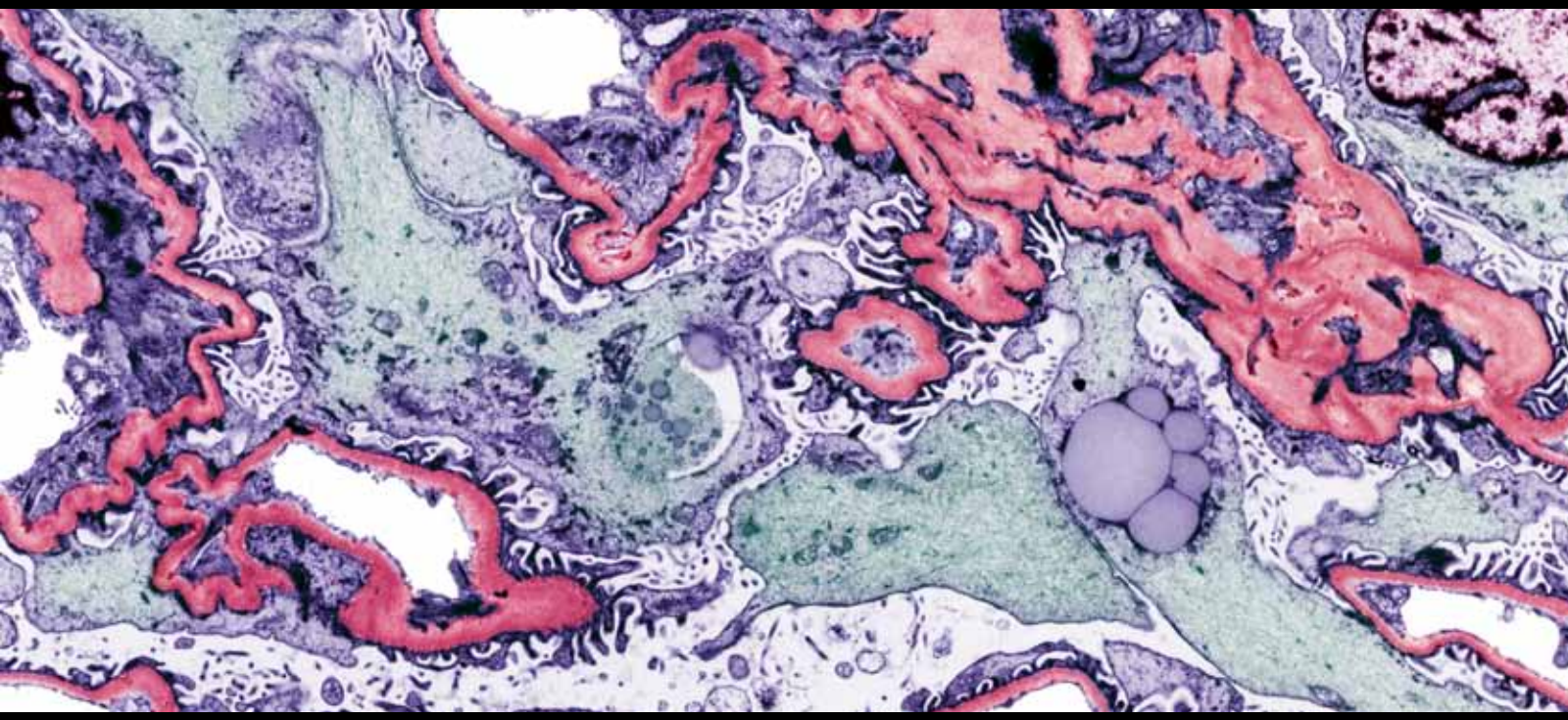
Multiple Sclerosis

Guest Editors: Sreeram Ramagopalan, David Dyment, Rachel Farrell, and Noriko Isobe



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Editorial Board

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Editorial
Multiple Sclerosis

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Multiple sclerosis (MS) is the commonest disease of the central nervous system (CNS) to cause permanent disability in young adults. Based on strong circumstantial evidence, MS is considered to be an organ-specific autoimmune disorder, but the exact cause is as yet unknown. It appears that the disease develops in a genetically susceptible population as a result of environmental exposures.

The incidence of MS has been documented to be increasing across the globe. The focus of this issue was therefore on studies with a goal of further understanding the aetiology of the disease. We were pleased to have received submissions on a wide variety of topics including the immunology of MS (research papers on the roles of Fc receptors by X. Z. Hu et al. and tumor necrosis factor-(TNF-) related apoptosis-inducing ligand (TRAIL) by A. L. O. Hebb and colleagues) and the genetics and epigenetics of MS (reviews on the future of MS genetics by S. V. Ramagopalan and D. A. Dyment and microRNAs in MS by K. U. Tufekci and coworkers). The potential role of vitamin D deficiency influencing susceptibility to and the clinical course of MS is gaining interest, and to this end, we have a review paper on this topic by J. Smolders and a research paper describing the novel role of a vitamin D receptor modulator on experimental autoimmune encephalomyelitis by S. Na et al. The role of mitochondria as potentially underlying CNS damage is reviewed by G. R. Campbell and D. J. Mahad, and J. Witherick and colleagues review a potential treatment measure, namely, mesenchymal stem cells. Finally, we have a perspective piece regarding the potential role of human endogenous retroviruses in MS by B. Krone and J. M. Grange, and finally G. Disanto and colleagues try to address the complex issue of heterogeneity of multiple sclerosis.

We hope that you enjoy reading this special issue as much as we enjoyed putting it together.

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Noriko Isobe

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Research Article

Inhibition of Experimental Autoimmune Encephalomyelitis in Human C-Reactive Protein Transgenic Mice Is FcγRIIB Dependent

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We showed earlier that experimental autoimmune encephalomyelitis (EAE) in human C-reactive protein (CRP) transgenic mice (CRPtg) has delayed onset and reduced severity compared to wild-type mice. Since human CRP is known to engage Fc receptors and Fc receptors are known to play a role in EAE in the mouse, we sought to determine if FcγRI, FcγRIIb, or FcγRIII was needed to manifest human CRP-mediated protection of CRPtg. We report here that in CRPtg lacking either of the two activating receptors, FcγRI and FcγRIII, the beneficial effects of human CRP are still observed. In contrast, if CRPtg lack expression of the inhibitory receptor FcγRIIB, then the beneficial effect of human CRP is abrogated. Also, subcutaneous administration of purified human CRP stalled progression of ongoing EAE in wild-type mice, but similar treatment failed to impede EAE progression in mice lacking FcγRIIB. The results reveal that a CRP $\rightarrow$ FcγRIIB axis is responsible for protection against EAE in the CRPtg model.

1. Introduction

C-reactive protein (CRP) is a widely used blood marker of inflammation [1], but it is increasingly apparent that the protein plays a causal role in host defense against microbial pathogens [2] and in cardiovascular disease [3]. Furthermore, in at least three different mouse models, human CRP has been shown to protect against autoimmune disease [4–6]. Importantly, we showed that human CRP transgenic mice (CRPtg) are resistant to experimental autoimmune encephalomyelitis (EAE) [6], an animal model of multiple sclerosis (MS). Thus in CRPtg compared to wild-type mice, EAE onset was delayed, its severity was attenuated, and infiltration of encephalitogenic T-cells and monocytes/macrophages into the CNS was prevented [6]. The encephalitogenic cells with which CRP interacts to manifest protection in EAE and the mode of action of human CRP on these cells were not identified. Since human CRP binds both human and mouse Fc receptors [7–10] and because there is growing evidence that Fc receptors play a major role in controlling the emergence of EAE and other autoimmune diseases [11–15], we sought to determine if FcγRs were required for human CRP-mediated protection against EAE in the mouse.

Here we show that for CRPtg mice lacking expression of the activating receptors FcγRI and FcγRIII, expression of human CRP delays onset and reduces severity of EAE as well as or better than it does in CRPtg with an intact FcγR repertoire. In contrast in CRPtg mice that lack expression of the inhibitory receptor, FcγRIIB, no human CRP-mediated protection from EAE is observed. Likewise, administration of purified human CRP to wild-type mice with ongoing EAE prevented the disease from worsening, whereas the same treatment failed to halt worsening of EAE for mice lacking FcγRIIB. The combined data suggest that human CRP $\rightarrow$ mouse FcγRIIB interaction and its presumed

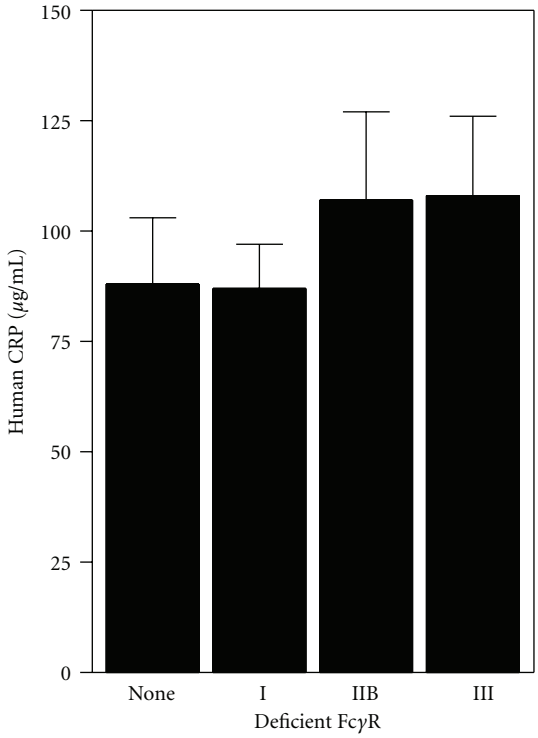

Figure 1: No effect of FcγR deficiencies on expression of human CRP by CRPtg mice. Each bar and each whisker are the mean and standard deviation, respectively, for human CRP serum concentration measured for $n = 5$ mice. Blood was obtained 24 hours after i.p. injection of 25 μg endotoxin, and human CRP was measured by ELISA, both as described in [6].

inhibitory consequences are essential for realizing human CRP-mediated protection against EAE in the CRPtg mouse model.

2. Materials and Methods

2.1. Animals. CRPtg mice have been described in detail elsewhere [16, 17]. The CRPtg strain (C57BL/6 background) carries a 31-kb human DNA fragment encoding the *CRP* gene, all the known *cis*-acting CRP regulatory elements (i.e., the entire human *CRP* promoter) and the *CRP* pseudogene [16]. *Cis*-acting regulatory elements within the transgene are responsible for both tissue specificity and acute phase inducibility of its expression, and the *trans*-acting factors required for its correct regulation are conserved from mouse to man [16, 17]. Human CRP is expressed as an acute phase reactant in CRPtg and reaches blood levels comparable to those observed in humans with inflammatory disease (up to 500 μg/mL) [17]. We showed earlier that human CRP level in the blood of CRPtg was elevated during the course of EAE [6].

CRPtg mice were mated to mutants (also C57BL/6) lacking functional expression of the genes encoding the α-chains of FcγRI (FcγRI$^{-/-}$ mice) [12], FcγRIIB (FcγRIIB$^{-/-}$ mice) [18], and FcγRIII (FcγRIII$^{-/-}$ mice) [19]. FcγR-deficient versus sufficient and CRPtg versus non-CRPtg progeny were obtained in the expected Mendelian ratios each genotype appeared phenotypically normal, and none of the FcγR deficiencies significantly altered expression of human CRP (Figure 1). To identify the various genotypes, we used *CRP* transgene-specific and *FcγR* mutation-specific PCRs, as described [12, 16–19]. All mice were fed a standard chow diet (Ralston Purina Diet) and maintained at constant humidity (60 ± 5%) and temperature (24 ± 1°C) with a 12-hour light cycle (6 AM to 6 PM). All protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and were consistent with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication 96-01, revised 1996).

2.2. Induction of EAE. An immunodominant myelin oligodendrocyte protein (MOG) peptide was used to immunize 10–12-week-old mice, as described in [6]. On days 0 and 7, mice received subcutaneously an injection of 150 μg MOG peptide emulsified in complete Freund's adjuvant containing 500 μg heat-killed *Mycobacterium tuberculosis* (Difco, Detroit, MI). On days 0 and 2, mice received an intraperitoneal injection of pertussis toxin (500 ng) (List Biological Laboratories, Campbell, CA). Development of EAE symptoms was monitored daily using a clinical scale ranging from 0 to 6 as follows: 0, asymptomatic; 1, loss of tail tone; 2, flaccid tail; 3, incomplete paralysis of one or two hind limbs; 4, complete hind limb paralysis; 5, moribund (in which case animals were humanely euthanized); 6, dead. Mice were observed for at least 30 days, and those with a score of at least 2 for more than 2 consecutive days were deemed to have developed EAE. The maximum clinical score achieved by each animal during the 30-day observation period was used to calculate average maximum clinical score (severity) for each experimental group. To study the time-course of disease, average clinical scores were calculated and plotted daily for each group of mice, and cumulative disease index was calculated by area under the curve. When determining the average day of onset of EAE, animals that did not develop any symptoms of EAE during the 30-day period were assigned a day of onset of 31.

2.3. Administration of Human CRP to Mice with EAE. EAE was induced as described above, and the development of symptoms was monitored. On the day their disease symptoms achieved or eclipsed a score of 2 (flaccid tail), each mouse received subcutaneously an injection of 50 μg of highly purified (95%–98%) human CRP (US Biological, Swampscott, MA). The disease course was then followed for an additional 10 days. The CRP preparation was sodium azide-free, contained <0.4 ng endotoxin/mg protein by *Limulus* amebocyte assay, and had pentameric integrity as judged by overloaded native polyacrylamide gel electrophoresis (data not shown). Control animals received 200 μg of heat-denatured (boiled for 5 minutes) human CRP.

2.4. Statistical Analyses. Among genotype differences in EAE, day of onset and maximum clinical score (mean ± sem)

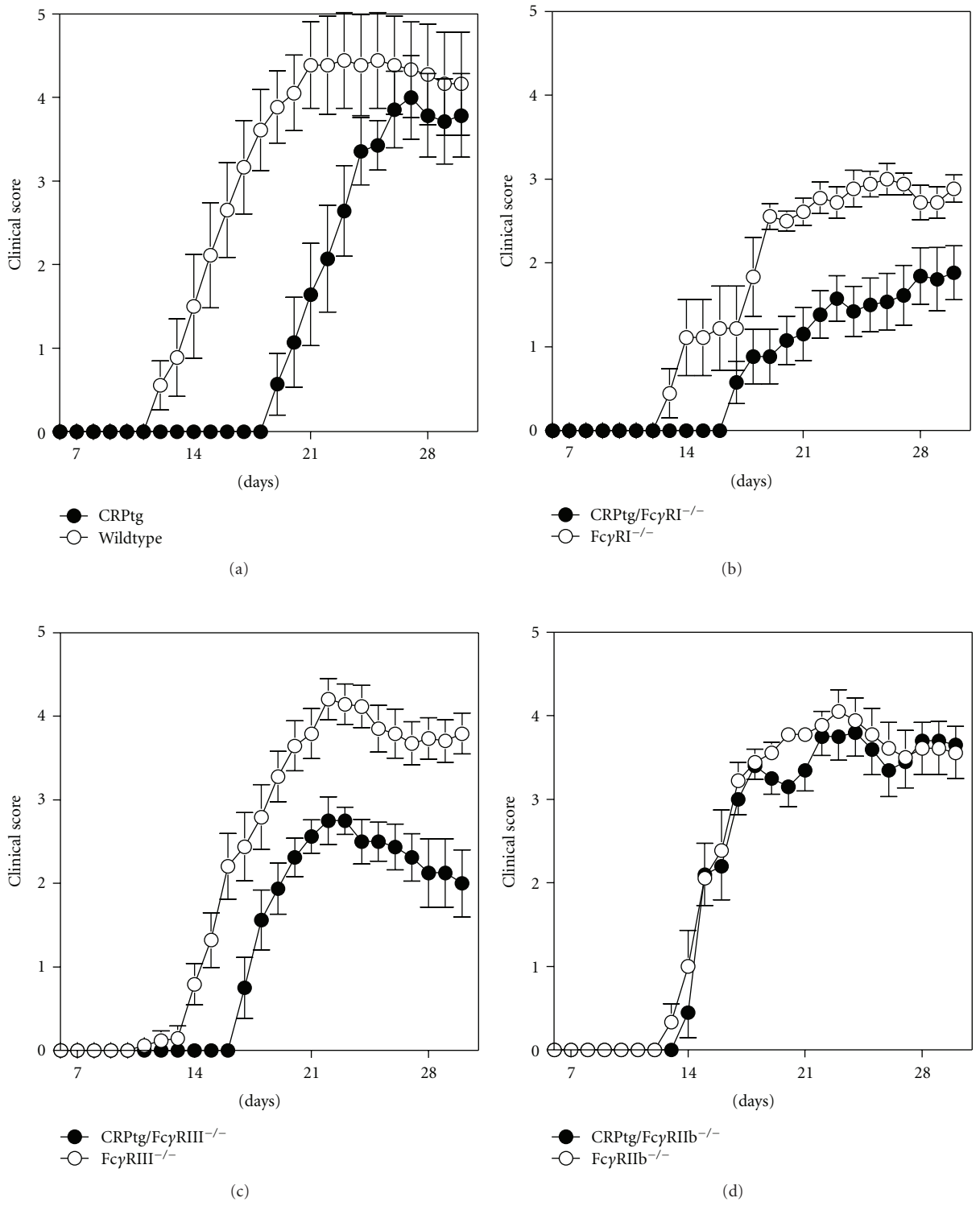

Figure 2: CRP-mediated protection from EAE requires FcγRIIB. CRPtg versus littermate wildtype (a) or their respective counterparts lacking expression of FcγRI (b), FcγRIII (c), or FcγRIIB (d) were injected with MOG peptide, and EAE symptoms were monitored. Presence of the CRP transgene (closed circles in each panel) delayed onset of EAE in mice with intact FcγRs (a) and delayed onset and reduced severity of EAE in mice lacking FcγRI (b) or FcγRIII (c). In contrast in mice lacking FcγRIIB (d), expression of human CRP had no beneficial effect. See Table 1 for sample sizes and statistical analyses.

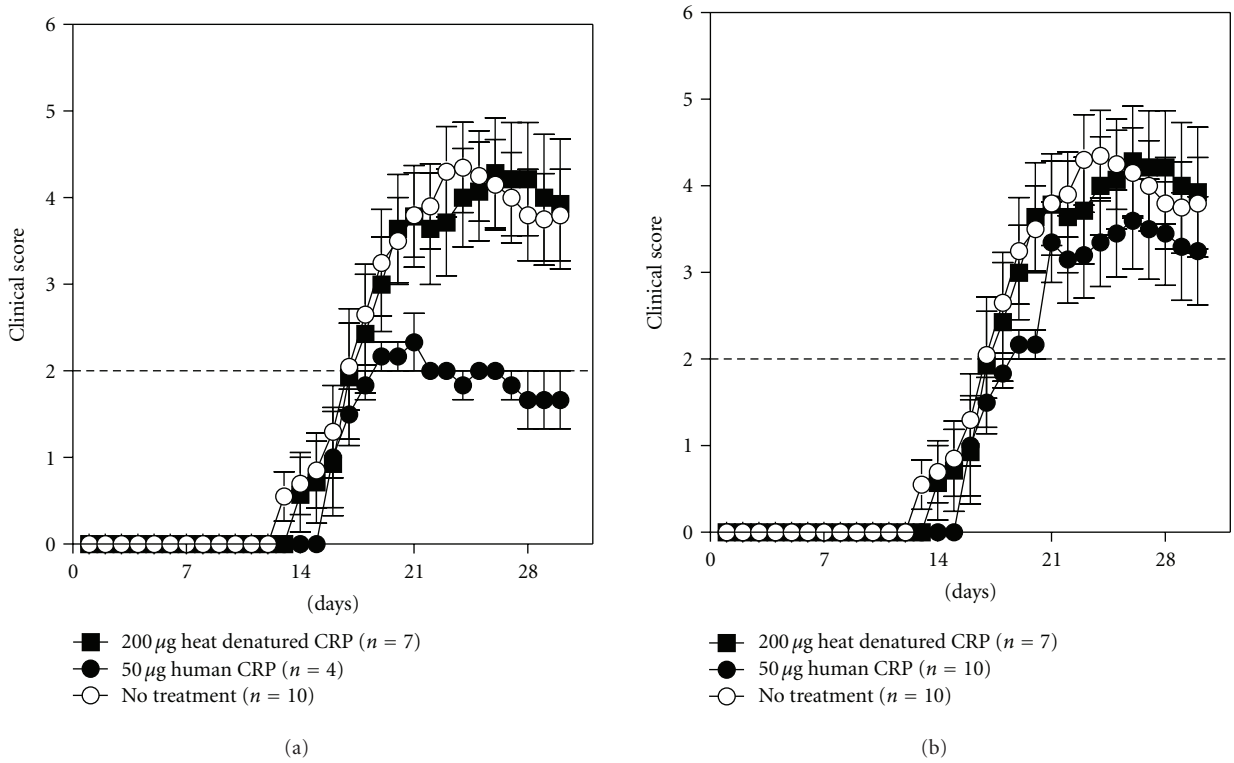

Figure 3: CRP treatment stalls progression of EAE in wild-type mice but not in FcγRIIB$^{-/-}$ mice. Wildtype (a) versus FcγRIIB$^{-/-}$ (b) with ongoing EAE were injected with 50 μg purified CRP s.c. when their clinical scores reached 2 (horizontal line), and EAE symptoms were monitored for 10 days. Controls received heat-denatured CRP.

were evaluated by one-way ANOVA and posthoc Neuman-Keul's multiple comparison tests. A P value less than .05 was considered significant.

3. Results and Discussion

As we reported previously in [6], onset of EAE was delayed by ~1 week for CRPtg compared to wild type mice (Figure 2(a) and Table 1; $P < .001$, t-test), and this delay led to reduced cumulative disease index (Table 1; 32.8 versus 46.55) even though average disease severity was not significantly lowered (Table 1 and Figure 2(a)). In comparison, for CRPtg that lacked expression of either FcγRI or FcγRIII (Figures 2(b) and 2(c), resp.), human CRP-mediated protection included not only a delay in EAE onset and a reduced cumulative disease index but also a significant reduction in disease severity (Table 1). In contrast, for CRPtg mice lacking the inhibitory receptor FcγRIIB, expression of human CRP conferred no protective benefit (Table 1 and Figure 2(d)).

Other groups showed that human CRP administered subcutaneously to mice can reverse autoimmune- and antibody-induced inflammation [5, 20], a beneficial effect that reportedly requires certain FcγRs [20]. To test if human CRP administration might likewise protect mice from EAE and to test if FcγRIIB was required, we administered purified human CRP to wildtype versus FcγRIIB$^{-/-}$ mice with ongoing disease. The results are summarized in Figure 3. We observed that for wildtype mice (Figure 3(a)) treatment with human CRP, but not treatment with heat-denatured CRP, halted progression of EAE. In contrast, no protective influence of CRP therapy was observed for FcγIIB$^{-/-}$ mice (Figure 3(b)).

It has been documented that some of the in vivo activities of human CRP likely result (directly or indirectly) from the proteins ability to bind FcγRs [20, 21]. FcγRs are a family of receptors of which most mammals express four main types: FcγRI, FcγRII, FcγRIII, and FcγRIV [14, 22, 23]. Each of FcγRI, FcγRIII, and FcγRIV is comprised of a ligand binding α-chain paired with a common γ-chain (Fc$R\gamma$) that encodes an immunoreceptor tyrosine-based activation motif (ITAM) essential to propagate cell activating signals. FcγRIIB on the other hand is comprised of a single α-chain and it carries a cytoplasmic tyrosine-based inhibitory motif (ITIM) that propagates cell inhibiting signals. Various investigators have reported that human CRP binds to one or more isoforms of FcγRI, FcγRII, and FcγRIII, in both mouse and man [7–10], and FcγRs reportedly influence EAE in the mouse [14, 24]. Thus in CRPtg, human CRP potentially could either exacerbate EAE by binding one of the inflammation-promoting FcγRs on encephalitogenic cells or dampen EAE by binding FcγRIIB. Using CRPtg mice with selective deletion of FcγRs, we were able to investigate if either capacity is realized in vivo.

Compelling evidence was obtained that the beneficial action of human CRP in mouse EAE depends mainly on

Table 1: Effect of transgenic expression of human CRP on the outcome of EAE in mice lacking various Fcγ receptors[a].

Strain	No. mice	Clinical measure of disease symptoms				CDI[b]
		Day of onset mean ± sem		Severity mean ± sem		
Wildtype	26	16.5 ± 0.6		3.9 ± 0.1		46.55
CRPtg	13	20.3 ± 0.8	$P < .001$[c,d]	4.1 ± 0.2	ns[c]	32.8
FcγRI$^{-/-}$	9	16.3 ± 0.9	ns[c]	3.3 ± 0.2	ns[c]	38.78
FcγRI$^{-/-}$/CRPtg	13	21.6 ± 1.3	$P < .001$[d]	2.7 ± 0.1	$P < .01$[d]	18.21
FcγRIIb$^{-/-}$	9	15.1 ± 0.4	ns[c]	4.2 ± 0.2	ns[c]	55.33
FcγRIIb$^{-/-}$/CRPtg	10	15.2 ± 0.3	ns[d]	4.1 ± 0.3	ns[d]	51.83
FcγRIII$^{-/-}$	16	15.6 ± 0.5	ns[c]	4.4 ± 0.2	ns[c]	50.58
FcγRIII$^{-/-}$/CRPtg	8	18.4 ± 0.5	$P < .05$[d]	3.1 ± 0.2	$P < .05$[d]	29.63

[a]EAE was induced with MOG peptide as described in Section 2.
[b]Cumulative disease index (area under the curve: arbitrary units as described in Section 2).
[c]Results of Neuman-Keuls multiple comparison test comparing indicated genotype to wildtype
[d]Results of Neuman-Keuls multiple comparison test comparing indicated CRPtg genotype to its non-CRPtg littermates.

expression of the inhibitory receptor FcγRIIB. Thus in FcγRIIB$^{-/-}$ mice, EAE is neither delayed nor dampened by transgenic expression of human CRP. In fact the tempo and severity of EAE in CRPtg/FcγRIIB$^{-/-}$ was not significantly different from that seen in wild type mice. In contrast, the human CRP-associated delay in EAE onset and attenuation of EAE symptoms were fully expressed in mice that lacked the activating receptors: FcγRI or FcγRIII. We did not formally rule out the possibility that FcγRIV might play a role, as FcγRIV$^{-/-}$ mice are not available to us, but we did perform experiments with mice that lack the FcR common gamma chain FcRγ, which are predicted to lack expression of FcγRI, FcγRIII, and FcγRIV [14]. Human CRP transgenic FcR$\gamma^{-/-}$ were obviously more resistant than wildtype mice (data not shown), nevertheless the contribution of FcRγ (and thus FcγRIV) to human CRP-mediated resistance to EAE remains unclear because FcR$\gamma^{-/-}$ mice *per se* are intrinsically very resistant to EAE [24]. Thus in their sum the data suggest that the EAE-protective effect of human CRP in the CRPtg mouse depends largely on the availability/expression of FcγRIIB. Presumably by binding FcγRIIB, human CRP expressed endogenously during the course of disease dampens the activation state of encephalitogenic (FcγRIIB-expressing) cells in CRPtg. Likewise, in nontransgenic mice, exogenously administered human CRP has the same effect as long as FcγRIIB is present.

4. Conclusions

For CRPtg mice, transgene-expressed human CRP inhibits EAE, and this beneficial action requires FcγRIIB. If as in CRPtg with EAE, a protective CRP → FcγRIIB axis exists in humans with MS, then CRP administration might be beneficial in the clinical treatment of patients with MS. Ongoing efforts in our laboratory are aimed at identifying the CRP-responsive FcγRIIB-expressing encephalitogenic cell(s) involved in this action, which we posit to be dendritic cells [25].

Acknowledgments

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Research Article

Effects of IFN-B on TRAIL and Decoy Receptor Expression in Different Immune Cell Populations from MS Patients with Distinct Disease Subtypes

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Using quantitative RT-PCR, we compared mRNA levels for TRAIL [tumor necrosis factor (TNF)–related apoptosis-inducing ligand] and its receptors in various immune cell subsets derived from the peripheral blood of untreated normal subjects (NS) and patients with distinct subtypes of multiple sclerosis (MS): active relapsing-remitting MS (RRA), quiescent relapsing-remitting MS (RRQ), secondary-progressive MS (SPMS) or primary-progressive MS (PPMS). Consistent with a role for TRAIL in the mechanism of action of interferon-β (IFN-β), TRAIL mRNA levels were increased in monocytes from patients clinically responsive to IFN-β (RRQ) but not those unresponsive to this therapeutic (RRA). TRAIL-R3 (decoy receptor) expression was elevated in T cells from untreated RRMS patients while IFN-β therapy reversed this increase suggesting that IFN-β may promote the apoptotic elimination of autoreactive T cells by increasing the amount of TRAIL available to activate TRAIL death receptors. Serum concentrations of soluble TRAIL were increased to a similar extent by IFN-β therapy in RRQ, RRA and SPMS patients that had not generated neutralizing antibodies against this cytokine. Although our findings suggest altered TRAIL signaling may play a role in MS pathogenesis and IFN-β therapy, they do not support use of TRAIL as a surrogate marker for clinical responsiveness to this therapeutic.

1. Introduction

Multiple sclerosis (MS) is a chronic neurodegenerative autoimmune disorder characterized by CNS inflammation, demyelination, and axonal injury resulting in clinical relapses and disability [1–3]. MS is considered to be a T cell-mediated disease [4, 5] in which failed apoptotic deletion of autoreactive T cells has been implicated as a pathogenic mechanism [6, 7]. Apoptosis plays an important role in immune system homeostasis by eliminating autoreactive immune cells that might otherwise promote autoimmunity [8]. Tumor necrosis factor- (TNF-)related apoptosis-inducing ligand (TRAIL) plays a key regulatory function in this regard by activating death receptors present on various cellular components of the immune system such as T cells, B cells, and monocytes [9]. As a result, a number of immune cell subtypes have been implicated in autoimmunity subsequent to the loss of TRAIL function [9]. Although $CD4^+$ T cells specific for myelin antigens are thought to initiate and exacerbate MS through secretion of proinflammatory cytokines, peripheral blood monocytes may also contribute to this disease by migrating to the CNS and releasing inflammatory mediators that trigger nerve and tissue damage [1, 2, 10–12]. In the case of B lymphocytes, three lines of evidence suggest these immune cells are involved in MS pathogenesis: increased myelin-specific antibodies, presence of B cells reactive against myelin, and the ability of the anti-CD20 antibody Rituximab to deplete B cells and reduce relapses and disease burden as assessed by MRI [11–14].

TRAIL, also known as Apo2 ligand (Apo2L), is a member of the TNF superfamily that shares 24% amino acid homology with the death receptor CD95 (Fas/ApoL) ligand [15]. TRAIL and CD95L can promote the apoptotic death of a number of cancer cells [15]. Despite TRAIL mRNA being

present in a wide variety of tissue types, most normal cells are resistant to TRAIL cytotoxicity [15]. CNS inflammation in MS is associated with elevated expression of TRAIL, both within the CNS and autoreactive immune cells [16–18]. TRAIL inhibits activated T cell proliferation through intricate interactions with various receptors for this cytokine [19]. The initial TRAIL receptor identified, death receptor 4 (DR4 or TRAIL-R1), transmits proapoptotic signals via a cytoplasmic death domain. DR5 or TRAIL-R2 also contains a DR4-like death domain that conveys apoptotic signaling [15]. TRAIL-R3 and TRAIL-R4 lack the cytoplasmic tails found in TRAIL-R1 and TRAIL-R2 necessary to trigger apoptosis and therefore act as decoy receptors [15]. These decoy receptors prevent TRAIL-induced apoptosis and represent an important mechanism for regulating the apoptotic sensitivity of immune cells. The selective expression of decoy receptors in normal tissues has led to the proposal that TRAIL may be useful for preferentially inducing the apoptosis of cancer cells [15].

TRAIL has been implicated in both MS pathogenesis and the mechanism of action of interferon-beta (IFN-β), a disease modifying therapy that has been used to treat MS for over twenty years [19–21]. Recombinant IFN-β therapy is typically employed for the treatment of relapsing-remitting MS (RRMS). Although the precise mechanism(s) responsible for the beneficial effects of IFN-β in the treatment of MS remain unclear, the abilities of this cytokine to inhibit T-cell activation and proliferation as well as facilitate the apoptotic elimination of autoreactive T cells are thought to be therapeutically relevant [22]. TRAIL/Apo2L-deficient mice subjected to myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis (EAE) display increased T-cell proliferative responses, more inflammatory lesions in the spinal cord and brain, and elevated clinical scores relative to wild-type littermates, while peripheral administration of recombinant TRAIL reduces EAE severity [23]. Moreover, IFN-β increases circulating levels of soluble TRAIL (sTRAIL) and the expression of membrane-bound TRAIL (mbTRAIL) in immune cells derived from the peripheral blood of MS patients [21, 24]. TRAIL may therefore contribute to the mechanism of action of IFN-β by promoting the apoptosis of autoreactive immune cells in MS patients. IFN-β is not curative but reduces disease progression as evidenced by decreased frequency and severity of relapses. However, some patients are unresponsive to IFN-β therapy and continue to experience relapses and disease progression while treated with this therapeutic [25]. The reasons why some patients respond to IFN-β therapy while others do not benefit remain unclear. The present study therefore sought to determine whether the expression of TRAIL and/or its receptors in peripheral blood immune cells from treated and nontreated MS patients discriminated IFN-β responders from IFN-β nonresponders.

2. Materials and Methods

2.1. Patient Selection and Blood Sampling. One hundred ninety-nine participants were recruited for this study including participants with RRMS ($n = 100$), SPMS ($n = 38$), and PPMS ($n = 30$) as well as thirty-one healthy control subjects from the community, matched for age and gender. Study inclusion criteria included definite MS (RRMS, SPMS, PPMS) according to consensus definitions, 18 years of age or older, and if being treated with IFN-β, patients were on a stable dose for 6 months having had a dose within 8–16 hours prior to blood draw. Several disease modifying therapies (DMTs) have been approved for relapse-onset MS (RRMS and SPMS with superimposed relapses). First line agents, include three different preparations of interferon-β (IFN-β) (IFN-β1a or Avonex, IFN-β1b or Betaseron, and IFN-β1 or Rebif) and glatiramer-acetate (Copaxone). Natilizumab (Tysabri) and mitoxantrone (Novantrone) are considered second line therapies and used for treatment of more aggressive forms of relapse-onset MS [26–28]. For the purpose of this study, RRMS patients were assigned into "active" and "quiescent" groups based on their clinical condition at the time of blood collection. Relapsing-remitting active (RRA) disease was defined in patients that had experienced one or more clinical relapse(s) or had Gd+ enhancing lesion(s) on MRI in the previous year ($n = 41$). Relapsing-remitting quiescent (RRQ) disease was defined in patients that had neither relapses within the previous year nor EDSS progression over the same time period ($n = 59$). These clinical definitions of active and quiescent MS are supported by differences in the expression of members of the Inhibitor of Apoptosis family in peripheral blood mononuclear cells and T cells derived from such patients [29].

Both MS patients and control subjects had an absence of other major medical illness including cancer and autoimmune disease. Study exclusion criteria for all subjects included treatment with immunosuppressive therapy or treatment with intravenous methylprednisolone within 3 months of study participation. All participants provided written informed consent prior to study participation according to the Declaration of Helsinki. All participants meeting inclusion/exclusion criteria provided a blood sample totaling 38 mL into four 8 mL BD Vacutainer sodium citrate Ficoll gradient tubes, one serum tube, and/or one Paxgene (Qiagen) whole blood RNA tube.

2.2. Cell Purification, RNA Extraction, and TRAIL Quantification. Whole blood RNA was isolated using the PAXgene whole blood RNA kit as per manufacturer's instructions and as previously described [29]. For the isolation of highly purified T cells, B cells or monocytes, 400 μL of RosetteSepT, RosetteSepB, or RosetteSep monocytes (Stem Cell Technologies, B.C.), respectively, was added to three separate Ficoll-gradient tubes (Becton, Dickinson and Company; BD Vacutainer CPT Cell Preparation Tube with Sodium Citrate). All tubes (T cells, B cells, monocytes) were centrifuged at 1650–1800 $\times$ g for 25 minutes in a swinging bucket centrifuge. The T cells, B cells, or monocytes were transferred to separate 15 ml conical centrifugation tubes, treated with ammonium chloride (StemCell Technologies, Vancouver, B.C.) to remove any remaining red blood cells, and centrifuged to pellet the cells. RNA was extracted from cell pellets using the RNeasy kit (Qiagen). FACS analyses were performed to confirm the purity (95–98%) of immune cell populations. Total RNA

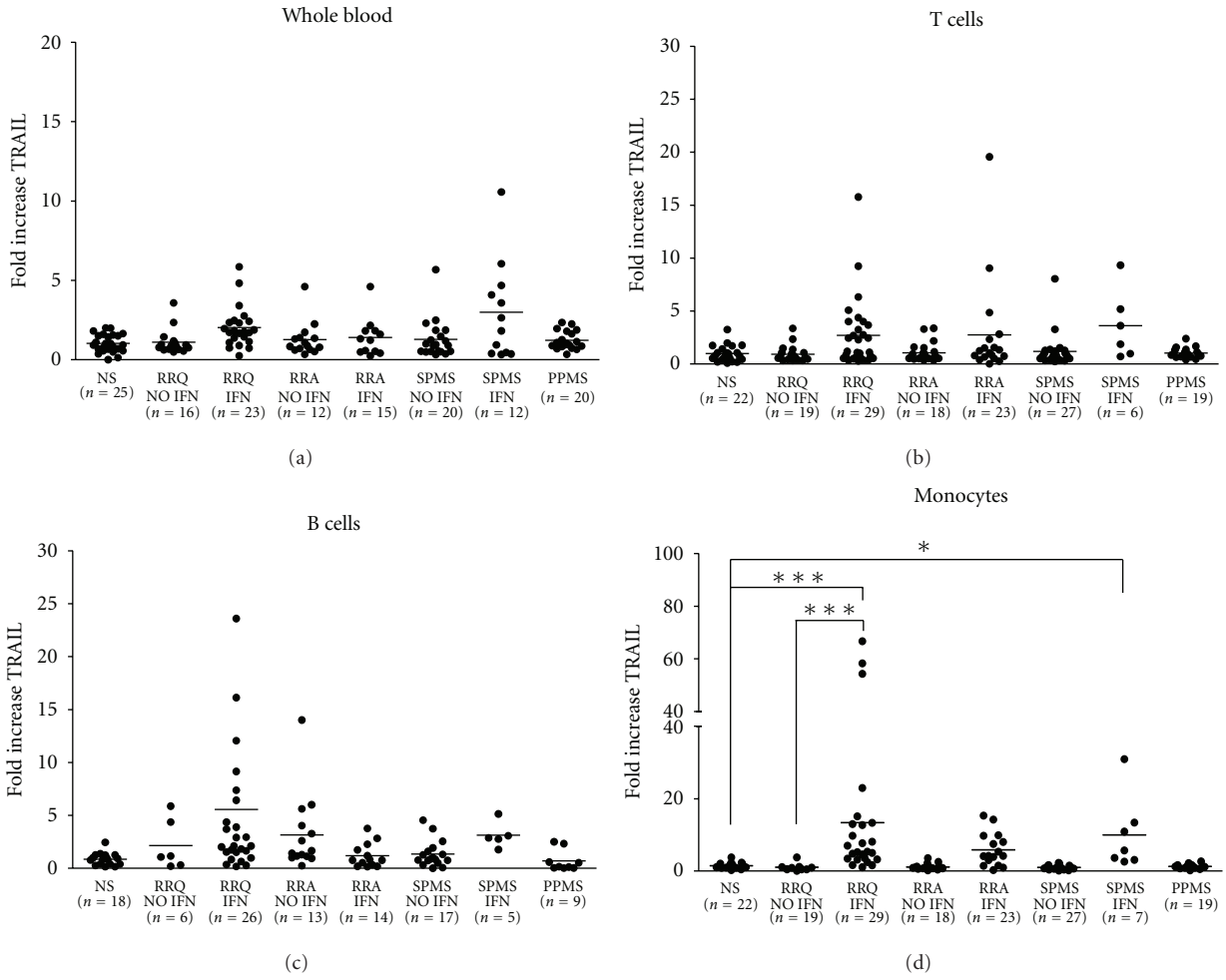

Figure 1: Relative quantification of TRAIL mRNA expression in RNA extracted from whole blood, T cells, B cells, and monocytes employing qRT-PCR. (a) In whole blood, TRAIL gene expression was no different between the various groups**.** (b) In T cells, TRAIL gene expression was also no different among NS and the various patient groups. (c) In B cells, TRAIL gene expression was the same in the various groups examined. (d) In monocytes, TRAIL gene expression was increased in RRQ IFN and SPMS IFN patients relative to NS. TRAIL mRNA was also elevated in RRQ IFN relative to RRQ NO IFN. $^{*}P < .05$,$^{**}P < .01$ and $^{***}P < .001$, Dunn's post hoc test.

yields were measured by ultraviolet (UV) absorbance, and RNA samples were diluted in RNAse free water to give a final concentration of 10 ng/μL.

2.3. Quantitative RT-PCR. Quantitative RT-PCR (qRT-PCR) was performed to confirm changes in TRAIL, TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (TRAIL decoy receptor), and TRAIL-R4 (TRAIL decoy receptor) mRNA levels in different subtypes of untreated and treated MS patients relative to normal control subjects. Data were first stratified according to MS disease subtype activity and IFN-β treatment. If statistical comparisons between these groups were not significant, data were pooled and analyzed according to overall disease classification (RRMS, SPMS, PPMS). Total RNA (50 ng) was reverse transcribed to yield first-strand cDNA and amplified using the Taqman one-step EZ RT-PCR Core reagents kit (Applied Biosystems, Foster City, CA, USA). The expression of beta (β) actin was used as an endogenous control reference (Applied Biosystems, Foster City, CA, USA). Primers for TRAIL and its receptors were made according to previously published sequences [30–32] (see Table 1). Although the intent was to compare TRAIL profiles amongst all samples collected, in some cases this was precluded due to decreased RNA yields. Results were expressed in Fold change relative to a "calibrator." The calibrator sample was the RNA sample from a normal age-matched female subject [33].

2.4. ELISA. Blood was collected in a 3 ml serum separator tube, allowed to clot at RT for 1 hour and centrifuged at 1000 × g for 20 minutes. Serum was transferred to two 1.5 ml eppendorf tubes and stored at −80°C until analyses. Enzyme linked-immunosorbent assay (ELISA) measured soluble TRAIL as per manufacturer's instructions (R&D systems, Minneapolis, MN USA). Serum (50 μl in duplicate) from normal healthy control subjects (NS, $n = 34$), RRMS and SPMS patients treated with IFN-β (RRMS IFN, $n = 41$; SPMS IFN, $n = 16$), RRMS and SPMS patients not

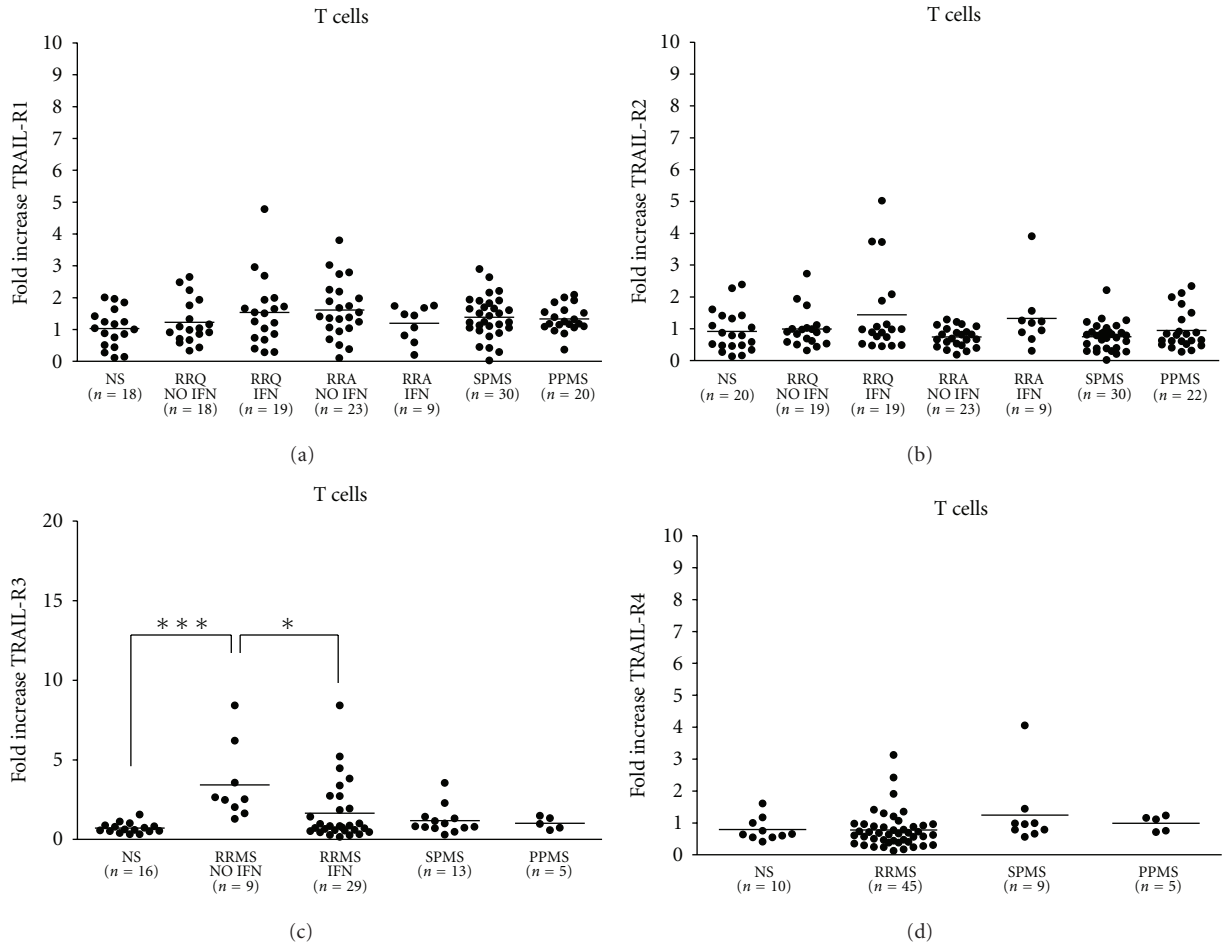

Figure 2: Relative quantification of TRAIL death (TRAIL-R1, TRAIL-R2) and decoy receptor (TRAIL-R3, TRAIL-R4) mRNAs in T cells. (a) In T cells, there were no differences in gene expression of TRAIL-R1 and (b) TRAIL-R2. (c) TRAIL-R3 gene expression was increased in RRMS NO IFN patients relative to the NS group while administration of IFN-β reversed this increase in RRMS patients (RRMS IFN). $^{*}P < .05$ and $^{**}P < .01$, Dunn's posthoc test.

Table 1: Human Taqman Probe and Primer Sequences. Taqman probe and primer sequences for TRAIL and its receptors (R) have been previously published.

Human gene	Human primer and probe sequences
TRAIL	Forward 5′-GCTCTGGGCCGCAAAAT-3′
	Reverse 5′-TGCAAGTTGCTCAGGAATGAA-3′
	Probe 5′-(FAM)ACTCCTGGGAATCATCAAGGAGTGGGC(TAMRA)-3′
TRAIL-R1	Forward 5′-TGTACGCCCTGGAGTGACAT-3′
	Reverse 5′-CACCAACAGCAACGGAACAA-3′
	Probe 5′-(FAM)TGTCCACAAAGAATCAGGCAATGGACATAAT(TAMRA)-3′
TRAIL-R2	Forward 5′-CACTCACTGGAATGACCTCCTTT-3′
	Reverse 5′-GTGCAGGGACTTAGCTCCACTT-3′
	Probe 5′-(FAM)TCACACCTGGTGCAGCGCAAGCAG(TAMRA)-3′
TRAIL-R3	Forward 5′-TCTCCACGCGCACGAAC-3′
	Reverse 5′-CCCCTTGCATCTCTGGTCAA-3′
	Probe 5′-(FAM)CAGCCAACGATTTCTGATAGATTTTTGGGAG(TAMRA)-3′
TRAIL-R4	Forward 5′-TTGGCGCTTTCGATCCAC-3′
	Reverse 5′-CGGTCGGGACGCTTTGT-3′
	Probe 5′-(FAM)CTCCTCCCTTCTCATGGGACTTTGGG(TAMRA)-3′

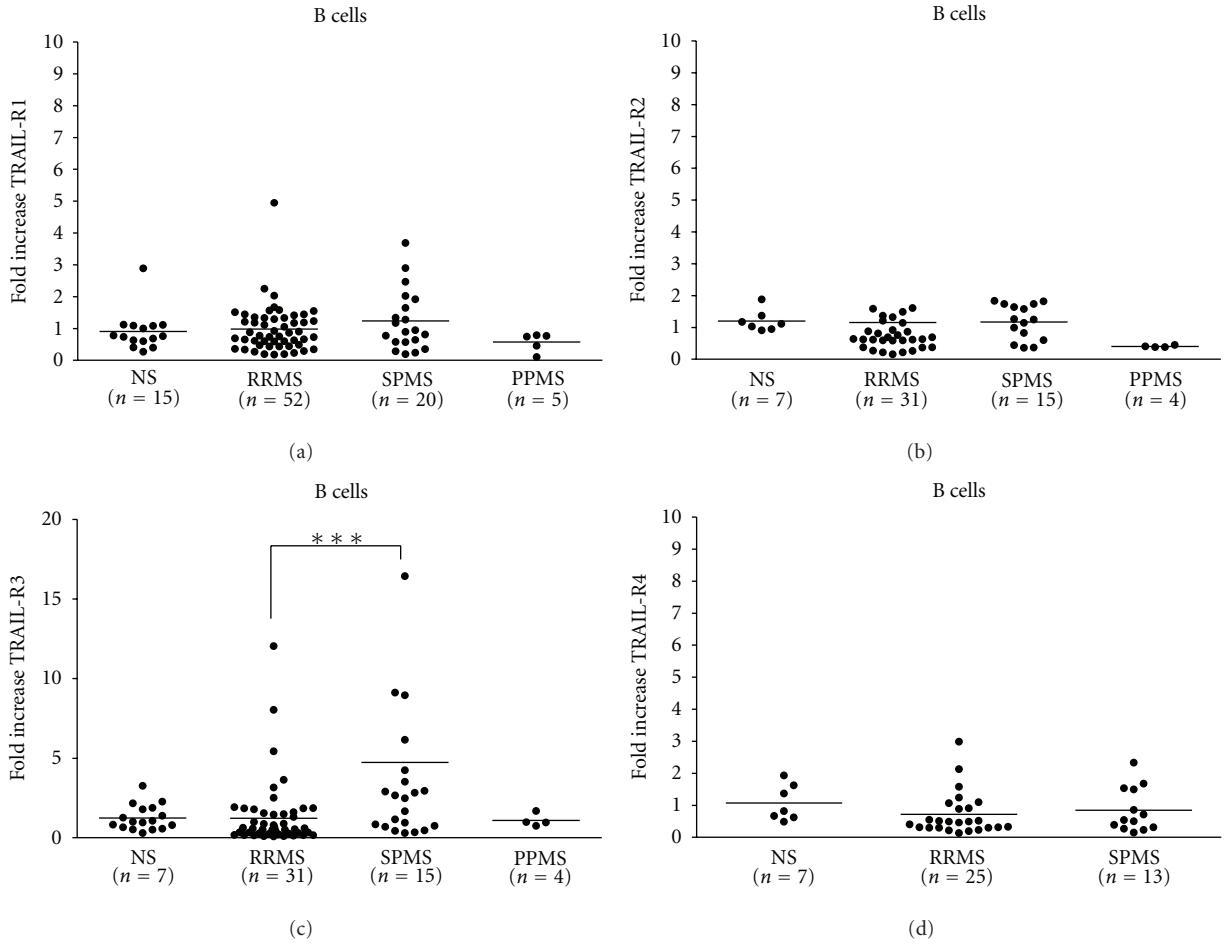

Figure 3: Relative quantification of TRAIL death (TRAIL-R1, TRAIL-R2) and decoy receptor (TRAIL-R3, TRAIL-R4) mRNAs in B cells. (a) TRAIL-R1, (b) TRAIL-R3 and (d) TRAIL-R4 mRNA levels were not altered by IFN-β treatment or different between the various groups (NS group and MS subtypes). Insufficient amounts of B cell RNA were isolated to permit analysis of TRAIL-R4 levels for PPMS patients. (c) TRAIL-R3 gene expression was increased in SPMS patients irrespective of IFN-β treatment condition (SPMS NO IFN and SPMS IFN groups not different and therefore pooled) relative to the RRMS group. $^*P < .05$, Dunn's post-hoc test.

treated with IFN-β (RRMS NO IFN, $n = 32$; SPMS NO IFN, $n = 12$), and PPMS ($n = 23$) were analyzed for soluble TRAIL (sTRAIL) using an ELISA with a monoclonal antibody specific for human TRAIL according to manufacturer's instructions (R&D Systems, Minneapolis, MN). All measurements were performed in duplicate.

2.5. Statistical Analyses. A nonparametric test (Kruskal-Wallis test) was used to compare potential differences in expression of mRNAs encoding TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4 in whole blood, T cells, B cells and monocytes derived from the peripheral whole blood of the various groups (NS and patients with subtypes of MS that were either treated or not treated with IFN-β). If significant at an alpha of $P \leq .01$, Dunn's posttest was used to determine whether differences existed between individual groups. The Mann Whitney U test was used to compare soluble TRAIL protein levels in blood serum from NAB(−) and NAB(+) MS patients. Statistical tests were performed using GraphPad Prism version 4 for Windows, GraphPad Software, San Diego California USA, http://www.graphpad.com/.

3. Results

3.1. IFN-β Increases TRAIL MRNA in Monocytes from RRQ and SPMS Patients. Using RNA derived from whole blood, T cells or B cells, no differences in TRAIL gene expression were observed between the different subtypes of IFN-β-treated and untreated MS patient populations (Figures 1(a)–1(c)). In peripheral monocytes, however, a significant increase in TRAIL gene expression was observed in both RRQ and SPMS patients treated with IFN-β relative to normal subjects (NS) (Figure 1(d)). Relative to untreated RRQ patients (RRQ NO IFN), a significant increase in TRAIL gene expression was also observed in IFN-β-treated RRQ patients (RRQ IFN).

3.2. TRAIL-R3 mRNA Is Elevated in T Cells from RRMS and This Increase Is Reversed by IFN-β Treatment. In T

cells, no differences in either TRAIL-R1 or TRAIL-R2 were observed between the various groups (Figures 2(a) and 2(b)). There was, however, a significant increase in TRAIL-R3 mRNA in T cells from untreated RRMS patients relative to NS (Figure 2(c)). Interestingly, IFN-β-treatment in RRMS patients reversed this increase in TRAIL-R3 mRNA such that expression levels of this gene were significantly decreased in T cells from IFN-β-treated RRMS patients relative to untreated RRMS patients. No differences in TRAIL-R3 mRNA were observed in whole blood or monocytes (data not shown). Quantitative RT-PCR analysis for TRAIL-R4 revealed no differences across disease subtypes in either T cells (Figure 2(d)), monocytes, or whole blood (data not shown).

3.3. TRAIL-R3 mRNA Is Increased in B Cells from SPMS Relative to RRMS Patients. In B cells, no differences in TRAIL-R1, TRAIL-R2, or TRAIL-R4 were observed (Figures 3(a), 3(b), and 3(d)). A significant increase in TRAIL-R3 mRNA expression was observed in SPMS patients relative to RRMS patients (Figure 3(c)). No effects of IFN-β treatment were noted in this analysis (data not shown).

3.4. IFN-β Increases Soluble TRAIL in Serum Regardless of MS Subtype or Disease Activity. The range of soluble TRAIL (sTRAIL) in serum was between 21.6 and 157.2 pg/mL (Figure 4(a)) for the NS group. In the three patient populations treated with IFN-β, (RRQ IFN, RRA IFN, and SPMS IFN), serum concentrations of sTRAIL were significantly increased relative to NS (Figure 4(a)). While not indicated on Figure 4(a), statistical significance ($P < .001$) was also observed between the RRQ NO IFN and RRQ IFN groups as well as the RRA NO IFN and RRA IFN groups. These findings confirm our earlier qRT-PCR results demonstrating that IFN-β-treated patients had elevated TRAIL mRNA in peripheral monocytes suggesting that a major source of sTRAIL is from this particular cell type. When patients were grouped according to the presence of neutralizing antibodies against IFN-β, a significant decrease in sTRAIL was observed in the NAB(+) relative to NAB(−) patients (Figure 4(b)).

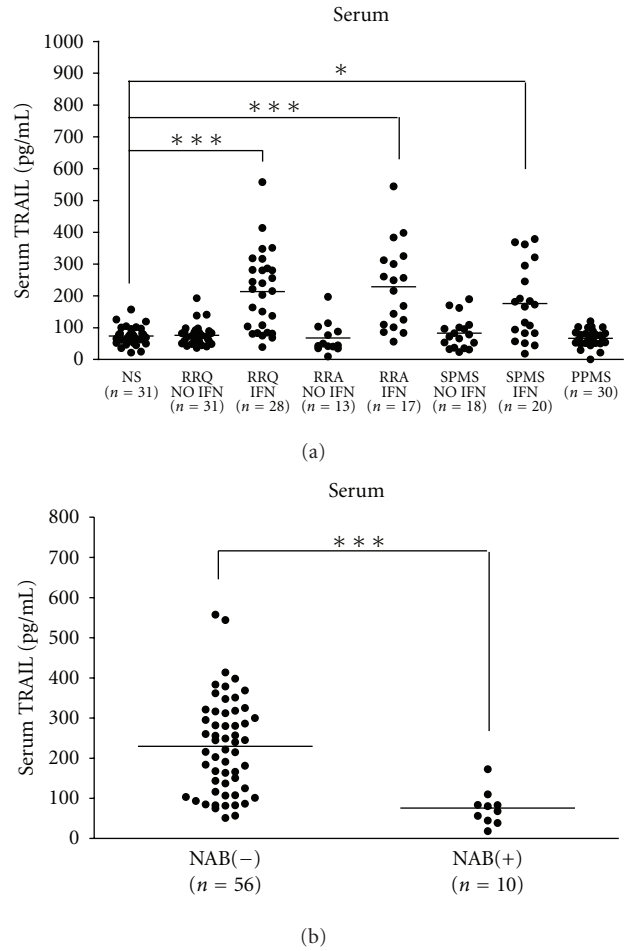

Figure 4: Absolute quantification of soluble TRAIL protein in peripheral blood serum. (a) Levels of soluble TRAIL protein were increased in serum from IFN-β treated-patients irrespective of subgroup (RRMS, SPMS) or disease activity (RRQ, RRA) relative to NS and untreated MS patients. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$, Dunn's post-hoc test. (b) Ten patients (RRQ IFN $n = 4$; RRA IFN $n = 3$; SPMS $n = 3$) that were NAB(+) showed attenuated sTRAIL levels relative to IFN-β NAB(−) MS patients. $^{***}P < .001$, Mann Whitney U test.

4. Discussion

In this retrospective study, we first compared levels of TRAIL mRNA in whole blood, T cells, B cells, and monocytes isolated from the peripheral blood of healthy normal subjects (NS) and MS patients treated with various preparations of IFN-β. Only in monocytes were significant differences in TRAIL gene expression detected between NS and MS patients that received IFN-β. Elevated TRAIL gene expression associated with IFN-β therapy was correlated with clinical responsiveness to this therapeutic in RRMS patients as suggested by higher levels of monocytic TRAIL mRNA in RRQ, but not RRA, patients relative to the NS group. However, TRAIL mRNA levels were not different between RRQ and RRA patients that received IFN-β, indicating that clinical responsiveness to this therapeutic cannot be distinguished purely on this basis. This finding was both supported and extended by our measurements of soluble TRAIL levels in peripheral blood serum. In this case, IFN-β treatment produced a comparable elevation of soluble TRAIL levels in both RRQ and RRA patients relative to the NS group. Furthermore, in patients that were NAB+, the ability of IFN-β to elevate soluble TRAIL levels was lost. Thus, while TRAIL induction appears to depend upon the availability of biologically active IFN-β, blood levels of this death receptor ligand did not distinguish RRA from RRA patients. These results are in agreement with findings from a recent prospective study that reported levels of soluble TRAIL in blood plasma failed to predict clinical responsiveness of RRMS patients to IFN-β [34].

TRAIL-R3 encodes a decoy receptor that suppresses the apoptotic activity of TRAIL [35]. In the present study, we found that expression of TRAIL-R3, but not that for

the death-inducing receptors TRAIL-1 and TRAIL-2 or the second TRAIL decoy receptor known as TRAIL-R4, is to be elevated in T cells from RRMS patients. TRAIL-R3 expression has also been found to be selectively elevated in T cell lines derived from MS patients compared to those from healthy donors [36]. Moreover, we observed in RRMS patients treated with IFN-β that expression of TRAIL-R3 was reduced to levels similar to that detected in healthy controls. We speculate that by reducing TRAIL-3 levels on T cells, IFN-β may enhance the proapoptotic and antiproliferative effects of TRAIL on these immune cells. In keeping with this line of reasoning, we also observed TRAIL-R3 expression in B cells from SPMS patients to be enhanced relative to RRMS patients that may reflect immune mechanisms which could conceivably contribute to disease progression. To the best of our knowledge, this is the first demonstration that TRAIL-R3 may be involved in both the mechanism of action of IFN-β as well as MS disease progression.

Acknowledgments

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Research Article

What is Next for the Genetics of Multiple Sclerosis?

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We review here our current understanding of the genetic aetiology of the common complex neurological disease multiple sclerosis (MS). The strongest genetic risk factor for MS is the major histocompatibility complex which was identified in the 1970s. In 2011, after a number of genome-wide association studies have been completed and have identified approximately 20 new genes for MS, we ask the question—what is next for the genetics of MS?

1. Introduction

Hermann Eichorst first recognized the familial clustering of multiple sclerosis (MS) late in the 19th century [1] but it was not until almost a century had passed that it was firmly established by population-based studies that relatives of MS patients have an increased risk for developing the disease [2]. In keeping with this observation, Davenport had already made it clear that northern Europeans had a higher frequency of MS, whereas the disease was much less common in Asians and Africans [1]. Twin studies and later an adoption study showed that the observed familial aggregation was due to shared genes versus any shared familial exposures [3].

Progress in identifying the genes responsible has, historically, progressed at a glacial pace, despite the early success with the major histocompatibility complex (MHC) association. In 1972, MS was shown to be associated with the MHC [4], later pinpointed to a specific allele, *HLA-DRB1*15*, of the class II gene *HLA-DRB1* [5]. The success in identifying this association was primarily due to the large effect size of this region (odds ratio (OR) >2). The next significant step forward occurred in 2007 when new genes were identified for MS by genomewide association studies (GWAS) [6]. Now at the dawn of 2011, we have approximately 20 genes conferring a mild to modest effect (OR < 1.3) on risk that are robustly associated to MS [7], as well as the long known HLA association. The question can therefore be posed, is this "job done" for MS genetics?

2. Discussion

Unfortunately far from it; the associated variants so far identified explain about 50% of the inherited risk of MS. There are several possible explanations as to the "missing" genetic basis of MS.

It is possible that the immune-related disease loci identified to date have more of an overall impact on MS risk than currently estimated. This can result when the marker SNP is an imperfect proxy for the actual causal mutation that led to the association signal. There is some evidence to support this hypothesis in complex disease. Recent resequencing of 63 GWAS-identified positional candidate genes in Crohn's disease identified three novel low-frequency coding variants in the *IL23R* gene [8]. The odds ratios conferred by these newly detected low frequency variants was approximately 2.4 on average [8]. Although this odds ratio is a possible

overestimation due to winner's curse, the value appears larger than the approximate 20% risk increase conferred by most common variants detected by GWAS. However, the fact that only 1 out of 63 genes had robustly associated rare variants and that these newly detected variants jointly explained only an extra 0.44% of the variance of Crohn's disease suggests that rare variants in GWAS-associated genes are not likely to make a large contribution to inherited predisposition to complex disease [8].

Perhaps there are additional disease loci than the roughly 20 or so associated genes? These other susceptibility genes could be identified by even larger scale GWAS involving tens or hundreds of thousands of MS patients and controls. The GWAS published so far in MS have not exceeded 2000 patients in the initial (screening) phase. Statistical modelling has suggested that 12,627 SNPs explains approximately 3% of the variance in MS risk [9]. We await the results of the MS GWAS funded by the Wellcome Trust which involves tens of thousands of participants to see if the vast resources expended in such a project are translated into novel insights into MS aetiology. It should be remembered that MS is phenotypically a heterogeneous disease [1], and while current GWAS have used unselected patient populations to identify disease associations, this may miss variants more important to certain patient groups.

Another explanation may be that some disease loci may contain only rare variants. In order to identify these genes a sequencing-based approach would be required. In the past this was not possible given the cost and technology available; however recent advances in next-generation sequencing technologies (whole exome and whole genome sequencing) could rapidly facilitate the identification of these variants that would be too rare to be picked up by GWAS. These rare variants would be expected to be causal and have a relatively large effect on risk (i.e., OR > 3). The 1000-Genomes project has highlighted the fact that each of us has 250 to 300 loss-of-function variants in our genes [10]. However, for complex diseases, power considerations will be an issue to cope with the wealth of data generated by whole genome sequencing. It has been suggested that whole-exome sequencing will be most fruitful in identifying rare disease causative variants in families that have multiple affected individuals [11].

SNPs are only one type of genetic variation. It has been observed that individual copies of the human genome contain large regions (tens to hundreds of kilobases in size) that are deleted, duplicated, or inverted relative to the reference sequence. These structural variants may contribute to MS aetiology but have not yet been adequately tested. However, a study by the Wellcome Trust Case Control Consortium observed that most common structural variation are well tagged by SNPs and so have been indirectly explored through genomewide SNP studies and therefore concluded that common structural variants are unlikely to contribute greatly to the genetic basis of common human diseases [12].

Moving on from single locus associations to consider biological systems, it may be that gene-gene and gene-environment interactions may play an important role in disease. Once patterns of association and interaction are better understood, the effects of specific gene and environmental exposures on developing MS may be significant. Indeed epistatic interactions exist between MHC haplotypes [13] and can greatly alter risk. For example, *HLA-DRB1*08,* interacts with *HLA-DRB1*15* to more than double the risk associated with a single copy of *HLA-DRB1*15* [13]. On its own, *HLA-DRB1*08* increases the risk of MS modestly, highlighting that a variant with a small marginal effect is not necessarily clinically insignificant; it may turn out to have a strong effect in certain genetic backgrounds. As yet, no functional explanation can be given for these interactions; understanding the mechanism of these interactions will be critical to further understanding MS aetiology.

Epigenetic contributions may also play an important role in MS. Epidemiological data strongly hints at a parent-of-origin effect in MS [14]. For example, maternal half-siblings have double the risk for MS as compared to paternal half-siblings (2.35% versus 1.31%) [14]. Risk for MS in maternal half-siblings compared to their full siblings does not differ significantly [14] suggesting that this maternal effect is a major component of familial aggregation of the disease. The mechanism of the increased risk conferred maternally remains to be elucidated but epigenetic mechanisms such as DNA methylation and histone modification may regulate genomic function in such a way to increase MS risk [15]. A recent study investigating these effects utilized next-generation sequencing in discordant twins. The investigators could not find evidence for any epigenetic differences between the twins to explain the MS discordance [16]. However, there were a number of limitations to the study design used, and it is of interest that DNA methylation differences have been shown to exist between twins discordant for systemic lupus erythematosus [17] and for parent of origin effects in type 2 diabetes [18].

3. Conclusion

As with all complex diseases, the genetics of MS has not yet been fully elucidated. While GWAS have been responsible for a wealth of new information these association studies have not provided all the answers for MS risk. We are now in an era of very exciting potential applications of sequencing technology. Next-generation sequencing platforms allow us to survey multiple levels of natural variation at unprecedented resolution and depth. As sequencing costs continue to decrease, and both laboratory and computational protocols improve, we will see ever increasing use of this technology, hopefully enabling us to completely unlock the complex genetic basis of MS. There is unlikely to have a single answer, with interactions, rare variants, epigenetic factors all likely to be contributing. Ultimately, well-performed functional studies will be required to understand how all these risk factors interact to predispose to MS. Against this it will be debated whether further genetic research will actually advance our understanding of MS. However, the motivation for future work is the need to understand disease mechanisms to derive safe and effective treatments and ultimately to prevent the disease.

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Review Article

MicroRNAs and Multiple Sclerosis

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MicroRNAs (miRNAs) have recently emerged as a new class of modulators of gene expression. miRNAs control protein synthesis by targeting mRNAs for translational repression or degradation at the posttranscriptional level. These noncoding RNAs are endogenous, single-stranded molecules approximately 22 nucleotides in length and have roles in multiple facets of immunity, from regulation of development of key cellular players to activation and function in immune responses. Recent studies have shown that dysregulation of miRNAs involved in immune responses leads to autoimmunity. Multiple sclerosis (MS) serves as an example of a chronic and organ-specific autoimmune disease in which miRNAs modulate immune responses in the peripheral immune compartment and the neuroinflammatory process in the brain. For MS, miRNAs have the potential to serve as modifying drugs. In this review, we summarize current knowledge of miRNA biogenesis and mode of action and the diverse roles of miRNAs in modulating the immune and inflammatory responses. We also review the role of miRNAs in autoimmunity, focusing on emerging data regarding miRNA expression patterns in MS. Finally, we discuss the potential of miRNAs as a disease marker and a novel therapeutic target in MS. Better understanding of the role of miRNAs in MS will improve our knowledge of the pathogenesis of this disease.

1. Introduction

MicroRNAs (miRNAs) represent a class of noncoding RNA molecules that play pivotal roles in cellular and developmental processes by regulating gene expression at the posttranscriptional level. miRNAs are endogenous, evolutionarily conserved, single-stranded RNAs approximately 22 nucleotides in length that suppress the expression of protein-coding genes by directing translational repression through base-pairing with complementary messenger RNA (mRNA)and/or by promoting degradation of target mRNA degradation [1, 2]. Since the identification of the miRNA lin-4 as a regulator of developmental timing in the nematode *Caenorhabditis elegans* (*C. elegans*) in 1993 [3, 4], more than 17000 miRNAs have been recognized in 142 species. Currently, 1048 human miRNAs are registered in the miRNA registry (miRBase) which is the most commonly used database for miRNA (September 2010, release 16, http://www.mirbase.org/) [5]. miRBase reports 672 miRNAs in mouse and 408 miRNAs in rat, with new miRNAs constantly being identified, though the biologic function of only a fraction of miRNAs has been elucidated. miRNAs are predicted to regulate up to one-third of all human protein-coding genes. Unraveling the miRNA translational silencing network remains a challenge in part because individual miRNAs typically target several transcripts rather just one specific gene and a single mRNA can be regulated by several distinct miRNAs that act cooperatively [2]. Ribosome profiling experiments showed that miRNAs mediate destabilization of target mRNAs resulting in reduced protein levels [6]. miRNAs play an important role in diverse biologic processes such as development, cell proliferation and differentiation, apoptosis, oncogenesis, metabolism, angiogenesis, and inflammation. The expression of miRNAs is initially controlled at the level of transcriptionby transcription factors that regulate the production of miRNA-containing primary transcripts in specific cell types during development or in response to different environmental signals. Dysregulation of miRNA expression and function is associated with a variety

of human diseases including cancer, neurodegeneration and autoimmunity [7, 8].

The regulation of mammalian immune responses by miRNAs is a concept currently evidenced by rapidly accumulating data [9, 10]. miRNAs have unique expression profiles in cells of the innate and adaptive immune systems and have pivotal roles in the regulation of both cell development and function. Recent studies focused on the networkwide role of miRNA or the functions of individual miRNAs have revealed that these small noncoding RNAs are involved in T and B cell differentiation in the thymus and bone marrow, respectively. During the effector phases of adaptive immunity, miRNAs contribute to the differentiation of T cells into functional lineages, class switching and germinal centre formation in B cells and activation of antigen-presenting cells (APCs) through pattern recognition pathways [11]. miRNAs are also directly involved in innate immunity and transduction signalling by Toll-like receptors (TLRs) and the ensuing cytokine response [12]. Up to one half of innate immune genes are predicted to be under the direct regulation of miRNAs. With the capacity of miRNAs to regulate the survival and death of T and B cells, control over miRNA expression is essential to prevent adaptive immune cells from unregulated proliferation leading to cancer or autoimmunity [13, 14]. miRNAs are differentially expressed in autoimmune diseases and miRNA regulation may have an impact on the development or prevention of autoimmunity. miRNA dysregulation is linked to autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, Sjögren's syndrome, psoriasis, and MS [15–17]. MS is the most common autoimmune disease of the central nervous system (CNS). It is a chronic, neuroinflammatory, and demyelinating disease in which myelin specific autoreactive CD4+ T cells become activated in the peripheral immune compartment, cross the blood-brain barrier (BBB), and promote neurological disability [18, 19]. Both genetic (HLA type) and environmental causes for MS have been suggested. Recently, genomewide association studies have identified additional potential MS susceptibility loci [20, 21]. Recent studies suggest that miRNA dysregulation may contribute to the pathogenesis of MS. Thus, better understanding of miRNA mechanisms might shed light, not only on the pathogenesis of MS but also on potential approaches for managing or even suppressing the disease. In this review, we briefly overview the biogenesis and action mechanisms of miRNAs and summarize recent advances in our understanding of both the intended functions of miRNAs in managing immune responses. We then review evolving knowledge on the role of miRNA in autoimmunity and emerging data regarding miRNA expression patterns in MS. Finally, we also discuss the potential of miRNAs as a diagnostic and prognostic indicators of disease type and status and as a novel therapeutic target in MS.

2. MicroRNAs

2.1. MicroRNA Biogenesis. All miRNAs are processed and maturated through a complex biogenesis process involving multiple protein catalysts, accessory proteins, and macromolecular complexes following a coordinated series of events. The reader is referred to excellent recent reviews for detailed discussions of miRNA biogenesis and its regulation [22–25]. MicroRNAs can be encoded by independent genes but may also be processed from a variety of different RNA species, including introns, 3′-UTR of mRNAs, long noncoding RNAs, transposable elements, and genomic repeats [26–32]. miRNAs are expressed as 21–23 nucleotide RNA molecules initially transcribed by RNA polymerase II as long primary miRNAs (pri-microRNAs). Although most of the miRNA genes are transcribed by RNA Polymerase II, a cluster of human miRNAs have recently been shown to utilize RNA Polymerase III for their transcription [33]. Pri-miRNAs are typically 3 to 4 kilobases long single-stranded RNAs with 5′ cap, 3′ poly(A) tail and complicated secondary structure [34–37]. Pri-miRNAs are processed in the nucleus into one or more precursor-miRNAs (pre-miRNAs) with an approximately 70-nt loop structure. Processing is performed by a protein complex named microprocessor complex consisting of the nuclease Drosha (nuclear RNase III) and the stranded RNA-binding protein, human DiGeorge syndrome critical region gene 8 (DGCR8) (also known as Pasha in flies) [35, 37–42]. Drosha functions as the catalytic subunit while DGCR8 recognizes the RNA substrate. Pre-miRNAs are exported from the nucleus to the cytoplasm by exportin-5 which specifically recognizes the characteristic end structure of pre-miRNAs [43–46]. In the cytoplasm, another RNase III, known as Dicer, further processes the pre-miR into mature miRNA, which is double stranded (miRNA duplex) [47, 48]. After Dicer processing, the miRNA duplex is unwound and a strand (known as miRNA strand or guide strand) binds to an Argonaute 2 (Ago 2) protein (eIF2C2 in human) in a process that is referred to as miRNA loading or assembly, while the complementary strand (known as miRNA* strand, star strand or passenger strand) is degraded. The effector complex that mediates catalytic mRNA cleavage is known as RNA-induced silencing complex (RISC), and the effector complex that mediates translational repression directed by miRNAs is known as micro-ribonucleoprotein complex (miRNP) [49–51]. The single stranded mature microRNA must associate with the RISC. Mature microRNAs are incorporated into a miRNP (Figure 1). In this complex, which includes the Dicer-transactivation-responsive RNA-binding protein (TRBP)-PACT-Ago 2, microRNAs can direct downregulation by two mechanisms: translational inhibition and target mRNA cleavage [52–55]. Perfect match with the target results in mRNA degradation whereas partial match leads to translational inhibition.

Inflammation has been reported to regulate miRNA biogenesis; TLR ligands, antigens, or cytokines can modulate miRNA expression level through regulation of specific transcription factors [2, 9, 56]. Cytokines have been shown to regulate Dicer expression resulting in alteration of pre-miRNA processing. Interferon-beta (IFN-β) has been shown to inhibit Dicer expression, which results in decrease of pre-miRNA processing, whereas IFN-γ induces pre-miRNA processing [57].

2.2. Detection of MicroRNAs. Information about miRNA and target expression patterns can help to assess the likelihood that a predicted miRNA-target relationship is relevant *in vivo* [58]. Expression of a miRNA can be measured by molecular biology techniques, such as Northern blotting, RNase protection assay, polymerase chain reaction- (PCR-) based techniques, and high throughput assays [59–61]. miRNA expression profiles were first generated by small RNA cloning and Northern blotting [4, 62–67]. The small size of miRNAs initially hampered PCR-based methods [61]. However, since the development of quantitative real-time PCR, PCR-based techniques have become very popular due to their high sensitivity [62, 68, 69]. *In situ* hybridization has provided further insight into the tissue-specific expression of pri- and mature miRNAs [62, 70–74]. Microarray techniques are widely used to comprehensively assay the entire miRNome (the global miRNA expression profile) in tissues or in cell lines [62, 68, 75–83]. In addition, serial analyses of gene expression (SAGE) adapted for small RNAs have been used to obtain miRNomes [84]. Interest in the SAGE approach was stimulated by recent innovations in next generation (deep) sequencing methods that provide a powerful tool for various genomics studies [85–87]. Overall, these technical improvements are expected to greatly widen the repertoire of known miRNAs in a variety of biological systems [61]. Emerging techniques for miRNA detection and quantification, including luminescence-based, fluorescence-based, electrochemical, colorimetric, and enzyme-based, and nanotechnology-based methods have recently been reviewed [88]. Whereas expression analyses are required to identify miRNAs with altered expression patterns in diseased tissues, functional analyses of the ability of these miRNAs to regulate expression of target mRNAs are essential to understand their impact on pathogenic pathways and processes.

3. MicroRNAs and Immunity

Clearly, both innate and adaptive immune responses are extremely highly regulated. Recent work from a number of laboratories has revealed that miRNAs play an important role in this intricate system (Table 1). miRNAs have unique expression patterns in immune cells and play a pivotal role in their development, maturation, and function.

3.1. Role of MicroRNAs in Immune Cell Development. miRNAs have an important role in regulating stem cell self-renewal and differentiation by repressing the translation of selected mRNAs in stem cells and differentiating into daughter cells. Such a role has been shown in embryonic stem cells, germline stem cells and various somatic tissue stem cells [89]. The first studies implicating miRNAs in immunological processes were originated from expression profiling of haematopoietic cells during their development. Haematopoietic stem cells reside mainly in the bone marrow and give rise to all blood cell lineages, including cells that constitute the immune system [9]. These cells must maintain a precise balance between self-renewal and differentiation into multipotent progenitors, which subsequently give rise to both the common lymphoid and common myeloid progenitors of the haematopoietic system [9, 90]. Systematic investigation of miRNA levels in hematopoietic cell lineages has identified miRNAs that are now considered as markers of these lineages [91–93]. Peculiar miRNA profiles in different haematopoietic organs and cell types suggest that miRNAs are dynamically regulated during early haematopoiesis, lineage commitment, and the development of immune cells and are involved in the regulation of these processes.

One of the first miRNAs described to have a role in immune cell development was miR-181a which is highly expressed in thymus cells and expressed at lower levels in the heart, lymph nodes, and bone marrow [91, 94]. In bone marrow-derived B cells, miR-181a expression has been shown to decrease during B cell development from the pro-B to the pre-B cell stage [91]. miR-181a inhibits the transition of pro-B to the pre-B cell stage. Moreover, miR-181a was identified as a positive regulator of B lymphocyte differentiation based on evidence that expression of miR-181a in hematopoietic stem and progenitor cells resulted in an increase in CD19+ B cells and a decrease in CD8+ T cells [91]. Interestingly, miR-181a is also involved in thymic T cell differentiation, by defining the activation threshold of T cell receptor (TCR) [94]. This miRNA modulates TCR signaling, thus affecting the sensitivity of T cells to antigens [94]. Other examples of miRNA-mediated regulation of immune cell development include miR-223 which was identified as an essential modulator of granulocytic differentiation [95] and miR-150 which has been shown to be critical for B cell differentiation [96, 97]. Collectively, these studies demonstrate that miRNAs play critical roles at distinct stages of immune cell development.

3.2. MicroRNAs in Adaptive Immune Responses. The adaptive or acquired immune system involves the selective recognition and removal of nonself by the TCRs on T cells and antibodies produced by B cells. The maturation, proliferation, differentiation, and activation of T and B cells are complex processes tightly controlled at different levels including miRNA-mediated posttranscriptional gene regulation [11]. Adaptive immunity refers to immune responses to antigens that undergo learning processes and provide specific memory. Once APCs capture a pathogen, they display foreign antigens complexed with major histocompatibility complexes (MHCs) on their surface to enable recognition of the antigen by naïve T cells to induce the adaptive immune response [19]. The combination of this interaction further drives the upregulation of both CD80 and CD86 on the surface of APCs. CD80 and CD86 identify two additional receptors, CD28 and Cytotoxic T-lymphocyte antigen 4 (CTLA4), on the surface of the T cells to provide a second signal to APCs [19]. CD28 is associated with activation of the T cell whereas CTLA4 is more regulatory. After this second signal, the T cells become activated, and APCs begin to secrete important cytokines, including IL-12 and IL-23, which bind to specific receptors on T cells and drive them to secrete different cytokines, such as IFN-γ or IL-17, depending on the cytokine milieu. T cells also begin to secrete IL-2, which

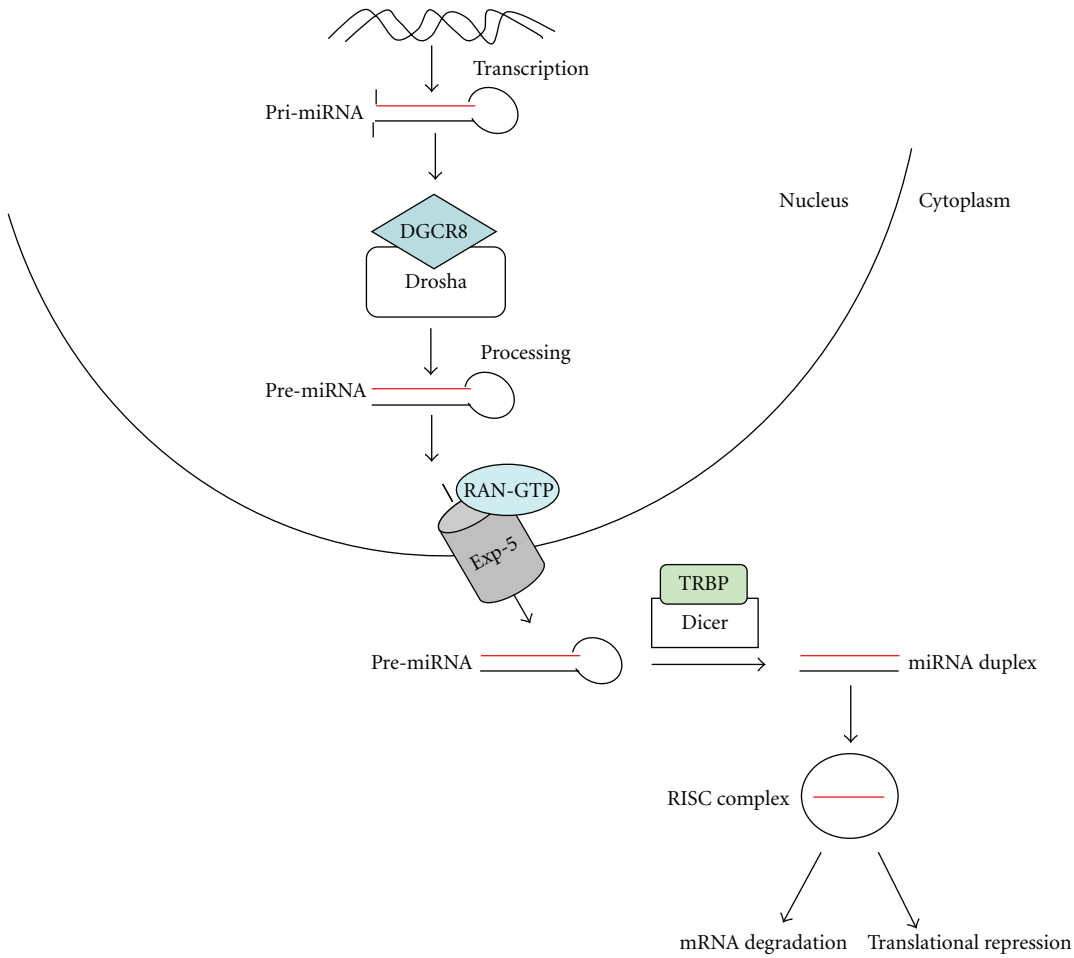

Figure 1: miRNA genes are transcribed in the form of Pri-miRNA. The DGCR8-Drosha complex processes in the form Pre-miRNA followed by transport into cytoplasm by Exportin-5. In cytoplasm, Pre-miRNA is processed by Dicer into miRNA duplex. Of miRNA duplex, one strand is loaded into RISC complex, which functions for either mRNA degradation or translational repression.

then activates its own IL-2 receptor [19]. Upon activation of their TCR in the presence of costimulatory molecules, naïve T cells differentiate into various subsets of effector T cells with distinct effector functions (e.g., Th1, Th2, Th17, Th9). This differentiation is directed by a specific cytokine milieu leading to the expression of transcription factors specific for the respective lineages. The expression levels of all molecules involved in adaptive immune responses (transcription factors, cell surface receptors, cytokines, and their receptors) may be regulated by miRNAs as discussed below.

3.2.1. T Cells. The development of T cells in the thymus and their activation in the periphery are controlled by complex protein signalling networks that are subject to regulation by miRNAs [9, 98]. miRNA expression profiles vary between T cell subsets and different developmental stages [92, 99]. Specific deletion of Dicer in the T cell lineage resulted in impaired T cell development and aberrant T helper cell differentiation and cytokine production [100]. A severe block in peripheral CD8+ T cell development was observed upon Dicer deletion in the thymus. However, Dicer-deficient CD4+ T cells, although reduced in numbers, were viable and could be analyzed further. These cells were defective in microRNA processing, and upon stimulation, they proliferated poorly and underwent increased apoptosis [100]. Deletion of Dicer at an early stage of T cell development compromised the survival of alpha-beta lineage cells whereas the numbers of gamma-delta-expressing thymocytes were not affected in developing thymocytes [101]. Mice with higher expression of miR-17–92 in lymphocytes developed lymphoproliferative disease and autoimmunity and died prematurely. Lymphocytes from these mice showed more proliferation and less activation-induced cell death. The miR-17–92 miRNA suppressed expression of the tumor suppressor PTEN and the proapoptotic protein Bim [102]. T cell sensitivity to antigen is intrinsically regulated during maturation to ensure proper development of immunity and tolerance. Increasing miR-181a expression in mature T cells augments the sensitivity to peptide antigens while inhibiting miR-181a expression in the immature T cells reduces sensitivity and impairs both positive selection and negative selection [94]. These effects are in part achieved by

the downregulation of multiple phosphatases, which leads to elevated steady-state levels of phosphorylated intermediates and a reduction of the TCR signaling threshold. T cell activation requires signaling through the TCR and costimulatory molecules, such as CD28. Costimulation-dependent upregulation of miR-214 promotes T cell activation by targeting the negative regulator Pten. Thus, the requirement for T cell costimulation is, in part, related to its ability to regulate expression of miRNAs that control T cell activation [103].

Recent data have also indicated a role for miRNAs in the differentiation of T cells into distinct effector T helper cell subsets. miR-155 has an important role in the mammalian immune system, specifically in regulating T helper cell differentiation and the germinal center reaction to produce an optimal T cell-dependent antibody response [104]. miR-155 exerts this control, at least in part, by regulating cytokine production. Many types of specialized Th cells, including Th1, Th2, Th17, Th9, follicular helper T, and Treg, have been identified. Different Th cells are committed to their paths but recent emerging evidence suggests that under certain conditions, seemingly committed Th cells possess plasticity and may convert into other types of effector cells [105]. There is growing evidence that clinically similar forms of autoimmune demyelinating disease can be driven by myelin-specific T cells of distinct lineages with different degrees of dependence on IL-17 production to achieve their pathological effects [106]. miRNAs play an important role in the development of Th17 cells [107]. Bcl-6, a transcriptional repressor, binds to the promoters of the Th1 and Th17 cell transcriptional regulators T-bet and RORgammat and represses IFN-γ and IL-17 production. Bcl-6 also represses expression of many miRNAs predicted to control the T follicular cell signature, including miR-17-92, which represses CXCR5 expression. Thus, Bcl-6 positively directs T follicular cell differentiation, through combined repression of miRNAs and transcription factors [108]. miRNAs are also essential in the development, differentiation, and function of Treg cells which are potent immune regulators [109]. Recent studies showed a crucial role for miRNAs in Treg cell biology and the prevention of spontaneous autoimmunity [110–112].

miR-155 deficiency in Treg cells results in increased suppressor of cytokine signaling 1 (SOCS1) expression accompanied by impaired activation of signal transducer and activator of transcription 5 (STAT5) transcription factor in response to limiting amounts of IL-2. Forkhead box P3-(Foxp3-) dependent regulation of miR155 maintains competitive fitness of Treg cell subsets by targeting SOCS1 [113]. miR-155-deficient mice have reduced numbers of Tregs, both in the thymus and periphery, due to impaired development. However, no evidence for defective suppressor activity of miR-155-deficient Tregs was found, either *in vitro* or *in vivo*, suggesting that miR-155 contributes to Treg development, but that additional miRNAs control Treg function [114]. The expression of miR-142-3p was recently shown to be repressed by Foxp3, leading to increased production of cyclic AMP and suppressor function of Treg cells [115]. Depleting miRNAs by eliminating Dicer reduces Treg cell numbers and results in immune pathology [116]. Dicer facilitates, in a cell-autonomous fashion, the development of Treg cells in the thymus and the efficient induction of Foxp3 by transforming growth factor-beta (TGF-β). These results suggest that Treg cell development involves Dicer-generated RNAs awaiting functional assessment. miR-31 negatively regulates Foxp3 expression by binding directly to its potential target site in the 3′-UTR of Foxp3 mRNA whereas miR-21 acts as a positive, though indirect, regulator of Foxp3 expression [117]. Finally, miR-155 inhibition sensitizes CD4+ Th cells for Treg-mediated suppression [118].

3.2.2. B Cells. The generation of B cells that express high affinity antigen receptors involves two main stages: antigen-independent development in the bone marrow and antigen-dependent selection in the secondary lymphoid organs, both of which are associated with dynamic regulation by miRNAs [9, 119]. Antigen receptors on the surface of B cells trigger adaptive immune responses after encountering their cognate antigens but also control a series of antigen-independent checkpoints during B cell development. These physiological processes are regulated by the expression and function of cell surface receptors, intracellular signaling molecules, transcription factors, and miRNAs [119]. Temporal regulation of several different miRNAs was observed and putative new cell type-specific miRNAs were identified in the development of B cells, suggesting the involvement of many, but undefined, regulatory pathways in B cell development and maturation [9]. The role of miRNAs in controlling the early development of B cells is now thought to involve the modulation of key protein factors that control these aspects of B cell development [97]. miR-181 is preferentially expressed in the B-lymphoid cells of mouse bone marrow and its ectopic expression in hematopoietic stem/progenitor cells leads to an increased fraction of B-lineage cells, without increase of T cells or myeloid cells in both tissue-culture differentiation assays and adult mice [91].

In contrast, mice with a conditional deletion of Dicer in B cells had a complete block in B cell development [120]. This block is related to dysregulated expression of the proapoptotic protein Bim, probably during the selection of effective antigen receptors. These results suggest a defect in the regulation of B cell selection. Regulation of apoptosis and cell cycle progression plays an essential role in the maintenance of B-cell homeostasis, because a fine balance of survival and expansion is critical for preventing lymphocytic disorders. Interestingly, the changes observed by gene expression profiling of Dicer-deficient B cell precursors are generally similar to those observed in B cells lacking the miR-17–92 family. Absence of miR-17–92 leads to increased levels of the proapoptotic protein Bim and inhibits B cell development at the pro-B to pre-B transition [121]. In addition to effects on antigen receptor selection, miRNAs also regulate the transcription factors involved in early B cell development [9]. Constitutive expression of miR-150, which is highly upregulated at the immature B cell stage, leads to a block at a proximal stage of B cell development, the pro-B to pre-B cell transition, indicating that miR-150 most likely downregulates mRNAs that are important for

pre- and pro-B cell formation or function [96]. miR-150 controls B cell differentiation by targeting the transcription factor c-Myb [97]. miR-125b also promotes B cell diversification in the germinal center by inhibiting premature utilization of essential transcription factors for plasma cell differentiation [122].

The contribution of miRNAs in the antigen-driven stages of the humoral response in secondary lymphoid organs has also been described [9]. miR-155 is required in B cell responses to thymus-dependent and -independent antigens [123]. B cells lacking miR-155 generated reduced extrafollicular and germinal center responses and failed to produce high-affinity IgG_1 antibodies. When transcription factor Pu.1 is overexpressed in wild-type B cells, fewer IgG_1 cells are produced, suggesting that loss of Pu.1 regulation is a contributing factor to the miR-155-deficient phenotype [123]. The miR-23a cluster is a downstream target of PU.1 involved in antagonizing lymphoid cell fate determination [124]. miR-155 represses activation-induced cytidine deaminase, which is required for immunoglobulin gene diversification in B lymphocytes [125, 126]. A recent study showed that numerous miRNAs were expressed in a stage- or transformation-specific fashion in B cells, suggesting specific functional or pathological roles [127].

3.3. MicroRNAs in Innate Immune Responses. The innate immune response provides the initial defense against infection by external pathogens and is predominantly mediated via myeloid cells such as macrophages, DCs, monocytes, neutrophils, as well as natural killer (NK) cells. The presence of pathogens is commonly detected by tissue APCs such as macrophages and DCs via families of pattern recognition receptors that bind nonself-antigens such as microbial products. Many families of pattern recognition receptors have been identified, although the best characterised are the TLR which are composed of 11 members and the interleukin IL-1 receptors which have 10 members. On ligation, the APC is activated by the Nuclear factor kappa B (NF-κB) pathway that leads to the production of type 1 IFNs, including IFN-β. These processes are stereotypical and do not generate immunological memory. The distinction between the body's cells and unwanted foreign invaders becomes obscured in autoimmune diseases. Thus, the innate immune system plays an important role in autoimmunity. Emerging data have identified an important contribution of miRNAs to the development and function of innate immune cells. Furthermore, studies investigating myeloid cell development and function have identified a common theme of a dynamic interplay between lineage-specific transcription factors and miRNAs. miRNAs involved in the regulation of granulocytes, monocytes, macrophages, DCs, NK, and natural killer T cells have been identified [9, 98].

Several studies have shown that transcription factors involved in monocytopoiesis are regulated by, and/or regulate, specific miRNAs, which indicates a connection between these molecular species during development [9, 98]. Studies in human umbilical cord blood CD34+ haematopoietic progenitor cells induced to differentiate into monocytes upon exposure to macrophage-colony stimulating factor (M-CSF) showed that monocytopoiesis is controlled by a circuitry involving sequentially three miRNAs (i.e., miR-17-5p, miR-20a, and miR-106a, members of the miR-17–92 and related miR-106a–92 families) and the transcription factor acute myeloid leukaemia-1 (AML1) [128]. During monocytic differentiation, the expression of these miRNAs is downregulated, whereas the transcription factor AML1 is upregulated at the protein but not mRNA level. Accordingly, this process promotes M-CSF receptor (M-CSFR) transcription, which therefore enhances the differentiation and maturation of monocytes. While these miRNAs target AML1, this transcription factor binds and transcriptionally inhibits expression of miR-17-5p, miR-20a, and miR-106a in a mutual negative feedback loop [128]. PU.1 is another transcription factor that is crucial for monocyte and macrophage differentiation [129]. PU.1 activates the transcription of miR-424, and this upregulation is involved in stimulating monocyte differentiation through miR-424-dependent translational repression of nuclear factor I/A (NFI-A). In turn, the decrease in NFI-A levels is important for the activation of differentiation-specific genes such as M-CSFR [129]. Translational repression of NFI-A by miR-233 is also involved in myeloid cell differentiation [95].

Neutrophils arise from granulocyte-monocyte progenitors under the influence of the transcription factor growth factor independent 1 (GfI1) [9]. GfI1 was recently shown to bind to the promoter regions of pri-miR-21 and pri-miR-196b and repress their expression [130]. The sustained expression of miR-155 can increase immature granulocyte numbers *in vivo*, and several of its targets, including SH2-domain-containing inositol-5-phosphatase 1 (SHIP1), are probably involved in this process [131, 132]. In addition to regulating neutrophil development, miRNAs also regulate granulocyte function. Genetic deletion of miR-223 can positively influence myeloid cell development and function *in vivo* [133]. miR-223 is induced by the myeloid transcription factors PU.1 and CCAAT/enhancer-binding protein-β (C/EbPβ), and it negatively regulates both the proliferation and activation of neutrophils. Myeloid Elf1-like factor 2C (*MEF2C*) has been shown to be a direct target of miR-223. TLR4-activated NF-κB rapidly increases the expression of miR-9 that operates a feedback control of the NF-κB-dependent responses by fine tuning the expression of a key member of the NF-κB family [134]. Brief exercise alters the miRNA profile in circulating neutrophils in humans [135].

miRNAs regulate distinct aspects of DC biology and so are involved in the crucial connection between innate and adaptive immune responses. miR-34 and miR-21 have been shown to be important for human myeloid-derived DC differentiation by targeting the mRNAs encoding Jagged1 and WNT1 [136]. Myeloid-derived DCs from Bic−/− (miR-155-deficient) mice showed defects in antigen presentation to T cells [137]. In addition, miR-155 downregulated expression of DC-specific ICAM3-grabbing nonintegrin (DC-SIGN; also known as CD209) by human monocyte-derived DCs through suppression of PU.1 expression [138]. DC-SIGN is a cell surface C-type lectin that binds pathogens, implicating miRNAs in the regulation of pathogen uptake by

DCs. In human myeloid-derived DCs, knockdown of miR-155 expression significantly increased protein expression of the proinflammatory cytokine interleukin-1β (IL-1β) [139]. miR-146a acts as a regulator of monocyte and DC activation but not myeloid/DC subset differentiation [140].

miRNAs have been implicated in the development and function of NK cells which are important components of immune surveillance against cancer and viral infection [9]. NK cells express the receptor natural killer group 2, member D (NKG2D), which recognizes ligands, MHC class I polypeptide-related sequence A (MICA), and MICB, expressed by cells undergoing stress triggered by events such as viral infection or cell transformation [9, 141]. Engagement of NKG2D on NK cells leads to direct killing of the target cell. A recent study showed that a set of miRNAs, many of which are overexpressed by various cancer cells, binds to MICA and MICB 3′-UTR sequences and maintains expression of MICA and MICB protein under a certain threshold and facilitates acute upregulation of MICA and MICB during cellular stres [142]. Certain herpesvirus family members, namely, cytomegalovirus, Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus, produce miRNAs that target MICB mRNA, suggesting a miRNA-based immunoevasion mechanism that appears to be exploited by human viruses [143]. Lipopolysaccharide (LPS) stimulation decreased expression of miRNAs, miR-17-5, miR-20a, and miR-93, which target MICA, implicating a novel role for miRNAs in NKG2D ligand expression. These results suggest that TLR stimulation allows expression of NKG2D ligands through multiple pathways, including downmodulation of specific miRNAs [144]. Invariant NKT (iNKT) cells are a class of innate-like T cells that express an invariant TCR that recognizes lipids presented by the MHC class I-like CD1d molecule and regulate diverse immune responses [9, 145]. Two recent studies showed that differentiation and homeostasis of iNKT cells require Dicer in a cell-autonomous fashion [146, 147]. Dicer deletion results in a substantial reduction of iNKT cells in thymus and their disappearance from the periphery. Without Dicer, iNKT cells do not complete their innate effector differentiation and display a defective homeostasis due to increased cell death.

Numerous studies clearly demonstrate that miRNAs play an essential role in the regulation of various aspects of innate immunity, including the regulation of direct microbial killing, the production of cytokines, and antigen presentation by MHC molecules. All of these mechanisms are important for host defense and are instrumental in initiating antigen-specific responses by cells of the adaptive immune system [9].

Both the induction and repression of miRNA expression in response to inflammatory stimuli can influence several biological processes and exert pro- or antinflammatory effects [16]. Microbial products are important proinflammatory stimuli and activation by TLR ligands has been shown to modulate several miRNAs including miR-9, miR-125b, miR-146a, and miR-155 [98]. Of these miRNAs, only miR-146a and miR-155 appear to be induced in multiple cell types. In macrophages and DCs, stimulation by TLRs ligands results in miR-155 induction via NFκB pathway and signalling through the c-jun-N-terminal kinase (JNK) pathway [148, 149]. The induction of miR-155 by LPS has also been demonstrated *in vivo* and is accompanied by a decrease in miR-125b expression [149].

The downregulation of miR-125b appears to be necessary in macrophages to prevent suppression of Tumor necrosis factor-alpha (TNF-α) during the inflammatory response. miR-155 is upregulated in murine macrophages by the synthetic triacylated lipopeptide Pam3CSK4, the synthetic double stranded RNA analog poly(I:C), LPS, and CpG oligonucleotides, suggesting that several TLR ligands can induce miR-155 expression and that miR-155 is involved in the regulation of both bacterial and viral innate immune responses [148]. Fas-associated death domain protein, IkB kinase ε and receptor interacting serine-threonine kinase 1 were experimentally validated as targets of miR-155 [139, 148]. Involvement of miR-155 in the TLR-induced antigen presentation pathway was confirmed by a study showing that miR-155-deficient DCs are unable to induce efficient T-cell activation, with impaired antigen presentation and costimulation [137]. miR-9 is upregulated in both polymorphonuclear neutrophils and monocytes after TLR4 activation. This miRNA is also induced by TLR2 and TLR7/8 agonists and by the proinflammatory cytokines TNF-α and IL-1β [134]. MiR-146, miR-147, and miR-21 are also upregulated after the activation of TLR4 upon stimulation via LPS [150–152]. However, in contrast to miR-155, these miRNAs are negative regulators of pattern-recognition response. miR-146a reduces the translation of tumour necrosis factor receptor-associated factor-6 (TRAF6) and IL-1 receptor-activated kinase-1 (IRAK1), which are two key components of the TLR signalling pathway [150]. These studies indicate an essential role of miRNAs as important regulators of inflammation.

4. MicroRNAs and Autoimmunity

The roles of miRNAs are only beginning to be explored in the context of autoimmunity, in which they may be involved in regulating immune responses against self-tissues [9]. Immune responses are normally targeted against microbial pathogens and not self-antigens by mechanisms that are only partially understood. Over the past few decades, multiple mechanisms have emerged that operate to prune the lymphocyte repertoire of self-reactive specificities and maintain immunological tolerance. miRNAs target immune transcripts to fine-tune gene expression and turn on negative feedback loops. Both of these actions are crucial to limit costimulation, set precise cellular activation thresholds, curtail inflammation, control lymphocyte growth, and maintain regulatory T cell homeostasis and suppressive function [153]. miRNA expression is tightly regulated during hematopoiesis and lymphoid cell differentiation and disruption of the entire miRNA network or selected miRNAs may lead to dysregulated immune responses. Dysregulation of single or a few miRNAs or miRNA clusters can result from genetic variation, hormonal influences, or environmental triggers including infections. In the light of this vast and promiscuous miRNA-mediated regulation of autoimmune

genes, it is anticipated that changes in miRNA levels or their target sequences may help explain susceptibility to complex autoimmune diseases. Abnormalities in miRNA expression related to inflammatory cytokines, Th17 and Treg cells, as well as B cells have been described in several autoimmune diseases [9, 13, 14, 154].

In 2007, the involvement of miRNA in a new pathway regulating autoimmunity was discovered in T lymphocytes in the sanroque mouse [155]. The sanroque mouse was originally selected from screening mutant mice derived from the chemical mutagen N-ethyl-N-nitrosourea and has been shown to result from a mutation in the gene Roquin that encodes a RING-type ubiquitin ligase [14]. In normal T cells, Roquin normally limits the expression of inducible T-cell costimulator (ICOS) by promoting the degradation of ICOS mRNA. In sanroque mice, however, the absence of this regulation leads to an accumulation of lymphocytes that is associated with a lupus-like autoimmune syndrome. Yu et al. reported that miR-101 is required for the Roquin-mediated degradation of ICOS mRNA [155]. Introducing mutations into the miR-101 binding sites in the 3′-UTR of ICOS mRNA disrupted the repressive activity of Roquin. These results revealed a critical miRNA-mediated regulatory pathway that prevents lymphocyte accumulation and autoimmunity. More recently, deletion studies showed that targeted deletion of miRNAs in hematopoietic stem cells or in thymus disrupts T cell homeostasis and results in autoimmunity and abnormal cytokine production. Recent studies revealed the importance of miRNA regulation in safeguarding Treg function in the prevention of autoimmunity. miRNA biogenesis is indispensable for the function of Treg cells. Specific deletion of either Drosha or Dicer phenocopies mice lacking a functional Treg cell-specific transcription factor Foxp3 gene or Foxp3(+) cells whereas deletion throughout the T cell compartment also results in spontaneous inflammatory disease, but later in life [112]. Treg cell-mediated immune tolerance is critically dependent on the Dicer-controlled miRNA pathway. Mice with conditional Dicer knockout within the Treg cell lineage rapidly developed fatal systemic autoimmune disease resembling the Foxp3 knockout phenotype [110, 111]. Although thymic Treg cells developed normally in Dicer-deficient mice, the cells exhibited altered differentiation and dysfunction in the periphery. Interestingly, Dicer-deficient Treg cells retained some suppressive activity, albeit reduced compared to wild-type mice [111]. However, under inflammatory conditions Dicer-deficient Treg cells were completely devoid of any suppressor activity and instead showed a robust *in vitro* proliferative response leading to autoimmunity suggesting that miRNAs preserve the Treg cell functional program under inflammatory conditions. These findings support a central role for miRNAs in maintaining the stability of differentiated Treg cell function *in vivo* and homeostasis of the adaptive immune system.

Further support for a causal relationship between specific miRNAs and the onset of autoimmunity has come from studies involving miR-17–92 overexpression in mice [102]. Mice with higher expression of miR-17–92 in lymphocytes developed lymphoproliferative disease and autoimmunity and died prematurely. miR-17–92 overexpression promoted marked lymphoproliferation, the presence of serum autoantibodies, and tissue changes such as lymphoid infiltrates and antibody deposition. T cells seem to develop normally in these mice, but the number of mature CD4+ T cells was markedly increased and they had a highly activated profile, suggesting a failure of peripheral tolerance. Lymphocytes from these mice showed more proliferation and less activation-induced cell death. The miR-17–92 miRNA suppressed expression of the tumor suppressor Phosphatase and tensin homolog (Pten) and the proapoptotic protein Bim [102]. This mechanism probably contributed to the lymphoproliferative disease and autoimmunity of miR-17–92-transgenic mice. Dysregulation of miRNAs involved in immune cell development may cause autoimmunity. A recent study has shown that inhibition of miR-181a in T cells during thymic development converts endogenous positively selecting peptides into autoantigens [156].

Emerging evidence has demonstrated that miRNAs are differentially expressed in autoimmune diseases and miRNA regulation may impact in the development or prevention of autoimmunity. miRNA dysregulation is linked to autoimmune diseases that include rheumatoid arthritis, systemic lupus erythematosus, primary biliary cirrhosis, ulcerative colitis, psoriasis, Idiopathic thrombocytopenic purpura, primary Sjögren's syndrome, and MS.

These molecules have also been shown to be useful as diagnostic and prognostic indicators of disease type and severity [15–17]. Many autoimmunity and disease susceptibility genes are targeted by several miRNAs [153, 157]. The precise mechanisms miRNAs use to promote or hinder autoimmunity have yet to be elucidated. However, several potential mechanisms deserve consideration, including loss or downregulation of miRNA expression due to mutation, epigenetic activation, aberrant processing, or transcriptional downregulation; overexpression of particular miRNA consequent to gene amplification or mutation, especially miRNA promoter regions, or due to transcriptional upregulation that may result in the suppressed production of its target proteins; and mutation at the 3′-UTR of the target mRNA or its gene [14]. In most cases the role of specific miRNAs in autoimmune diseases has been established *in vitro* by association, and that causal roles *in vivo* remain a matter of investigation [14].

Clinical characteristics along with pathological heterogeneity make MS appealing to study many aspects of miRNAs in an organ-specific autoimmune disease, such as their potential as diagnostic or prognostic biomarkers and their role in pathogenesis of autoimmunity, neuroinflammation, and organ dysfunction. Thus, we will focus on the involvement of specific miRNAs in MS pathogenesis following the general overview of the immunopathobiology of the disease.

5. MicroRNAs and Multiple Sclerosis

5.1. Genetic and Epigenetic Factors in Multiple Sclerosis. MS is a chronic inflammatory demyelinating disease of the CNS that primarily affects young adults. Prevalence

rates for MS vary between 2 and 160 per 100,000 in different countries, and more than 2 million individuals are affected by this disease worldwide [158]. Autoreactive T cell-mediated autoimmune response to myelin antigens results in both inflammation and axonal degeneration accounting for the disability of patients with MS [19]. The exact factors that initiate inflammation are unknown, but it is generally believed that MS is caused by environmental factors in a genetically susceptible host that trigger a T-cell autoimmune response against the CNS [18]. In the literature, several genetic factors have been described to influence the development and severity of MS and are responsible for disease susceptibility [20]. The major genetic factor in MS is the major histocompatibility complex [159], however recent genomewide association studies revealed new susceptibility alleles for MS that are all related with immune functions (e.g., CASP8, CD58, STAT3, interleukin 7 receptor (IL7RA; CD127), interleukin 2 receptor A (IL2RA)) [20, 160, 161]. Nevertheless, no locus has been detected of constant form in all the studies, suggesting the existence of genetic heterogeneity. MS is likely to be the result of interactions between environmental stimuli (e.g., infection), susceptibility genes (which predispose individuals to the development of neuroinflammation), and modifier genes (which affect disease phenotype in susceptible subjects). Although viruses may trigger MS relapses, there is no definitive evidence that there is an MS virus or an ongoing chronic infection of the nervous system. It is possible, however, that a self-limited CNS infection in childhood could trigger MS, and epidemiological evidence suggests that Epstein-Barr virus (EBV) may play a key role in MS [18]. Other nongenetic but nevertheless gene-regulation factors including epigenetic mechanisms such as DNA methylation and histone modification and miRNA-mediated posttranscriptional gene regulation might individually influence both susceptibility and severity of the disease [162].

Experimental allergic encephalomyelitis (EAE) serves as the primary and most widely used animal model for MS and can be induced in susceptible rodent strains by active immunization of myelin antigens [163, 164]. Different types of the model have been developed that mimic virtually all the clinical features of MS including relapsing, relapsing remitting, progressive, and opticospinal forms. The majority of treatments for MS have stemmed from studies in the EAE model, further supporting the concept that autoimmune processes in the EAE model are relevant to MS [18]. However, there are also examples of mechanisms that have worked in EAE but have failed in the clinic, such as the TNF-α antagonists and anti-p40 (a subunit of IL-12 and IL-23) [163, 164].

5.2. Immunopathobiology of Multiple Sclerosis. The clinical course of MS varies, with 80% of patients presenting with episodes of disability followed by a period of recovery classified as relapsing-remitting while 10%–15% exhibit a more progressive disease without remission, namely, primary progressive [165, 166]. The patient has a yearly risk of about 3% for a transition from the relapsing-remitting phase to the chronic, progressive form of MS. Over a period of 10 years, roughly half of relapsing-remitting patients enter a secondary progressive stage of disease characterized by accumulating disability while recovery between episodes diminishes.

There is consensus that a dysregulated immune system plays a critical role in the pathogenesis of MS. Relapses are driven by the adaptive immune system and involve waves of Th1, Th17, and CD8+ cells that infiltrate the CNS and provoke an attack. These cells are modulated by Treg and B cells. MS is initiated and maintained by continuous migration of inflammatory immune cells from the periphery into the target organ. The three ways that lymphocytes can enter the CNS include entry from the bloodstream across the choroid plexus into the Cerebrospinal fluid (CSF), from the blood in the subanachroid space into the CSF, or directly into the parenchyma under permissible conditions, such as inflammation, controlled by cell adhesion molecules and cytokines [167]. Subpopulations of T cells may employ different trafficking mechanisms [163]. Infiltration of T cells into the CNS initiates a complex immunological cascade consisting of epitope spreading, which triggers new attacks, and activation of the innate immune system composed of microglia, dendritic cells, and astrocytes [18]. The secondary progressive phase is due to neurodegeneration triggered by neuroinflammation and is driven by the innate immune system. The loss of axons and their neurons in the course of chronic neuroinflammation is a major factor determining long-term disability in patients and neurodegeneration as the major cause of irreversible neurological disability in MS patients. Thus, in the relapsing stage, a proinflammatory milieu that combines both the innate and adaptive immune system is present whereas in the progressive stage abnormalities of the innate immune system predominate [18].

5.2.1. Adaptive Immune Responses in Multiple Sclerosis

Pathogenic T Cells. Among cells isolated from the inflammatory infiltrate in actively demyelinating MS lesions, approximately 10% are T cells [168, 169]. There, multiple T-cell subsets have been implicated: CD4+ Th1 and Th17, γ/δ T cells, CD8+, and Treg cells [163]. CD4+ T cells are the most prominent cells in active MS lesions but are not present in chronic MS lesions [170]. It is generally believed that the acute MS lesion is initiated by a myelin-reactive CD4+ T cell that is stimulated in the periphery and enters the brain and spinal cord [18]. Recent research has focused on the different roles of subsets of CD4+ T cells in MS and other autoimmune diseases. Th1 cells classically express IFN-γ, TNF-β, IL-2, and nitric oxide [171] and activate macrophages to stimulate cell-mediated immunity [168]. Th2 cells release IL-4, IL-5, IL-6, IL-10, IL-13, and TGF-β [168]. These cytokines may be associated with disease recovery in MS. Th17 cells are CD4+ T cells subtype that are associated with autoimmune diseases. Th17 cells are dependent on IL-23, TGF-β, IL-6, and IL-1 [163]. Th17 cells produce IL-17A and IL-17F, which are upregulated in chronic lesions [172, 173], and IL-22 which is also involved in MS pathogenesis. It is now recognized that

Th17 cells play a crucial role in autoimmunity in the EAE model [174]. However, recent work by Haak et al. [175] has demonstrated that overexpression of IL17 in T cells did not exacerbate EAE. If Th17 cells are given with Th1 cells, then full disease induction occurs [176]. These results suggested that pure Th17 cells are not pathogenic. Both types of cells (Th1 and Th17) may play a role in MS and could account for the immunological and clinical heterogeneity of the disease [18, 177].

Most TCRs are composed of two linked polypeptides, α and β, which participate in the recognition of foreign antigen plus self-MHC [168, 178]. However, a small subtype of circulating lymphocytes expresses γ/δ TCR polypeptides which function in both innate and adaptive immunity [168, 179]. Clonal expansion of activated lymphocytes bearing the γ/δ TCR has been demonstrated in samples isolated from the CSF of patients with recent-onset MS but not from patients with chronic MS [180]. Recently, investigators demonstrated that γ/δ T cell-deficient mice were unable to recover from EAE [168, 179, 181]. Histopathologically, there was a prolonged presence of monocytes and lymphocytes in the CNS [179, 181]. CD8+ T cells are also implicated in MS pathology. Within MS plaques, clonal and oligoclonal expansion of CD8+ T cells reactive to myelin antigens has been observed [182]. A new effector T cell subset, Th9 cells, has been identified. Jager et al. showed that Th9 effector cells participate in induction of EAE [183]. These results suggested that Th9 cells may participate in MS pathogenesis.

Regulatory T Cells. Defects in Treg-cell function have been described in MS, and a major goal of MS immunotherapy is to induce regulatory cells in a physiological fashion [184–186]. Clinical studies in MS patients showed that Treg cell dysfunction occurred in the initial stages of the disease [168]. In addition, experimental data suggest that regulatory cells may not be effective if there is ongoing CNS inflammation [187].

B Cells and Antibodies. MS is generally thought to be a T cell-mediated immune disease although there is an important role of humoral immunity in pathogenesis of MS. Intrathecal antibody synthesis is a hallmark of the disease process and, in most of cases, consists of oligoclonal IgG production [18, 188]. A direct correlation has been reported between levels of immunoglobulin production and MS disease severity [189, 190]. Antibodies to self-antigen such as Myelin Basic Protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) have been identified in the serum of patients with MS and clinically isolated syndromes (CISs) [191, 192]. B cells and plasma cells have been detected in brains and CSF of patients with MS [189]. Characterization of the B-cell compartment within the CSF of MS patients shows that short-lived plasmablasts, not plasma cells, are the predominant antibody-secreting cell in MS CSF [189, 193] and the B cell to monocyte ratio correlates with the rate of disease progression. B cells are also potent APCs and may play a prominent role in T-cell antigenic stimulation. Thus, B cells may well be active participants in initiating and maintaining disease [168].

5.2.2. Innate Immune Responses in Multiple Sclerosis. The innate immune system consists of monocytes, dendritic cells, and microglia. The innate immune system plays an important role in the immunopathogenesis of MS. The secondary progressive phase of MS has been believed to be related to neurodegenerative changes in the CNS [18]. Furthermore, chronic microglial activation occurs in MS [194]. The peripheral innate immune system changes cause the transition from the relapsing-remitting to the progressive stage. This raises important questions regarding the pathogenesis and treatment of different stages of MS. A major question is whether aggressive and early anti-inflammatory treatment will prevent the secondary progressive form of the disease. There are no specific therapies designed to affect the innate immune system in MS. Furthermore, like the adaptive immune system, there are different classes of innate immune responses, for example, protective and tolerogenic versus pathogenic and proinflammatory [18]. This fact should be kept in mind for new drug development studies that target innate immunity.

Antigen Presenting Cells. Macrophages are the major MHC Class II positive cell in the CSF. Macrophages in EAE have an integral role in initiating disease, and depletion of macrophages significantly inhibits disease [195]. Macrophages are not the only class II positive cells that can present myelin antigens. Monocytes, DCs, microglia, and astrocytes have all been implicated in presenting antigen and involved in MS pathogenesis [163]. Greter et al. demonstrated that mice with MHC class II expression limited to DC can still develop disease [196]. DCs can be further subdivided into myeloid (mDCs) and plasmacytoid DCs (pDCs) depending on their lineage, and they also differ in function [163]. pDCs are the major CNS-infiltrating DC population during EAE and pDCs have both stimulatory and regulatory effects on T cells [197]. pDCs negatively regulate CD4+ inflammatory responses in the CNS [197]. Depletion of pDCs during either the acute or relapse phase of EAE resulted in exacerbation of disease severity [163]. In MS patients, pDC from peripheral blood showed an immature phenotype. The pDC had a lower capacity to secrete IFN-α upon TLR-9 stimulation. This may indicate why common infectious agents trigger MS attacks [198]. mDCs within the CNS activate myelin specific T cells that are recruited to the inflamed tissue and facilitate differentiation into Th1 and Th17 cells [199, 200]. However, Deshpande et al. reported that mDC isolated from the peak of disease are less efficient APCs than those isolated at disease onset, suggesting that changes in DC phenotype may contribute to remissions [200].

Microglial cells seem to be crucial for maintaining autoimmune responses in the CNS. It has been demonstrated that both a microglial cell-specific deficiency of CD40 expression and a transient inactivation of microglial cells reduce disease severity [201].

Astrocytes also express MHC Class II after IFNγ exposure and it has been reported that astrocytes can present antigen [202]. Astrocytes from mice deficient in Class II transactivator (CIITA) failed to activate MOG-specific CD4+ T cells due to a lack of MHC Class II expression [163, 203]. However, CIITA-deficient mice still were susceptible to EAE [203]. However, human astrocytes do not effectively activate encephalitogenic T cells in vitro [204]. They may also influence the disease by secretion of cytokines and chemokines.

5.3. Neurodegeneration in Multiple Sclerosis. The identification of MS susceptibility loci, of which at least 15 have a primary function in immunological systems, favors early immune dysregulation followed by secondary neurodegenerative processes [163]. Indeed, MS is not exclusively a white matter disease. Specific cognitive deficits such as memory impairment, attention deficit, and reduced mental reasoning are increasingly being explained by damage to neurons in the gray matter, which affects 45%–65% of MS patients [205]. Although the precise trigger for MS remains elusive, it is understood that autoimmune mechanisms underlie the pathology, and furthermore that activated T cells migrate through the BBB where they accumulate and proliferate because of antigen restimulation. These cells release a host of proinflammatory molecules, which, in turn, further activate microglia or infiltrated macrophages and B cells. Axonal and neuronal injury occurs as an early event in the disease and is strongly correlated with the degree of inflammation in the brain [206–208]. In MS, neurons in the cortex and spinal cord are also affected, albeit to varying extents [209, 210]. The latest events in the chain of neuronal damage processes following focal axonal lesions include axon degeneration and atrophy of neuronal cell bodies and dendrites [165]. The loss of neurons and their processes is the leading cause of atrophy and is the primary determinant of long-term disability in MS patients. This chain of events produces a marked inflammatory response, which causes axonal injury through various antigen specific and bystander mechanisms.

In MS, both soluble factors and surface molecules could participate in neurodegeneration. Besides injurious proinflammatory molecules, proapoptotic factors produced by T cells, including FasL, granzyme B, soluble TNF-related apoptosis-inducing ligand (TRAIL), glutamate, nitric oxide, and free radicals, are possible mediators of injury [208, 211–214]. Accumulating evidence suggests that the increased energy demand of impulse conduction along excitable demyelinated axons and reduced axonal ATP production induce a chronic state of virtual hypoxia in chronically demyelinated axons, ultimately leading to excessive stimulation of Ca^{2+}-dependent degradative pathways [215]. Glutamate and nitric oxide can lead to enhanced expression of chemokine (C-C-motif) ligand 2 (CCL2) on astrocytes, which, in turn, leads to infiltration of CD11b cells and additional tissue damage [216]. Antiexcitotoxic compounds have an ameliorating effect in EAE model [18]. Another important component of neurodegeneration relates to changes in Na^{+} channels, and these are targets of therapy [217].

Axonal injury can be directly caused by immune cells. CD4+ and CD8+ T-cell subsets, once activated, are highly neurotoxic. These effects are mediated through a variety of contact-dependent mechanisms involving cell surface molecules such as FasL, LFA-1, and CD40. Th1 and Th17 proinflammatory classes of CD4+ T cells are neurotoxic whereas the anti-inflammatory Th2 subset is not [218]. Although activated T cells can clearly harm neurons, the converse has also been observed. Activated T cells underwent apoptosis that was mediated through neurons via a FasL-dependent mechanism [219]. In another context, neurons may induce encephalitogenic T cells to convert to T-regulatory cells that inhibit encephalitogenic T-cell action and suppress EAE [220]. It is likely that the adaptive immune system orchestrates the attack against CNS cells and drives microglia and macrophages to attack oligodendrocytes and neurons. Activated microglia and peripherally derived macrophages are shifted towards a strongly proinflammatory phenotype and produce apoptosis-inducing molecules such as the TRAIL and the proinflammatory cytokines TNF-α and IL-1β as well as potentially neurotoxic substances including nitric oxide, oxygen radicals and proteolytic enzymes [221, 222].

5.3.1. Neurodegeneration and MicroRNAs. Many recent studies provide a link between miRNA function and neurodegeneration [223–225]. Complete loss of miRNA expression in the brain leads to neurodegeneration in several animal models. Evidence from patient material is emerging that miRNA dysregulation could, indeed, contribute to neurodegenerative disorders. The translation of proteins previously implicated in familial forms of disease seems to be under control of miRNAs, and changes in miRNAs might explain how these proteins become affected in sporadic neurodegeneration. Thus, miRNAs are rapidly moving to center stage as key regulators of neuronal development and function as well as important contributors to neurodegeneration. The link between miRNAs and axonal neurodegeneration in the context of MS has not been focused on to date.

Endogenous tissue repair mechanisms such as myelin repair, gliogenesis, and neurogenesis in MS may also be modulated by specific miRNAs. Enhancing such repair mechanisms is an important, and increasingly realistic, therapeutic goal in MS [226]. Neurogenesis is defined as a process that includes the proliferation of neural stem/progenitor cells (NPCs) and the differentiation of these cells into new neurons that integrate into the existing neuronal circuitry. Recent studies point to the importance of miRNAs in regulating lineage-specific gene expression and determining neuronal identity during neurogenesis [227, 228]. These new observations suggest that miRNAs could function at many levels to regulate self-renewal of neural stem cells and neuronal fate specification, implicating miRNAs in the complexity of neurogenesis. miRNAs are also involved in adult neurogenesis which may imply the possible role of some miRNAs in endogenous repair mechanisms in MS

[229, 230]. In addition, cross talk between miRNA and epigenetic regulation contributes to the modulation of adult neurogenesis [231]. The modulation of miRNAs involved in adult neurogenesis may stimulate the differentiation of NPCs into mature neurons that can replace neurons lost through the disease process in MS. Patient studies also suggest the presence of neuronal precursor cells in MS lesions [232].

Within the CNS, myelin is produced by oligodendrocytes. Developmentally, the oligodendrocyte lineage arises from subventricular zone progenitors that give rise to oligodendrocyte progenitor cells (OPCs), which divide and migrate throughout the CNS before terminally differentiating to generate mature oligodendrocytes which myelinate receptive axons [233]. Each step of progression along the lineage is under tight transcriptional control; elucidation of this control is vital for understanding developmental myelination and for developing strategies to promote repair in demyelinating diseases.

Remyelination following CNS demyelination restores rapid saltatory conduction of action potentials and contributes to the maintenance of axonal integrity [234]. Chronic demyelination predisposes axons to atrophy, an irreversible event that is a major pathological correlate of progressive functional decline. Remyelination in MS is in most cases insufficient, leading to irreversible disability. Different and nonexclusive factors account for this repair deficit [235]. Local inhibitors of the differentiation of OPCs might play a role as well as axonal factors impairing the wrapping process. Alternatively, a defect in the recruitment of OPCs toward the demyelinated area may be involved in lesions with oligodendroglial depopulation. Deciphering the mechanisms underlying myelin repair success or failure should open new avenues for designing strategies aimed at favoring endogenous remyelination [235]. The few treatments that are available for combating myelin damage in MS, which largely comprise anti-inflammatory drugs, only show limited efficacy in subsets of patients. More effective treatment of myelin disorders will probably be accomplished by early intervention with combinatorial therapies that target inflammation and other processes—for example, signaling pathways that promote remyelination [236]. However, the integration of these pathways with transcriptional and post-transcriptional regulatory networks is not fully understood. The interplay of transcription factors and epigenetic modifiers including histone modifications, DNA methylation, and miRNAs during development is essential for the acquisition of specific cell fates [237]. Recent studies have identified a number of new transcriptional regulators and miRNAs as having key roles in oligodendrocyte (OL) differentiation and CNS myelination, providing new targets for myelin repair [233].

Selective deletion of miRNA-processing enzyme, Dicer, in oligodendrocyte lineage cells results in severe myelinating deficits despite an expansion of the oligodendrocyte progenitor pool [238, 239]. Dugas et al. identified the miRNA pathways responsible for myelination using Dicer1-deleted transgenic mouse model [238]. In this study, they found the inhibition of OPC-OL miRNA processing resulting in defects in mature miRNA processing. They also identified three miRNAs: miR-219, miR-138, and miR-338. Of these miRNAs, miR-219 is important for OL differentiation, directly repressing PDGFRalpha, Sox6, FoxJ3, and ZFP238 which promote OPC differentiation [238]. Postnatal Dicer ablation in mature OLs results in inflammatory neuronal degeneration through increased demyelination, lipid accumulation, and peroxisomal and oxidative damage and therefore indicates that miRNAs play an essential role in the maintenance of lipids and redox homeostasis in mature OLs [240]. A small subset of miRNAs (e.g., miR-9, miR-23, miR-206, miR-219, miR-338, and miR-17-92 cluster), is important to orchestrate the switch from OPCs to myelin-forming oligodendrocytes [238–244]. Transcription factors, myelin proteins, signaling molecules, and cytoskletal proteins were identified as validated targets of these miRNAs. Interestingly, the highest differentially expressed miRNAs demonstrated a similar pattern of expression throughout all stages of differentiation, suggesting that they potentially regulate a common target or set of targets in this process [245].

Dysfunction of the BBB is a major hallmark of MS and may impair tissue homoeostasis, which may have effects on disease progression, repair mechanisms, and drug delivery [246–248]. Thus, restoration of BBB permeability may help endogenous tissue repair. Although the pivotal role of miRNAs in angiogenesis is well established [249–251], these molecules have not been focused on in the context of MS, BBB integrity, and cerebral angiogenesis. Only one study showed that a proapoptotic miRNA, miR-15a, was downregulated by peroxisome proliferator-activated receptor delta in brain endothelial cells [252]. Peroxisome proliferator-activated receptor delta is a nuclear receptor whose agonists have been shown to inhibit EAE [253–255]. However, the contribution of vascular protection by peroxisome proliferator-activated receptor delta through miRNA regulation in the recovery process is not known.

5.4. MicroRNA Studies in Multiple Sclerosis. Little is known about what drives the differential control of the immune system in MS patients compared to unaffected individuals. Thus, it is important to reveal the aberrant miRNA expression profiling in MS patients. To our knowledge there have been only seven publications investigating the role of miRNAs in MS, six of which focus on the immune system in MS and the other on active and inactive MS lesions (Table 2). Differences in miRNA expression patterns have been documented in MS compared to healthy controls and in relapse versus remission of the disease.

Studies in peripheral blood mononuclear cells (PBMCs) of patients with MS revealed different expression patterns compared to control individuals. Using qPCR, a pilot study of the expression of 346 miRNAs in PBMCs obtained from a small number of MS patients during relapse and remission, versus healthy controls, demonstrated differences in gene expression patterns not only between the MS patients and healthy controls but also between patients with and without active disease [256]. Two miRNAs (miR-18b and miR-599) have been shown to be associated with relapse whereas another miRNA (miR-96) was found to be involved in

the remission of the disease. The genes targeted by miR-96 are involved in immunological pathways such as interleukin signaling and other pathways as wnt signaling [256]. In another recent study, Keller et al. [257] investigated the expression profiles of 866 human miRNAs; in whole blood cells of MS patients 165 miRNAs were identified that were significantly up- or downregulated in patients with RRMS as compared to healthy controls. The best single miRNA marker, miR-145, allowed discriminating MS patients from controls with a specificity of 89.5%, a sensitivity of 90.0%, and an accuracy of 89.7%. The authors concluded that single miRNAs, and even more so miRNA expression profiles, may have the potential to serve as diagnostic biomarkers for RRMS. However, MS patients in that study were treated with either glatiramer acetate or interferon-beta while one patient was not treated with anything. One of the difficulties of studying MS is the acquisition of samples unaffected by the influence of immunomodulatory treatment. These studies do not provide information about miRNA expression in various cell subpopulations and their importance during the differentiation and activation of lymphocytes in MS.

The recent study by Du et al. [258] identified a Th17 cell-associated miRNA, miR-326, as a major determinant of MS in a Chinese population but not of neuromyelitis optica. Its expression was highly correlated with disease severity in patients with MS and mice with EAE. *In vivo* silencing of miR-326 resulted in fewer Th17 cells and mild EAE, and its overexpression led to more Th17 cells and severe EAE. Du et al. also found that miR-326 promoted Th17 differentiation by targeting Ets-1, a negative regulator of Th17 differentiation [258, 259]. These results suggest a critical role for miR-326 in the regulation of Th17 differentiation and the pathogenesis of MS. Although a more recent study did not identify any statistically significant change in whole blood miR-326 expression between MS patients and controls [260], one of the three most upregulated miRNA detected in active MS lesions is miR-326 lending further support to the relevance of this miRNA for MS pathogenesis [261]. The discrepancies between the results of clinical studies may be caused by differences observed in MS patients from Asian or Caucasian origin [260]. In a group of MS patients in relapse, glucocorticoid treatment downregulates miR-326 expression indicating that this miRNA is under control of disease-modifying drugs and thus may be used in the monitoring of therapy responses [258]. Further exploration of the function of miR-326 in other cell types may be of great importance for understanding the immunopathogenesis of MS.

Although it is known that specific miRNAs are involved in each step of the maturation of pluripotent hematopoietic stem cells into the various blood cell lineages including B and T cells [262], little is known about miRNA involvement in the differentiation during T-cell activation under disease conditions such as MS. A recent study has analyzed the expression of 365 miRNA and revealed different miRNA expression profiles in CD4+, CD8+, and B cells of peripheral blood from eight RRMS patients compared with ten healthy volunteers and they have also validated miRNA in CD4+ cells with qPCR [263]. Importantly, all the patients had no immunomodulatory or other MS specific treatments in the six months before or during the study. Ten miRNAs in CD4+, four miRNAs in CD8+, and six miRNAs in B cells were differentially expressed in MS patients. Lindberg et al. found distinct and cell-specific expression patterns of miRNA in all cell subpopulations, which is well in line with reports about diverse miRNA expression in immune cells. Furthermore, the expression of potential target genes of these miRNA was altered. miR-17-5p, which is known to be involved in the development of autoimmunity and in numerous lymphoproliferative diseases [264], was detected in CD4+ lymphocytes of MS patients [263]. Functional experiments with a synthetic inhibitor of miR-17 also supported the link between miRNA expression and the altered target gene expression. Moreover, authors have found that miRNAs were also differentially expressed in the two study groups following *in vitro* stimulation of CD4+ T cells with anti-CD3/CD28. miR-17-5p and miR-193a were strongly upregulated, in contrast to the downregulation of miR-497, miR-1, and miR-126. This was correlated with alterations in the expression of potential target genes of miR-17-5p, that is, phosphatase and tensin homology and phosphatidyl-inositol-3-kinase regulatory subunit 1, which were downregulated upon stimulation of CD4+ cells *in vitro*. Other deregulated miRNAs did not respond to the stimulation probably due to other, non-T-cell activation related, mechanisms in their mode of action. These results support the role of miRNA-dependent regulatory mechanisms in the immunopathogenesis of MS. However, in a larger and more recent study, Cox et al. showed that miR-17 is underexpressed in MS whole blood [260]. This discrepancy between the studies may be due to methodological differences. Another cause of the discrepancy may be the material analyzed in those two studies, such as cell types. Patient number and disease activity status may also change the outcome of the analyses. In the study by Cox et al., the transcriptome of currently known miRNAs was investigated using miRNA microarray analysis in peripheral blood samples of 59 MS patients that were free of disease modifying therapy for at least 3 months before the study and 37 healthy age-matched controls. Of the patients, 18 had a primary progressive, 17 a secondary progressive, and 24 a relapsing remitting disease course. In all MS subtypes miR-17 and miR-20a were significantly underexpressed in MS, confirmed by qPCR. It was demonstrated that these miRNAs modulate T cell activation genes in a knock-in and knock-down T cell model. The same T cell activation genes are also upregulated in MS whole blood mRNA, suggesting that miR-17 and miR-20a are implicated in the development of MS [260].

It is known that Tregs play a key role in the autoimmune balance and their improper function may facilitate the expansion of autoreactive T cell clones. $CD4^+CD25^+Foxp3^+$ Treg cells play a pivotal role in the maintenance of self-tolerance and controlling autoimmunity [109].

Recent evidence has been provided for a potential functional defect of $CD4^+CD25^+Foxp3^+$ Treg cells in patients with RRMS [265]. The fact that ablation of miRNAs in Treg cells completely phenocopies the loss of Foxp3 cells clearly indicated that multiple immunosuppressive mechanisms used by Treg cells are ultimately controlled by miRNAs

[109]. The miRNA expression profile in Treg cells from treatment naive RRMS patients has recently been analyzed by De Santis et al. [266]. The suppressive capacity of isolated $CD4^+CD25^+$ has been verified by *in vitro* suppression assays. When the expression levels of 723 human miRNAs were compared in $CD4^+CD25^+$ T cells obtained from 12 MS patients and 14 healthy donors using microarray assay, 23 human miRNAs were differentially expressed between study groups. Among the deregulated miRNAs, members of miR-106b-25 were found to be downregulated in the Treg cells of MS patients when compared to healthy donors as confirmed by qPCR. Unexpectedly, in a preliminary experiment performed in a very small number of subjects, the ratio between Treg cells ($CD4^+CD25^+CD127^{DIM}$)/T effector cells ($CD4^+CD25^+CD127^{high}$) showed an enrichment of these miRNA in Treg cells derived from patients as compared to healthy controls [266]. miR-106b and miR-25 modulate the TGF-beta signaling pathway through their action on cell cycle inhibitor CDKN1A/p21 and the proapoptotic gene BCL2L11/Bim. TGF-β is involved in Treg cell differentiation and maturation [267]. Therefore, the deregulation of this miRNA cluster may alter Treg cell activity during the course of MS, by altering TGF-β biological functions.

A recent *in situ* and *in vitro* study extends the current concepts of MS lesion activity to the level of miRNA-regulated gene expression and may have broad implications for the regulation of macrophage activation in autoimmune and inflammatory diseases in general [261]. In the study which used laser capture microdissection from active and inactive MS lesions to pool single cells and in vitro cultures, differentially expressed miRNAs were assigned to specific cell types by qPCR. Tissue specimens were obtained from the brains of 20 MS pateints and nine control subjects without any known neurological disease. In active MS lesions 20 miRNAs, including those that are associated with immune responses such as miR-155 and miR-146a, were at least twofold more abundant and eight miRNAs were at least twofold less abundant than in normal white matter. Eight of the active MS brain specimens are derived from MS cases with a very fulminant disease course called Marburg's variant. Some miRNAs were more prominently regulated in Marburg's variant than those in the other active MS lesions, probably reflecting the more intense tissue destruction in Marburg's variant. In inactive MS lesions, 22 miRNAs were at least twofold more abundant and 13 miRNAs at least twofold less abundant than in normal white matter. Among the significantly altered miRNAs, some showed differential regulation in active versus inactive lesions whereas others were modified in the same direction. Junker et al. found that three of the most upregulated miRNAs in active MS lesions, namely, miR-155, miR-326, and miR-34a, target CD47, which was one of the downregulated transcripts in the active lesions in comparison to normal brain white matter. CD47 is a membrane glycoprotein and mediator of macrophage inhibition via its receptor signal-regulatory protein alpha on myeloid cells. Using laser dissection microscopy combined with qPCR, CD47 gene expression was found to be downregulated in the center of chronic active and inactive MS lesions [268]. CD47 has been considered as a "don't eat me signal" and its reduction in brain cells of MS could promote phagocytosis of myelin by macrophage activation. Active MS lesions are defined by the presence of myelin degradation products in macrophages, and phagocytosis of myelin by activated macrophages/microglia is a crucial step in tissue destruction in MS. The results of the study by Junker et al. suggest that miRNAs dysregulated in MS lesions reduce CD47 in brain resident cells, releasing macrophages from inhibitory control, thereby promoting phagocytosis of myelin [261]. This mechanism may have broad implications for miRNA-regulated macrophage activation in inflammatory diseases.

Altered miRNA profiles detected in MS active lesions may reflect the presence of infiltrating immune cells, changes in brain resident cells such as glial cells, or both. MiRNA profiling in isolated cells by laser capture microdissection from active and inactive MS lesions showed that the most prominently upregulated miRNAs in active MS lesions, miR-155, miR-650, miR-34a, and miR-326 were detected in both microdissected astrocytes and infiltrating immune cells [261]. Novel techniques that allow detection of miRNAs and their targets at the same tissue sections may be used to confirm these results [269, 270]. Under *in vitro* culture conditions, human astrocytes contained all 10 miRNA that were most strongly upregulated in active MS lesions, including miR-155, which is known to modulate immune responses in different ways but so far had not been assigned to CNS resident cells [104, 137, 271, 272]. When cultured astrocytes were stimulated with various cytokines (i.e., IL-1β, TNF-α, IFNγ, and TGF-β), miR-23a, miR-146a, and miR-155 were strongly induced in vitro [261]. These results were also confirmed with cultured astroglial cells from miRNA-$155^{-/-}$lacZ mice expressing lacZ reporter instead of miR-155 [104, 261].

Although Junker et al. have focused on only three upregulated miRNAs in active MS lesions (i.e., miR-155, miR-326, and miR-34a) and their common target CD47, other upregulated miRNAs, especially miR-146a and miR-34a deserve further mention. These miRNAs are known to modulate immune responses in different ways. They are also implicated in other CNS disorders accompanied by chronic neuroinflammatory conditions such as epilepsy, Alzheimer's disease (AD), and schizophrenia. miR-34a upregulation was determined in peripheral blood cells of sporadic AD patients, cerebral cortex of APPswe/PSDeltaE9 mice, and prefrontal cortex of schizophrenic patients [273–275]. Cortical expression of miR-34a was inversely correlated with the protein level of Bcl2 in a double transgenic mouse model of AD. *In vitro* experiments in SH-SY5Y neuronal cells verified anti-apoptotic gene *bcl2* as a target of miR-34a [274]. The meaning of these results in the context of MS is currently unknown. Interestingly, mood stabilizators such as lithium and valproate modulate the expression level of miR-34a both *in vitro* and *in vivo* [262, 276, 277]. Metabotropic glutamate receptor 7, which is an important regulator of glutamatergic function and of fear, aversion, and cognition, was identified as a target of miR-34a [277, 278]. Although changes in miR-34a expression levels are in opposite directions (downregulation in rat hippocampus and upregulation in cultured

Table 1: miRNA in immune functions.

miRNA	Expressing cells	Functions	Targets
Let-7e	macrophages	Innate immune response	TLR4
miR-9	myeloid cells	Immune response	NFK B1
miR-17-5p	myeloid cells	monocyte proliferation and differentiation	RUNX1
miR-17-92	B and T cells	B and T cell development	BIM, PTEN
miR-21	myeloid cells	macrophage activation	IL12a, PTEN, PDCD4
miR-34	DC and B cells	Myeloid DC differentiation	FOXP1, JAG1, WNT1
miR-125b	monocyte	Innate immune response, TLR signaling	TNF-α
miR-126	HSC	expansion of progenitor cells	HOXA9, PLK2
miR-132	monocyte	Innate immune response	not determined
miR-142	Treg cell	Suppresor function of Treg cells	AC9
miR-146a	monocyte	Innate immune response, TLR signaling	IRAK-1, IRAK-2, TRAF6
miR-150	B and T cells	mature B-cell production, T-cell activation	Myb
miR-155	B and T cells, DC	Innate and adaptive immune response	AID, BACH1, CEBPB, CSFR
		macrophages germinal center response	c-MAF, FADD, IKK, JARID2,
		Ig G class-switch	PU.1, Ripk1, SOCS, TAB2
		Peripheral T cell development	
miR-181a	T cells	T cell receptor signaling	AID, BCL2, CD69, DUSP5
		B cell development	DUSP6, PTPN22, SHP2
miR-181b	macrophages, B cells	B cell class switch	AID
miR-196b	HSC	Hematopoietic stem-cell homeostasis	HOX
miR-223	myeloid cells	Granulopoiesis	MEF2C
miR-326	T cells	TH-17 cells development	ETS1
miR-424	myeloid cells	monocyte differentiation and maturation	*NFIA, PU.1*

AC9: adenylate cyclase 9; AID: Activation-Induced Cytidine Deaminase; BACH1: BTB and CNC homology 1, basic leucine zipper transcription factor 1; BCL2: B-cell lymphoma 2; BIM: BCL2-like 11; CEBPB: CCAAT/enhancer-binding protein beta; CSFR: Colony stimulating factor receptor; c-MAF: musculoaponeurotic fibrosarcoma oncogene homolog; DC: dendritic cell; DUSP5: Dual specificity protein phosphatase 5; DUSP6: Dual specificity protein phosphatase 6; ETS1: v-ets erythroblastosis virus E26 oncogene homolog 1; FADD: Fas-Associated protein with Death Domain; HSC: haematopoetic stem cell; HOX: Homeobox protein; HOXA9: Homeobox protein Hox-A9; FOXP1: Forkhead box P1; IKK: inhibitor of NF-kappaB kinase; IL12a: Interleukin-12 subunit alpha; IRAK-1: Interleukin-1 receptor-associated kinase 1; IRAK-2: Interleukin-1 receptor-associated kinase 2; JAG1: jagged 1; JARID2: Jumonji; Myb: Myb oncogene-like; MEF2C: Myocyte-specific enhancer factor 2C; NFIA: Nuclear factor 1 A-type; PDCD4: Programmed cell death protein 4; PTEN: phosphatase and tensin homolog; PLK2: pololike kinase 2; PTPN22: Tyrosine-protein phosphatase non-receptor type 22; PU.1: spleen focus forming virus (SFFV) proviral integration oncogene spi1; Ripk1: Receptor-interacting serine/threonine-protein kinase 1; RUNX1: Runt-related transcription factor; SHP2: SH2 domain containing protein thyrosine phosphatase; SOCS: Suppressor of cytokine signaling; TAB2: TAK1-associated binding protein 2 TRAF6: TNF receptor associated factor-6; TLR: Toll-like receptor; WNT1: wingless-related MMTV integration site 1.

lymphoblastoid cells) [262, 277], these results suggest that miRNAs and their predicted effectors may be targets for the action of psychotherapeutic drugs.

miR-146a has been recently identified as a potentially endogenous regulator of TLR and cytokine receptor signalling, suggesting a link between miRNAs and human inflammatory diseases [279]. In contrast to the emerging role of miR-146a in innate immunity, a role of this miRNA in the adaptive immune response has recently been identified. MiR-146a is among the most highly expressed miRNAs in murine Tregs, thus suggesting a possible role for miR-146a in maintaining differentiated T-cell lineages [116]. miR-146a modulates activation-induced cell death (AICD), acting as an antiapoptotic factor, and it is known that Fas-associated death domain (FADD) is a target of miR-146a [280]. Furthermore, miR-146a-enforced expression impairs both activator protein 1 (AP-1) activity and IL-2 production induced by TCR engagement, suggesting a role of this miRNA in the modulation of adaptive immunity. NF-κB and c-ETS binding sites were identified as required for the modulation of miR-146a transcription upon TCR engagement.

Recently, specific miRNAs have been shown to be significantly upregulated in response to cytokine stress and in affected regions of AD brain. The brain-enriched miRNA-146a is currently thought to be a key regulator of the immune and inflammatory signaling systems in both health and disease [150, 279, 281]. Inflammatory processes contribute to the onset, progression, and propagation of this common disorder and amyloid beta peptides, key pathological lesions of AD, are important inflammatory mediators, as are upregulated IL-1β and increased oxidative stress.

The established role of miR-146a in innate immunity responses may also contribute to the pathogenesis

of neuroinflammatory CNS diseases such as MS and AD. miRNA-146a controls TLR and cytokine signaling through a negative feedback regulation loop involving downregulation of TRAF6 and IRAK1 levels [150]. In neuroinflammation, TLRs provide a critical link between immune stimulants and the initiation of host defense, and TLR activation modulates the release of inflammatory cytokines [282, 283]. TLRs are expressed on cells of the CNS and can influence local CNS immune responses. There is a marked increase in expression of TLRs in MS brain lesions and CSF mononuclear cells as well as in EAE brain lesions [284, 285]. The secondary progressive phase is characterized by progressive accumulation of disability in the absence of clinical attacks and is driven by the innate immune system [18]. However, the exact role of specific miRNAs in these processes is unknown.

The miRNA profiling of microglial cells in both unstimulated and stimulated conditions has not been reported. Our preliminary study using microarray and qPCR revealed that the expression levels of a set of miRNA were deregulated upon LPS stimulation in N9 murine microglial cell line (Table 3). Predicted target genes of upregulated miRNAs detected in this preliminary study were also found to be downregulated in a microarray study with N9 cells [286]. Validation experiments for the predicted target genes are ongoing. Interestingly, deregulated microglial miRNAs are somewhat different from those detected in murine primary macrophages or mouse Raw 264.7 macrophages upon LPS stimulation [149, 151, 287]. Further studies for the profiling of microglial miRNA expression in MS and EAE are still warranted.

6. Diagnostics and Therapeutic Perspectives

In patients with MS, intensive efforts are directed at identifying biomarkers in body fluids related to underlying disease mechanisms, disease activity and progression, and therapeutic response [288]. Without biomarkers, the clinical and pathological heterogeneity of MS makes treatment difficult. Thus, identification of biomarkers appears desirable for an improved diagnosis of MS as well as for monitoring of disease activity and treatment response. Biomarkers are defined as parameters that are objectively measurable biological characteristics, which can be used as indicators of physiologic or pathologic processes. A valuable biomarker requires robust and reproducible assays that work in clinically available samples as well as archived material. As miRNAs are more stable than mRNAs, they are good candidates for use as disease biomarkers and their use as biomarkers has gained growing interest in the last few years [289]. Blood serum and plasma are important sample types for investigating miRNAs as biomarkers. Blood biomarkers are attractive because blood samples are easy to collect, cheaper, and noninvasive. However, miRNA profiles in all body fluids such as CSF and their content including hematopoietic cells, exosomes, microvesicles, and microparticles can be used as diagnostic, prognostic, or monitoring marker [290, 291]. miRNA profiling has been established only in an AD study [292] and has not been evaluated in the context of MS. Besides magnetic resonance imaging parameters [166], CSF biomarkers provide important and specific information since changes in the CSF composition may reflect disease mechanisms inherent to MS [288, 293]. miRNA profiling in CSF may provide valuable information about key pathological processes of MS such as inflammation, demyelination, neuroaxonal injury, gliosis, and regeneration. In recent years, the field of biomarker discovery has gradually shifted from the aim of finding a single perfect surrogate marker to the construction of composite markers with higher performance. miRNA profiles may be coupled with diagnostic evaluation of miRNA targets, mRNAs, and protein output; therefore comparison of miRNA analysis with transcriptomic and proteomic studies represents one of the major challenges for clinical application. Another major challenge is represented by technological aspects of miRNA detection aimed at high throughput, sensitivity, and accurate analysis. The level of miRNAs in body fluid samples is very low and efficient and reproducible recovery of miRNA may be problematic. Due to their short length and high sequence similarity within miRNA families, reliable and accurate quantification is still a challenge. In addition, RNA-purified plasma can also contain inhibitors that affect qPCR efficiency [294]. Analyses of miRNA-associated SNPs (e.g., SNP in miRNA genes, in miRNA binding sites in the target mRNA, or in miRNA biogenesis pathway genes) are also potential biomarkers of the diseases associated with miRNA dysregulation [295–298]. While still not fully validated, profiling of blood cells, exosomes, or body fluid miRNAs would represent a tremendous and promising advance in noninvasive diagnostics of CNS disorders. Identification of suitable miRNA-based biomarker sets for MS based on parameters in peripheral blood is only in its infancy.

While we have only just begun to gain insights into miRNA biology, their apparent association with the onset and progression of human diseases such as MS has produced great interest in assessing the feasibility of therapeutic regulation of miRNAs [299]. miRNA-based therapies could involve administration of a specific miRNA mimic to downregulate target genes or antisense oligonucleotide for the blocking of certain miRNA to increase expression of target genes. Importantly, anti-miRNA strategies may be preferred over antisense mRNA strategies in complex human diseases because of the potential of miRNA to affect the regulation of multiple disease-related genes. However, because manipulation of one miRNA may have impact on multiple mRNAs and because one mRNA may be regulated by multiple miRNAs, it is important to guard against off-target effects. Also, miRNAs, instead of causing translational repression or mRNA degradation, may relieve translational repression and promote transcription [300, 301]. Another challenge is the risk of triggering a cellular immune response with RNA therapy. A very promising approach may be the use of LNAs (locked nucleic acids). These molecules comprise a class of bicyclic conformational analogues of RNA, which exhibit high binding affinity to complementary RNA molecules and high stability in blood and tissues *in vivo* [302]. Recent reports on LNA-mediated miRNA silencing in primates support the potential of LNA-modified

Table 2: Differential miRNA expression in Multiple Sclerosis.

Sampe type	Number of patients and disease status	Specificity of patients and treatment	Number of tested miRNA	Results	Target genes	Reference
Whole bood	59 MS (18 PP, 17 SP, 24 RR) and 37 controls	Causian No IMT	733	miR-17 and miR-20a downregulated	ND	Cox
CD4+CD25+	12 MS (RR) and 14 controls	No IMT	723	miR-106b, MiR-19a, MiR-19b and miR-25 upregulated	TGF β signaling	De Santis
CD4+, CD8+, B	8 MS (RR) and 10 controls (microarray)	No IMT	365	miR-17-5p upregulated in CD4+ cells	ND	Lindberg
	15 MS (RR) and 10 controls (qPCR)					
Peripheral blood leukocytes	43 MS (RR)	Chinese	ND	miR 326 upreguated in CD4+ cells	Ets-1	Du
	40 control			miR-326 promotes Th-17 differentiation		
	11 NMO					
Whole bood	20 MS (RR)	glatiramer acetate (9)	866	miR-145 upregulated in MS	ND	Keller
	19 controls	interferon-b (10)				
Whole bood	21 MS (9 remission, 4 relaps)	ND	364	miR-18b and miR-599 upregulated in relapse	interleukine signaling	Otaegui
	8 control			miR-96 upregulated in remission	Wnt, glutamate	
Brain tissue	20 MS (16 active, 5 inactive)	ND	365	miR-34a, miR-155 and miR-326 upregulated in active lesions	CD47	Junker
	9 controls					

Ets-1: v-ets erythroblastosis virus E26 oncogene homolog 1; IMT: Immunmodulatory treatment; MS: Multiple sclerosis; ND: not determined; NMO: neuromyeliis optica; PP: primary progressive; RR: Relapsing remitting, Secondary progressive.

oligonucleotides in studying miRNA functions *in vivo* and in the future development of miRNA-based therapeutics [303, 304]. LNA-modified miR-122 inhibitor has entered the clinic and it is in phase I trials with the goal of treating hepatitis C infection [299]. miRNAs could also be promising potential targets or tools for new therapeutic strategies in the treatment/prevention of autoimmunity. However, to date, no miRNA therapies have been tested *in vivo* for the treatment of autoimmune diseases.

7. Conclusion

The field of study of miRNAs is a very rapidly evolving new field in molecular biology. miRNAs are important regulators of gene expression, and they function by repressing specific target genes at the posttranscriptional level. miRNA-mediated regulation is essential for immune homeostasis and the prevention of autoimmune diseases. miRNA expression is tightly regulated during hematopoiesis and lymphoid cell differentiation and disruption of the entire miRNA network or specific miRNAs may lead to dysregulated immune responses. Abnormalities in miRNA expression related to inflammatory cytokines, Th17 and Treg cells, as well as B cells have been described in several autoimmune diseases. Emerging evidence suggests that miRNA dysregulation may contribute to the pathogenesis of MS. In the near future, further understanding of the role of miRNAs in intracellular signaling, the expression of proteins involved in immune responses, modulation of cytokines and chemokines, adhesion and costimulatory molecules and the interplay between the immune system and CNS should help to define the role of miRNAs in autoimmunity, and provide an exciting framework for developing new biomarkers and new therapeutic interventions in MS. It is reasonable to assume that future studies concerning the function of miRNAs involved in immune responses will extend our understanding about the complex regulatory networks in autoimmune diseases and MS. These efforts might allow the invention of novel strategies for the treatment of MS. miRNAs are promising reliable biomarkers of human diseases due to their stability

Table 3: Significantly altered miRNAs upon stimulation LPS in N9 microglial cells.

MicroRNA	Microarray		qPCR		Targets
	Fold change	*P*	Fold change	*P*	
mmu-miR-105	0.35	.02	ns		not determined
mmu-miR-125b-3p	2.81	ns	0.16	.047	IL-1β, IL-13, TNF-α
mmu-miR-191	3.12	ns	0.21	.032	CCL9, CRP, IL-6, TLR-3
mmu-miR-193*	0.26	.03	0.28	.047	CCL6, IL-10, IL-12Rγ
mmu-miR-208a	3.01	ns	0.12	.015	CD8, IL-18BP, IL-24
mmu-miR-224	3.73	ns	0.09	.033	CD53, CXCL-14, IL-11
mmu-miR-297c*	0.31	ns	0.12	.033	not determined
mmu-miR-324-3p	0.33	ns	0.18	.049	not determined
mmu-miR-376c	2.99	.01	ns		not determined
mmu-miR-421	0.35	ns	0.03	.033	not determined
mmu-miR-431*	4.62	ns	0.22	.034	CD5, CD81, DICER, IRAK1, TRAP 1
mmu-miR-669g	3.48	ns	0.15	.015	not determined
mmu-miR-1190	0.28	.01	0.12	.016	not determined
mmu-miR-1894-5p	0.34	ns	0.08	.017	not determined

CCL9: chemokine (C-C) motif ligand 9; CXCL-14: chemokine (C-X-C) motif ligand 14; CRP: c reactive protein; IL-1β: interleukin 1-β; IL-6: interleukin-6; IL-10: interleukine-10; IL-11: interleukin-11; IL-12Rγ: interleukin 12 receptor γ; IL-13: interleukin-13; IL-18BP: interleukin-18 binding protein; IL-24: interleukin-24; IRAK1: interleukin-1 receptor associated kinase-1; TLR-3: Toll-like receptor-3; TRAP 1: TNF receptor associated protein 1; TNF-α: tumor necrosis factor-α.

being less susceptible to chemical modification and RNase degradation. Although there is much to be learned in the field, the role of miRNAs in regulating a great variety of targets and, as a consequence, multiple pathways makes their use in diagnostics a powerful tool to be exploited for early detection of MS, assessment for risk disease, and monitoring both disease progression and therapeutic responses to disease-modifying drugs.

8. Take-Home Messages

(i) miRNAs have recently emerged as a new class of modulators of gene expression at the posttranscriptional level and are thought to play a critical role in many biological processes.

(ii) miRNAs are involved in the development, maturation, and the functions of immune cells, which suggest that they are implicated in the development of autoimmune diseases.

(iii) Changes of expression of some miRNAs have been reported in autoimmune pathologies such as rheumatoid arthritis, systemic lupus erythematosus, and MS.

(iv) MS serves an example of a chronic and organ-specific autoimmune disease in which miRNAs modulate immune responses in the peripheral immune compartment and the neuroinflammatory process in the brain.

(v) The differential expression of miRNAs and their role in MS have been investigated by several studies.

(vi) miRNAs have the potential to serve as biomarkers for the assessment of disease activity and therapeutic response to disease-modifying drugs in MS.

Conflict of Interest Disclosure

The authors declare no competing financial interests.

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Review Article

Vitamin D and Multiple Sclerosis: Correlation, Causality, and Controversy

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The last years, many studies reported associations between correlates of vitamin D exposure and several correlates of multiple sclerosis (MS) disease activity. This review discusses studies on vitamin D status, Expanded Disability Status Scale (EDSS) score, and relapse activity of MS. Furthermore, several considerations for intervention studies on vitamin D supplementation in MS are provided.

Vitamin D is hot in multiple sclerosis (MS) research. The geographical distribution of MS prevalence, which increases when approaching the poles, sparked the interest in a poor vitamin D exposure as a risk factor for developing MS [1]. Now, half a century of research further, a large body of clinical observations and experimental work *in vitro* and in animal models of MS has been reported [2]. As reviewed recently, a limited exposure to sunlight and other correlates of vitamin D exposure has been associated consistently with an increased risk on developing MS [3]. The prospect of a potential tool to prevent MS is tempting, yet challenging to investigate in an intervention study since it would require a huge population to measure any effect on MS incidence. However, vitamin D supplementation has not only been proposed to prevent MS, but also to attenuate disease activity of MS [4].

In patients with established MS, earliest observations show a seasonal fluctuation of several disease characteristics. In Switzerland, more relapses were recorded during winter and spring than during summer [5]. More gadolinium enhancing lesions on T1 MRI have been reported in Germany in spring and early summer, and less in autumn [6]. Regrettably, these observations were not related directly to serum concentrations of 25-hydroxyvitamin D (25(OH)D), the vitamin D metabolite that reflects vitamin D status best [7], and prove difficult to reproduce. Other studies did correlate measures of MS severity directly with vitamin D status. The most widely used score to quantify disability due to MS is the Expanded Disability Status Scale (EDSS) [8]. EDSS-score has been reported to correlate negatively with serum 25(OH)D levels [9, 10]. Van der Mei et al. showed elegantly that EDSS-score also correlated negatively with recent sun exposure [10]. Therefore, it remains to be seen whether vitamin D status contributes to disability progression in MS, since (prospective) studies on this issue are at present lacking. Interestingly, however, Burton et al. performed a small phase I/II study which compared two groups of MS patients on supplementation with either high (up to 40.000 IU/d) or low doses ($\leq$4.000 IU/d) of vitamin D_3 for 52 weeks [11]. They reported a smaller proportion of patients with any progression on the EDSS-scale in the high- versus low-dose vitamin D group (2/25 versus 9/24, resp.). Although premature, this finding encourages further research on the effect of vitamin D on disease progression of MS. Relapse activity has also been related to vitamin D status. During relapse, lower serum 25(OH)D levels have been reported in several MS populations, when compared to remission of MS [12–14]. However, also this information is prone to many interpretations. We observed that patients with <5 years relapsing remitting MS (RRMS) and $\geq$1 relapses in the 2

years prior to serum sampling had markedly lower serum 25(OH)D levels when compared to relapse-free patients [9]. Interestingly, a large prospective longitudinal study in RRMS patients with a wider range of disease duration showed that an increase of serum 25(OH)D levels with 10 nmol/L was associated with a 9–12% reduction of the hazard on relapses [15]. Additionally, a prospective study in children with RRMS calculated a comparable 14% decrease in hazard on relapses for each 10 nmol/L increase of vitamin D status [16]. These studies suggest that supplementation with vitamin D may protect RRMS patients against relapses. Interestingly, although not statistically significant, a smaller proportion of patients with relapses was described by Burton et al. in the high- versus low-dose vitamin D_3 supplementation group [11].

Altogether, the evidence that is available on vitamin D status and disease activity of MS shows a consistent picture. To consolidate a possible role of vitamin D supplementation in the treatment of MS, randomized placebo-controlled clinical trials are needed. Supplementation of high doses of vitamin D could easily elevate serum 25(OH)D levels with 100 nmol/L [11, 17]. When a linear relationship between vitamin D status and reduction of relapse-risk is assumed [15], the effect of this intervention on relapse-risk could be substantial [15, 16]. However, there are several considerations. First of all, vitamin D status predicts the hazard on relapses, but vitamin D status itself is dependent on sun exposure and outdoor physical activity. There is some evidence available that both these factors contribute individually to disease activity of MS [18]. Additionally, the inverse linearity of the relationship between vitamin D status and hazard for relapses in the supraphysiological range of serum 25(OH)D levels is uncertain [15]. Therefore, the effect in intervention studies may not be as large as predicted from observational studies. Secondly, the amount of vitamin D which should be supplemented is uncertain. In most clinical trials with a new treatment, the difference between presence and absence of a specific substance is compared. In studies on vitamin D, an effect of supraphysiological versus physiological levels of vitamin D exposure will be assessed. Supplementation of low doses may fail to reduce the risk on relapses significantly when compared to the physiological range of vitamin D status in control groups. This problem could be tackled by selective inclusion of patients with a poor vitamin D status. Alternatively, high doses of vitamin D could be supplemented. Although short-term exposure to high doses of vitamin D showed no side-effects [11, 17], there are some concerns on long-term safety and efficacy. Thirdly, the groups of patients which should be supplemented with vitamin D are ill defined. The most fundamental choice is the inclusion of either RRMS or progressive MS patients. Progressive MS patients display the poorest vitamin D status [9], yet an effect of vitamin D on EDSS progression is less well consolidated. Since an effect on relapse activity is most likely [15, 16], studies in RRMS patients are most obvious. Mechanistic approaches may also help to specify the patients to be included. We showed that, in RRMS patients with a short disease duration of <5 years, vitamin D status correlated with peripheral T cell homeostasis [19]. Additionally, supplementation of vitamin D reduced the T cell proliferative response to myelin antigens in RRMS patients [11]. Alternatively, 25(OH)D is also present in the central nervous system of MS patients and may be involved in local immune regulatory systems in progressive MS [20]. Fourthly, response of patients to vitamin D supplementation may depend upon the genetic profile of both vitamin D-related genes [21] and MHC class II genotype [22]. Intervention studies should allow analyses on genetic profiles associated with response to vitamin D. Lastly, studies should not only focus on classical outcomes as MRI parameters, relapse activity, and disability progression. Other symptoms found in MS have also been associated with a poor vitamin D status, including presence of depressive symptoms [23] and cognitive decline [24]. Including also these parameters could provide a complete overview of the impact of vitamin D supplementation in MS.

There are many different opinions about vitamin D in MS, ranging from scepticism to a firm belief in the new panacea. These opinions reflect the current state of evidence: there are a lot of indications for a therapeutic role of vitamin D in MS, but these remain all circumstantial evidence. Everyone will agree, however, that these indications provide at least an excellent reason to develop well-designed clinical trials on vitamin D supplementation in MS. Currently first clinical trials are starting up and will undoubtedly cast more light on the issue.

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Research Article

A Nonsecosteroidal Vitamin D Receptor Modulator Ameliorates Experimental Autoimmune Encephalomyelitis without Causing Hypercalcemia

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Vitamin D receptor (VDR) agonists are currently the agents of choice for the treatment of psoriasis, a skin inflammatory indication that is believed to involve an autoimmune component. 1,25-dihydroxyvitamin D3 [1,25-$(OH)_2D_3$], the biologically active metabolite of vitamin D, has shown efficacy in animal autoimmune disease models of multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, and type I diabetes. However, the side effect of 1,25-$(OH)_2D_3$ and its synthetic secosteroidal analogs is hypercalcemia, which is a major impediment in their clinical development for autoimmune diseases. Hypercalcemia develops as a result of the action of VDR agonists on the intestine. Here, we describe the identification of a VDR modulator (VDRM) compound A that was transcriptionally less active in intestinal cells and as a result exhibited less calcemic activity *in vivo* than 1,25-$(OH)_2D_3$. Cytokine analysis indicated that the VDRM not only modulated the T-helper cell balance from Th1 to Th2 effector function but also inhibited Th17 differentiation. Finally, we demonstrate that the oral administration of compound A inhibited the induction and progress of experimental autoimmune encephalomyelitis in mice without causing hypercalcemia.

1. Introduction

Experimental autoimmune encephalomyelitis (EAE), an inflammatory demyelinating disease induced in mice by immunization with myelin components, displays pathological and clinical resemblances to the human demyelinating disease multiple sclerosis (MS). EAE and MS are characterized clinically by neurodegeneration and paralysis and pathologically by demyelination and infiltration of lymphocytes and monocytes into the CNS [1]. Epidemiological studies have shown a global north-south gradient of MS incidence and mortality rates. In other words, geographic distribution of MS prevalence increases with increasing latitude on both sides of the equator [2, 3]. A strong correlation between latitude and MS incidence could be explained by the decreased exposure of susceptible population to UV radiation. Since UV light is required for vitamin D synthesis in the skin, a number of studies have explored the connection between vitamin D and MS. In a prospective epidemiological study (Nurses' Health Study) involving 187,000 women from 1980 to 2001, intake of vitamin D from supplements was inversely associated with the risk of MS [4]. The notion that vitamin D could be involved in the regulation of disease activity of MS is further strengthened from the observation that lower serum 25-hydroxyvitamin D levels were observed during MS relapses than those during remission [5]. Importantly, pharmacological doses of the biologically active form of vitamin D (1,25-dihydroxyvitamin D3; 1,25-$(OH)_2D_3$) greatly reduced the incidence of disease in the EAE model [6].

1,25-$(OH)_2D_3$ is being increasingly recognized as an important immunomodulatory agent apart from its classical

role in mineral homeostasis and maintenance of skeletal architecture. 1,25-$(OH)_2D_3$ and its synthetic analogs exert these effects by binding to the vitamin D receptor (VDR) that belongs to the steroid/thyroid hormone nuclear receptor superfamily [7, 8]. VDR, a ligand-dependent transcription factor, functions as a heterodimer with another nuclear receptor, namely, retinoid X receptor (RXR). Upon ligand binding, VDR undergoes a conformational change that promotes RXR-VDR heterodimerization [9, 10]. Liganded RXR-VDR heterodimer translocates to the nucleus, binds to the vitamin D responsive elements (VDREs) present in the promoter regions of responsive genes, and recruits chromatin modifying enzymatic activities through interaction with coactivators and DRIP complex, which ultimately leads to the initiation of transcription [11]. 1,25-$(OH)_2D_3$ and its synthetic analogs act as immunomodulators with immunoregulatory and anti-inflammatory properties [3, 12, 13] and as a result have shown efficacy in various *in vitro* and *in vivo* models of autoimmune diseases (arthritis, multiple sclerosis, inflammatory bowel disease, and EAE). However, the major hurdle facing the translation of basic and applied research to therapeutic ligands is hypercalcemia associated with the current generation of VDR ligands. Therefore, there is a clinical unmet need for the identification of novel VDR ligands that exhibit an improved therapeutic index.

1,25-$(OH)_2D_3$ is a secosteroidal compound and most of the VDR ligands that have been described to date have a secosteroidal backbone [14]. Administration of VDR ligands results in hypercalcemia by increasing calcium absorption from the intestine. Consistent with this view, VDR-null mice display marked hypocalcemia [15]. 1,25-$(OH)_2D_3$ action on duodenal enterocytes induces calcium transport protein 1/transient receptor potential vanilloid epithelial calcium channel 6 (CaT1/TRPV6) expression, which channels calcium from the intestinal lumen into the cell [16]. VDR ligands also induce the expression of an EF-hand-containing carrier protein, calbindin-9k that ferries the bound calcium from the apical to the basolateral membrane [16]. Therefore, a tissue selective/cell-context-dependent VDR ligand that is transcriptionally less active in intestinal cells but a potent agonist in immune cells may exhibit reduced hypercalcemia liability and a better therapeutic index required for the treatment of MS. Since nonsteroidal structures have provided tissue selective estrogen receptor modulators (SERMs) that are agonists in bone and antagonists or transcriptionally inactive in breast and uterine cells [17, 18], we have identified and characterized a nonsecosteroidal analog of vitamin D, compound A, as a nonsecosteroidal VDRM. We demonstrate that compound A functions as a potent and efficacious agonist in human peripheral blood mononuclear cells (PBMCs) and osteoblasts but exhibits attenuated transcriptional activity in intestinal cells. In addition, compound A modulates the balance of Th1 versus Th2 cytokine profile. A plausible mechanism of this shift could be due to the induction of GATA3, a master regulator of Th2 differentiation. The cell-context-dependent activity of compound A also translated *in vivo* in reduced hypercalcemic liability in a murine model of hypercalcemia. We also demonstrate that in a preclinical murine EAE model of MS, compound A delayed the onset of EAE and reduced the severity of the disease at a noncalcemic dose. Finally, splenocytes obtained from VDRM-treated MOG-induced EAE animals showed attenuated T cell proliferation response to the MOG peptide antigen and showed increased IL-10 and reduced interferon-γ (IFN-γ) production. Furthermore, VDRM significantly inhibited Th17 differentiation. Thus, compound A represents a novel class of VDRMs that could be efficacious for treating autoimmune diseases such as MS without hypercalcemia side effect.

2. Methods

2.1. Cell Culture and Transfections. For the RXR-VDR heterodimerization assay, Saos-2, cells maintained in DMEM supplemented with 10% FBS were plated at 5000 cells/well in a 96-well plate. The next day, cells were transfected using 0.5 μL of fugene (Roche Diagnostics, Indianapolis, IN), 100 ng of luciferase reporter vector pFR-Luc (Stratagene, La Jolla, CA) and 10 ng each of pVP16-VDR-LBD and pGal4-RXRα-LBD expression vectors/well. For HeLa and Caco-2 one-hybrid mammalian transactivation assay, HeLa and Caco-2 cells, maintained in DMEM supplemented with 10% FBS, were plated at 5000 cells/well in a 96-well plate. Cells were transfected using 0.5 μL of fugene (Roche Diagnostics, Indianapolis, IN), 100 ng of luciferase reporter vector pFR-Luc (Stratagene, La Jolla, CA), and 10 ng of pGal4-VDR-LBD expression vectors/well. Total DNA amount was kept constant by adding empty vector DNA as needed. Cells were treated with the ligand 24 hours after-transfection, and luciferase activity was quantitated the next day using Steady-Glo luciferase detection reagent (Promega, Madison, WI).

2.2. Rat Osteocalcin Luciferase (OCN-Luc) Assay. The activation of osteocalcin VDRE by VDR ligands was evaluated in a rat osteoblast-like cell line (ROS 17/2.8) stably expressing rat osteocalcin promoter (1.154 kb) fused with luciferase reporter gene. The development of the stably transfected ROS 17/2.8 cell line (RG-15) containing OCN-Luc has been described [19]. Confluent RG-15 cells maintained in DMEM/F-12 medium (3 : 1) containing 5% FBS, 300 μg/mL G418 at 37°C were trypsinized (0.25% trypsin) and plated into white opaque 96-well cell culture plates (25000 cells/well). After 24 hours, cells (in DMEM/F-12 medium containing 2% FBS) were treated with the indicated concentrations of the compounds. After 48 hours of treatment, the medium was removed, cells were lysed with 50 μL of lysis buffer (from luciferase reporter assay system, Roche Diagnostics, Indianapolis, IN) and assayed for luciferase activity using the Luciferase Reporter Gene Assay kit from Roche Diagnostics. Aliquots (20 μL) of cell lysates were pipetted into wells of white opaque microtiter plates (Dynex Technologies, Chantilly, VA) and placed in an automated injection MLX microtiter plate luminometer. The luciferase reaction mix (100 μL) was injected sequentially into the wells. The light signals generated in the reactions were integrated over an interval of two seconds and the resulting luminescence values were used as a measure of luciferase activity (relative units).

2.3. TRPV6 Quantitative RT-PCR (Q-PCR) Assay. Human colon carcinoma, Caco-2, cells, maintained in DMEM (high glucose with 25 mM Hepes buffer; Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA), were plated at 5500 cell per well in a 96-well plate in a total volume of 100 μL/well. The cells were kept in the 96-well plate for 6 days to differentiate them into small intestinal cells that express TRPV6/CaT1. On day 3 after plating, spent media were removed and replaced with fresh media (150 μL/well). On day 6, the spent media were removed again and the cells were maintained in treatment media (180 μL/well) (DMEM (low glucose, without phenol red; Invitrogen, Carlsbad, CA) containing 10% charcoal-stripped FBS (Hyclone, Logan, UT)). The cells were treated with various concentrations of VDR ligands prepared in treatment media (20 μL/well). Twenty hours after-treatment, total RNA was prepared by the RNeasy 96 method, as described by the manufacturer (Qiagen, Valencia, CA). The RNA was reversetranscribed and amplified for human TRPV6 and GAPDH mRNAs by quantitative RT-PCR using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Optimized primer pairs and probes for human TRPV6 and GAPDH genes were obtained commercially (Applied Biosystems, Foster City, CA). Each 20 μL quantitative RT-PCR reaction in a 384-well Taqman PCR plate consisted of forward and reverse primers (900 nM), Taqman probe (200 nM), total RNA (4 μL for each well of the 96-well culture plate), and 10 μL of Taqman Universal PCR Master Mix (Roche Diagnostics, Indianapolis, IN). Reactions were incubated at 48°C for 30 minutes, followed by 10 minutes at 95°C, and subjected to 40 cycles of PCR (95°C for 15 seconds followed by 60°C for 1 minute).

2.4. Cytokine Assays in PBMCs. Peripheral blood mononuclear cells (PBMCs) were isolated from normal human donors by sedimentation on Ficoll-Hypaque. Cells were resuspended in RPMI-1640 supplemented with charcoal-treated FBS (2%). PBMCs (25×10^6/T75 flask) were treated with PHA (10 μg/mL) and TPA (100 ng/mL). The cells were cultured in the presence of various concentration of 1,25-$(OH)_2D_3$ or compound A prepared in RPMI-1640 containing 2% charcoal-treated FBS. Cytokine levels were quantitated by multiplex ELISA using antibodies specific for IL-4, IL-5, IL-13, and IFN-γ. Twenty four hours after-treatment, total RNA was prepared by the RNeasy 96 method and the RNA was reversetranscribed and amplified for human IL-2, IFN-γ, IL-4, IL-10, GATA-3, and GAPDH mRNAs by quantitative RT-PCR as described in the TRPV6 Q-PCR assay section.

2.5. In Vivo Hypercalcemia Assay. Female, 6-7 weeks old, DBF1 mice, weighing ~25 g, were purchased from Harlan Industries (Indianapolis, IN). Mice were housed with ad libitum access to food (TD 5001 with 0.95% calcium and 0.67% phosphorus, vitamin D3 4500 IU/kg; Teklad, Madison, WI) and water. Compounds were given daily orally via gavage for 6 days. Dosing volume was 100 μL/mouse with 4 mice in each group. Serum ionized calcium was examined at 6 hours after last dosing using a Ciba-Corning 634 Ca^{++}/pH Analyzer (Chiron Diagnostics Corp., East Walpole, MA).

2.6. EAE Model. All mice (C57B6) were age- and sex-matched (6- to 10-week-old females) at the start of experiments. Mice were immunized subcutaneously at two sites on the back with 300 μg MOG_{35-55} peptide (MEVGWYR-SPFSRVVHLYRNGK, Peptides International, Louisville, KY) emulsified in a total of 200 μL Complete Freund's Adjuvant (CFA; Difco, Detroit, MI) containing 500 μg *M. tuberculosis* H37 Ra (Difco) on days 0 and 7, supplemented with intraperitoneal injections of 500 ng pertussis toxin (Calbiochem, San Diego, CA) on days 0 and 2. Clinical symptoms of EAE were scored daily by a blinded observer using the following scale: 0: no symptoms, 0.5: distal weak or spastic tail, 1: completely limp tail, 1.5: limp tail and hind limb weakness (feet slip through cage grill), 2.0: unilateral partial hind limb paralysis, 2.5: bilateral partial hind limb paralysis, 3.0: complete bilateral hind limb paralysis, 3.5: complete hind limb and unilateral partial forelimb paralysis, and 4.0: moribund or death. The data was recorded as the mean daily clinical score. MOG-immunized mice were administered orally with vehicle (sesame seed oil), 1,25-$(OH)_2D_3$ (0.5 μg/kg/d) or compound A (10 μg/kg/d) for 21 days in a total volume of 200 μL.

2.7. Splenocyte Cell Culture and Proliferation Assay. Splenocyte cell suspensions were isolated from MOG_{35-55}-immunized mice at day 28 by homogenizing spleens between frosted glass slides (Fisher, Pittsburgh, PA) and removing RBC with ACK lysing buffer (BioWhittaker, Walkersville, MD). Pooled splenocytes of 6 individual mice from the same group were plated in triplicate in 96-well round bottom plate at 2×10^5 cells/well in 200 μL complete RPMI 1640 medium (Invitrogen) supplemented with 2 mM L-glutamine, 25 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, 5.5×10^{-5} M 2-ME, and 5% FCS (all supplements from Invitrogen) containing either 0 and 80 μg/mL of MOG_{35-55} (Peptides International) or control $OVA_{323-339}$ peptide (ISQAVHAAHAEINEAGR, Research Genetics, Inc., Huntsville, AL) and cultured at 37°C, 5% CO_2. Proliferation was measured by incorporation of [^{3}H]-methylthymidine (1 μCi/well, ICN Radiochemicals, Irvine, CA) during the last 8 hr of culture using a filtermate harvester (Packard Instrument Co., Downers Grove, IL) and a 1450 microbeta liquid scintillation counter (Pharmacia Biotech AB). Results were determined as mean ± SE from triplicate cultures. Cytokine levels produced by cultured splenocytes or purified CD11c+ dendritic cells from splenocytes (Miltenyi Biotech.) from MOG_{35-55}-immunized mice were analyzed by removing 100 μL of cell culture supernatant per well after 60 h of culture as described above. Supernatants were filtered using Millipore plates (Cat # Mabvnob) and stored at −80°C. Cytokines were analyzed with LINCOplex mouse cytokine kit (St. Charles, Miss).

2.8. Naïve $CD4^+$ T Cell Th17 Differentiation In Vitro. Naïve mouse $CD4^+$ T cells were purified with AutoMACS and

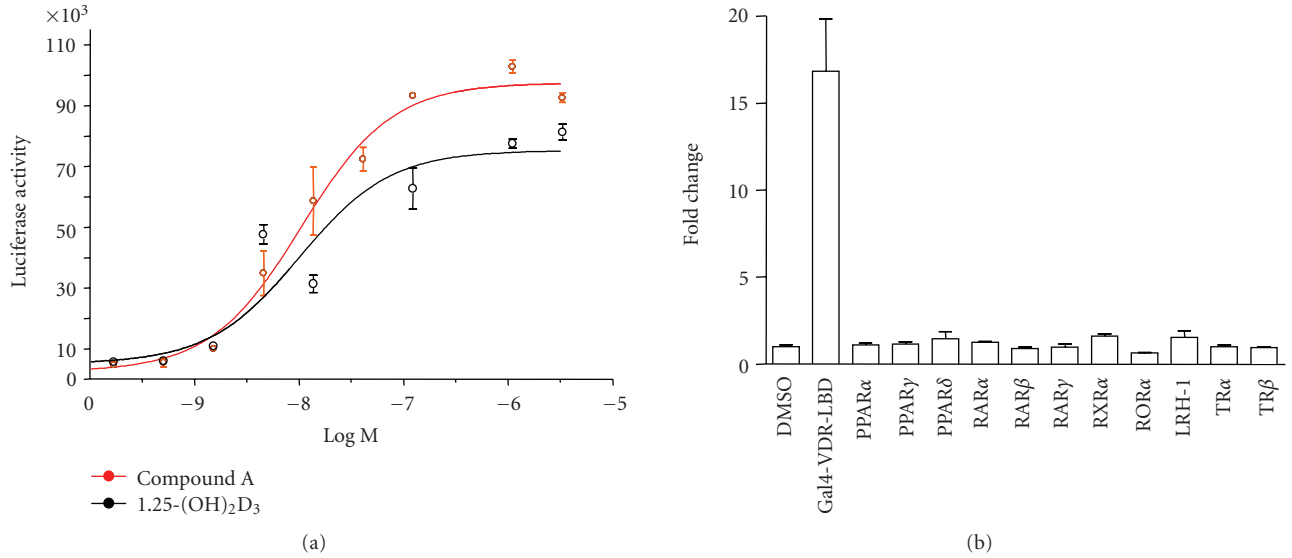

Figure 1: Compound A is a nonsecosteroidal VDR ligand. (a) Compound A is a potent agonist in RXR-VDR heterodimerization assay. SaOS-2 cells were cotransfected with expression vectors encoding Gal4-RXRα-LBD and VP16-VDR-LBD along with a Gal4-responsive luciferase reporter. After transfection, cells were treated with vehicle or various concentrations of 1,25-$(OH)_2D_3$ or compound A, and the reporter activity was expressed as light units ±SE (standard error). A schematic of the RXR-VDR heterodimerization-based ligand-sensing assay and the chemical structures of 1,25-$(OH)_2D_3$ and compound A are also presented. (b) Compound A is selective for VDR-dependent transactivation. HeLa cells were cotransfected with various nuclear receptor-Gal4 DNA-binding domain chimeras and the reporter pFR-Luc. Cells were treated with 100 nM compound A. Cell extracts were subsequently assayed for luciferase activity. Data are expressed for each receptor as fold induction of luciferase activity relative to vehicle-treated cells and represent the mean of four replicates ± SE.

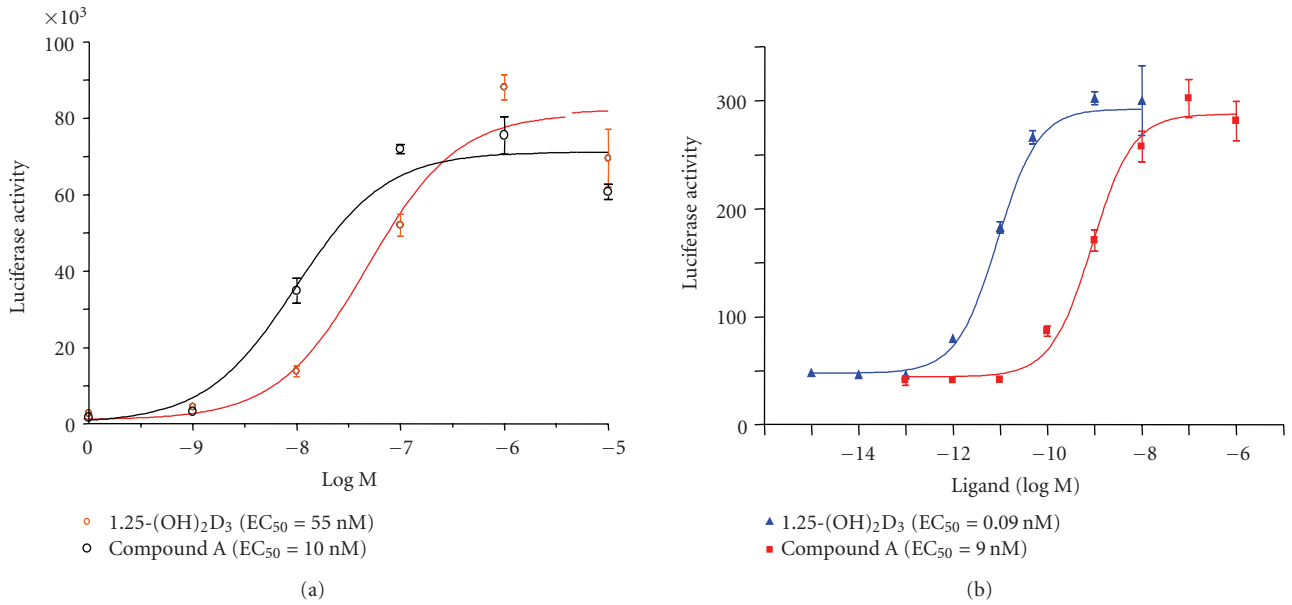

Figure 2: Compound A induces VDR-mediated gene expression. (a) Compound A is as potent as 1,25-$(OH)_2D_3$ in inducing VDR-LBD-dependent gene expression. Luciferase activity (±SE) of HeLa cells transfected with Gal4-VDR-LBD in a mammalian one-hybrid setting in the presence of vehicle or various concentrations of 1,25-$(OH)_2D_3$ or compound A is shown. Results are in arbitrary light units obtained from experiments performed in triplicate. (b) Compound A induces VDRE-dependent expression of the rat osteocalcin promoter in osteoblasts. ROS17/2.8 cells stably transfected with rat osteocalcin reporter (OCN-Luc) were treated with various concentrations of 1,25-$(OH)_2D_3$ or compound A. Results are shown in percentage of the luciferase activity obtained by treating the cells with 1 μM 1,25-$(OH)_2D_3$. All the transfections were performed in triplicate.

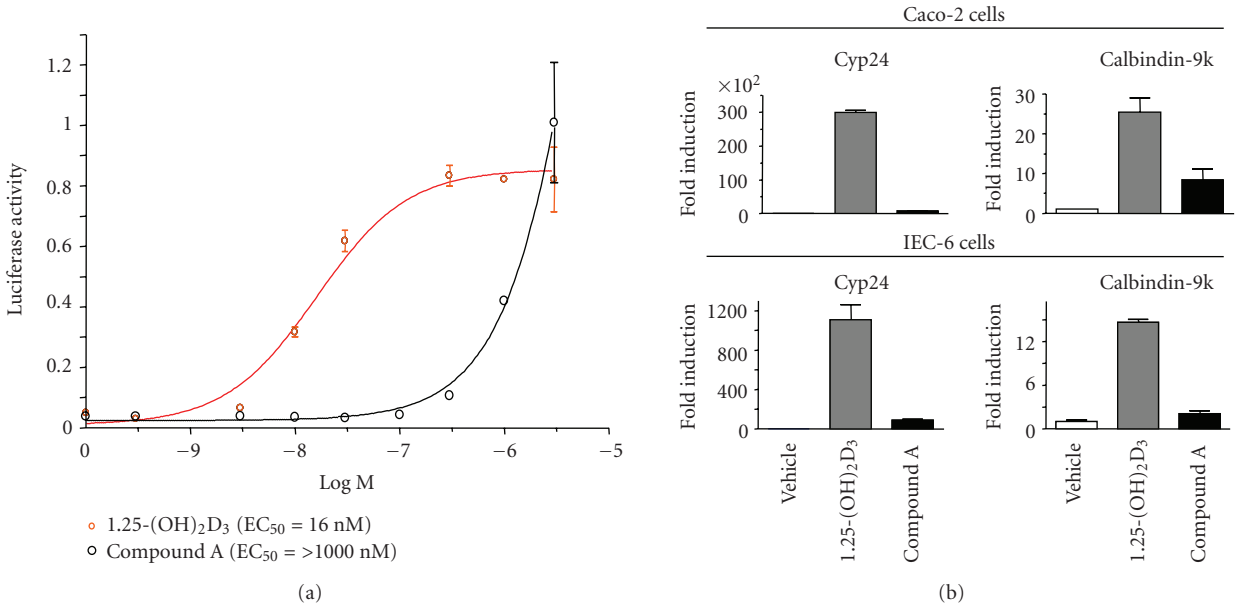

Figure 3: Compound A is less potent and efficacious in inducing the expression of vitamin D-responsive genes in intestinal cells. (a) Compound A shows weak potency in inducing the expression of endogenous CaT1 gene in differentiated Caco-2 cells. Taqman Q-PCR was performed on total RNA prepared from differentiated Caco-2 cells treated with various concentrations of 1,25-$(OH)_2D_3$ or compound A for 24 hours. Levels of GAPDH mRNA were measured in all the samples, and the results were normalized and presented (±SE) as relative light units (RLU) after normalization with the GAPDH transcript levels. (b) Compound A is a poor inducer of endogenous vitamin D-responsive genes in differentiated Caco-2 and rat duodenal cells. Taqman Q-PCR was performed on total RNA prepared from Caco-2 and rat duodenal IEC-6 cells treated with vehicle or 100 nM each of 1,25-$(OH)_2D_3$ or compound A for 24 hours. The fold induction of CYP24 and calbindin-9k transcripts relative to GAPDH transcripts is shown as mean ± SE of quadruplicate experiments.

differentiated *in vitro*. To induce Th17 differentiation, T cells were incubated with plate-bound mAbs of anti-CD3 and anti-CD28 with soluble neutralizing mAbs of anti-IL-4 (5 μg/mL), anti-IFNγ (5 μg/mL), recombinant IL-6 (10 ng/mL), IL-1β (5 ng/mL), and TGF-β (5 ng/mL) (R&D System Inc., MN). Cells were then incubated at 37°C in the presence or absence of 1,25-$(OH_2)D_3$ and compound A for 4 days. Differentiated Th17 cells were then washed, and equal numbers of Th17 cells were restimulated with plate-bound anti-CD3 mAb for 18 h and cell supernatants were used for measuring levels of IL-17 and IL-22 by ELISA (R&D System Inc. MN).

2.9. Statistical Analysis. Statistical significance versus control was defined as $P < .05$ in Dunnett's test. *In vitro* concentration-response curves were fit using the sigmoidal/variable slope fitting option in GraphPad Prism (GraphPad Software, Inc.).

3. Results

3.1. Compound A is a Nonsecosteroidal VDR Agonist. 1,25-$(OH)_2D_3$ and its synthetic analogs induce heterodimerization of VDR with RXR, resulting in the formation of RXR-VDR heterodimers that are the transcriptionally active functional units of vitamin D signaling pathway [9, 10, 13]. This assay is a surrogate for VDR ligand binding, since VDR ligands and not RXR ligands drive heterodimerization between RXR and VDR [10, 13]. The RXR-VDR heterodimerization-based ligand sensing assay was performed by cotransfecting SaOS-2 cells with Gal4-RXRα-LBD and VP16-VDR-LBD expression vectors, along with a Gal4-responsive reporter. 1,25-$(OH)_2D_3$ and compound A were equipotent in inducing RXR-VDR heterodimerization with EC_{50} (concentration of the ligand required for 50% of the maximal activity) value of 10 nM (Figure 1(a)). The receptor specificity of nonsecosteroidal VDR ligands was confirmed by transfecting SaOS-2 cells with the Gal4-DNA binding domain chimeras of various nuclear receptor-LBD constructs, along with a Gal4-responsive luciferase reporter. Compound A induced the expression of the Gal4-dependent reporter only through Gal4-VDR-LBD and not through Gal4-TRα, Gal4-TRβ, Gal4-RARα, Gal4-RARβ, Gal4-RARγ, Gal4-PPARα, Gal4-PPARδ, Gal4-PPARγ, Gal4-RXRα, Gal4-RORα, or Gal4-LRH1 LBDs (Figure 1(b)). The structures of compound A and its diaryl analogs have been reported in a US patent (no. 7772425).

To determine whether compound A also induces VDRE-dependent gene expression, a mammalian one-hybrid assay was performed in HeLa cells to compare the potencies of the VDR ligands in mediating Gal4-VDR-LBD-dependent transactivation of a Gal4-luciferase reporter construct. Both 1,25-$(OH)_2D_3$ and compound A were potent in inducing VDR-LBD-mediated transactivation with EC_{50} values of 55 and 10 nM, respectively (Figure 2(a)). To further confirm that

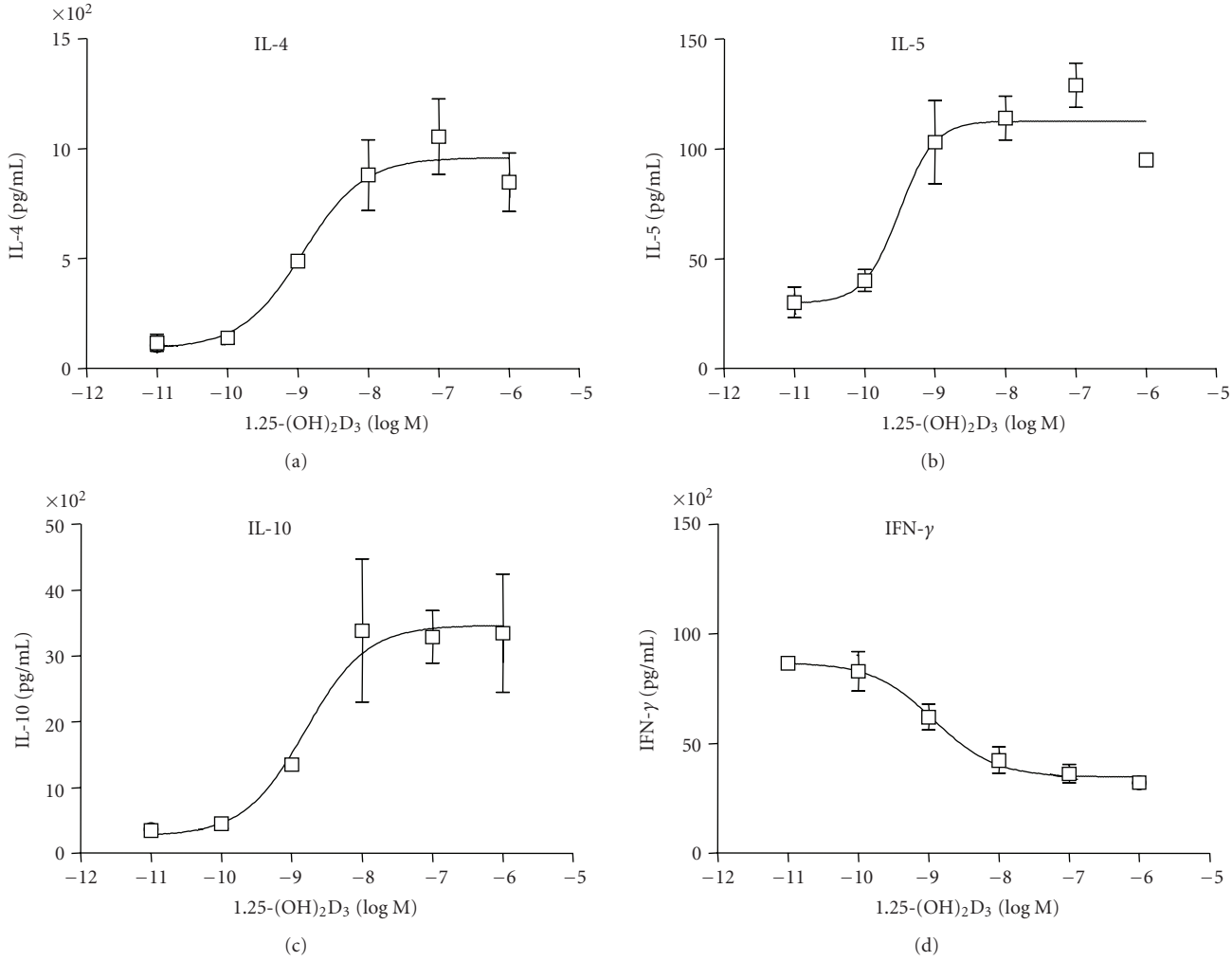

Figure 4: 1,25-$(OH)_2D_3$ shifts the balance from Th1 to Th2 cells in activated PBMCs. Primary cells isolated from donors were stimulated with TPA (100 ng/mL) and PHA (25 μL/mL) and treated with vehicle or various concentrations of 1,25-$(OH)_2D_3$ for 24 hours. Cytokines were measured by LINCOplex human cytokine kit on supernatants obtained from vehicle-treated or VDR ligand-treated samples using antibodies for human IL-4, IL-5, IL-10, and IFN-γ. The amount of IL-4, IL-5, IL-10, and IFN-γ protein levels is shown as mean ± SE of triplicate experiments.

compound A was potent in inducing VDRE-dependent gene expression of an endogenous gene, Ros 17.2 rat osteosarcoma cells permanently transfected with a rat osteocalcin promoter luciferase reporter, OCN-Luc (15) were used. 1,25-$(OH)_2D_3$ and compound A induced the expression of the rat OCN-Luc reporter with EC_{50} values of 0.09 and 9 nM, respectively (Figure 2(b)).

3.2. Compound A Is a Less Potent Agonist than 1,25-$(OH)_2D_3$ in Intestinal Cells. VDR ligands result in hypercalcemia by increasing calcium absorption from the intestine. 1,25-$(OH)_2D_3$ has been shown to induce the expression of epithelial calcium channel, TRPV6, that absorbs calcium from the intestinal lumen into the duodenal enterocyte [15, 16]. TRPV6 is a vitamin D-responsive gene *in vitro* and *in vivo*, and its expression is drastically reduced in VDR knockout mice [15, 16, 20]. Therefore, a cell-context-dependent VDR ligand that is transcriptionally less active in intestinal cells but a potent agonist in target cells (immune cell) may exhibit reduced hypercalcemic liability. We next examined the expression of endogenous TRPV6 gene in Caco-2 cells after treatment with VDR ligands. Although Caco-2 cells are colon cancer cells, upon density-dependent growth (6–14 days of culture), they differentiate into small intestinal like cells that express many of the markers of small intestine, including TRPV6, which is normally expressed in the duodenum [20]. These cells upon differentiation also acquire the machinery required for VDR ligand-dependent transepithelial calcium transport (apical to basolateral), similar to that of enterocytes [20, 21]. 1,25-$(OH)_2D_3$ (EC_{50} = 16 nM) was a potent inducer of TRPV6 message in differentiated Caco-2 cells (Figure 3(a)). In contrast, compound A (EC50 = >1000 nM) showed attenuated potency in inducing the expression of the endogenous TRPV6 gene in differentiated Caco-2 cells

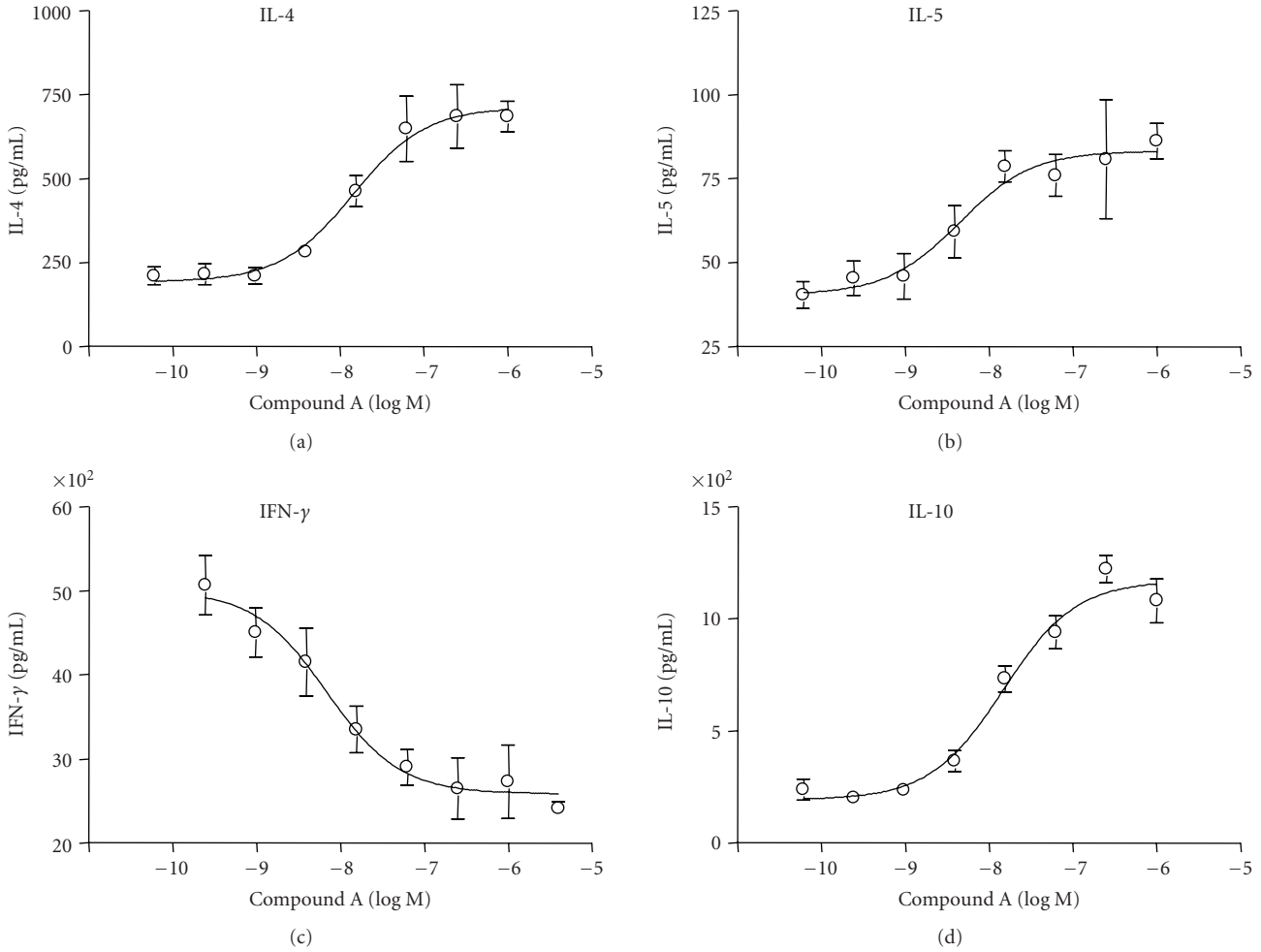

Figure 5: Compound A augments Th2 and inhibits Th1 cytokines in activated PBMCs. Primary cells isolated from donors were stimulated with TPA (100 ng/mL) and PHA (25 μL/mL) and treated with vehicle or various concentrations of compound A for 24 hours. Cytokines were measured by LINCOplex human cytokine kit on supernatants obtained from vehicle-treated or VDR ligand-treated samples using antibodies for human IL-4, IL-5, IL-10, and IFN-γ. The amount of IL-4, IL-5, IL-10, and IFN-γ protein levels is shown as mean ± SE of triplicate experiments.

(Figure 3(a)). These results indicate the cell-type selectivity of compound A since it was less potent than 1,25-$(OH)_2D_3$ in Caco-2 cells.

We also compared compound A with 1,25-$(OH)_2D_3$ for its effect on the expression of two VDRE-dependent genes, namely, CYP24 and calbindin-9k in differentiated Caco-2 and IEC-6 (rat duodenal crypt cell line) cells. Treatment of Caco-2 and IEC-6 cells with 1,25-$(OH)_2D_3$ (100 nM) for 24 hours resulted in a robust induction of human and rat CYP24 and calbindin-9k gene expression (Figure 3(b)). In contrast, compound A was significantly less efficacious than 1,25-$(OH)_2D_3$ in inducing the expression of endogenous CYP24 and calbindin-9k in these cells (Figure 3(b)). All these observations further support the notion that compound A is a cell-context-dependent VDRM.

3.3. Compound A Is a Potent Agonist in PBMCs. The lesions of Multiple Sclerosis have shown an increased expression of proinflammatory Th1 cytokines and decreased expression of Th2 anti-inflammatory cytokines IL-4 and IL-10. The disease is also ameliorated by IL-4 and IL-10 cytokine therapy in an EAE murine model of multiple sclerosis [20, 22]. 1,25-$(OH)_2D_3$ affects the Th1-Th2 balance, and it has been shown to augment Th2 cell development which is accompanied by increased production of IL-4 and IL-10 cytokines *in vitro* and *in vivo* [8, 13, 23, 24]. The effect of 1,25-$(OH)_2D_3$ and compound A on cytokine secretion from PHA/PMA-activated human PBMCs was examined by multiplex ELISA. 1,25-$(OH)_2D_3$ increased IL-10 protein levels in PHA/PMA-activated human PBMCs with an EC_{50} value of 2 nM (Figure 4). Compound A also increased IL-10 cytokine levels and showed EC_{50} value of 14 nM (Figure 5). Both 1,25-$(OH)_2D_3$ and compound A induced the protein levels of Th2 cytokines IL-4, IL-5, and IL-10 in a dose-dependent manner (Figures 4 and 5). However, the levels of Th1 cytokine IFNγ were decreased in a dose-responsive

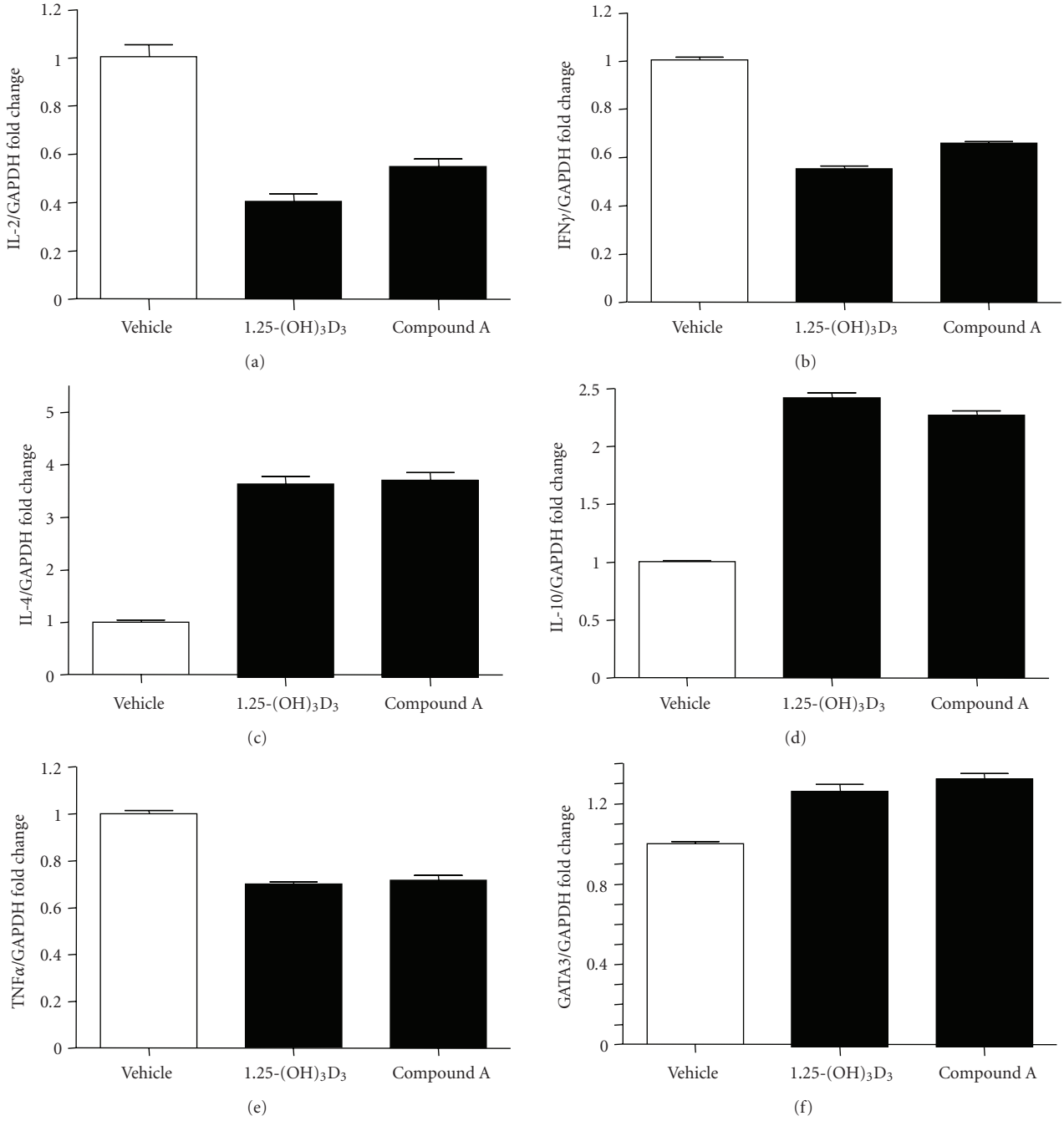

Figure 6: Nonsecosteroidal VDRM is as efficacious as 1,25-$(OH)_2D_3$ in modulating cytokine gene expression in activated human PBMCs. Primary cells isolated from donors were stimulated with TPA (100 ng/mL) and PHA (25 μL/mL) and treated with vehicle or 100 nM each of 1,25-$(OH)_2D_3$ and compound A for 24 hours. Taqman Q-PCR was performed on RNA obtained form vehicle-treated or VDR ligand-treated samples using primer pairs and probes for IL-2, IL-4, IL-10, IFN-γ, TNF-α, GATA3, and GAPDH. The amount of IL-2, IL-4, IL-10, IFN-γ, TNF-α, and GATA3 transcripts relative to GAPDH transcripts is shown as mean $\pm$ SE of quadruplicate experiments.

manner after 1,25-$(OH)_2D_3$ and compound A treatments (Figures 4 and 5). The EC_{50} values for IL-4, IL-5, and IL-10 induction and IFNγ inhibition were 1 nM, 0.3 nM, 2 nM, and 1 nM, respectively for 1,25-$(OH)_2D_3$. The corresponding EC_{50} values for compound A were 14 nM, 4 nM, 7 nM, and 14 nM, respectively (Figures 4 and 5). These results indicate that compound A is not only a potent agonist in PBMCs but also shifts the balance from proinflammatory Th1 to anti-inflammatory Th2 phenotype.

Furthermore, 1,25-$(OH)_2D_3$ and compound A were equally efficacious in decreasing the mRNA expression of Th1 cytokines IL-2 and IFN-γ and increasing the mRNA expression of Th2 cytokines IL-4 and IL-10 in activated PBMCs (Figure 6). In addition, the VDR ligands showed

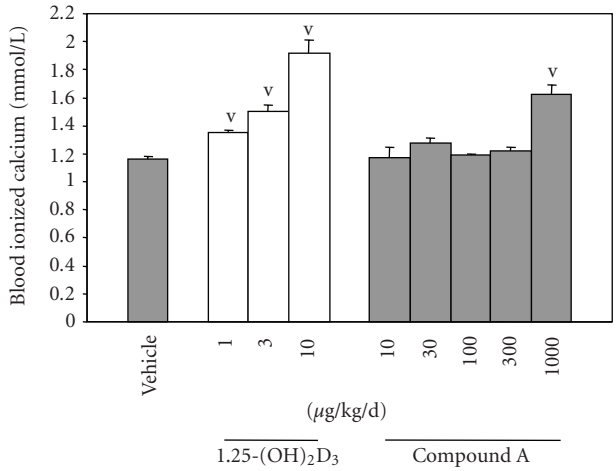

Figure 7: Compound A is less calcemic *in vivo*. 1,25-$(OH)_2D_3$ or compound A was administered in sesame seed oil at indicated doses to mice by gavage for 6 consecutive days, and blood ionized calcium was measured 6 hours after the last dose.

equivalent efficacy for decreasing TNF-α expression in activated PBMCs (Figure 6). 1,25-$(OH)_2D_3$ has also been shown to induce the expression of GATA-3 [13], a master regulator of Th2 differentiation [24]. Interestingly, compound A was also as efficacious as 1,25-$(OH)_2D_3$ in augmenting GATA-3 expression (Figure 6). Therefore, one of the plausible mechanisms of VDR ligand-mediated Th1 to Th2 shift might be their ability to induce the expression of basic helix-loop-helix transcription factor GATA-3. Therefore, compound A exhibited similar immunomodulatory effects as 1,25-$(OH)_2D_3$ in effector T cell functions.

3.4. Compound A Is Less Calcemic In Vivo. The hypothesis that the decreased VDR-mediated transcriptional activity of compound A on TRPV6 gene expression in differentiated Caco-2 cells would translate to less calcemic activity *in vivo* was tested after oral administration of VDR ligands to mice in a 6-day murine model of hypercalcemia. Mice were treated for 5 days with the VDR ligands, and blood-ionized calcium was measured 24 hours after the last dosing. 1,25-$(OH)_2D_3$ caused hypercalcemia in mice when dosed at 1 μg/kg/d. In contrast, compound A showed statistically significant hypercalcemia only at 1000 μg/kg/d but not at 300 μg/kg/d dose (Figure 7). Therefore, compound A is at least 300 times less calcemic than 1,25-$(OH)_2D_3$ *in vivo* when administered orally.

3.5. Compound A Inhibits Mouse EAE Induction and Severity In Vivo. Since 1,25-$(OH)_2D_3$ and its secosteroidal analogs have shown efficacy in a number of murine autoimmune disease models, including EAE [6, 25–27], we next examined whether oral administration of compound A could also affect the pathogenesis of EAE without inducing hypercalcemia. EAE was induced in C57B6 mice by immunization with MOG peptide and the animals were dosed orally with 1,25-$(OH)_2D_3$ (0.05 μg/kg/d) or compound A (10 μg/kg/d) daily for 21 days starting on the day of immunization. Vehicle control mice were immunized with MOG to induce the disease, and they were treated with vehicle (sesame seed oil). CFA control mice were mock immunized with CFA without MOG peptide and were not treated with vehicle or VDR ligands. Both 1,25-$(OH)_2D_3$ and compound A delayed the appearance of clinical signs of EAE induced by the MOG peptide. 1,25-$(OH)_2D_3$ initially reduced the severity of the disease until day 17 of the treatment. However, after day 19, the severity of EAE in 1,25-$(OH)_2D_3$-treated group was indistinguishable from the control groups (Figure 8(a)). In contrast, compound A treatment significantly resulted in less severe course of disease throughout the treatment period (Figure 8(a)). Microscopic evaluation of spinal cord neuropathology revealed that compound A treatment prevented demyelination that was readily visible as demyelinated plaques containing infiltrating mononuclear cells in the spinal cord sections of vehicle-treated sample (Figure 8(b)). Demyelinated areas were reduced in the spinal cord sections of compound A-treated animals (Figure 8(b)). Since the major problem associated with 1,25-$(OH)_2D_3$ treatment is hypercalcemia, we also measured serum calcium levels at the end of the study. 1,25-$(OH)_2D_3$ (0.05 μg/kg/d) resulted in hypercalcemia whereas serum calcium levels were within the normal range after compound A (10 μg/kg/d) treatment. The difference of serum Ca^{++} level between 1,25-$(OH)_2D_3$-and compound A-treated animals was statistically different whereas there was no statistically difference between compound A and vehicle group (Figure 8(c)).

To determine if compound A can modulate antigen T cell function in EAE, total splenocytes from diseased mice were stimulated *ex vivo* with either MOG peptide or ovalbumin peptide at the indicated concentrations, and T cell proliferation was measured by ^{3}H-thymidine incorporation. Compared with vehicle-treated mice, *in vivo* treatment with compound A suppressed the specific recall response to the encephalitogenic MOG peptide used in the EAE model (Figure 9(a)). The recall response of MOG-immunized animals was specific for the MOG peptide and was not observed for ovalbumin (OVA) peptide (Figure 9(a)). Furthermore, both 1,25-$(OH)_2D_3$ and compound A decreased Th1 cytokine IFN-γ production in MOG-stimulated splenocytes (Figure 9(b)). Interestingly, both 1,25-$(OH)_2D_3$ and compound A also induced IL-10 cytokine production in isolated $CD11c^+$ dendritic cells stimulated with 100 ng/mL LPS (Figure 9(b)), indicating that compound A modulated immune response *in vivo* in autoimmune pathogenic conditions. Th17 cells have recently been demonstrated to be the essential pathogenic cells involved in EAE model. In order to test whether 1,25-$(OH)_2D_3$ and compound A had the direct effect on Th17 differentiation or Th17 secretion, we performed Th17 differentiation assay *in vitro* in the presence of these compounds. As shown in Figure 10, both 1,25-$(OH)_2D_3$ and compound A significantly inhibited both IL-17 and IL-22 expression, indicating that Th17 differentiation was efficiently inhibited by these compounds. Interestingly, once Th17 cells were fully differentiated, the restimulation of these differentiated Th17 cells by anti-CD3 mAb to produce IL-17 and IL-22 was only slighly affected by these compounds (data not shown),

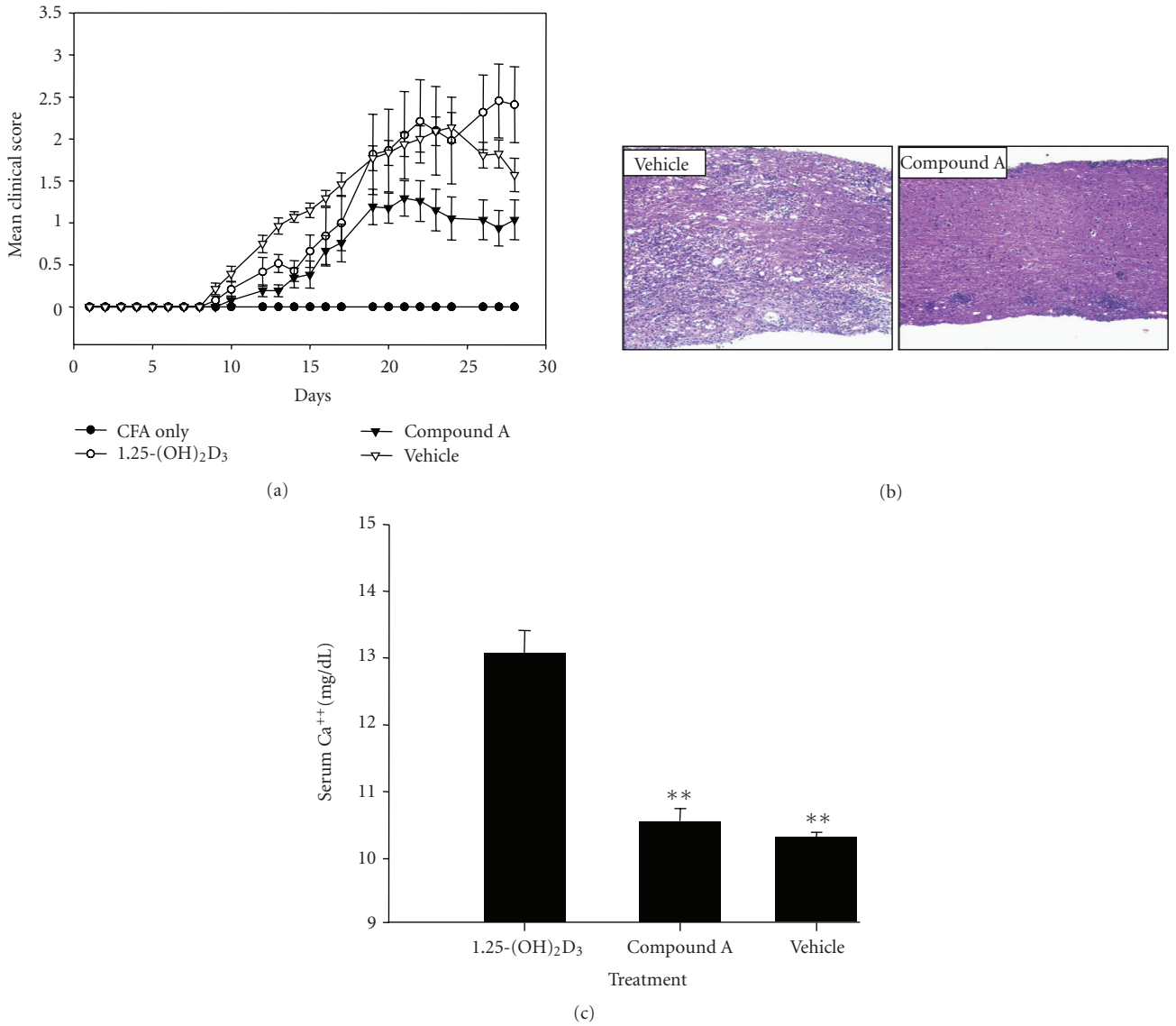

Figure 8: Oral administration of VDRM is therapeutically efficacious in MOG-induced EAE. (a) Clinical course of EAE after treatment with VDR ligands. MOG immunized C57B6 mice were orally administered with vehicle, 1,25-$(OH)_2D_3$ (0.5 μg/kg/d), or compound A (10 μg/kg/d) for 21 days. Each point represents the mean clinical score for particular day in vehicle or VDR ligand-treated ($n = 15$) groups. Error bars represent mean ± SE. Vehicle group consisted of MOG-immunized mice treated with vehicle (sesame seed oil). CFA group was mock immunized with CFA only (without MOG peptide) and was not treated with any ligands. There was statistically significant reduction of overall EAE disease score between the compound A-treated group and vehicle group and between compound A-treated and 1,25-$(OH)_2D_3$-treated group ($P < .001$) whereas the difference between 1,25-$(OH)_2D_3$-treated group and vehicle group is nonsignificant. (b) Compound A improves spinal cord pathology in EAE. On day 28, mice spinal cords were harvested and subjected to histological analysis. Spinal cord sections of vehicle and compound A-treated MOG-immunized mice were analyzed for demyelination by eosin-hematoxylin staining. (c) Compound A does not cause hypercalcemia at therapeutically efficacious dose. At the end of the EAE study, blood ionized calcium was measured 6 hours after the last dose. Value shown represents mean values ± SD of 15, 12, and 6 individual mice of vehicle group, compound A group, and 1,25-$(OH)_2D_3$-treated group, respectively; $^{**}P < .01$.

indicating that VDRMs were mainly involved in Th17 differentiation stage.

4. Discussion

The success of nonsteroidal SERMs in limiting the side effects of estrogen on breast and uterus while still retaining therapeutic efficacy in bone [17] prompted us to pursue identification of nonsecosteroidal VDRMs for the treatment of autoimmune diseases, such as MS. In this study, we have identified a cell-context-dependent VDRM that shows attenuated calcemic liability *in vivo* relative to 1,25-$(OH)_2D_3$. We also show that the nonsecosteroidal VDRM modulates the balance between Th1 and Th2 cells as well

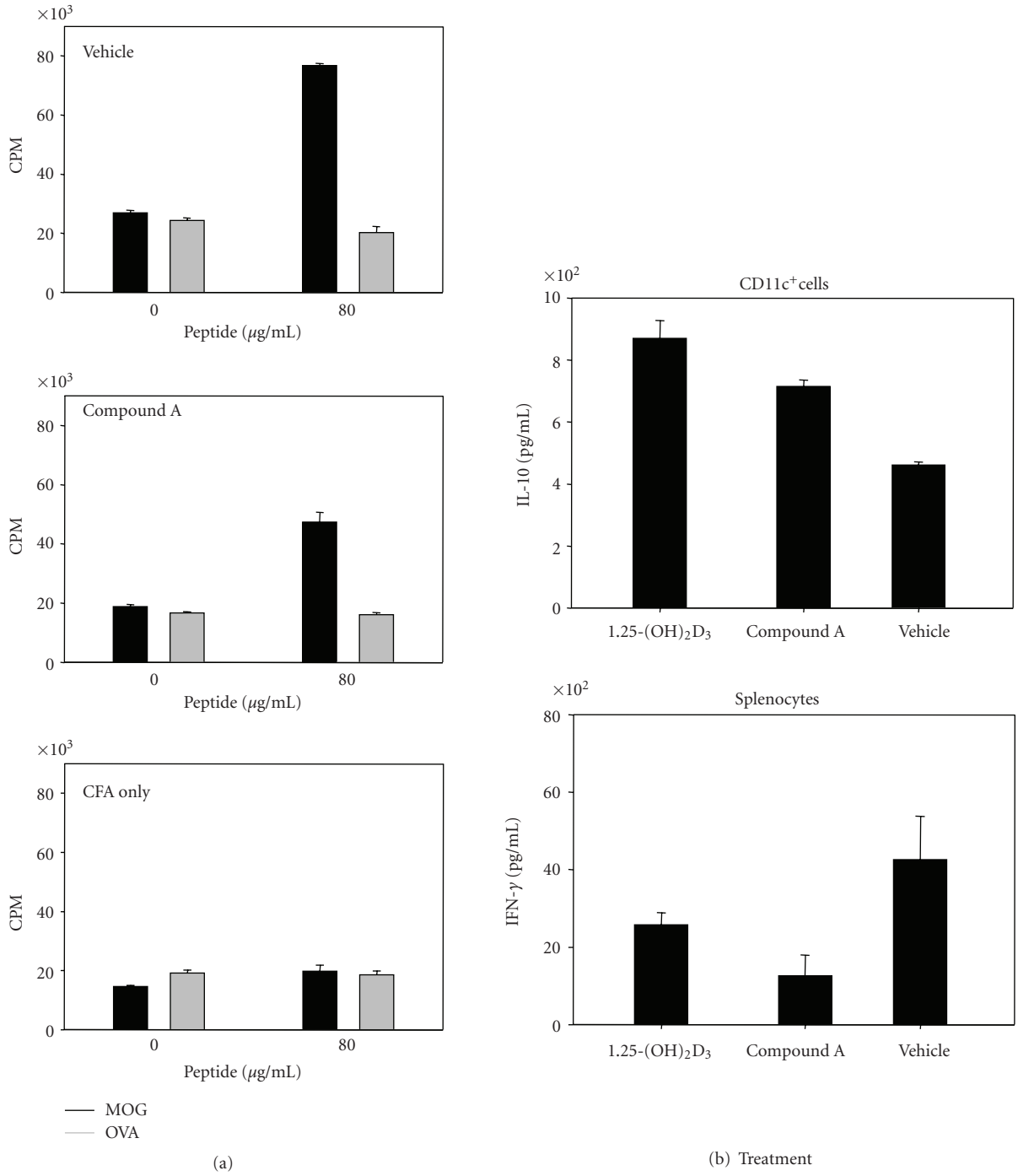

Figure 9: VDR ligands modulate peptide-specific recall responses from MOG-immunized mice. (a) Compound A inhibits the proliferation of MOG-specific splenocytes. Splenocytes from groups ($n = 5$) of MOG-immunized mice were harvested at day 28 and analyzed ex vivo for proliferative responses to MOG at the concentrations indicated. Cells were cultured in triplicate in 96-well plates for 60 hours, and proliferation was measured by ^{3}H-thymidine incorporation during the final 8 hours of culture. Values represent the mean ± SE of triplicate for each peptide concentration. Ovalbumin (OVA) peptide was used as specificity control since the mice were immunized with MOG peptide. Vehicle group consisted of MOG immunized mice treated with vehicle (sesame seed oil). CFA group was mock immunized with CFA only (without MOG peptide) and was not treated with any ligands. (b) Effect of VDR ligands on cytokine elaboration in MOG-immunized animals. For dendritic cell IL-10 production, CD11c$^+$ cells were purified on day 28 from splenocytes obtained from MOG-immunized animals. The effect of VDR ligands on IL-10 levels from splenic CD11c$^+$ cultures stimulated for 24 hours with 100 ng/mL LPS is shown. VDR ligands decreased IFN-γ elaboration from splenocyte cultures stimulated for 48 hours with 80 μg/mL MOG peptide. IL-10 and IFN-γ protein levels were measured by ELISA.

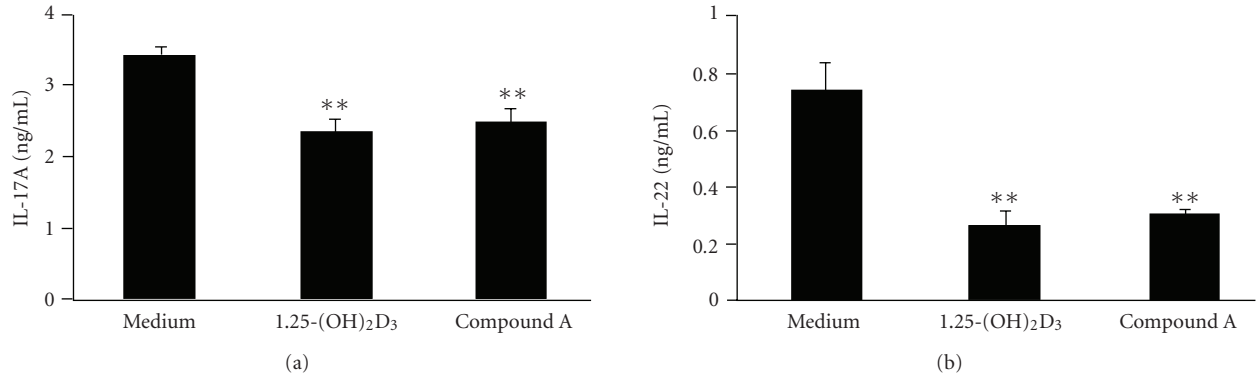

Figure 10: VDRM inhibits Th17 differentiation in vitro. Purified mouse naïve CD4+T cells were differentiated into Th17 cells in vitro under Th17 differentiation conditions for 4 days in the presence or absence of VDR ligands. Differentiated Th17 cells were then washed and restimulated with plate bound anti-CD3 mAb for 18 h. The supernatant was then measured for the expression of IL-17 (a) and Il-22 (b) by ELISA. Value shown represent the mean values of triplicate cultures and error bars represent the standard deviation (**$P < .01$.)

as Th17 differentiation, since compound A inhibited Th1, Th17 cytokine production and augmented the production of Th2 cytokines. Furthermore, the nonsecosteroidal ligand described herein also displayed therapeutic activity in the EAE model at a nonhypercalcemic dose. Our results extend the observations that VDR ligands are efficacious in the treatment of murine EAE and demonstrate for the first time that a nonsecosteroidal VDRM is therapeutically effective at a nonhypercalcemic dose.

$CD4^+$ T-helper cells could be broadly divided into different effector cells such as Th1, Th2, and Th17 cells based upon their cytokines elaborated by these cells upon antigenic/mitogenic stimulation. The recent evidence indicates that Th17 cells are the key T cells involved in the pathogenesis of autoimmune diseases, whereas Th2 cells are involved in the pathology of allergic indications and produce IL-4, IL-5, IL-10, and IL-13 cytokines [24]. 1,25-$(OH)_2D_3$ is regarded as an immunoregulatory hormone that in addition to its classical role on mineral homeostasis and maintenance of skeletal architecture also exhibits beneficial effects on Th17-mediated autoimmune diseases. 1,25-$(OH)_2D_3$ has shown efficacy in several autoimmune disease models, namely, systemic lupus erythematosus in lpr/lpr mice [28], type I diabetes in nonobese diabetic mice [29, 30], collage-induced arthritis [25], EAE [6], experimental autoimmune uveitis [31], and inflammatory bowel disease [26, 32]. Although 1,25-$(OH)_2D_3$ has demonstrated efficacy in preventing the incidence and progression of disease in the EAE model, the therapeutic activity was associated with accompanying hypercalcemia (Figure 8).

The VDRM, compound A, displayed many of the biological actions of 1,25-$(OH)_2D_3$. It induced RXR-VDR heterodimerization (Figure 1(a)), augmented VDR-LBD-dependent gene expression in HeLa cells (Figure 2(a)), upregulated the expression of a VDRE-dependent gene, osteocalcin in osteoblasts (Figure 2(b)), inhibited IFN-γ, while augmenting IL-4, IL-5, and IL-10 cytokine elaboration in human PBMCs (Figure 5), and inhibited the expression of proinflammatory cytokines (IL-2, IFN-γ, and TNFα) and induced the expression of anti-inflammatory cytokines IL-4 and IL-10 (Figure 6). It also induced the expression of GATA-3 (required for Th2 cell differentiation) in activated human PBMCs (Figure 6). More importantly, both 1,25-$(OH)_2D_3$ and compound A significantly inhibited Th17 cell differentiation (Figure 10), which is consistent with the recent observation made by Chang et al. (34). However, it was significantly less potent than its secosteroidal counterpart in inducing the expression of vitamin D-responsive genes (TRPV6, Cyp24, and calbindin-9k) in differentiated Caco-2 as well as rat duodenal cells (Figure 3). These results indicate an attenuation of the VDR signaling pathway to compound A in intestinal cells. At the same time, the vitamin D signaling pathway still responds to 1,25-$(OH)_2D_3$-complexed VDR for vitamin D-dependent gene expression in Caco-2 cells (Figure 3).

Studies with the VDR knockout animals have indicated duodenal TRPV6 to be a major mediator of 1,25-$(OH)_2D_3$-mediated calcium absorption from intestine and hypercalcemia [15]. Therefore, the weak agonist activity of compound A in human intestinal cells predicted that it might be less calcemic than 1,25-$(OH)_2D_3$ *in vivo*. In order to test this hypothesis, compound A was administered orally to mice in a 6-day murine model of hypercalcemia. The nonsecosteroidal VDRM was found to be at least 300 times less potent than 1,25-$(OH)_2D_3$ in inducing hypercalcemia by the oral route (Figure 7). The reduced calcemic liability of compound A prompted us to test it in a murine EAE model of MS. 1,25-$(OH)_2D_3$ and its secosteroidal analogs have been shown to ameliorate EAE [6, 27]. Here, we demonstrate that treatment of MOG-immunized animals with the nonsecosteroidal VDRM, compound A, delayed the onset of EAE and resulted in a less severe course of disease during the entire treatment period (Figure 8(a)). 1,25-$(OH)_2D_3$ on the other hand showed efficacy initially (till day 17 of the treatment) and delayed the onset of the disease. Interestingly, compound A did not raise the serum calcium levels above the normal range whereas 1,25-$(OH)_2D_3$ treatment of MOG immunized animals resulted in hypercalcemia (Figure 8(c)).

A plethora of epidemiological and pharmacological data demonstrating the connection between vitamin D and MS, coupled with our observation that a nonsecosteroidal VDRM ameliorates EAE, strongly supports the use of noncalcemic VDRMs as attractive candidates for the prevention and treatment of MS. Since VDR ligands have different mechanism of action from currently approved MS treatment, they may be more efficacious and useful in a combination therapeutic regimen.

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Review Article

Mitochondria as Crucial Players in Demyelinated Axons: Lessons from Neuropathology and Experimental Demyelination

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Mitochondria are the most efficient producers of energy in the form of ATP. Energy demands of axons, placed at relatively great distances from the neuronal cell body, are met by mitochondria, which when functionally compromised, produce reactive oxygen species (ROS) in excess. Axons are made metabolically efficient by myelination, which enables saltatory conduction. The importance of mitochondria for maintaining the structural integrity of myelinated axons is illustrated by neuroaxonal degeneration in primary mitochondrial disorders. When demyelinated, the compartmentalisation of ion channels along axons is disrupted. The redistribution of electrogenic machinery is thought to increase the energy demand of demyelinated axons. We review related studies that focus on mitochondria within unmyelinated, demyelinated and dysmyelinated axons in the central nervous system. Based on neuropathological observations we propose the increase in mitochondrial presence within demyelinated axons as an adaptive process to the increased energy need. An increased presence of mitochondria would also increase the capacity to produce deleterious agents such as ROS when functionally compromised. Given the lack of direct evidence of a beneficial or harmful effect of mitochondrial changes, the precise role of increased mitochondrial presence within axons due to demyelination needs to be further explored in experimental demyelination *in-vivo* and *in-vitro*.

1. Introduction

Axons are unique structures in the central nervous system with much of their cytoplasm found at great distances from the neuronal cell body. Myelination of axons is essential for fast conduction of action potentials and their metabolic efficiency [1]. Axonal degeneration in demyelinating diseases such as multiple sclerosis (MS) indicates the importance of trophic support that myelin lends to axons whilst also providing protection from various extracellular insults. Mitochondria, the ubiquitous energy-producing organelles, are found within axons. They are vastly dynamic and locate to areas in which they are most needed and, as we will discuss in this review, appear highly adaptable to subtle energy changes within the axon. We review mitochondrial changes that follow demyelination and indicate the damaging consequences of mitochondrial failure for axons.

2. Mitochondria

Mitochondria are charged with supplying the vast amount of ATP required in eukaryotic cells. Other important roles in calcium buffering and apoptosis cannot be underestimated [2]. The respiratory chain which is responsible for the process of oxidative phosphorylation, ultimately resulting in the production of ATP from ADP, consists of complex I–complex IV and an additional ATP synthase.

Mitochondria contain the only extranuclear DNA in the cell (mtDNA). The mitochondrial genome holds 13 protein encoding genes which incorporate into complex I,

complex III, complex IV, and ATP synthase. Complex II is the only complex with all subunits encoded by nuclear DNA, which, from an investigative point of view, is highly advantageous [3, 4]. MtDNA mutation (point mutations and deletions) led energy deficiency states frequently affect the central nervous system (CNS) in patients with primary mitochondrial diseases.

3. A Question of Energy for Axons

The vast energy requirement of axons is highlighted by the location of the sodium potassium ATPase (Na^+/K^+ ATPase) which extends along myelinated segments of the axons (internodes) [5]. The Na^+/K^+ ATPase facilitates the rapid exchange of sodium for extracellular potassium, through the process of active transport and is thus a major consumer of energy in the CNS [6]. Sodium channels play an important role in axons, and their clustering at the nodes of Ranvier facilitate the fast propagation of action potentials or "saltatory conduction" and allow influx of sodium into the axon. Different isoforms exist, but the accepted forms known to exist in neurons are $Na_v1.1$, $Na_v1.2$, and $Na_v1.6$. Those expressed on axonal membranes are predominantly $Na_v1.2$ and $Na_v1.6$ [7]. The importance of the persistent sodium influx allowed by $Na_v1.6$ is highlighted by the redistribution of the channel along nonmyelinated axons to maintain action potentials [8]. During development in the premyelination state, $Na_v1.2$ channels support action potentials [9] which are soon replaced following myelination with $Na_v1.6$ channels, identified to allow a persistent current of sodium. Myelin also induces the clustering of sodium channels at nodes of Ranvier [10]. In the CNS, mitochondria were presumed to reside in the nonmyelinated segments, nodes of Ranvier, based on findings in the peripheral nervous system (PNS); however, recent evidence suggests that mitochondria preferentially locate in the internodes [11], at least in small diameter axons, which would fit with the energy demand hypothesis. Evidence for the need of precise location of mitochondria within axons is observed in growth cones. An elegant study by Morris and Hollenbeck in 1993 showed that the presence of mitochondria in neurons is coordinated with axonal outgrowth. They showed that by blocking the growth of a number of axons and then visualising the mitochondrial content, the preferential location of the mitochondria was in the outgrowing axons, particularly in the terminal ends [12]. Whilst ATP can readily diffuse into the cytosol, it appears that the precise location of mitochondria is important.

Axons, both myelinated and unmyelinated, are an excellent forum to understand the relationship between mitochondria and the differing energy demands of the CNS, given the difference in ion channel expression in these axons [13]. The lamina cribrosa is a region of the optic nerve that is unmyelinated with myelination of fibres occurring at the posterior border. Complex IV activity was assessed in fibres of the lamina cribrosa [3]. Complex IV consumes 90% of cellular oxygen [2] and is involved in proton pumping across the inner mitochondrial membrane. Perhaps, importantly, numerous inhibitors of complex IV exist including nitric oxide, a competitive inhibitor [14], whilst its more toxic derivative, peroxynitrite, can irreversibly inhibit both complex I and complex IV [15]. Complex IV activity was found to be vastly increased in the unmyelinated segment compared to the myelinated segment which corresponded with an increase in complex IV subunit II protein level [16, 17]. The distribution of complex IV activity correlated with certain isoforms of sodium channels. Both $Na_v1.1$ and $Na_v1.6$ were found to increase in intensity with lack of myelin whilst there was little predilection for $Na_v1.2$ in either region [18]. These findings in control subjects suggest a physiological role for the increased presence of mitochondria in the unmyelinated segments compared with myelinated segments of optic nerve axons.

4. Loss of Compartmentalisation of Electrogenic Machinery and a Question of Energy for Demyelinated and Dysmyelinated Axons

Several groups have predicted metabolic changes within axons based on changes in ion channel distribution following demyelination and dysmyelination (Figure 1) [19–21]. There has been a number of studies with animal models in which genes for myelin proteins have been knocked out or toxic insults to myelin have been introduced.

In a mouse model of hypomyelination caused by gene knockout encoding the myelin basic protein gene, complex IV activity was found to be increased associated with an increase in mitochondrial density suggestive of adaptive changes to this change in myelin [20]. Furthermore, in a proteolipid protein (PLP) overexpressing mouse model, dysmyelination at 1 month followed by complete demyelination at 4 months was associated with an increase in mitochondrial density and complex IV activity compared to wild type [19]. Axonal degeneration was not evident at this time point. In a hemizygous PLP overexpressing mouse model predisposing to only partial demyelination, whilst mitochondrial density increased, complex IV activity remained similar to wild type. Electron microscopy revealed the presence of degenerating axons suggesting a lack of compensation from complex IV predisposed to the phenotype. These animal models show the relationship between disruptions to myelin and changing energy demands compensated for by alterations in mitochondria.

In one particular model in which antiserum to galactocerebroside was used to selectively demyelinate cat optic nerve, a significant increase in mitochondrial number was observed which reached a peak at seven days after injection [21]. The authors suggest that this is an adaptive feature of the demyelinated axons particularly as this coincided with a change from conduction block to slowed conduction [22]. In a demyelination model induced by Theiler's murine encephalomyelitis virus (TMEV), neurological dysfunction results from progressive nervous system demyelination. Mitochondrial numbers were found to be significantly increased in demyelinated axons compared to controls [23]. Interestingly, mitochondrial numbers correlated well

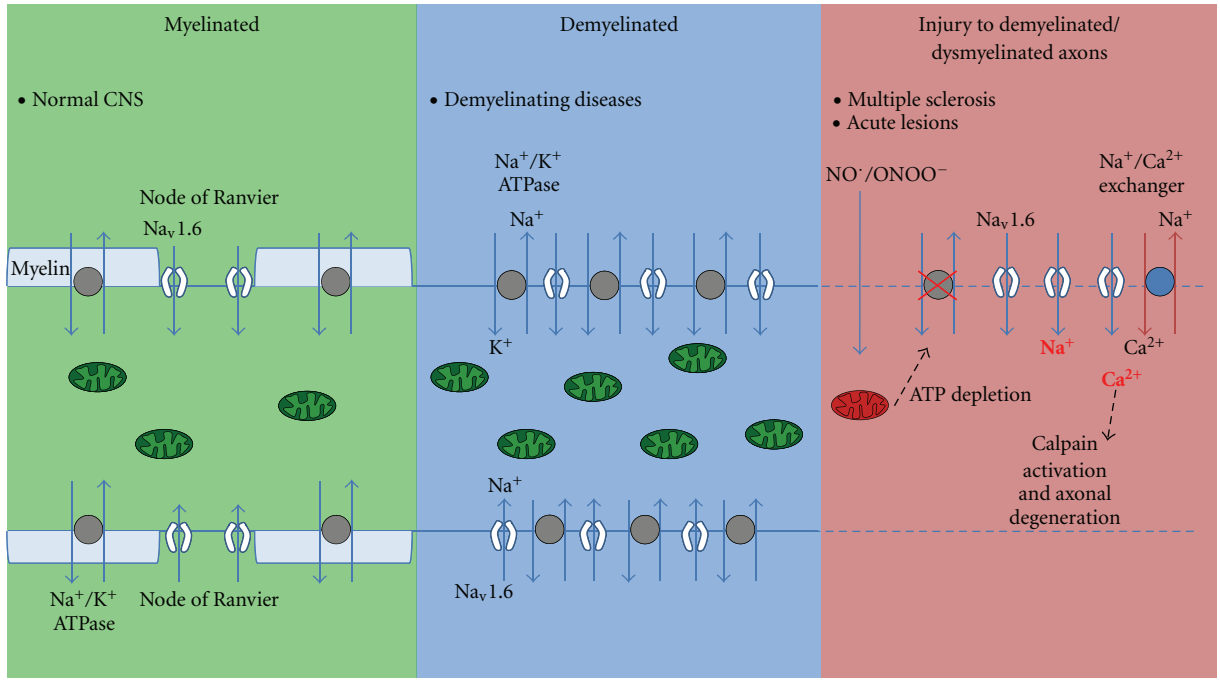

Figure 1: Mitochondrial changes within axons in response to demyelination and further cytoskeletal injury. Sodium channels ($Na_v1.6$) are located at nodes of Ranvier in the CNS whilst the Na^+/K^+ ATPase extends the myelinated segments (internodes). To facilitate the extended energy demand of the axon, mitochondria have been shown to distribute throughout the axonal cytoplasm in small axons in the CNS *(green)*. The redistribution of sodium channels facilitates the continuation of action potentials along the demyelinated axons *(blue)*. Mitochondrial dysfunction can leave the axon vulnerable as observed in acute and chronic stages of MS *(red)*. It is hypothesised that the failure of the Na^+/K^+ ATPase, possibly due to mitochondrial dysfunction, can lead to increased sodium concentrations in the axoplasm. Reversal of the Na^+/Ca^{2+} exchanger can ensue, resulting in toxic calcium levels and downstream processes with consequent axonal degeneration. Mitochondrial dysfunction (red) possibly by inflammatory products such as nitric oxide (NO) and peroxynitrite ($ONOO^-$) can lead to membrane depolarisation and retrograde transport toward the neuronal soma (arrow to the left). A population of axons have been shown to exist without the Na^+/K^+ ATPase which would also have a similar effect to an energy defect.

with the percentage of total spinal cord demyelination and strong correlations were found between the number of degenerating axons and intra-axonal mitochondria in demyelinated axons, raising the possibility that increases of intra-axonal mitochondria may be detrimental to axons. Mitochondrial density was still increased after 6 months. Our view, partly supported by observations in unmyelinated axons, is that an increase in mitochondria *per se*, as long as the function is not compromised, is not damaging to the axons. However, mitochondrial defects in situations of elevated energy needs, as seen in some demyelinated axons, may be even more detrimental due to the toxic effects of dysfunctional mitochondria.

Mitochondrial defects in experimental demyelinating models implicate the organelle in the pathogenesis of axonal degeneration. Post-translational modification of mitochondrial respiratory chain subunits as well as transcriptionally regulated changes in mitochondrial function have been observed in animal models of MS [24–27]. Moreover, the addition of reactive oxygen species scavengers resulting in decreased optic nerve degeneration suggests a role for mitochondrial injury via oxidative stress and identifies mitochondrially targeted agents as potential therapy in MS [28].

5. The Importance of Mitochondrial Transport, Fusion, Fission, and Biogenesis for Demyelinated Axons

The observation of an increased mitochondrial presence within demyelinated axons raises a number of important questions including how the mitochondrial changes occur. The possible explanations include an increase in anterograde transport, decrease in retrograde transport, increase in fusion, decrease in fission, and mitochondrial biogenesis within axons.

Neurons have developed a highly sophisticated transport system to meet the needs of the axon with machinery for both transport of proteins away from the cell body, into the far reaches of the axon, anterograde transport, and a system to transport aberrant proteins back to the cell body for degradation, retrograde transport. Two pools of mitochondria exist in axons, those that are stationary, which comprise 70% of axonal mitochondria, and those that are mobile. Mobile mitochondria have been found to move on both microtubules and actin-microfilaments within the axon. A 1995 study used toxic agents which were applied to axons that disrupted either microtubules or actin-microfilament [29]. The authors concluded that

mitochondrial velocity within axons is determined by co-ordination of microtubule and actin-microfilament based movements.

There are two motor transport systems responsible for anterograde and retrograde movements of mitochondria, kinesins and dyneins, respectively. Kinesin motors are responsible for anterograde transport whilst dynein motors are responsible for retrograde transport along microtubules. Kinesins of the kinesin-1 family associate with mitochondria as well as vesicles containing, among other proteins, amyloid precursor protein (APP). Sites of APP accumulation in postmortem tissues are readily used to detect disruption of fast axonal movement [30, 31]. Inhibition of kinesin-1 in *Drosophila melanogaster* has been shown to inhibit mitochondrial transport [32]. The importance of anterograde mitochondrial transport is highlighted by mutations of one member of the kinesin family, Kif1β, shown to transport mitochondria. These mutations can lead to the axonal form of Charcot-Marie-Tooth disease (CMT2a). Heterozygous knockout mice showed symptoms similar to CMT2a with progressive muscle weakness. The ATPase activity of the motor was found to be reduced, thus, suggestive of a functional loss of motor activity [33]. Retrograde movement of microtubules appears to require dynein motors [34], although it is known that several kinesin motor families have retrograde movement, however, the velocity at which these move indicates they are unlikely to be involved in axonal mitochondrial transport. Myosin motors are implicated on actin-microfilaments due to the bidirectional movement of mitochondria. Myosin V is a likely candidate as it has been shown to move organelles at similar rates as observed with mitochondria in axons [29].

It is clear from recent studies that retrograde movement of mitochondria occurs in situations of mitochondrial dysfunction. For instance in familial amyotrophic lateral sclerosis where mutations in the antioxidant enzyme Cu/Zn superoxide dismutase-1 (SOD1) which accounts for 20% of all familial cases, it has been shown that this mutation inhibits anterograde transport but not retrograde transport [35]. Furthermore, when the complex III inhibitor, antimycin, was used in a cell culture model of neurons derived from dorsal root ganglion, the number of mitochondria associated with retrograde transport significantly increased [36].

There was understood to be a mitochondrial docking protein for the stationary pool in axons although the actual identity of this protein remained unknown for some years. In a recent study, syntaphilin, a neuron-specific protein was identified as this docking protein [37]. The authors show that mitochondria and syntaphilin colocalise within axons, syntaphilin immobilises the organelle, and in syntaphilin knockout mice mitochondrial motility increased but their number significantly reduced in axons.

Mitochondria are far more dynamic organelles than depicted in textbook images, existing in networks rather than in isolation. Large networks of mitochondria can form through the process of fusion and can be broken up through the action of fission. Mitochondrial fusion machinery consists of the mitofusins MTF1 and MTF2 as well as the GTPase OPA1, responsible for both outer and inner membrane fusion, respectively [38]. DRP1 is the key regulator of mitochondrial fission [39]. The importance of these processes can be observed again in CMT2a which is associated with a number of mutations in MFN2 [40]. Similarly, mice that lack MFN1, MFN2, or OPA1, all responsible for mitochondrial fusion, do not survive beyond midgestation [40–42]. OPA1 mutations in humans cause autosomal dominant optic atrophy (ADOA) characterised by the degeneration of retinal ganglion cells and the optic nerve axons [43].

It was long assumed that axons lack the protein synthesis machinery required for organelle and protein biogenesis and thus the neuronal cell body would operate as this site, probably close to the nucleus, followed by distribution of the organelle to the axons [44]. However, recent evidence suggests that local protein synthesis can occur in axons [45]. Newly synthesised mtDNA has been shown to be present not only in the cell body but throughout axons. The authors also show that Drp1, an essential mitochondrial fission protein, is present in axonal mitochondria. It is probable that newly synthesised mitochondria, supported by an increase in anterograde transport, fuse with already immobile mitochondria at specific energy-demanding sites. How important each of the above processes are for the mitochondrial changes following demyelination needs to be further investigated.

6. Mitochondrial Changes in Multiple Sclerosis

MS is the most common demyelinating disease of the central nervous system and, as such, much can be learnt from studies of the demyelinated axons [46]. Demyelination in MS classically occurs with inflammation and is associated with axonal loss which underlies neurological impairment in MS [47]. Demyelinated lesions associated with various degrees of inflammation, represent the pathological hallmarks of the disease which have been studied since the 19th century. Axonal degeneration is most prevalent in active lesions where inflammation is greatest. In chronic lesions, many surviving axons remain chronically demyelinating with only a few undergoing degeneration at any given time point. Axonal injury is highlighted by dephosphorylation of neurofilament side arms and has been observed in models of de/dysmyelination [48] and in acute and chronic MS lesions [49]. In addition, accumulation of beta-amyloid precursor protein (β-APP) has been shown to be a marker for axonal damage in cases of diffuse axonal injury [50]. The protein is known to be transported by fast axonal transport which is disrupted in axonal injury leading to accumulation of organelles and intra-axonal proteins around the site of injury [51]. It is only recently that mitochondrial changes have been studied in these axons.

Mitochondrial activity in the form of complex IV has been shown to be increased in inactive areas of chronic lesions associated with an increase of mitochondrial mass (Figure 1) [52, 53]. The increase in complex IV has been noted using several biochemical techniques [54]. Besides the activity and density, the mobility of mitochondria within

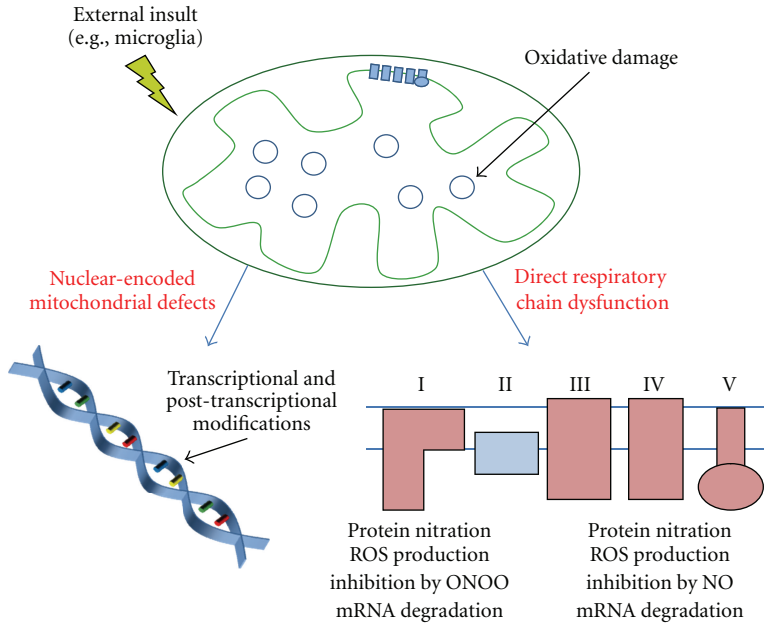

Figure 2: Mitochondrial dysfunction in MS and its models. Inflammation and the resulting toxic environment can have multiple effects on mitochondria including mtDNA damage (left arrow) and respiratory chain modifications (right arrow). Increased mtDNA copy number and mtDNA deletions have been observed in MS, which may be a related phenomenon. Actual respiratory chain defects are observed where all but complex II (blue) have both nuclear and mitochondrial DNA-encoded subunits. Defects include protein nitration affecting complexes I and IV observed in EAE, reduction in complex I activity in chronic lesions with reductions in complex I and III in nonlesional motor cortex. Complex IV activity is increased, along with mitochondrial mass, in chronic axons within nonpathological axons whilst a reduction is observed in pathological axons. The recognised production of reactive oxygen species particularly stemming from complex I and III, along with complex inhibition by nitric oxide and peroxynitrite, a result of chronic inflammation, points to the high vulnerability of mitochondria in MS.

axons may also be influenced by demyelination. The expression of axon-specific mitochondrial docking protein, syntaphilin, in chronic lesions indicates a potentially immobile reservoir that supplies the necessary energy in demyelinated axons [52]. These axons were morphologically intact, except for demyelination, and did not show an accumulation of β-APP. The fact that the above mitochondrial changes were observed in approximately 50% of morphologically intact axons that were phosphorylated provides support for the adaptive or compensatory theory.

Complex IV defects have been noted in both nonphosphorylated and APP-positive chronically demyelinated axons with an associated decrease of mitochondrial mass in the former but not latter case, suggesting differing mechanisms (Figure 2) [52]. Another study finds agreement with the reduction in ATP synthase expression in MS lesions [55]. Enhanced immunoreactivity of the mitochondrial heat shock protein (mtHSP70) in chronic lesions has been noted indicating an environment of oxidative stress [53, 56].

Although not directly located to axons, other mitochondrial defects have been shown in MS tissue including complex I dysfunction in white matter lesions [54] as well as reduced complex I and complex III dysfunction in nonlesional motor cortex [57]. This study based on gene expression profiles, revealed a decrease in nuclear encoded subunits of complex I, complex III, complex IV, and ATP synthase (Figure 2). Whilst this was reflected in the reduced activities of complex I and complex III whilst complex IV activity remained the same suggesting a compensatory mechanism. Furthermore, in white matter MS lesions, oxidative damage to mtDNA has been reported [54]. The compensatory mitochondrial changes in chronic lesions appear to be an adaptive process to the demyelination-induced energy changes that we have discussed previously. The increase in complex IV, which has been shown in a number of studies, may also result from a reduction in other complex activities, though whether this is enough to sustain ATP production over a period of time is unknown. The adaptive changes of mitochondria in demyelinated axons, however, will increase the vulnerability of these axons to further energy defects. The mitochondrial defects are likely to compromise the function and structure of the surviving demyelinated axons in MS, contributing to the neurological disability and its progression.

7. Mechanisms of Axonal Degeneration Resulting from Demyelination

MS is viewed as a disease of two stages with vast axonal loss in the acute stage, associated with inflammation [30, 49]. Direct T-cell cytotoxicity, matrix metalloproteins, and cytokines have all been implicated in axonal transaction [58–60]. Incomplete and failure of remyelination results in gradual slow-burning loss of chronically demyelinated

axons which significantly contributes to global axonal loss [61], a mechanism that has also been observed in the normal-appearing white matter [62]. In the chronic stage of disease, a cumulative loss of axons over many years is observed despite a decrease in inflammatory activity. Thus, it is understood that this represents a slow-burning axonal disruption phenomenon to the point where the CNS cannot compensate. The exact mechanism through which chronically demyelinated axons degenerate is not known.

There is no doubt that sodium channels have an important role to play, both in restoring conduction in demyelinated axons and in their degeneration (Figure 2). It is hypothesised that the accumulation of sodium in the axon can lead to reversal of the sodium-calcium exchanger resulting in potentially lethal calcium levels [63]. Evidence for this initial mechanism is observed in postmortem tissue of acute MS lesions where APP positive axons are associated with co-localisation of $Na_v1.6$ channels and the Na^+/Ca^{2+} exchanger [64]. Elevated calcium levels are further exacerbated by an increase in expression of N-type voltage-gated calcium channels in MS [65]. Calcium can activate multiple pathways including those of calpains of which high levels are recognised to be a final common pathway to cell death [13, 66]. Calpain inhibitors, preventing axonal loss, identify the calcium-mediated pathway as the final step toward axonal degradation [67]. Evidence for the importance of sodium channels in axonal degeneration stems from a number of *in vivo* studies which show axonal protection as a result of the use of sodium channel blockers [68–71]. Loss of axons was ameliorated in the corticospinal tract of EAE animals when phenytoin treatment was given [70] which coincided with improving clinical outcomes. A protection against reduced conduction velocity was also observed. Flecainide and lamotrigine have also shown this effect in the same model with an improved functional outcome and reduction in neurological deficit, respectively, [68, 69]. It has been noted that exacerbation of EAE followed the withdrawal of two sodium channel blockers, phenytoin and carbamazepine [72] which was probably due to the associated marked increase in inflammatory cells.

A recent clinical trial of lamotrigine in secondary progressive MS patients produced intriguing results [73]. The primary outcome measure, white matter volume, and whole brain volume, showed an unexpected loss of partial cerebral volume in the treatment group. This difference between treated group and controls was no longer apparent once lamotrigine was discontinued, the whole brain volume increased in the treated group. As sodium channels are expressed in other cell types including immune cells, the outcome measurement of brain volume may be effected by oedema and loss of inflammatory cells as well as direct effects on neurodegeneration. For instance, the administration of phenytoin decreases the number of inflammatory cells by 75% [74], which may explain brain volume decreases in the treatment group.

Complementary to the hypothesis of increased axonal sodium via persistent sodium entry through $Na_v1.6$, is that of the lack of the sodium-potassium ATPase on some chronically demyelinated axons. This is not a rare phenomenon with more than half of chronic lesions containing axons in which only 50% express the Na^+/K^+ ATPase [5]. In these axons, already high sodium axonal levels are exacerbated by the failure to extrude sodium via the Na^+/K^+ ATPase [75]. The fact that these axons exist at all in chronic lesions is interesting. One of the reasons may be the lack of $Na_v1.6$ channels along the axolemma, as only a third of axons in chronic lesions express $Na_v1.6$ and do so only in a patchy rather than continuous pattern. These axons do not appear to be degenerating, highlighted by the lack of positive APP staining. Further evidence comes from the lack of association with the sodium-calcium exchanger with these axons[76]. It is probable that rather than suffering from degeneration, they lack the capacity to electrically conduct as the Na^+/K^+ ATPase is responsible for returning the axonal membrane potential to normal following an action potential.

Mitochondrial adaptations in demyelinated axons appear crucial to maintaining the axonal integrity and to preventing oxidative damage [52]. This is a point often missed but may turn out to be just as important as an increase in energy demand. In those axons with cytoskeletal changes induced by demyelination mitochondrial function is of high importance in order to facilitate the operation of the sodium-potassium ATPase. An energy-deficient state mediated by any mode of mitochondria dysfunction will render the axon nonconductive. Indeed nitric oxide, an inhibitor of complex IV, has been shown to contribute to axonal dysfunction and degeneration [55, 77].

Mitochondrial changes may have an underlying yet under appreciated role in MS and may explain the lack of consistent relationship between inflammation, demyelination, and axonal loss. Indeed, conduction block rather than axonal loss has been shown to occur following demyelination [78]. Energy deficiency or mitochondrial defects may cause conduction block particularly in demyelinated axons [79]. The preferential loss of small-diameter axons in MS with relative preservation of large axons may also be explained by these mitochondrial changes. The relatively reduced volume-to-surface-area ratio in small axons may indicate that small-diameter axons do not have the same capacity for mitochondrial changes as their large axon counterparts.

8. Conclusions

Mitochondria's multiple functions implicate them as crucial players in a healthy central nervous system, and their functional failure, therefore, can result in catastrophic events, as evident in primary mitochondrial disorders. Energy changes associated with demyelination and dysmyelination including the redistribution of ion channels and pumps requires an associated increase in mitochondria. Neuropathological studies and evidence from animal models suggest that demyelinated axons are heavily reliant on mitochondria to fulfil both energy demands and oxidative stress protection. Indirect observations point to the fact that this is an essential requirement of the axon rather than a pathogenic event. An energy deficit is likely to lead to an accumulation of sodium in axoplasm, reversal of sodium/calcium exchange,

and axonal degeneration. An energy deficit may be the result of not just oxidative phosphorylation defects but also altered mitochondrial transport, fission, fusion, and biogenesis. Given the limitations of sodium channel blockade and the important role of mitochondria in CNS disorders with demyelination, protection of axonal mitochondria seems a priority to preserve demyelinated axons, and developing treatments warrants further investigation.

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Review Article

Mechanisms of Oxidative Damage in Multiple Sclerosis and a Cell Therapy Approach to Treatment

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Although significant advances have recently been made in the understanding and treatment of multiple sclerosis, reduction of long-term disability remains a key goal. Evidence suggests that inflammation and oxidative stress within the central nervous system are major causes of ongoing tissue damage in the disease. Invading inflammatory cells, as well as resident central nervous system cells, release a number of reactive oxygen and nitrogen species which cause demyelination and axonal destruction, the pathological hallmarks of multiple sclerosis. Reduction in oxidative damage is an important therapeutic strategy to slow or halt disease processes. Many drugs in clinical practice or currently in trial target this mechanism. Cell-based therapies offer an alternative source of antioxidant capability. Classically thought of as being important for myelin or cell replacement in multiple sclerosis, stem cells may, however, have a more important role as providers of supporting factors or direct attenuators of the disease. In this paper we focus on the antioxidant properties of mesenchymal stem cells and discuss their potential importance as a cell-based therapy for multiple sclerosis.

1. Introduction

In recent years, clinical trials of stem cell therapies for neurological disorders have begun. Specifically in multiple sclerosis (MS), a number of trials studying the potential of bone-marrow-derived stem cell therapies have been published [1–3]. The initial experimental rationale was to regard stem cells as multipotential cells capable of differentiating into central nervous system cells able to replace lost or damaged cells in diseased tissue. Indeed, major research programmes of myelin repair are ongoing [4]. A further, and potentially more clinically applicable, function of stem cells is their ability to modulate disease processes. Mesenchymal stem cells (MSCs) have potent immune modulatory effects in experimental models [5]. Furthermore, MSCs are able to secrete a variety of substances that may attenuate disease processes or provide trophic support for the diseased nervous system [6, 7]. In MS, oxidative stress is associated with significant damage to myelin and axons, which in turn leads to clinical symptoms [8]. A major research strategy for many years has been to develop therapies which reduce the damage caused by oxidative stress and thus reduce tissue injury. This paper will focus on stem cells, and specifically MSCs, as providers of antioxidant function for central nervous system cells.

2. Mechanisms of Tissue Damage in MS and Experimental Models of CNS Inflammation

2.1. The Immunology of Multiple Sclerosis. Multiple sclerosis has classically been thought of as a T-cell-dependent process associated with macrophage-mediated demyelination driven by myelin-specific autoantigens. Evidence for the central role of T cells includes the presence of Th1 (T helper) cytokines, receptors, and cells in the CSF, circulation, and lesions of MS patients [9–11]. Furthermore, $CD4^+$ T cells polarized to Th1 phenotype play a central role in the animal model of MS, experimental autoimmune encephalomyelitis (EAE) [12]. In recent years, however, it has become clear that the

immunological interplay in MS is much more complicated than first thought. Evidence countering the central role for $CD4^+$ T cells includes the fact that MHC class 1-restricted $CD8^+$ cells are the predominant cell type found in active MS lesions [13]; lymphocytes may not be present in early demyelinating lesions and perivascular inflammatory cuffs can occur in normal appearing white matter [14]. In addition, therapies such as anti-interleukin 12p40 that target $CD4^+$ T-cell function have proved ineffective in clinical trials [15]. These and other developments have led to the need for further interrogation of the underlying immunology of the condition and redirected efforts to focus on alternative cell types that may contribute to the pathogenesis of MS. Previously, unknown contributors to the disease process include Th17 cells (producing IL 17), B cells, $CD8^+$ cells, and both $CD4^+$ and $CD8^+$ T-regulatory cells. Other effector populations include $CD56^+$ natural killer cells, invariant NK cells, and stem cells [16]. There is also evidence for the role of humoral immunity in MS demonstrated by the presence of immunoglobulin on macrophages actively phagocytosing myelin [17], immunoglobulin and complement in degenerating myelin sheaths [18], and by the occurrence of plasma cells in plaques [19].

2.2. Patterns of Tissue Injury in MS and Experimental Demyelinating Models. Pathological changes noted in postmortem or (more rarely) biopsy tissue from patients suffering from MS have revealed some of the mechanisms of tissue damage. Several patterns of tissue injury have been demonstrated. Although only able to offer a "snap shot" of tissue damage, certain common themes, some specifically relating to oxidative damage, have emerged.

(i) *Classical Actively Demyelinating Lesions.* There is a broad spectrum of immunological findings with some lesions dominated by T cells and macrophages, whilst others are notable for their immunoglobulin and complement components. Experimental demyelinating lesions can be induced by cytotoxic T cells, autoantibody generation, and genetic abnormalities giving further indications of the variety of factors that may determine an in vivo lesion [20]. In EAE, inoculation with myelin components, including myelin basic protein (MBP), proteolipoprotein, myelin oligodendrocyte glycoprotein, myelin-associated glycoprotein, and S100 protein, elicits an immunological response mediated via $CD4^+$ MBP-reactive T cells resulting in paralytic signs in the host organism. In EAE, demyelinating lesions require the presence of T cells, myelin autoantibodies, and complement activation though the relevance of this observation to MS is not fully established [21].

(ii) *Slowly Expanding Lesions of Progressive MS.* These are notable for the presence of a rim of activated microglia at their periphery with associated active demyelination. Macrophages containing myelin degradation products are not found within these lesions. T-cell infiltrates are located perivascularly but are not prevalent [22].

(iii) *Cortical Demyelination.* Cortical lesions are most commonly found in the deep sulcal structures in band-like subpial lesions [23]. Within these lesions, profound microglial activation can be viewed largely in the absence of lymphocytes [24]. The presence of a multitude of inflammatory cells within the meninges overlying these lesions has led some to hypothesise that they are driven by soluble factors released by the overlying cells [20].

(iv) *Diffuse White Matter Injury.* The so called "normal appearing white matter" (NAWM) (defined on magnetic resonance imaging) in fact demonstrates pathological abnormalities in MS patients, particularly in the progressive stages of the disease [25]. There is a predominance of $CD8^+$ MHC class I-restricted T lymphocytes in a diffuse inflammatory infiltrate found particularly in the perivascular space. There is widespread activation of microglia expressing footprint activation antigens of radical production, and microglial activation is closely associated with diffuse axonal injury/loss. There is no doubt that some Wallerian degeneration of axons occurs following focal demyelinating lesions, but within the NAWM there is a greater degree of inflammation and microglial activation than would be expected with Wallerian degeneration alone [20]. Many of the mechanisms leading to diffuse injury in NAWM are poorly understood; within activated microglia, frequently found in NAWM, there is expression of type-II nitric oxide synthases suggesting that oxygen and nitric oxide radicals are involved in the process; nitric oxide/oxygen free radicals inhibit enzymes of the respiratory chain causing mitochondrial dysfunction [26] which may be an important factor driving axon dysfunction; the size-dependent axonal loss in NAWM suggests energy deficiency which may also play an important role in the progressive axon loss characterising the latter stages of the disease [27].

2.3. Oxidative and Nitrative Stress. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated as part of normal cellular physiology. However, if there is overproduction of ROS or a failure of antioxidant mechanisms, these species can cause damage to lipids, proteins, and nucleic acids and may lead to cell death. CNS neurones are constantly exposed to low levels of these oxidative/nitrative species which can easily be dealt with by inherent repair and protection mechanisms. In the inflammatory state, however, these defences can become overwhelmed leading to oxidative/nitrative stress and damage to the basic structural and functional elements of the cells. Reactive oxygen and nitrogen species include superoxide ions, hydrogen peroxide, nitric oxide, and peroxynitrite, all of which are produced as part of the inflammatory response and have a potential role in tissue damage in multiple sclerosis. High levels of NO, peroxynitrite, and superoxide have all been demonstrated in spinal fluid from patients with MS [28].

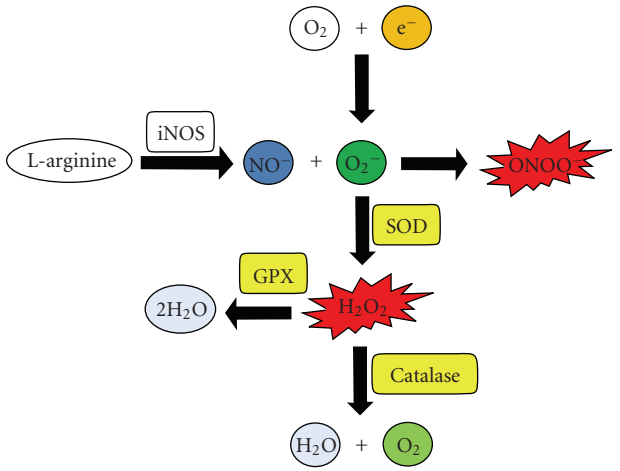


Figure 1: Cellular detoxification of reactive oxygen and nitrative species. Oxygen is reduced to superoxide (O_2^-) during inflammation, and nitric oxide (NO^-) is generated by the action of inflammatory nitric oxide synthase (iNOS) on L-arginine. In the absence of detoxifying enzymes, NO^- and O_2^- react to produce the highly toxic peroxynitrite ($ONOO^-$). Superoxide dismutase (SOD) competes for the superoxide anion and dismutes it to form hydrogen peroxide (H_2O_2) which can then be removed by the enzymes catalase and glutathione peroxidase (GPX).

There are several reasons why the CNS is particularly vulnerable to oxidative damage. These include the fact that brain tissue is very active in oxidative metabolism leading to relatively high levels of intracellular superoxides: the limited ability of the CNS to engage in anaerobic respiration resulting in high levels of superoxides in a hypoxic environment [29]; cellular features predisposing to oxidative damage within the oligodendrocyte population including low levels of antioxidant defences, membrane elaborations, and high iron content; the composition of myelin as a preferential target of ROS due to high protein : lipid ratio [30].

2.3.1. Reactive Nitrogen Species. Nitric oxide (NO) is produced in the nervous system in response to inflammation through the induction of inflammatory nitric oxide synthase (iNOS). It has been demonstrated that there is increased iNOS production, and by implication NO species, in the CNS of animals with EAE, [31]. There is also evidence of increased proinflammatory cytokine production in MS, and by extension NO [9]. Further indirect evidence of NO production includes the following: TNFα and IFNγ have been identified in astrocytes within both CNS lesions and CSF white blood cells of patients with MS [32]; higher levels of NO have been demonstrated within the peripheral monocytes of patients with MS compared with control subjects [33]; nitrite and nitrate levels are elevated in the CSF of patients with MS [34]. Direct evidence includes the fact that iNOS mRNA has been identified in MS plaques [35] with evidence that macrophages, astrocytes, and microglia within active lesions express high levels of iNOS and endothelial NOS [36]. There is also evidence for the presence of reactive nitrogen species in MS through nitrotyrosine, a marker of the presence of peroxynitrite, which is found in the diseased brain particularly in areas of demyelination and inflammation [37].

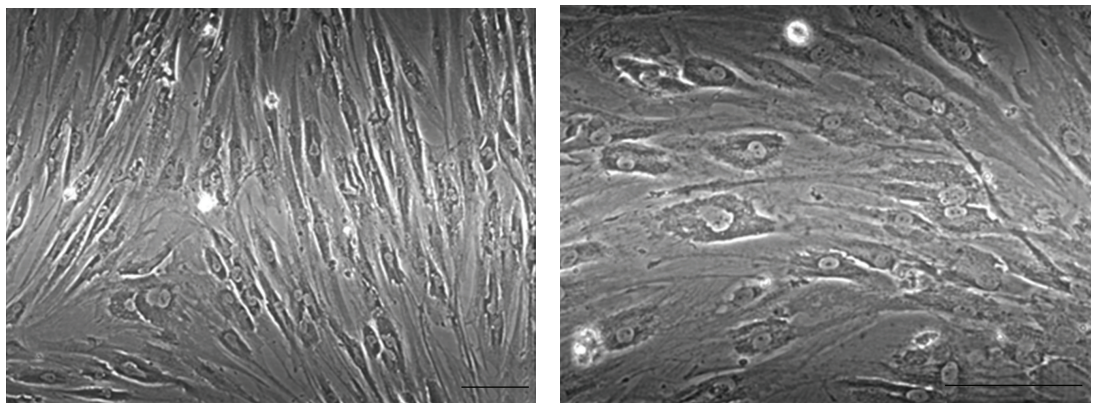
Reactive nitrogen species have a wide variety of effects on cells through the modification of protein structure and function: they inhibit several enzymes involved in respiration thereby disrupting mitochondrial function and reducing ATP content as demonstrated in neurons exposed to NO [32]; NO is known to affect several of the enzymes involved in oxidative defence including catalase [38]; it has been hypothesised that oxidation also results in the production of epitopes which may provoke autoimmune responses [39]; peroxynitrite can lead to cell death through a number of mechanisms including affecting cell signalling and through DNA breakdown [40]; NO can also deaminate DNA [41] and inhibit repair mechanisms [42]. Both reactive nitrogen and oxygen species also affect lipid peroxidation and consequently membrane function/permeability, which has implications for the function of embedded proteins within the lipid bilayer [43].

Oligodendrocytes show particular susceptibility to NO species and can even be lysed by the levels of NO produced by activated microglial cells. Experiments have shown that this lysis can be prevented in coculture by the addition of antagonists of NO production [44].

High levels of phosphorylation within axon neurofilaments protect against proteolysis. Dephosphorylation renders the axons more susceptible to proteolytic damage and axonal degeneration [45]. It has been shown that inflammatory agents, such as nitric oxide (NO), reduce neurofilament phosphorylation levels and thereby facilitate axon destruction. It is also known that inflammation causes conduction block and has been demonstrated *in vivo* that NO/derivatives can block conduction in both central and peripheral axons and that demyelinated axons are particularly vulnerable to NO-mediated block [46].

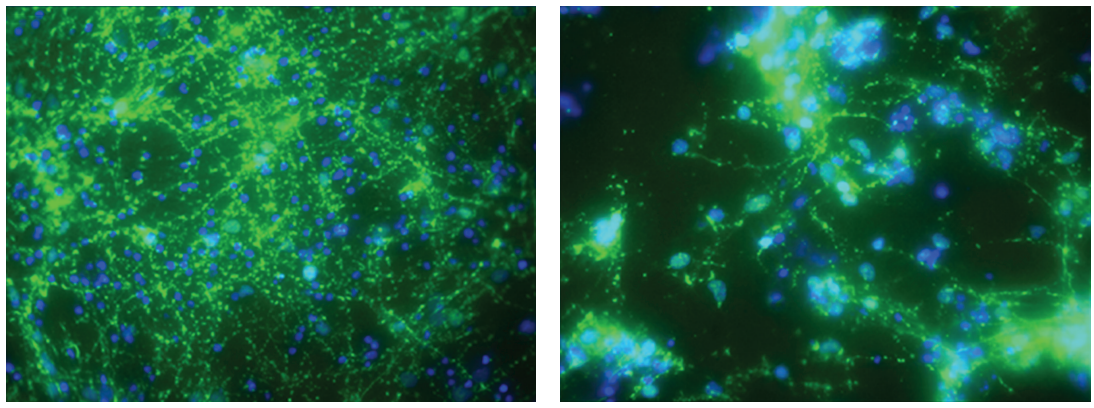
2.3.2. Reactive Oxygen Species. Extensive evidence implies increased ROS production in inflammatory demyelinating diseases. Human microglia are one of the most potent producers of superoxide [47], and it is known that during inflammatory demyelinating disease cells such as these are recruited to lesions within the CNS. Neurons also produce ROS in response to electrical activity following eicosanoid production driven by calcium cellular influx [48]. Direct evidence of lipid peroxidation has been demonstrated in postmortem brain tissue with findings pointing to a pivotal role in early plaque evolution [49]. There is also evidence for increased ROS production in EAE with macrophages and microglial cells exhibiting high levels compared with controls [50] and for higher levels of superoxide throughout affected brain areas [51]. It has also been noted that peroxynitrite is formed very early in the course of EAE and correlates with disease activity [52].

Oligodendrocytes are susceptible experimentally to ROS-mediated damage at levels which do not affect astrocytes or macrophages [53]. The high levels of iron found in oligodendrocytes, reacting with hydrogen peroxide and



(a) (b)

Figure 2: Human bone-marrow-derived mesenchymal stem cells in culture (scale bar = 100 microns).



(a) (b)

Figure 3: Nitric oxide promotes axonal injury to cerebellar neurones in vitro. Immunofluorescent images depicting cerebellar axonal morphology (a) pre- and (b) posttreatment with 0.1 mM NO. Green: axonal marker SMI 312. Blue: DAPI nuclear stain.

leading to the formation of the highly toxic peroxynitrite, may explain this susceptibility. In addition, low oligodendrocyte levels of glutathione, the failure of expression of Mn-SOD, and low levels of metallothionein, all important antioxidants, may contribute [54–56]. Hydrogen peroxide is produced in peroxisomes which are particularly abundant in oligodendrocytes during the period of active remyelination, contributing to the failure of long-term repair of myelin and the axon loss associated with the progressive stages of the disease. Furthermore, preoligodendrocytes appear to be significantly more sensitive to oxidative stress compared to mature oligodendroctes [57] providing further barriers to repair and remyelination. ROS also have direct effects on the lipid and protein components of myelin, directly through peroxidation and indirectly through the production of matrix metalloproteinases which have been shown to degrade myelin basic protein.

2.3.3. Detoxification of Reactive Oxygen and Nitrogen Species. Cells possess a diverse array of defence mechanisms to reduce potentially harmful build-up of ROS, specifically a number of antioxidant enzymes including superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase (Figure 1). The balance between ROS/RNS and detoxifying enzymes within inflamed tissue may be crucially important in determining the extent of cellular damage and tissue injury.

The cause of axonal degeneration in secondary progressive disease is unknown, but recent evidence has suggested a role for central nervous system peroxisomes in axonal maintenance [58]. Peroxisomes are cellular organelles which are involved in a number of anabolic and catabolic reactions and may have an important role in the detoxification of inflammatory compounds such as ROS. Both SOD and catalase are produced by peroxisomes, and catalase is specific to peroxisomes. Inherited abnormalities in peroxisomal function cause a variety of neurological disorders, including X-linked adrenoleucodystrophy and adrenomyeloneuropathy, which share many clinical, radiological, and neuropathological features with multiple sclerosis [59]. Furthermore, defects in peroxisomal function within the central nervous

system have been implicated in experimental models of neuroinflammatory and neurodegenerative disease [60]. Thus, peroxisomes may have vital functions in multiple sclerosis of limiting tissue damage. We have previously shown that nitric oxide causes significant axonal and neuronal damage in cell culture models (Figure 3) [61] and that the addition of recombinant SOD to cultures protects cerebellar neurons from nitric-oxide-mediated injury [62, 63].

3. Cell-Based Approaches to Combat Oxidative Damage in Multiple Sclerosis

A number of different cell-based approaches to repair and protect against tissue damage in MS have been postulated and are the subject of intense research. Examples include haematopoietic stem cells which have been used in an immune-reprogramming capacity and neural stem cells which are predominantly being explored for their regenerative potential. Meanwhile, mesenchymal stem cells (MSCs) (Figure 2) have emerged as promising candidates in the protection of neurones against the oxidative damage encountered in MS. Here we describe the biology of MSCs and the antioxidant properties which may have future relevance to the treatment of the disease.

3.1. Mesenchymal Stem Cells. The potential therapeutic applications of MSCs for neurological disorders have generated great interest. To date, human MSC transplantation has been shown to improve outcome in a variety of animal models of neurological disease including that of experimental autoimmune encephalomyelitis, stroke, amyotrophic lateral sclerosis, Krabbe's disease, and spinal cord injury [64–77]. Pilot translational studies of MSC therapy have also commenced in stroke [78], multiple system atrophy [79], amyotrophic lateral sclerosis [80], metachromatic leukodystrophy, and Hurlers syndrome [81], as well as in multiple sclerosis [82]. Administration of MSC is an attractive therapeutic option in these disorders as MSCs are easily isolated from various anatomical sources, have versatile growth and differentiation potential, and their immunosuppressive properties make it probable that allogeneic as well as autologous cell therapy could be considered. This is of importance since in clinical therapies the use of an allogeneic source for cellular therapy is likely to be more convenient and feasible than an autologous population which would take weeks to prepare [83]. Despite having a favourable safety profile in comparison with some other types of stem cells, there are undoubtedly risks associated with MSC treatments which should not go unexplored. The use of an allogenic MSC bank, for example, would carry with it the risks of chromosomal abnormalities associated with long-term cell culture and development of therapies will require the provision of careful and considered safety studies and protocols.

MSCs were first identified in 1966 in studies by Friedenstein et al., who isolated bone-/cartilage-forming progenitor cells from rat bone marrow cells with fibroblast-like morphology [84]. Since this discovery, the most studied and accessible source of MSCs has been the bone marrow, although MSCs have been isolated from a number of tissues including the liver, foetal blood, cord blood, and amniotic fluid [85–93]. Unlike the haematopoietic stem cells and their progeny, cultured MSCs express a number of nonspecific surface markers, none of which, individually or in combination, have been shown to achieve high levels of MSC isolation and enrichment [94–98]. Within the bone marrow, MSCs comprise 0.001–0.1% of the total population of nucleated cells [85]. MSCs may be considered multi-potent stem cells that have the ability to differentiate down both mesenchymal and nonmesenchymal lineages. Human MSCs contribute to the regeneration of mesenchymal tissues such as bone, cartilage, muscle, ligament, tendon, adipose, and stroma [85] and can also give rise to cells with ectodermal and endodermal phenotypes [98–104].

Research suggests that transplantation of MSCs has the potential to be an effective treatment for neurodegenerative disorders through a multitude of different mechanisms including replacement of lost cells by differentiation into functional neural tissue, modulation of the immune system to prevent further neurodegeneration, and provision of trophic support for the diseased nervous system [105]. Bone-marrow-derived MSCs are able to evade the allogeneic immune system, as well as suppress immune responses directed against third-party cells following intravenous infusion [106–109]. In addition, when infused into the circulation, MSCs have the capacity to migrate specifically to sites of brain injury, thus targeting sites for neural repair [75–77]. Although MSCs display a plethora of neuroprotective and regenerative properties, increasing evidence implies that the major mechanistic neuroprotective role of bone-marrow-derived MSCs is their capacity to secrete a diverse range of potentially neuroprotective factors including antioxidants [5, 6, 105, 110–112]. With many antioxidant drugs emerging as potential therapeutic agents for neurodegenerative disorders [113, 114], these findings emphasise the potential for bone-marrow-derived MSCs as therapeutic agents for CNS neurodegenerative disorders, especially disorders in which oxidative damage is a key aetiological component.

3.2. Antioxidant Properties of Mesenchymal Stem Cells. MSCs have direct antioxidant activity that is conducive to neuroprotection both *in vivo* and *in vitro* [5, 7]. *In vitro* studies have shown that MSC-conditioned media can confer a neuroprotective effect against oxidative insult to both primary cortical and cerebellar neurons, and also neuroblastoma cell lines [5–7, 63]. Evidence suggests that one method by which MSCs exert a neuroprotective effect against oxidative stress is through the modulation of signalling pathways involved in antioxidant and stress-related processes. Nitric oxide, whilst performing many physiological roles at low levels, has been shown to induce apoptosis in a variety of cultured peripheral and central neurons and be involved in degeneration during central nervous system inflammation [115, 116]. It has been established that these effects are mediated through the p38 MAP kinase pathway [61]. Secreted protein factors, including neurotrophic factors and cytokines, are thought to have the

ability to inhibit death-inducing pathways and also activate cell survival pathways [117, 118]. MSCs have been shown to protect neurons against toxic insults via modulation of both the PI_3kinase/Akt and MAP kinase pathways [7].

Another mechanism by which MSCs can exert a direct antioxidant effect is through the secretion of antioxidant molecules. We have recently shown that bone-marrow-derived MSCs secrete the extracellular antioxidant molecule superoxide dismutase 3 (SOD3) [7, 63]. The superoxide dismutase family member SOD3 is the only antioxidant enzyme that scavenges superoxide in the extracellular compartment [119] and limits the formation of strong neurotoxic oxidants including both the hydroxyl radical and peroxynitrite in the extracellular space. It has been demonstrated in a variety of studies that SOD3 can attenuate tissue damage and inflammation [120–128]; in addition, SOD3 secretion by human MSCs has been shown to provide direct neuroprotection in cerebellar neurons exposed to nitric oxide and activated microglia [7, 63]. Experiments show that SOD3 secretion by human bone-marrow-derived MSCs is regulated synergistically by the inflammatory cytokines TNFα and IFNγ [63]. Both TNFα and IFNγ are important mediators of the immune system and inflammatory processes in the CNS and are therefore present in the diseased brain. In EAE and other central nervous system inflammatory disorders, both IFNγ and TNFα are upregulated and are critically involved in the initiation and amplification of the local immune response [129]. These cytokines also enhance the secretion of the superoxide ions by a variety of immune and nonimmune cells [130–133]. In summary, SOD3 secretion by MSCs is a potentially valuable and regulatable therapeutic anti-inflammatory property that may be of relevance to treatment strategies for inflammatory disease of the CNS.

Several studies have looked at the effects of transplanted MSCs on both the clinical course and immunopathology of EAE. MSC transplantation confers significant therapeutic capacity to modulate autoimmune processes, resulting in significant reductions in demyelination and lesion size within the CNS and also reductions in the number of cellular infiltrates in the brains of the host organism [68, 69, 134–137]. Inflammatory processes within the CNS involve activated microglia, astrocytes, macrophages, and lymphocytes, releasing a plethora of anti- and proinflammatory cytokines [138]. Evidence from both *in vitro* and *in vivo* studies suggests that MSCs have the ability to inhibit microglial activation and therefore attenuate inflammation [139–142]. It has also been shown that MSCs are capable of significantly decreasing inducible nitric oxide synthase (iNOS) expression within microglia [140], thus enhancing their antioxidative effect. Further supporting evidence has recently shown that MSCs inhibit molecules associated with neuronal damage and display a variety of antioxidant effects when administered to EAE mice [5].

4. Conclusions

MSCs possess a diverse range of properties making them attractive candidates for cell-based therapies. In particular, antioxidant functions may be utilised as a strategy to reduce inflammation-driven oxidative stress. Cell-based therapies for MS are currently in development, and the diverse modes of action of stem cells make them attractive candidates. Further work to enhance delivery and targeting of cells, plus optimising and regulating their antioxidant properties, is required, but stem cell therapies for MS may form an important part of the therapeutic armoury for the disease in years to come.

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Review Article

Multiple Sclerosis: Are Protective Immune Mechanisms Compromised by a Complex Infectious Background?

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The immunological background of multiple sclerosis (MS) manifests as an altered reactivity against a diverse range of infections, particularly with the Epstein-Barr virus. Although this could be only an epiphenomenon of a more generalised dysfunction of the immune system in MS, it is also possible that a complex infectious background forms the basis of a specific immune dysregulation finally causing the disease. It is thus suggested that the complex infectious background bears the key for an understanding of the immune pathogenesis of the disease. It appears probable that improved standards of hygiene cause regulatory defects in the immune system, allowing the abnormal expression of human endogenous retroviral (HERV) genes. On the basis of epidemiological observations we describe how a failure of expansion or an eclipse of a subfraction of self-antigen-specific $CD8^+$ T cells mediating immune repair, and a deleterious mode of action of HERV gene products, could underlie the pathogenesis of MS.

1. Introduction

Studies on the viral aetiology of disease have traditionally focused on exogenous viruses, but more recently attention has turned to the role of human endogenous retroviruses (HERVs) which comprise around 8% of the human genome [1]. Although a number of diseases are associated with abnormal formation of HERV-encoded gene products, it is difficult to establish whether such genetic expression is a cause or an effect of the disease process. Multiple sclerosis (MS), the pathogenesis of which is poorly understood [2], provides an ideal opportunity for the study of the way in which HERVs may be involved in the various stages in the evolution of a disease [3, 4].

Although it is widely accepted that there are strong endogenous and exogenous components to the aetiology of MS [5, 6], extensive studies failed to incriminate a single endogenous or exogenous agent [7, 8]. Instead they point to a complex infectious background to the pathogenesis of the disease involving a multitude of exogenous infectious agents and to a compromised immunological protective background [9, 10]. The causal relationship of any given agent to the aetiology of MS is difficult to determine as most or all the putative agents infect most human beings at some stage of their lives. On the other hand, the HERVs coded for by the human genome are part of the genetic background of all humans [3, 4, 11]. Accordingly, any causative agent may well be obscured.

2. Human Endogenous Retroviruses in MS

Human endogenous retroviruses (HERVs) have entered the human germ line successively over a period of millions of years [3, 4, 11], and are present in the genome of all host cells. Most HERVs are defective, having been inactivated by negative selection and an accumulation of mutations (deletions, termination codons, and frame shifts) and thus most of their genetic loci are inactive [12, 13]. Some reading frames have, however, maintained open and code for a complete protein, such as the ERVWE1 locus on chromosome 7q21 which codes for syncytin-1, and one member of the HERV-W family, the multiple sclerosis-related virus (MSRV), can form complete virions under certain circumstances [14]. Moreover, it is well established that

certain exogenous human retroviruses, HTLV-I and HIV, as well as endogenous retroviruses in other species, such as rodents, ovines, and birds, can be cause of neurological and malignant diseases [15].

In this respect it is of interest that the presence of HERV sequences, and in particular those of the γ-retroviruses HERV-W/MSRV and of HERV-H/F, have been found in association with MS [12, 16, 17], that the expression of ERVWE1/MSRV in brain tissue from MS patients has been reported [18, 19], and that the presence of such sequences in the CSF has been claimed to be higher in MS than in controls [14, 20, 21]. Another group claimed that it is only syncytin that is expressed at higher levels in brains of MS patients [22], although the techniques used to show this have been criticised [23]. In addition, increased levels of antibody reactivity to specific γ-retroviral HERV Gag and Env epitopes have been found in serum and CSF from MS patients [12], and these antibody levels are related with the activity of the disease [4]. The HERV-W encoded Env (ERVWE1, syncytin) is upregulated in glial cells in active MS lesions and, when expressed as a construct in astrocytes, has been shown to induce oligodendrocyte cell death via redox-reactants [24], as well as inducing an autoimmune cascade [3].

Despite of 20 years of research, the role of HERVs as pathogens is still controversial but, nevertheless, they are the leading candidates for a link between genetic predisposition and environmental factors in a disease such as MS.

3. Are Exogenous Viruses and Chlamydia Involved in the Pathogenesis of MS?

Besides the HERVs as described above, there are at least three groups of exogenous infectious agents that can be grouped according to the nature of the observations that putatively link them to MS. The first group is exemplified by measles, varicella, and herpes simplex viruses, but it may contain many more potential members. The second group comprises the human herpes virus 6 (HHV-6) and *Chlamydia pneumonia* and the third group is represented only by the Epstein-Barr virus (EBV) which has emerged as the leading candidate among the putative pathogens.

In the premeasles vaccination era it was observed that, in the history of MS patients, there were more clinically manifest cases of measles that were experienced only later in life, as compared with controls [7, 8]. Moreover, the concentration of measles-specific antibodies was shown to be consistently higher in MS patients [7, 8]. The introduction of measles vaccination in early childhood, however, had no striking impact on the epidemiology of MS [8, 25]. A similar situation is observed with infection by varicella as, in contrast to previous studies on mostly adult patients, observations on a cohort of children showed that a history of chickenpox was associated with a reduced risk of MS (Odds ratio 0.58) [26].

Since the differences between prevalence of infectious disease or of a specific antibody between the patient and control groups are small, large groups must be studied to reveal significant differences. On this basis, at least 12 candidate infections, including measles and varicella, could have an association with MS [7–9, 27]. Problems of interpretation of such findings are illustrated by herpes simplex. Although MS patients have a higher prevalence of antiherpes simplex type 2 (HSV-2) antibody as compared with controls, this does not reflect a higher prevalence of HSV-2 infections in the patients; the reason being an elevated concentration of specific antibodies generated by infection with herpes simplex virus 1 (HSV-1) that cross-react more strongly in MS patients than in controls with the diagnostic HSV-2 antigen [9]. Moreover, a recent study on patients with MS commencing in childhood permitted a check in parallel for an association of specific infections to MS by means of elevated specific antibody. This identified measles, varicella, and herpes simplex viruses ($P < .0001$) as the leading candidates, whereas an MS association for others including influenza, parainfluenza and rubella viruses ($P = .035$, .029 and .054, resp.), was less clear, questionable, or not confirmed [9]. Although there are interpretational problems, these observations find a parallel in studies on the synthesis of specific antibodies in the brain against the same range of pathogens, namely measles, varicella, herpes simplex, and rubella [28–30].

Association of infections with human herpes virus 6 (HHV-6) and *Chlamydia pneumoniae* (Cpn) to MS are weaker but serological parameters differ qualitatively from controls and it is possible that active, possibly chronically active, infections with these pathogens synergize with EBV to produce a dysregulated immune response. These two infections are significantly associated with MS. Thus elevated IgM antibody levels against Cpn in paediatric MS patients are indicative of current or recent active infection at the time of onset of MS [9]. A meta-analysis of 26 studies showed that, despite interstudy variability, patients with MS were significantly more likely than healthy controls or those with other neurological diseases to have detectable levels of Cpn DNA in their CSF, although the authors concluded that this finding did not establish an aetiological relationship [31]. In the case of HHV-6, repeated phases of coinciding virus activity with EBV in patients with MS have been described [32]. Moreover, there is an interesting series of systematic studies on targets of specific B and T cells that are detected more frequently in MS as compared with controls [33, 34]. These studies were originally intended to identify the potential target(s) of autoimmune processes. It was, however, not possible to confirm the higher frequency of such B and T cells specificities in subsequent studies, but other specificities became apparent and another aspect emerged. All the MS-associated epitopes had sequences homologous to those in proteins of EBV, HHV-6 as well as of Cpn, indicative of some interrelationship between the immune responses against the three different pathogens [10].

4. Observations on EBV Infection

In recent years interest has focused on the role of infection with EBV in the aetiology of MS [35–38]. A meta-analysis has established that >95% of all patients with MS have serological evidence of prior EBV infection, compared to 87% in control subjects [39]. There is, however, no serological evidence of

reactivation or active EBV infection in the patients at the time of onset of MS [9, 37, 40], and claimed extensive EBV infection of lymph follicles in the brain of MS patients [41] was not confirmed in subsequent studies [42–44] and remains an open question [45]. Furthermore, there is a lag phase of several years or a decade or more between EBV infection in the patients and the clinical manifestation of MS [46]. During this rather long time period, EBV-specific antibody may well have dropped below detectable levels in some persons. Also, taking into account the distribution of specific antibody concentrations in the study groups, it is not only possible but indeed likely that in the patient group there are virtually no EBV-naïve persons and, accordingly, past EBV infection has been postulated as a necessary condition for development of MS [9, 10, 37]. Accordingly, primary EBV infection appears to be extremely rare in those with established MS [46], and in a longitudinal followup of a large cohort of EBV-negative young adults, MS was observed to occur only subsequent to EBV infection [47].

In addition some qualitative differences were apparent in the antibody response to EBV in the patients with MS and the controls; in particular, the former had notably elevated antibody levels against the EBNA-1 protein of EBV [30, 37, 40, 48, 49]. The local synthesis of anti-EBNA1 antibody in CSF has been described in several studies [30, 49–53]. It was also demonstrated that the risk of developing MS increases with the level of antibody to this virus [48]. The link between EBV infection and the risk of MS is, however, unclear and is not easily determined in an adult population since the great majority of controls are, from the third decade of life, likewise infected by this virus.

Notwithstanding, there are a number of hypothetical mechanisms by which EBV might induce MS [36, 54–56], though none of those suggested convincingly explain the pathogenesis of the disease, and it appears more likely that other infections synergize with EBV to produce a dysregulated immune response years or a decade before the clinical onset of MS. Moreover, the pathology of MS, characterised by widespread blood-brain barrier defects and a multifocal involvement of grey and white matter, argues against a conventional role for a presumed viral aetiology [57].

5. Darwinian Medicine

Attention has recently focused on environmental factors associated with the increase in the incidence of several classes of disease in the industrially developed nations. The concept, forming the basis of the emerging discipline of "Darwinian medicine" [58], is that hygiene-related factors isolate the human population from micro-organisms, both pathogens and, probably more importantly, commensals, that are crucial to the establishment of beneficial immunoregulatory networks. Thus, in principle, an "interkingdom cross-talk" between microbes and the human host can establish patterns of immune reactivity that prevent various allergic, autoimmune, and inflammatory diseases while a failure of such cross-talk can facilitate them [59]. One consequence of improved hygiene is that certain infections that were previously regularly encountered in infancy now occur at a much later time in life and after other infections may have altered the patterns of immune responsiveness. Infection by EBV is a good example, and it has been postulated that various other infections acquired before EBV may affect immunoregulatory networks, thereby leading to an attrition or eclipse of those regulatory T cells (T_{reg}s) that would otherwise protect against MS [10]. In this context, T_{reg}s, though essential to immune function, may in some circumstances induce harmful effects and have therefore been termed a "dangerous necessity" [60].

Accordingly, a critical determinant of MS risk could be a compromised number or activity of protective T_{reg}s [61, 62]. During an active and specific T cell-mediated immune response there could well be a competition with other kinds of T cells, most likely T-helper-cells, recognising the same epitope as the T_{reg}s or epitopes closely spatially situated on the relevant antigen(s). As a consequence certain T-helper cell populations, that induce production of specific antibody, could become expanded and, thus, account for the diverse rise in antibody levels as epiphenomena with little or no pathologic importance. Notwithstanding, the local production of measles, varicella, or rubella-specific antibodies in the central nervous system can be useful for diagnosis [28–30], and the production of anti-HERV antibodies may become of use as prognostic factor for MS disease [4, 63].

6. Target Epitopes and HLA-Polymorphism

On the supposition that the diverse MS-associated infectious agents express epitopes that generate regulatory and effector T cells involved in the prevention of, or an enhancement of risk of, MS, the challenge was to identify the relevant epitope or epitopes.

The EBV EBNA-1 protein is the most likely candidate to express an epitope that could affect T_{reg}s, since the concentration of specific antibody against this protein is, as mentioned above, particularly and significantly elevated in MS, in both children and adults [9, 64]. Moreover, the EBNA-1 protein is expressed in latent EBV infection, and T cells recognising EBNA-1, play a key role in immune control of EBV in healthy persons [64]. Under these circumstances the number of epitopes of T-helper cells recognised on EBNA-1 is very limited but in MS many more epitopes across the entire C-terminal domain of the protein are recognised [65]. This "epitope spreading" could well be induced by prior and/or simultaneous infections by the candidate pathogens mentioned above, notably HHV-6 and *Chlamydia pneumoniae*, as these bear many homologies to the additional epitopes on the EBNA-1 protein [10]. This altered epitope recognition could divert the immune response away from the single putatively protective T_{reg} epitope (which is likely to be harboured within the sequence FENIAEGLRALLARSHVER) and to the generation of an alternative range of competing T-helper cells.

The further challenge is to determine the host target for protective and altered, nonprotective, patterns of immune

reactivity, and a likely candidate would be an epitope of a HERV-W peptide since, beside the few functional HERV-W proteins mentioned above, there are some open reading frames that code for hypothetical HERV peptides [10]. A candidate HERV epitope with the amino acid sequence MPVPSAPST was identified on a hypothetical HERV peptide and shown to have homologies in *all the pathogens* to which elevated antibody concentrations are found in MS patients, including EBV, measles, varicella, HSV, *Chlamydia*, HHV-6, influenza, parainfluenza, rubella, and others [10]. It was postulated that the effector T cell specific for this candidate epitope would most readily cooperate with the above-mentioned T_{reg}s to support a supposed MS-protective immune response when the homologies of the respective specific target sequences are present on the same viral protein. This situation is realized with three pathogens: measles, varicella/zoster, and herpes simplex virus (type 1 and 2) [10]. Interestingly, on the basis of elevated antibody concentration, measles, varicella/zoster, and herpes simplex virus 2 have a very clear association with MS in children ($P < .0001$, with Bonferroni-Holm correction for multiple testing), whereas the MS association of the other pathogens is dubious or unconfirmed [9]. It must be stressed that gene transcripts of 21 of 25 open-reading frames with an initiating start codon for the hypothetical HERV peptide have been found in association with MS [10]. This gene transcript is coded on the complementary strand of the HERV-W Env gene region. To our knowledge this would be the first example of genetic information on the noncoding DNA strand of the human genome being implicated in immune processes relevant to health and disease!

It is therefore possible in principle to use epidemiological data and genetic databases to identify candidate targets of the relevant immune cells, and verification may come from future studies on HLA polymorphism. The relevance of the HLA polymorphism, for example, in MS has been demonstrated very clearly [66, 67], but though over 99% of individuals appear to be genetically incapable of developing MS, no specific gene making a major contribution to susceptibility to MS has yet been identified. In the light of the framework presented here, there is a need to investigate whether the critical determining factor in such susceptibility is a genetically determined absence of, or defect in, HLA molecules able to present the relevant peptides for supposed MS-protective T_{reg}s [61, 62] and T-effector cells and the presence of HLA molecules presenting homologous peptides to competing T cells with other functions.

7. Immune Repair Protecting against Disease Progression, Malignant Transformation, and Autoimmunity

The suggested mechanism of pathogenesis of MS presented here, based on the infectious background of the patient, is a novel one, but it may have a precedent in the pathogenesis of what, at first view, appears a completely unrelated disease, namely, melanoma [68–70]. There are parallels between the two diseases as in both it has been postulated that a HERV associated pathology, based on expression of HERV-encoded genes and the presentation of HERV-encoded peptides by HLA molecules, may be of critical relevance to pathogenesis [68, 69]. As proposed for MS, there is evidence that immune recognition of, and response to, such peptides contribute to the immune surveillance of the initiating events of the pathogenesis of melanoma years or decades before the onset of clinical disease. On the other hand, there are major differences between the two diseases, with MS being more complex. In the case of melanoma, the available evidence indicates that a major component of immune-mediated protection results from the induction, by a range of natural infections and vaccinations, of populations of effector T cells that cross-react with a HERV-encoded epitope, HERV-K-MEL, on a peptide coded for by an open reading frame of a HERV-K, and expressed on the surfaces of malignantly transformed melanocytes [69–71]. Indeed, vaccination with BCG or vaccinia early in life, or with yellow fever vaccine in adults, confers around 50% protection against melanoma later in life [68, 72, 73]. There is, however, no evidence that the mechanism for eclipse of protective regulatory cells as postulated for MS plays a role in the pathogenesis of melanoma.

It must be emphasised that HERV-encoded peptides are self-antigens and thus, in principle, able to be recognized by self-specific $CD8^+$ T cells, the developmental biology and function of which has attracted considerable interest in recent years [74–76]. These cells belong to a distinct genetic lineage and have different developmental requirements than those of conventional $CD8^+$ T cells. They undergo clonal proliferation when activated by infectious agents or vaccines bearing homologous epitopes [77], and may persist as self-specific memory cells for long periods of time. It is, however, important to note that self-specific $CD8^+$ T cells do not necessarily result in autoimmune reactions deleterious for the host but can instead result in immune repair.

In this context, while much attention has been paid to cell-mediated immune reactions resulting in cytotoxicity, much less interest has been shown towards mechanisms of cell repair, particularly in circumstances where conservation of cells, such as in the central nervous system, is of great importance. One such repair mechanism that has been studied involves gangliosides of the neolacto series, especially LM1, which are transferred from leucocytes to target cells by direct cell-to-cell contact [78–81]. In this context, gangliosides may be involved in the process of methylation of DNA which is the most important means by which a somatic cell can repress or silence the genetic expression of HERV-encoded genes [82, 83]. Thus, differential display analysis of gene expression established that LM1 mediates suppression of retroviral RNA [68, 79]. A mechanism for this is suggested by the finding that LMI induces S-adenosyl-homocysteine-hydrolase, an enzyme essentially involved in the generation of active methyl groups required for the process of methylation, as well as inducing a kelch-1-like protein, one of a family of proteins that mediate transcriptional repression [84].

8. Overexpression of HERV Env Proteins

The observations summarised above raise the question of how abnormal HERV expression can induce pathological changes at the cellular level and how these changes can be prevented [3, 70, 84]. One aspect that is dealt with in the various papers in this special issue of the journal is the diversity of mechanisms that induce autoimmune phenomena in MS, an aspect that has been critically reviewed elsewhere [85]. Another aspect is disturbed redox processes [24, 86, 87], as oxidative stress is certainly implicated in demyelinating disorders [88].

Retroviral Env proteins are glycoproteins that are able to cause neuroinflammation, neurodegeneration, and endoplasmic reticulum stress [86]. In MS the ERVWE1 Env, syncytin-1, is particularly overexpressed in glia where it induces endoplasmatic reticulum stress leading to the induction of free radicals that damage nearby cells [24, 86, 87]. The involvement of different cell components has been demonstrated, notably ASCT1, a receptor for syncytin-1, and a neutral amino acid transporter which were selectively suppressed in astrocytes. Syncytin-1 induces the expression of the endoplasmatic reticulum stress sensor (OASIS) and overexpresion of this sensor in astrocytes increases levels of inducible NO synthase with ensuing oligodendrocyte injury. Studies on transgenic mice gave insights into the role and mechanisms of HERV Env proteins in causing neuroinflammation and autoimmune processes, and confirmed human observations [86].

In melanoma, epidemiological observations led to the suggestion that HERV-K Env induced biosynthesis of "melanoma-melanin" via reduced levels of glutathione-peroxidase [68, 88, 89]. This modification of melanin is able to incorporate more heavy metal ions as compared with physiological eumelanins, in particular mercury, and tends to become oxidatively charged. Melanoma-melanin, responsible for the black colouration of melanomas, is detectable in dysplastic and congenital naevi cells, the presumed precursors of melanoma, but not in normal melanocytes. Melanin pigments have a very long life span and oxidatively charged melanoma melanin can thus catalyse the formation of harmful long-living reactive oxygen species and radicals over long periods of time, whereas normal melanin destroys them. Affected cells are therefore very vulnerable to any kind of oxidative stress, paving the way to cell degeneration and to malignant transformation through chromosome damage [90].

Moreover, in melanoma, interest has focussed on a distortion of the immune response caused by expression of HERV-encoded proteins leading to tumour escape and autoimmune processes [69], although questions of cause and effect remain.

These considerations on melanoma raise the question of whether similar mechanisms involving HERV-encoded proteins and melanin pigments could operate in MS. Although there is no direct evidence, there are some suggestive factors. Among the several environmental risk factors for MS [6], a leading one is a low level of bioactive vitamin D3, but it is still controversial whether any and, if so, which of the many actions of this vitamin is critical for MS [91]. It has been suggested that low levels of this vitamin cause, via γ-glutamyl-transpeptidase, a reduction in the intracellular levels of glutathione, as has been demonstrated in rat astrocytes [92]. The risk-enhancing effect of low vitamin D3 levels appears to commence during early *in utero* development as persons born in November/December have the least MS risk while those born in May/June have the highest risk. This difference is statistically significant [93]. The long-lasting impact of this effect may result from favouring the peroxide driven biosynthesis of melanoma-like melanin and by the long-living nature of the polymeric melanin redox pigments including pro-oxidative variants. Cigarette smoking, particularly when started early in life, is a risk factor for MS [94, 95], and for an early conversion to clinically definite multiple sclerosis [96]. The mechanism of this risk could be the generation of reactive oxygen species.

9. Conclusions

In view of the possible mechanistic link between the pathogenesis of melanoma and MS, further studies on the role of HERVs in MS, and their comparison with HERV-K in melanoma are indicated.

Although the two diseases under consideration, MS and melanoma, are quite different, there are parallels in that both conditions develop in tissues originating from the neural crest and that both can be explained in terms of "Darwinian medicine" [58, 69]. A complex pattern of exogenous infections and activation of endogenous retroviruses is apparently underlying the aetiology of both conditions.

Combining the analysis of epidemiological data with genetic data bank entries of infectious pathogens and of humans, it is possible to identify deficits within the regulatory networks of the immune system. We are convinced that on this basis it will become possible in the future to establish rational preventive and therapeutic measures for MS.

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Review Article

Heterogeneity in Multiple Sclerosis: Scratching the Surface of a Complex Disease

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Multiple Sclerosis (MS) is the most common demyelinating disease of the central nervous system. Although the etiology and the pathogenesis of MS has been extensively investigated, no single pathway, reliable biomarker, diagnostic test, or specific treatment have yet been identified for all MS patients. One of the reasons behind this failure is likely to be the wide heterogeneity observed within the MS population. The clinical course of MS is highly variable and includes several subcategories and variants. Moreover, apart from the well-established association with the HLA-class II DRB1*15:01 allele, other genetic variants have been shown to vary significantly across different populations and individuals. Finally both pathological and immunological studies suggest that different pathways may be active in different MS patients. We conclude that these "MS subtypes" should still be considered as part of the same disease but hypothesize that spatiotemporal effects of genetic and environmental agents differentially influence MS course. These considerations are extremely relevant, as outcome prediction and personalised medicine represent the central aim of modern research.

1. Introduction

Multiple Sclerosis (MS) is a debilitating disease of the central nervous system (CNS) pathologically characterized by myelin loss and axonal degeneration. Although more than 100 years have passed since Charcot, Carswell, Cruveilhier, and others described the clinical and pathological characteristic of MS, both the etiology and the pathogenesis of this disease are not yet conclusively known [1].

With no reliable diagnostic test currently available, MS remains a clinical diagnosis with supportive paraclinical evidence. The basis of diagnosis is to clinically establish that disease activity has affected more than one part of the CNS and on more than one occasion (dissemination in time and space). This may be supplemented by investigations such as MRI, cerebrospinal fluid (CSF) electrophoresis, and evoked potential testing [1].

Both genetic and environmental factors have been shown to increase the risk of MS and only a few features are shared by most MS patients: the presence of inflammation, demyelination, and axonal loss within the CNS, a history of Epstein-Barr virus (EBV) infection and the detection of non-specific oligoclonal IgG bands in the CSF which have been shown in up to 95% of the MS patients [2, 3].

However, no common target antigen has been identified, no single diagnostic test is currently available and reliable biomarkers of disease activity are also lacking. Additionally, MS is characterized by a very broad and extensive heterogeneity in terms of clinical features, genetics, pathogenesis and responsiveness to treatments. Taken together,

Table 1: Classic MS and its variants.

Classic MS	MS variants
(i) Relapsing-remitting (RRMS): 85% of all MS cases at onset	(i) Neuromyelitis Optica (NMO)
(ii) Second ary progressive (SPMS): 70%–80% of RRMS cases after 10 years from disease onset	(ii) Balo's concentric sclerosis
(iii) Primary progressive (PPMS): 15% of all MS cases at onset	(iii) Margburg's MS variant
(iv) Progressive-relapsing (PRMS): very small percentage	(iv) Schilder's MS variant

these observations have raised the question of whether MS is more a spectrum of diseases rather than a single entity. In this paper we aim to provide an updated analysis of the clinical, genetic, pathological, and immunological heterogeneity in MS.

2. Clinical Features

The differential diagnosis of MS is not straightforward. Several conditions such as infections, cerebrovascular diseases and autoimmune diseases can mimic the clinical features and the white matter changes seen in MS. Moreover, a few disorders are considered as MS variants and patients suffering from these conditions can either later develop a classic form of MS or show a disease course which is indistinguishable from that of classic MS. Thus, within the MS spectrum we can distinguish between classic MS (and its subcategories) and MS variants (Table 1) [4].

2.1. Classic MS. The clinical course of classic MS is highly variable, ranging from individuals showing occasional sensory nuisance to patients with fulminant course and death within months after disease onset.

Approximately 85% of MS patients present with a clinically isolated syndrome (CIS) and later develop the relapsing-remitting form (RRMS), in which acute exacerbations are followed by periods of remission of symptoms. With time, recovery from each episode is incomplete and persistent symptoms accumulate. Approximately 70% to 80% of RRMS cases will enter the secondary progressive phase (SPMS) [1, 4]. About 15% of MS patients develop the primary progressive form of MS (PPMS), which is characterized by a gradually progressive clinical course from disease onset. Finally, a small group of patients are diagnosed with progressive relapsing MS (PRMS) in which only partial or no recovery occurs after exacerbations and disability accumulates in a stepwise manner.

Further complicating this clinical scenario, the MS course is highly variable even within subgroups. The clinical outcome of RRMS cases varies from very mild forms of disease, wherein only minimal disability (Expanded Disability Status Scale, EDSS < 3) is attained over a period greater than 20 years from disease onset (mild MS) to rapidly progressive forms in which secondary progression is achieved in a few years (malignant MS) [5]. Moreover, during the secondary progressive phase of MS, disability progression can be acquired either because of a failure to recover from relapses (relapsing SPMS) or in the absence of clinically evident relapses (non relapsing SPMS) [6, 7]. Variability in disease outcome is also present in PPMS. In a recent study, the time to reach an EDSS of 6 was measured in a large cohort of PPMS patients. Interestingly, the rate of progression was shown to be slower than in other previous studies (14 years versus 7.1 years and 8.5 years to an EDSS of 6). Moreover, a marked variability was found within the same PPMS cohort with 25% of the patients reaching an EDSS of 6 in less than 7.8 years and another 25% in more than 27 years [8–10].

Poor outcome variables include male gender, frequent relapses in the first two years, a short period between the first and second attack, the absence of full recovery after the first attack, a high baseline T2 load on MRI, motor and cerebellar clinical signs, and African ethnicity [1, 4, 5, 11]. However, the reasons behind this variability are still unknown and although patients with benign disease for 10 years or longer tend to remain stable and not progress, the long-term clinical outcome of MS remains largely unpredictable [12].

2.2. MS Variants. Four conditions are known to closely resemble the classic form of MS and as yet it is not clear to what extent MS and its variants share common etiological and pathological features.

Neuromyelitis optica (NMO) or Devic's disease is a severe demyelinating disease of the CNS which preferentially affects the spinal cord and the optic nerve [13]. Although several epidemiological and clinical features discriminate between NMO and MS, whether these two conditions were two completely different entities or two faces of the same coin has long been debated. An important distinguishing finding was the detection in the serum of NMO patients of a specific antibody binding to aquaporin 4, a channel playing a central role in water homeostasis in the CNS [14]. The consequent detection of the same antibody in patients suffering from the Asian optical-spinal form of MS has led to the hypothesis that NMO and this particular form of MS may represent the same entity [13].

Marburg's variant of MS is characterized by fulminant demyelination and severe axonal loss which rapidly leads to extreme disability and sometimes death. A similar disease course is present in Balo's concentric sclerosis in which the pathological hallmark is the presence of lesions (detectable by MRI) characterized by concentric rings of demyelinated and normal tissue. Finally, Schilder's disease is a demyelinating disorder typically affecting children and characterized by large and confluent white matter lesions. Further details on MS variants can be found elsewhere [15, 16].

The presence of these variants and the fact that NMO is now acknowledged as a separate entity from MS raise the question as to whether analogous differences may be responsible for further stratification within the MS spectrum.

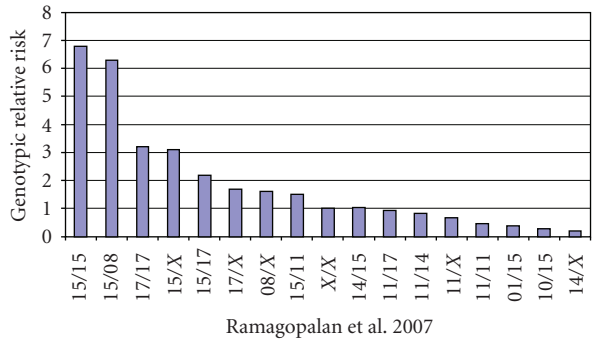

Figure 1: The relative risk of MS is determined by *trans epistasis* between different HLA-DRB1 alleles.

3. Genetics

3.1. Heterogeneity at Susceptibility Loci. A major role in determining genetic susceptibility to MS is played by the Human Leukocyte Antigen (HLA) genes which reside within the major histocompatibility complex (MHC) region. Each HLA allele is characterized by sets of digits separated by colons. The first set of digits describes the allele group, which often corresponds to the serological antigen. The second set of digits is used to distinguish alleles which are part of the same group but differ in the amino acid sequence of the encoded protein.

An association between MS and the MHC was demonstrated for the first time in the 1970s [17]. The association was later fine mapped to the extended class II haplotype HLA-DRB5*01:01-HLA-DRB1*15:01-HLA-DQA1*01:02-HLA-DQB1*06:02 in north Europeans [18] and it is now widely acknowledged that a predominant role is played by the HLA-DRB1*15:01 allele. Notably, this allele has been found to increase the risk of MS in nearly all the populations studied and an admixture scan of an African American cohort further suggested a major role for HLA-DRB1 [19–21].

On the other hand, several HLA-DRB1 alleles have been either positively or negatively associated with MS and these associations vary significantly across populations [22–29]. For example, in Sardinians MS is associated with the DRB1*03:01, DRB1*04:05 and DRB1*13:03 alleles [29]. Conversely, other allele groups such as DRB1*01, DRB1*10, DRB1*11 and DRB1*14 in Canadians and DRB1*09 in Japanese have been shown to exert a protective effect [26–28, 30]. Additionally, several studies have investigated the presence of HLA-class I alleles acting independently of class II loci. HLA-A*02, HLA-B*44 and HLA-Cw*05 alleles have been shown to decrease the risk of MS after conditioning on the presence of DRB1*15:01 [31–34]. A current list of HLA-class I and class II MS-associated alleles is provided in Table 2.

This scenario is further complicated by the extensive linkage disequilibrium of the MHC region and the presence of *cis* and *trans epistasis* between different HLA-class II genes (Figure 1) [26, 27, 35, 36].

However, the MHC is not the only a genetic region associated with MS susceptibility. Recent genome wide association (GWA) studies revealed the existence of multiple non-MHC MS susceptibility loci of modest effect [37–54]. A current list of the well-established associated variants is shown in Table 3.

The vast majority of these genes are involved in the immune system, and this supports the hypothesis that MS is an immune-mediated disorder of the CNS. However, as evidenced by their wide expression profile (see Table 3), different pathways in both the innate and adaptive immune responses are likely to be involved in MS pathogenesis. Intriguingly, another MS-associated gene (*KIF1B*) encodes a kinesin superfamily member which is believed to be responsible for axonal transport of mitochondria and synaptic vesicles precursors, suggesting that also a primary neurodegenerative component may play a role in MS [47].

In addition to these genes, several others have been associated with MS but currently lack replication. However, this does not necessarily mean false positive association. A careful ascertainment of cases and controls is a fundamental requirement which is not easily achieved, especially in a heterogeneous disease such as MS. Moreover, even in a perfectly designed study, the lack of replication could be simply explained by a diverse role played by the same variant in different populations. Genes such as *STAT3* and *CBLB* have been associated with MS in the Finnish and Sardinian MS populations respectively, but have not been replicated by other studies. Interestingly, STAT3 is a transcription factor involved in the differentiation of naïve CD4+ T cells into Th17 cells, while CBLB has been shown to negatively regulate both T and B cell receptor activations [55, 56]. Although a false positive association may well be responsible for this inconsistency, the immunological role played by these genes raises the hypothesis that some genetic variants may be either more easily identified or etiologically more relevant in certain isolated populations.

3.2. Heterogeneity at Outcome Loci. Several studies have also investigated the association between genetic variants and clinical outcome. In a Canadian report, the HLA-DRB1 allele frequencies were compared between mild (RRMS with EDSS $\leq$ 3 over a period >20 years) and malignant (PPMS or RPMS with EDSS $>$ 6 within 5 years of disease onset) MS cases. DRB1*01 was shown to be protective against a severe disease course in both sporadic and familial MS. Intriguingly, in the familial cases the protective effect of DRB1*01 was only significant when it was part of the DRB1*01-DRB1*15:01 genotype. HLA-DRB1*15:01 was instead equally distributed between mild and malignant MS patients, although a greater proportion of DRB1*15:01 homozygous patients was found in the malignant group [57]. A protective role for DRB1*01 was then confirmed in an Australian cohort of 984 RRMS and 246 PPMS patients, but only in the presence of DRB1*15 on the other allele (similarly to the Canadian familial cases). Additionally, DRB1*04 was also negatively associated with PPMS [58].

Conversely, in a Spanish MS cohort, both DRB1*01 and DRB1*04 were found to be associated with a shorter time to reach an EDSS of 6 [59]. Finally, in a large French study, a positive correlation between DRB1*15:01

Table 2: Reported HLA class II and class I associations across the world.

	Population	Approximate OR	Reference
HLA-DRB1 alleles			
*01	Canada	0.6	[26, 27]
	Sweden		[32]
	UK, US		[31]
*03	Canada	1.7	[26, 27]
	Sweden, UK, US, Italy, Spain		[24] [25]
	Sardinia		[29]
*04	Sardinia	2.2	[29]
*07	Italy	0.6	[22]
*08	Canada	1.7	[26, 27]
	UK, US, Italy, Spain	(15/8 genotype)	[25]
*09	Japan	0.4	[28]
*10	Canada	0.7	[26, 27]
*11	Canada	0.7	[26, 27]
*13	Sardinia	2	[29]
	Israel		[23]
*14	Canada, UK, US, Italy, Spain	0.3	[26, 27] [25]
*15	Near-universal	3	
HLA-class I alleles			
A*02	Sweden	0.6	[33]
	Italy		[31]
B*44	UK, US	0.4	[34]
Cw*05	UK, US	<1	[32]

and disease progression was shown in the RRMS but not in the PPMS groups [60]. While these findings seem conflicting it may be due to differences in study design: comparing PPMS with RRMS may fail to elicit important outcome effects given the tremendous clinical variability within the MS subgroups. Also, as mentioned previously, the same variant may play diverse roles in different populations.

HLA genes are thought to be involved in immune-mediated diseases through their role in antigen presentation. Thus one reason different HLA-DRB1 alleles may lead to different outcomes among MS patients may be due to antigen specificity. The myelin sheath is a complex structure comprised of various types of lipids (glycosphingolipids, cholesterol, and phospholipids) and proteins including proteolipid protein (PLP), myelin basic protein (MBP), myelin-associated glycoprotein (MAG), myelin-oligodendrocyte glycoprotein (MOG), and 2′ 3′-cyclic-nucleotide-3′phosphodiesterase (CNP) [61]. All of the above components have been suggested as candidate antigens, but to date there is no verified antigen for MS [61]. The complexity of the disease together with the heterogeneity of the MHC associated alleles would suggest that the different myelin components or the entire complex structure of the myelin sheath may be the target of the immune reaction. Differences in antigen specificity and the role played by the protein within the myelin sheath may lead to differences in clinical outcome in a patient-specific manner.

Non-MHC loci have also been investigated and a number of genes have been associated with different markers of disease phenotype such as age of onset, disease severity, lesion load and brain atrophy. Interestingly, a gene-ontology analysis showed that many of these genes were involved in neural processes and several cellular mechanisms, but further studies are needed to confirm these findings [62].

4. Pathology

4.1. Relapsing versus Progressive MS. The pathological hallmark of MS is the sclerotic plaque, which represents the end stage of a process involving inflammation, demyelination, remyelination, astrocytosis, and axonal degeneration. However, the order in which these processes take place is still unknown [1].

In the relapsing-remitting phase, the classical pathological finding is active white matter plaques in which inflammatory demyelination clearly plays a central role. Myelin-laden macrophages and (to a lesser extent) CD8+ T cells dominate the lesions, while CD4+ T cells (both Th1 and Th17) are found primarily in the perivascular regions and with relatively smaller numbers in the parenchyma [63–66]. Cortical demyelinating lesions are also present and have been shown to correlate with cortical atrophy, disease progression, physical disability, and cognitive impairment at later stages [67–70]. Interestingly, cortical demyelination

Table 3: List of established non-MHC MS-associated genes.

Gene	Proposed function	CH	OR	UCSC Microarray expression data	References
IL7Ra Interleukin 7 receptor	Cytokine receptor	5	1.18	CD4+ T cells ++++, CD8+ T cells ++++, CD56+ NK +++, BCDA4+DCs ++, CD14+ Monocytes+	[38–42, 44]
IL2Ra Interleukin 2 receptor	Cytokine receptor	10	1.19	CD4+ T cells ++, CD8+ T cells +, CD56+ NK +	[37, 38, 40, 42]
CLEC16A C lectin domain A	Sugar binding C type lectin	16	1.18	CD19+ B cells +, CD56+ NK +, BCDA4+DCs +	[45, 48, 49, 53]
CD58	Ligand of CD2/T cell activation	1	1.30	CD56+ NK ++++, CD14+ Monocytes++++, CD8+ T cells +++, CD19+ B cells++, CD4+ T cells ++, BCDA4+DCs ++	[37, 38, 43, 45, 49]
CD6	Cell signaling/T cell activation	11	1.18	CD4+ T cells ++++, CD8+ T cells ++++, CD56+ NK +++, BCDA4+DCs +	[54]
IRF8 Interferon regulatory factor 8	Interferon regulatory factor	16	0.80	CD19+ B cells ++++, BCDA4+DCs ++++, CD56+ NK ++, CD14+ Monocytes ++, CD4+ T cells +, CD8+ T cells +	[54]
CD226	Cell-cell adhesion	18	1.11	CD56+ NK ++	[50, 53]
TNFRSF1A Tumor necrosis factor receptor 1	Tumor necrosis factor receptor	12	1.20	CD14+ Monocytes +++, CD56+ NK ++, BCDA4+DCs +, CD4+ T cells +, CD8+ T cells +	[54]
EVI5 Ecotropic viral integration site 5	Cell cycle regulation	1	1.1	BCDA4+DCs +, CD14+ Monocytes +, CD19+ B cells+	[37, 45, 51]
CD40	Tumor Necrosis Factor receptor Super family member 5	20	1.20	CD56+ NK +, CD14+ Monocytes +, BCDA4+DCs +	[45]
TYK2 Tyrosine kinase 2	Cell signaling	19	1.32	CD56+ NK +++, CD14+ Monocytes +++, BCDA4+DCs +++, CD8+ T cells ++, CD19+ B cells ++, CD4+ T cells ++	[44, 45]
KIF1B Kinesin family member 1B	Axonal transport	1	1.34	Whole brain ++++	[47]

[+] Increasing number of crosses correspond to increasing expression levels.

seems to be present since the relapsing-remitting phase but becomes more prominent during the secondary progressive phase [71]. Moreover, in contrast with those of the white matter, grey matter lesions typically show a very low grade of both T and B inflammatory infiltrates [67].

In the progressive phase of MS (both PPMS and SPMS), neurodegeneration proves the main pathological finding and occurs on the background of a compartmentalized pathological immune reaction which seems to act independently from the central immune system [64, 71]. T cells are still

Table 4: Patterns of demyelination described by Lucchinetti et al. 2000 [7].

Pattern of white matter demyelination	Pathology
(i) Macrophage mediated	(i) Perivenous distribution of lesions (ii) T cell and macrophage infiltrates (iii) Shadow plaques (remyelination) (iv) Sharp lesion edges
(ii) Antibody mediated	(i) As pattern I lesions (ii) Deposition of immunoglobulin and activated complement
(iii) Distal oligodendrogliopathy	(i) Important oligodendrocyte apoptosis (ii) T cell, macrophage, and microglia infiltrates (iii) Degeneration of distal oligodendrocyte processes (iv) Ill defined lesion edges (v) Preferential loss of myelin associated glyco-protein (MAG) (vi) Concentric Balo-like lesions
(iv) Primary oligodendrocyte damage	(i) Similar to pattern I (ii) Massive oligodendrocyte loss

Table 5: Types of cortical lesions described by B∅ et al. 2003 [83].

Typeof cortical lesion	Extension
Type I	Extension through both white and gray matter
Type II	Lesion delimited within the cortex. Neither the brain surface nor the subcortical white matter is involved
Type III	Extended subpial lesions
Type IV	Extension throughout the full width of cerebral cortex but white matter is not involved

the main cell population found within chronic lesions but they are sparse and mainly located in perivascular spaces, while microglia, B cells, and plasma cells become increasingly prominent [72, 73]. Additionally, some studies have shown the presence of clusters of B cells resembling the structure of germinal centers inside the meninges [74, 75]. These B cells have been reported to bear EBV, although this finding lacks replication [76, 77]. Finally, inflammatory infiltrates are also detected in the normal appearing white matter (NAWM) in which T cells (mainly CD8+) and profound microglia activation are associated with diffuse axonal injury and do not correlate with the number, size, location, and destructiveness of active lesions [64, 71, 78].

4.2. Pathological Heterogeneity. The presence of heterogeneity in active white matter lesions has been largely debated since Lucchinetti et al. defined four distinct types of active plaques from a number of autopsy ($n = 32$) and biopsy ($n = 51$) samples, strongly suggesting a multiple disease hypothesis (Table 4) [7].

However, these findings must be interpreted with caution for several reasons: (1) Biopsy data are bound to be less representative and reliable than autopsy material [79]. (2) The pathological criteria used to define the activity of the plaques still lack a confident validation and this is likely to undermine the entire classification. (3) Complement activation (pattern II) is not easy to interpret in formalin-fixed tissue and has been shown to be an invariable and nonspecific feature of not only MS but also other white matter conditions [79–81]. (4) Apoptotic oligodendrocytes (pattern III) could be either mistaken for other apoptotic cells, in particular lymphocytes, or merely be the consequence of confounding factors such terminal hypoxia [79]. (5) Partial Balo lesions (pattern III) are a common finding in relapsing remitting patients and have been shown also in other patterns of MS lesions [81, 82]. (6) Finally, it is not clear to what extent these pathological findings should be seen in the lesions in order to confidently define them as part of a specific pattern.

Taken together, these observations suggest that these different types of white matter lesions are more likely to be part of the same spectrum or reflect different stages of demyelination rather than representing single and distinct pathological entities [63].

It is now widely acknowledged that disease progression depends on accumulated neuronal degeneration and cortical atrophy. Whether these are reached as a consequence of inflammation and demyelination or represent an independent neurodegenerative process has long been debated. Theoretically, five pathways may be involved and responsible for neuronal damage: (1) white matter demyelinating lesions, (2) grey matter demyelinating lesions of which four different types have been described (Table 5) [83], (3) diffuse inflammation of the NAWM, (4) B cell follicles located in the meninges which have been shown to correlate with areas of cortical atrophy [64, 74], and (5) a primary independent neurodegenerative process [84, 85].

Rather than acting independently, these mechanisms are likely to act together but to a different extent in a patient specific manner. These differences would then lead to the pathological heterogeneity seen in MS.

5. Immunological Phenotype

5.1. Cell Type Complexity. For a long time, MS has been generally considered as a CD4+ T helper cell-(Th-) mediated immune disorder. This concept primarily arose from the HLA-class II association with MS susceptibility and from the central role played by Th cells in experimental autoimmune encephalomyelitis (EAE), the rodent model of MS, in which an MS-like demyelinating disease is induced by the injection of myelin-specific CD4+ T cells [86]. However, while the treatment with an antibody against the p40 subunit of IL-12, which is important for Th1 cell differentiation, could prevent EAE [87], the use of ustekinumab (another antibody for the same subunit) produced no benefit in Phase II clinical trials [88]. These results highlight the much greater complexity of MS immunopathogenesis when compared to the EAE model.

Interestingly, the most consistent immunological feature in MS is the presence of IgG oligoclonal bands which are detected in the CSF of up to 95% of the MS patients [3]. Although their specificity remains to be resolved, their presence stands for an abnormal B cell activation within the CNS. Other recent studies suggest a relevant role played by B cells in MS pathogenesis in terms of T cell activation, CIS conversion to MS, and development of disease progression [76, 77, 89–91]. The central role played by B cells in MS is further supported by the significant reduction of inflammatory lesions and clinical relapses observed when B cells are depleted using the anti-CD20 monoclonal antibody Rituximab [92, 93].

T cells are also important and several recent studies were aimed at the identification of the T cell subtypes primarily involved in the immunopathogenesis of MS.

CD8+ T cells represent the largest T cell subset both in acute and chronic MS lesions. Moreover, they show oligoclonal expansion within the CNS strongly suggesting their contribution to MS pathogenesis [94–97].

Interleukin 17 (IL-17) producing T helper cells (Th17 cells) have been recently identified as a distinct subset of T cells strongly involved in autoimmunity [98, 99]. A central role for Th17 cells in MS has been suggested by several studies reporting: (1) the presence of IL-17+ T cells in active MS lesions [66], (2) an increased ability of CD4+ T cells taken from MS patients to produce IL-17 upon polyclonal mitogen or myelin-specific antigen stimulation [100], (3) higher frequency of Th17 in the CSF of CIS and RRMS patients in the relapsing rather than remitting phase [101], (4) higher expression of the transcription factor STAT 3 (which regulates the differentiation of CD4+ T cells into Th17 cells) during the relapsing phase of MS [102], and (5) the upregulation of miR-326 (a positive regulator of Th17 differentiation) in RRMS patients experiencing a relapse in comparison with remitting cases and healthy controls [103]. However, although these findings strongly support a role for Th17 cells in MS, whether these cells are causative or merely a marker of disease activity remains a challenging question.

The role of the main type of regulatory T cells (CD4+ CD25+ FOXP3+ Treg) in MS has also been extensively investigated. In RRMS patients, these Tregs display an impaired capacity to suppress both polyclonally activated and myelin-specific T cells as compared with controls [104–106]. Interestingly, a correlation between their suppressive function and vitamin D levels has also been reported giving a potential explanation for the association between vitamin D levels and relapse rate [107, 108]. Additionally, recent thymic emigrating Tregs seem to play a major role as they were shown to be reduced and to contain a significantly lower number of T cell receptor excision circles in RRMS as compared to normal controls [109, 110]. Finally, it must be noted that the CD4+ CD25+ FOXP3+ Tregs only represent one regulatory cell type and that other subsets have also been shown to be involved in MS. Further details on regulatory T cells in MS can be found elsewhere [111, 112].

These studies confirm the presence of a great immunological heterogeneity in the MS immune system with several different cell types all likely to be involved. Moreover, it has to be emphasized that in all the studies mentioned, differences between cases and controls are often very subtle and no immunological finding can at present be used as biomarkers of disease activity.

5.2. Individual Complexity. Most of the data for immunological phenotyping derives from studies performed in a limited number of patients, usually those with RRMS. However, even in these limited sets, heterogeneity can be appreciated. A recent study extensively investigated the cytometric profile of a large cohort of RRMS and CIS patients. Interestingly, both RRMS and CIS cases showed a decreased frequency of $CD8^{low}$ CD56+ CD3− CD4− cells which have a natural killer (NK) profile, adding to the hypothesis that NK regulatory properties may also be reduced in MS [113]. Moreover, in the same study, both RRMS and CIS patients were shown to cluster into three distinct groups: the first was characterized by the lower frequency of $CD8^{low}$ CD56+ CD3− CD4− cells while the second and third by changes in the frequencies of large granular and CD14+ cells, respectively [114].

Another recent study using EAE and RRMS patients showed differential response to interferon beta (IFN-β) treatment. Interestingly, IFN-β was more effective in Th1 as compared to Th17-induced EAE. Similarly, in RRMS patients a higher IL-17F concentration in serum was found in nonresponders as compared to responders. Non-responders also showed worse disease with steroid administration and had a higher number of relapses [115].

Finally, when considering sources of immunological variation in MS, it is interesting to note that the differentiation of Th17 and CD4+ CD25+ FOXP3+ Treg cells are tightly related. The differentiation of CD4+ naive T cells into Th17 cells or Tregs has been shown to be dependent on TGF β stimulation during antigen presentation. High levels of TGF β promote Treg production, while a low dose of TGF β exerts the opposite effect by increasing the expression of the Th17 transcription factor ROR$\gamma\tau$ leading to the production of Th17 cells. The flexibility of the Th17-Treg system is further confirmed by the capacity of TGF β and IL6 to actually reprogram Tregs into Th17 cells through the ROR$\gamma\tau$ and STAT3 pathways, respectively [111, 116]. Therefore, an immune system that was preferentially skewed towards the production of Th17 or Treg subsets may represent a further

source of interindividual heterogeneity in MS and lead to a more or less severe relapse rate and clinical course.

Taken together, these studies strongly suggest that different cell types are likely to be involved in a patient-specific manner and that these differences are able to influence disease course and response to treatments.

6. Conclusions and Perspectives

We have seen how MS clinical features, genetics, pathology, and immunological phenotype show a high degree of variability between individuals and ethnicities. Notably, no single pathway, reliable biomarker, diagnostic test, and specific treatment have yet been identified for all MS patients. However, there are several commonalities among the MS subtypes: the association of HLA-DRB1*15:01 allele has been shown across wide variety of populations and within clinical subtypes of MS [20]; similarly, low vitamin D level is now an established environmental MS risk factor [108]; furthermore, it is striking that more than 99% of the MS patients have been found to have been infected with EBV [117]. These observations lead us to conclude that despite the wide heterogeneity, there is insufficient evidence to maintain that MS represents a spectrum of etiologically different disorders. We believe that genetic and environmental factors play a central role not only in triggering the onset but also in modifying the course of the disease by influencing individual neurological susceptibility and immunological responses. This is likely to lead to the wide clinical, pathological, and immunological heterogeneity observed in MS patients.

The differences described in this review remain important considerations for accurate study designs as well as the ultimate goal of personalised treatments for MS patients. At present, the response to the currently approved therapeutic agents (IFN β, glatiramer acetate, mitoxantrone and natalizumab) varies significantly across the MS population. Moreover, no treatment is able to halt disease progression [118]. A clearer understanding of the heterogeneity within the MS phenotype is required in order to achieve effective treatment for all patients with MS.

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