All these mechanisms may impair axonal mitochondrial metabolism, maybe via Ca⁺²-mediated excitotoxicity, leading to axonal degeneration [152]. As mentioned above, NMO is now envisaged as primary astrocytopathy with secondary demyelination, since pathological studies have demonstrated the extensive loss of immunoreactivities for the astrocytic proteins, AQP4 and glial fibrillary acidic protein in active NMO lesions in contrast to increased AQP4 in active MS lesions, thus supporting the notion that astrocytes are selectively targeted in NMO. In addition, recent experimental studies have confirmed that the AQP4 antibody, the hallmark of NMO diagnosis, is indeed

pathogenic, since it results in selective astrocyte destruction and dysfunction in vitro, ex vivo and in vivo [153]. In mice lacking sphingosine-1 phosphate (S1P1) receptor on glial fibrillary acidic protein-expressing astrocytes but not on neurons, the efficacy of fingolimod, a known S1P1 receptor modulator, is lost, and attenuation of EAE severity has been observed, despite a normal response to fingolimod with respect to lymphocyte trafficking [154], thus pointing to astrocytes as possible targets for therapy. Finally, a new oral molecule, laquinimod, which is being tested as an immunomodulatory therapy for RRMS, has been found to markedly reduce NF- κ B (a transcription factor essential for the rapid regulation of cellular responses) activation, both in human astrocytes (but not in microglia) and in cuprizone-treated mice [155].

Novel or Emerging MS Therapies and Neuroprotection

Although existing first-line therapies in MS (i.e. IFN- β and GA) seem to succeed in ameliorating the inflammation that heralds the demyelination process, there is an unmet need for therapies aiming at axonal degeneration that would hinder/hamper disability. It seems this is being partially addressed by some novel drugs that were recently added to our therapeutic armamentarium or are going to be approved in the near future. To this end, fingolimod, apart from its main immunomodulating role (i.e. the prevention of lymphocyte egress from lymph nodes) upregulates the in vitro microglial production of brain-derived neurotrophic factor (BDNF) and glial-cell-derived neurotrophic factor, suggesting the promotion of the neuroprotective effects of microglia [156]. A putative neuroprotective role, apart from its anti-inflammatory properties, has also been ascribed to laquinimod, as it has been associated with increased serum levels of BDNF [157]. In addition, in the MOG-induced EAE model, laquinimod treatment, apart from reducing glutamatergic transmission while increasing GABAergic transmission, also preserved cannabinoid CB1 receptor sensitivity, which is normally lost during EAE induction, thus limiting glutamate excitotoxicity and possibly axonal damage [158]. Dimethyl fumarate, a molecule previously used as fumaric acid esters in psoriasis therapy, has recently proved beneficial in MS. Although hypothesized to exert its immunologic effects by shifting cytokine production from a 'Th1' pattern to a 'Th2' pattern (the basis of its usage in dermatology) novel mechanisms of action have recently been proposed. In this respect, the applica-

tion of dimethyl fumarate was associated with activation of the transcription factor nuclear (erythroid-derived 2)-related factor (Nrf2) and subsequently with the protection of glial cells against oxidative stress (possibly via glutathione recycling) [159]. Furthermore, it was recently suggested that activation of Nrf2 may be partially attributed to the modified astrocyte expression of histone deacetylases regulated by dimethyl fumarate [160]. Both the new monoclonal antibodies introduced in MS treatment seem to have a neuroprotective function. On the one hand, natalizumab reduced the CSF neurofilament level 3-fold, which is a biomarker of axonal damage [161]. On the other hand, lymphocytes reconstituting/regenerating after alemtuzumab treatment produced increased concentrations of BDNF, platelet-derived growth factor and ciliary neurotrophic factor, which may contribute to the sustained improvement of disability, thereby supporting the notion of neuroprotective autoimmunity [162]. Based on the concept that impaired energy balance underlies axonal degeneration and the progression of disability (especially in primary/secondary progressive MS), which, in part, is caused by excess accumulation of intra-axonal Na⁺ and Ca²⁺ ions, targeting voltage-gated sodium channels would exert a neuroprotective effect. In this regard, blocking acid-sensing ion channel 1 (ASIC1) with amiloride in patients with primary progressive MS results in a significant reduction in the rate of whole-brain atrophy. Immunohistochemical studies on postmortem spinal-cord tissue acquired from primary progressive MS patients have demonstrated the increased expression of ASIC1 in axons and oligodendrocytes in chronic inactive lesions [163]. Similar results in terms of protection against axonal degeneration in 2 EAE models were obtained with 2 additional sodium channel blockers, sefinamide and flecainide. Notably, both potently suppressed microglial activation via an independent mechanism [164]. Another trial with topiramate, a partial Na⁺ channel blocker is underway. The potential of neuroprotection with sodium channel blockers seems especially pertinent with the provocative news that a high salt concentration promotes the differentiation of CD4⁺ T cells into Th17 cells in vitro and exacerbates the course of EAE in vivo [165]. Finally, another promising strategy that favors/serves both neuroprotection and remyelination is to antagonize LINGO-1 (leucine-rich repeat and immunoglobulin-domain-containing 1) a CNS-specific protein expressed on both neurons and oligodendrocytes. LINGO-1 forms a complex with the Nogo receptor and inhibits neurite outgrowth, but has recently been documented as a key negative regulator

of the differentiation and myelination of oligodendrocyte progenitor cells [166]. A phase-I trial with anti-LINGO-1 antibody BIIB033 is currently underway (ClinicalTrials.gov identifier: NCT01244139).

Conclusions

In spite of the major advancements in the mechanisms involved in the re-establishment of immune homeostasis in EAE, the developing protocols aimed at the specific suppression of the autoimmune response are still in their infancy and cell-based therapies hold the greatest promise for achieving these aims. As we learn more about the

cells, molecules and pathways involved in the induction of tolerance during an autoimmune disease, we will progress toward developing novel therapeutic protocols that could result in the prevention or even reversal of unwanted immunity. The satisfaction derived from new findings on MS regulatory mechanisms will only be fully realized when such insights are translated into new therapeutic protocols.

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