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Therapeutic potential of statins in multiple sclerosis: immune modulation, neuroprotection and neurorepair

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Abstract

Statins as inhibitors of 3-hydroxy-3-methyl glutaryl coenzyme A reductase are widely used as cholesterol-lowering drugs. Recent studies provide evidence that the anti-inflammatory activity of statins, which is independent of their cholesterol-lowering effects, may have potential therapeutic implications for neuroinflammatory diseases such as multiple sclerosis (MS), Alzheimer's disease and brain tumors, as well as traumatic spinal cord and brain injuries. Studies with animal models of MS suggest that, in addition to immunomodulatory activities similar to the ones observed with approved MS medications, statin treatment also protects the BBB, protects against neurodegeneration and may also promote neurorepair. Although the initial human studies on statin treatment for MS are encouraging, prospective randomized clinical studies will be required to evaluate their efficacy in the larger patient population.

Keywords

blood-brain barrier; immunomodulation; inflammation; multiple sclerosis; neurodegeneration; neuroinflammatory diseases; neurorepair; pleiotropic effects; statins

Multiple sclerosis (MS) is a chronic CNS disease with a chronic inflammatory response directed against the myelin antigens [1,2]. Immunological studies indicate that autoreactive CD4⁺ and CD8⁺ lymphocytes migrate into the CNS following antigen recognition in the peripheral circulation. Once in the CNS, T cells undergo reactivation by abundant myelin antigens and perpetuate an inflammatory response that leads to demyelination and axonal loss

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through multiple effector mechanisms [2]. MS is one of the best-characterized neurological diseases, with effective therapies targeting specific steps in disease development [3]. Available treatments target peripheral auto-reactive T-cell activation (glatiramer acetate [GA]), inflammatory cell migration to the CNS (IFN- β and natalizumab) and suppress the inflammatory cell proliferation (mitoxantrone) [3]. In spite of effective suppression of the inflammatory response, the disease often progresses, which reflects its complex immune dysregulation and neurodegenerative processes, which are only partially blocked by targeting the above steps in the disease's development. Therefore, new therapies and combinations thereof are actively sought, supported by a more comprehensive understanding of the disease pathogenesis.

Statins are inhibitors of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, an enzyme that catalyzes the conversion of HMG-CoA to mevalonate, an early and rate-limiting step in cholesterol biosynthesis (Figure 1). Statins are effective cholesterol-lowering agents, and have been extensively used for primary and secondary prevention of cardiovascular disease. In addition to their cholesterol-lowering effects, more recent studies have reported on their previously unrecognized immunomodulatory effects [4,5]. In order to discuss statins' pleiotropic mechanisms of action, which have therapeutic potential in treating CNS inflammatory diseases [6–9], we will outline statins' mechanisms of action that target specific steps in the pathogenesis of MS.

Recent observations of statin-mediated protection of neuroprogenitor cells from inflammatory insult and the resulting enhanced remyelination during experimental allergic encephalomyelitis (EAE), an animal model of MS, suggest that, in addition to immunomodulatory activity [8, 9], statins mediate neuroprotection and possibly neuroregeneration [10]. They affect BBB integrity and inhibit the infiltration of inflammatory cells into the CNS [7]. They also prevent neurodegenerative changes and may promote neurorepair in EAE [10]. Therefore, statins represent a promising monotherapy, as well as a combination therapy with presently approved and new experimental therapies for MS.

Pharmacology & mechanisms of action of statins

Statin-induced reduction in cardiovascular morbidity and mortality has established the clinical benefits of lowering cholesterol levels [11,12]. However, recent clinical and experimental studies have documented a variety of statins' pleiotropic effects, such as anti-inflammatory, antiproliferative, antithrombotic and antioxidant activities in various pathological conditions [7–17].

Statins bind to HMG-CoA reductase at nanomolar concentrations as competitive inhibitors and replace the natural substrate HMG-CoA. The inhibition of HMG-CoA reductase by naturally occurring (lovastatin, mevastatin and simvastatin) and synthetic statins (fluvastatin, atorvastatin and rosuvastatin) inhibits the mevalonate pathway leading to the reduction of mevalonate pathway biologically active metabolites, including isoprenoids, dolichol, ubiquinone and cholesterol (Figure 1). The efficacy of different statins varies depending upon their bioavailability governed by their hydrophobicity and their turnover by the cytochrome P450 enzyme system [12]. Secondly, the bioavailability of statins is also affected by polymorphisms in genes coding for ApoE, ABC drug transporters, HMG-CoA reductase and the cytochrome P450 enzyme system [18].

Cholesterol is a major component of lipid rafts, which float between lipid bilayers of cellular membranes. Rafts are small platforms composed of sphingolipids and cholesterol in their exoplasmic leaflets and phospholipids and cholesterol in their inner cytoplasmic leaflets. They contain proteins such as GPI-anchored proteins, acylated proteins and various receptors [19]. Lipid rafts can cluster to form large platforms where functionally related proteins interact to

provide effective signal transduction, such as T-cell receptors and costimulatory molecules that form an immunological synapse, and ceramide/sphingomyelin and receptors that mediate cellular signaling [20]. Therefore, depletion of cholesterol in lipid raft domains could alter their structure and function, with a significant effect on cellular activation and signaling pathways [21].

Alternatively, restriction of the cellular availability of dolichol may interfere with the glycosylation of glycoproteins. Therefore, statin treatment may interfere with the biosynthesis of glycoproteins and their cellular functions [22]. However, most of the pleiotropic effects of statins are mediated by reduced levels of isoprenoids, which are required for isoprenylation of proteins and thus for their optimal function (Figure 1). Isoprenoids, farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), bind to proteins during their post-translational modification and serve an important role in the functional targeting of proteins to different cellular sites, which are important during their biological activity [15]. Small GTPases (Ras, Rho, Rac and CDC 42) and G proteins are the major substrates for such modifications [15,16]. The reversal of statin-mediated inhibition by mevalonate demonstrates that effects of statins are mediated by inhibition of HMG-CoA reductase. Reversal of the inhibitory effects of statins by FPP or GGPP but not by cholesterol or squalene, and the ability of FPP or GGPP to overcome the inhibition by inhibitors of isoprenyltransferases that transfers isoprenoids to proteins, document the role of isoprenylation in inflammatory diseases [4,17,23–26]. Therefore, inhibition of isoprenylation is reported to play a role in the statin-mediated cholesterol-independent pleiotropic effects targeting inflammation and oxidative stress in various disease states, including MS.

Statins target multiple steps in the pathogenesis of multiple sclerosis

Statins inhibit the activation of autoreactive CD4⁺ lymphocytes

Statins affect the innate immune response—Current studies indicate that MS is a CD4⁺ cell-mediated chronic inflammatory CNS disease [27]. Figure 2 provides a diagram outlining the steps involved in the pathogenesis of MS and mechanisms of action of statins in targeting disease development.

An inflammatory response is activated in the peripheral circulation by environmental triggers, most likely by ubiquitous viral or bacterial infections. The innate immune response is a first line of defense against infectious pathogens and provides immediate protection. It also regulates the activation of autoreactive T cells, which initiates a chronic autoimmune response targeting CNS autoantigens [27]. T cells involved in the innate immune response recognize pathogen-associated molecular patterns via pattern-recognition receptors, most notably Toll-like receptors (TLRs). Mehte *et al.* have reported that statins inhibit TLR-4 expression in monocytes via inhibition of isoprenylation [28]. This results in downstream inhibition of IRAK phosphorylation, and LPS-induced IL-6, IL-12, TNF- α and CD80 expression (Figure 2.1). The effect of statins on TLR signaling inhibits the activation of MAPKs and downstream activation of NF- κ B and the JAK/STAT signaling pathway, affecting the expression of multiple costimulatory molecules and cytokines.

The mechanisms underlying the pleiotropic effects of statins are mediated by the inhibition of isoprenylation, a post-translational protein modification, whereby the attachment of lipid isoprenoids ensures proper protein membrane attachment, activation, and optimal function [29]. This lipid modification is required for activation of the small GTPases Rho, Rac and Ras, which are involved in signal transduction, kinase activation and the transcription of proinflammatory cytokines and chemokines [15,16].

Statins inhibit antigen presentation by antigen-presenting cells in the peripheral circulation—

Dendritic cells (DCs) play a critical role in both the innate and adaptive immune response and probably bridge the two. DCs are the most efficient antigen-presenting cells (APCs); they activate T lymphocytes at low antigen concentrations and at low APC:lymphocyte ratios. Most importantly, they induce the primary T-cell response and play a role in the polarization of the adaptive immune response. DC-mediated T-cell differentiation depends on the state of maturation of DCs and on the cytokine milieu during lymphocyte priming. Several studies have addressed the effects of statins on APCs in humans. Kwak *et al.* first demonstrated that statins inhibit IFN- γ -induced MHC class II expression in human monocytes, DC precursors, in a dose-dependent manner via inhibition of class II transactivator (CIITA) [5]. Our studies confirmed that statins inhibit MHC class II expression in human monocytes, which translated into decreased antigen presenting ability in a mixed lymphocyte reaction [30]. Yilmaz *et al.* reported that simvastatin treatment of human immature DCs inhibits their maturation by lowering the expression of MHC class II DR, CD83, CD40, CD86 and CCR7 [31]. Preincubation of immature DCs with statins reduced their ability to stimulate T cells (Figure 2.1). While several studies confirmed that statins inhibit DC maturation in humans [31], the mechanisms of statin-induced inhibition of DC maturation are not well characterized. Results from our recent studies demonstrate that statins significantly increase the expression of suppressors of cytokine secretion (SOCS)3 and -7 in the peripheral blood mononuclear cells (PBMCs) and monocytes derived from patients with relapsing–remitting (RR) MS and healthy controls (HCs) [32]. In support of the finding that simvastatin-mediated upregulation of SOCS3 may inhibit DC maturation, Li *et al.* have reported that SOCS3-transfected DCs express decreased levels of MHC class II and CD86, inhibit the production of IL-12 and IL-23, and bias T-cell differentiation towards the Th2 phenotype in myelin oligodendrocyte glycoprotein-specific T cells [33]. Moreover, the transfer of SOCS3-transfected DCs to naive mice prevented the development of EAE. Qin *et al.* reported that SOCS3-transfected macrophages inhibit LPS-induced STAT-1 phosphorylation and CD40 gene expression [34]. Consistent with our results, Huang *et al.* have demonstrated that statins induce SOCS3 in mice macrophages, which was reversed by isoprenoid precursors [35]. However, the identity of isoprenylation targets and the linkage between the isoprenylation and SOCS3 gene expression have not yet been elucidated.

In systemic lupus erythematosus (SLE), a B-cell-mediated systemic autoimmune disease, atorvastatin reduced the expression of MHC class II molecules and the costimulatory molecules CD80 and CD86 on B cells. Consequently, statin-treated B cells had an impaired capacity to present antigens and to initiate the T-cell response. In the animal model of SLE, atorvastatin significantly ameliorated disease activity [36].

Statins modify T-cell differentiation—

While inhibition of DC maturation by statins inhibits MHC class II and costimulatory molecule expression, and therefore inhibits effective antigen presentation, the effect of DCs on T-cell differentiation is most significantly mediated by their cytokine secretion. Multiple studies have reported that statins inhibit proinflammatory cytokine production by monocytes and DCs in animal models of autoimmune diseases [37–39], and in humans [31,40,41]. These studies detected an overall inhibitory effect of statins on monocytes' proinflammatory cytokine secretion in healthy individuals, and in patients with Th1-mediated (rheumatoid arthritis and MS) and Th2-mediated diseases (asthma). However, more detailed studies of simvastatin-induced changes in cytokine expression in human monocytes detected a complex pattern: statins inhibit IL-6 and IL-23, while they induce IFN- γ , IL-27 and IL-4 [32]. These findings are suggestive of differential effects of statins on cytokine production in monocytes, and require further studies on the selected cell subsets, in particular DCs. We recently reported that simvastatin-induced changes in monocytes' cytokine production affect T-cell differentiation (Figures 2.2 & 2.3)[32].

Statins inhibit inflammatory T-cell proliferation & cytokine production—Studies of EAE disease have indicated that atorvastatin pretreatment of cells inhibits antigen-specific proliferative responses and inflammatory cytokine secretion [42]. Atorvastatin inhibited STAT4 phosphorylation and IFN- γ , IL-2, IL-12 and TNF- α (Th1) cytokine production (Figure 2.4), while it induced STAT6 phosphorylation and IL-4, IL-5 and IL-10 (Th2) cytokine secretion as depicted in Figure 2.5 [8]. Atorvastatin treatment also reduced CNS infiltration [8], as described previously with lovastatin treatment of an EAE rat model [7]. These effects of atorvastatin on immunomodulation in EAE were mediated by metabolites of the mevalonate pathway [23]. GGPP mediates proliferation, and FPP mediates Th-1 differentiation of myelin-reactive T cells via the MAPK pathway [23].

Furthermore, Nath *et al.* have demonstrated that lovastatin inhibits the expression of T-bet, a master transcription factor for multiple Th1 cytokines, and it induces expression of GATA3, a master switch for Th2 cytokines in the lymphocytes derived from mice with EAE [9]. We also demonstrated for the first time in patients with MS a decreased T-bet expression in PBMCs upon treatment with simvastatin, while GATA3 expression was unchanged [30]. While previous studies have demonstrated a statin-selective effect on activated cells [43], it is not established that statins selectively inhibit only Th1 cells. In fact, our recent studies indicate that simvastatin most significantly inhibits IL-17 transcription and secretion by the CD4⁺ cells derived from both RRMS patients and HCs (Figure 2.3) [32]. Our results also indicate that simvastatin inhibits expression of the retinoic acid receptor-related orphan receptor (RORC), a transcription factor that mediates the differentiation of Th17 cells and the secretion of IL-17. Recently, IL-17-producing cells have been recognized to play a critical role in autoimmunity [44,45]. The regulation of this cytokine, which has recently been discovered to play an important role in the development of human autoimmune diseases, remains to be investigated in response to treatment with statins.

Statins inhibit the migration of activated lymphocytes into the CNS

The BBB controls cell migration into the CNS through the regulation of selectins, addressins and adhesion-molecule expression. Its permeability changes, depending on the microenvironment, specifically, induction of adhesion molecules by activated cells, and also by cytokines and chemokines, which stimulate the migration of immune-competent cells into the CNS (Figure 2.6). Inflammatory insult leading to disruption of the BBB, the infiltration of inflammatory cells into the CNS and the resulting neurodegeneration are key events in the pathogenesis of MS [27]. The appearance of Gd contrast-enhancing lesions on MRI scans marks the disruption of the BBB and represents the initial changes leading to MS-lesion formation. The anti-inflammatory and BBB protection activity of statins was documented by the decreased number and volume of Gd contrast-enhancing lesions in an open-label study that evaluated 30 patients with active RRMS after 6 months of high-dose oral simvastatin treatment [46].

Singh and associates first reported on the inhibition of infiltration of immune cells by lovastatin treatment in a rat model of EAE [7], which was subsequently confirmed by other laboratories in different models of EAE [8,24,47–51]. Based on the observed physical interaction of statin with lymphocyte function-associated antigen (LFA)-1 [49], statins were proposed to inhibit leukocyte function in autoimmune disease by physical interaction with the integrin site of LFA-1 adhesion molecules on the surface of activated lymphocytes by a mechanism that does not involve cholesterol-lowering effects nor the inhibition of isoprenylation. However, subsequent studies from various laboratories did not support the inhibitory mechanism of statin binding to the L-site on LFA-1 molecule. Studies by Greenwood *et al.* reported that treatment of brain endothelial cells *in vitro* with lovastatin inhibits RhoA-mediated lymphocyte transmigration and that this effect is regulated by metabolites of the mevalonate pathway

[47]. Inhibitory effects of statins on transmigration of inflammatory cells were not the result of the effect on lymphocytes but rather the modification of endothelial cell activity and function [24,25,47,48]. Moreover, the use of inhibitors of protein prenylation suggested that the inhibitory effects of statins are mediated by attenuation of isoprenylation of endothelial cell proteins such as Rho GTPase [24,25]. *In vitro* studies with cultured rat [24,25,47] and human brain endothelial cells [51] reporting the inhibition of binding of monocytes to endothelial cells and their transmigration by treatment with statins support the *in vivo* observation in the EAE studies [7]. Statins were found to be effective at very low concentrations ($E_{50} = 9.5 \times 10^{-8}$ – 1.0×10^{-7} M) in reducing the transmigration of monocytes [51].

Although the inhibition of transmigration of inflammatory cells into the CNS by statins is well established, the mechanisms of action of statins on the BBB under inflammatory conditions are not well understood. Statin treatment of human endothelial cells reduced secretion of chemokine monocyte chemoattractant protein-1/CCL2 and IFN- γ -inducible protein-10/CXCL10 but did not affect the expression of ICAM-1, VCAM-1, E-selectin or CD90 [50]. Another study reported that lovastatin treatment inhibits the binding of monocytes to endothelial cultures by down-regulation of expression of VCAM-1 and E-selectin [25]. This inhibition was mediated by the inhibition of the PI3K/protein kinase B (Akt)/NF- κ B pathway in the endothelial cells [25]. Lymphocyte migration through the brain endothelial cell monolayer was reported to involve signaling through the brain endothelial ICAM-1 via a Rho-dependent pathway [52]. These findings suggest that lovastatin downregulates the pathways affecting the expression and interaction of adhesion molecules on endothelial cells, which in turn restrict the migration and infiltration of peripheral blood inflammatory cells into the CNS. The additional mechanism by which statins inhibit inflammatory cell transmigration through the BBB is by reducing the activity of matrix metalloproteinases [53–55] that facilitate inflammatory cell migration through the otherwise impermeable BBB. These effects were reversed by the addition of mevalonate, suggesting that they were mediated by HMG-CoA reductase inhibition [56]. Treatment with statins attenuates the expression of matrix metalloproteinase (MMP)9 in endothelial cells by the RhoA/ROCK pathway through an isoprenoid-dependent mechanism [53]. Therefore, statin-induced changes in signaling of cytokines/chemokines and Rho-mediated signaling affect function of adhesion molecules and extracellular proteases, regulate BBB integrity and thus the transmigration of inflammatory cells into the CNS in EAE and MS.

Statins affect antigen presentation within the CNS

Local antigen presentation is a critical requirement for the initiation and perpetuation of the chronic inflammatory response within the CNS. While the CNS is devoid of professional APCs, MHC class II antigens and costimulatory CD80 and CD86 molecules are upregulated on microglia and peripheral blood-derived macrophages in response to local inflammatory cytokine production. It was only recently demonstrated that DCs may migrate from the peripheral circulation into the CNS. Serafini *et al.* identified myeloid dendritic cells (mDCs) in MS lesions localized preferentially in perivascular cuffs together with T cells, suggesting that they play a role in antigen presentation within the CNS [57]. Most recently, Bailey *et al.* demonstrated that peripherally-derived mDCs accumulate in the CNS during relapsing EAE [58]. mDCs localize in the central parts of active lesions and induce IL-17 production by CD4 cells. This effect on T-cell differentiation was mediated by mDCs' ability to produce large amounts of IL-6, TGF- β and IL-23, which promote the differentiation of Th17 cells. The relative abundance of DCs in the CNS correlated with clinical disease activity. This study clearly demonstrated the central role of peripheral myeloid dendritic cells mDCs in antigen presentation within the CNS, and justifies their therapeutic targeting in the peripheral circulation. CNS resident astrocytes can also upregulate MHC class II following activation by IFN- γ , and have the potential to present antigens in a costimulation independent manner.

Pahan *et al.* first reported in 1997 that lovastatin *in vitro* treatment of CNS-resident APCs, microglia and astrocytes, results in decreased expression of TNF- α and IL-1 β [4]. Further studies from the author's laboratory have demonstrated that lovastatin treatment decreases mononuclear cell infiltration into the CNS of Lewis rats with EAE, and ameliorates the clinical severity of the disease [7,9]. Youssef *et al.* have reported that atorvastatin reduces CNS MHC class II expression [8]. Treatment of microglia inhibited IFN- γ -inducible transcription at multiple MHC CIITA promoters. MHC class II inhibition was reversed by mevalonic acid, indicating that the effect of statins was mediated through the inhibition of small GTPase isoprenylation, which is critical for their optimal function. The expression of CD40, CD80 and CD86 was also inhibited by the *in vitro* treatment of microglia and macrophages, and significantly inhibited their ability to present antigen.

Statins promote neuroprotection by inhibiting demyelination & loss of oligodendrocytes

In a large pathological study, Lucchinetti *et al.* described four patterns of demyelination, on the basis of myelin protein loss, the geography and extension of MS lesions, the patterns of oligodendrocyte destruction and the immunopathological evidence of complement activation [59]. Therefore, the mechanisms involved in myelin damage are uncertain and may vary between patients. However, cytokines (TNF- α and IFN- β), proteolytic enzymes, oxygen and nitrogen radicals, and excitatory amino acids produced by activated macrophages and resident microglia may all be contributing factors in myelin and axonal damage (Figure 2.7) [60–62].

In addition to myelin damage, loss of oligodendrocytes and their progenitors is a well-described hallmark of MS pathology. However, the mechanism of oligodendrocyte loss in MS lesions is not well understood. Direct injury by the cytotoxic cytokines TNF- α , IFN- γ and LT- α and reactive oxygen and nitrogen species, apoptotic cell death mediated by Fas/FasL signaling, CD8-mediated cytotoxicity and lysis by $\gamma\delta$ -cells (which recognize HSP64 on the oligodendrocyte surface) are among the many factors considered responsible for the observed cell death during the acute phase of the disease [63–66]. Moreover, deprivation of growth factors may also play a role in the observed oligodendrocyte loss characterized by Tunnel-positive apoptotic oligodendrocytes in chronic MS lesions. The detection of IL-17 and IL-17-producing mononuclear cells in the peripheral blood, and IL-17 mRNA in MS lesions, suggests that IL-17 plays a role in the pathogenesis of MS [67]. However, the precise role of these factors in the loss of oligodendrocytes and myelin in MS is not well understood. Recent studies reporting reduced loss of myelin proteins and lipids in the brains and spinal cord of animals with EAE treated with lovastatin [10], or a lovastatin and 5 aminoimidazole-4-carboxamide ribonucleoside (AICAR) combination [68], suggest the possible neuroprotective activity of lovastatin against loss of oligodendrocytes and myelin in EAE.

Statins support remyelination

Remyelination occurs in the initial stages of MS lesions, and correlates with the presence of oligodendrocytes and their progenitors [61–63]. Failure to remyelinate may be due to the depletion of remyelinating oligodendrocytes and their progenitors, inhibited recruitment of oligodendrocyte precursor cells and enhanced expression of myelination inhibitory signaling molecules. Therefore, success in remyelination may be achieved by downregulation of an ongoing inflammatory response, protection of endogenous oligodendrocytes and their progenitors and the suppression of myelination inhibitory molecules. Pathological studies of chronic MS lesions indicate the presence of insufficient numbers of oligodendrocyte progenitor cells and oligodendrocytes for myelin formation [62–63]. The expression of antibodies against oligodendrocytes and their progenitors [64,65], and activation of AMPA/kainite receptors by increased amounts of glutamate [66], are some of the factors that are reported to account for the observed demyelination in inflammatory areas in MS plaques. Reparative therapies with a potential to enhance remyelination, such as statins, provide a promising approach for myelin

repair [10,68,69]. Higher numbers of oligodendrocyte progenitors in the spinal cords of animals with EAE treated with lovastatin and their observed proliferation and differentiation into myelin-producing cells document the potential of statins to repair demyelinated lesions [10]. These *in vivo* studies are also supported by *in vitro* studies of mixed glial cultures, in which lovastatin treatment protected the oligodendrocyte progenitor cells against the inflammatory insult, and induced their differentiation into myelin-producing cells by Rho inactivation via an isopenylation-dependent mechanism (Figure 3) [10]. Subsequent studies with purified oligodendrocytes also reported that short-term treatment with simvastatin also induced differentiation to the mature phenotype via inactivation of RhoA. However, longer term treatment was associated with process retraction and cell death [69]. The observed increase in oligodendrocyte precursors, their differentiation and expression of myelin-related genes in mixed glial cultures and in EAE [10], as compared with the effects observed with purified oligodendrocytes [69], all indicate that trophic molecules secreted by astrocytes and microglia may potentiate the effects of statins. The observed increased expression of transcription factors and trophic factors responsible for proliferation of oligodendrocyte progenitors, and transcription factors for their differentiation into myelin producing oligodendrocytes in lovastatin-treated animals during disease remission, may contribute to the observed remyelination in animals with EAE treated with lovastatin [10]. Therefore, the best way to prevent disease progression in MS is to prevent demyelination, protect oligodendrocyte progenitors and promote remyelination. Lovastatin treatment of EAE rats has been reported to meet these criteria [10,68].

Statins inhibit axonal loss

Trapp *et al.* have reported that axonal transection is not only a late event secondary to demyelination, but also occurs in early MS lesions. Axonal loss is related to the intensity of the inflammation and correlates with neurological disability in MS. The precise cause of axonal loss is not well understood. Different mechanisms may apply at different time points during MS lesion development. Axonal degeneration as a consequence of demyelination is due to the loss of trophic signals to the axon provided by myelin and to glutamate-mediated calcium toxicity. Axons are also vulnerable to the proteolytic enzymes, cytokines, oxidative products and free radicals produced by a local inflammatory response [70,71].

Glutamate-mediated calcium toxicity-induced mitochondrial dysfunction and the associated loss of energy has been proposed as one of the mechanisms of axonal loss in MS [72,73]. Under limited energy supply due to either decreased production during calcium overload, or due to excessive utilization for conductance by demyelinated axons, the collapse of ion gradients across the membrane and calcium-activated protease-mediated proteolysis of neurofilaments result in axonal damage in MS brain lesions. These findings have clinical and therapeutic implications, since early axonal damage may be amenable to treatment with anti-inflammatory and neuroprotective agents.

Recent studies reporting protection against apoptosis via upregulation of expression of Bcl-2 in neurons isolated from guinea pigs following chronic administration of simvastatin suggest that simvastatin treatment may provide a neuroprotective effect [74]. An *in vitro* study reported neuronal protection by atorvastatin in glutamate-induced toxicity [75]. Moreover, the observed neuroprotection and recovery in animal models of spinal cord injury [55] and traumatic brain injury [76–78] following statin treatment suggest statin-mediated neuroprotection in these animal models of brain injury. However, the mechanisms of action of statin-mediated neuroprotection and possible repair are not understood at present. Increased migration of Th2 lymphocytes across the BBB [79] and a possible role of Th2 mediators in neuroprotection and repair have recently been reported [80,81]. In addition to immunomodulatory activity of statins, lovastatin was also reported to induce the expression of PPAR- γ [82], whose activation is

considered to be a pharmacological target in neurodegenerative diseases [83]. These observations suggest that statin treatment may also provide neuroprotection by attenuation of inflammatory disease-induced glutamate/calcium-mediated demyelinating axonal damage in MS.

Treatments for multiple sclerosis

Presently, six medications are approved by the US FDA as immunomodulatory therapies for RRMS. Since the widespread use of IFN- β 1b (Betaseron[®]), IFN- β 1a (Avonex[®] and Rebif[®]) and GA (Copaxone[®]) over the past decade has changed the disease course of RRMS patients, we will briefly outline here the results of the studies leading to their approval. Pivotal trials for those immunomodulatory therapies were performed in the early 1990s; however, many of their mechanisms of action are still not completely elucidated. The 1993 pivotal study for IFN- β 1b involved 372 patients with clinically active disease and an expanded disability status scale (EDSS) less than 5.5. Both primary outcome measures were significantly improved by treatment – the relapse rate decreased by 33% and the proportion of relapse-free patients increased from 16 to 31% in patients treated with IFN- β 1b in comparison with the placebo arm after 5 years. MRI study also reported a significant reduction in T2 lesion load, and decreased frequency of new lesions in comparison with a placebo group [84]. Since this first study did not show an effect on disability progression, it prompted extensive discussion concerning how relevant the decrease in relapse rate is in modifying long-term disease progression.

The second, 2-year study of intramuscular IFN- β 1a (Avonex) used a disability change defined as a sustained deterioration by one or more points in the EDSS, as a primary outcome measure. The study enrolled 301 RRMS patients with clinically active disease and a lower EDSS of 1–3.5. The study reported a significant decrease in the sustained disability progression ($p = 0.02$) and an 18% decrease in the relapse rate in IFN-treated patients. MRI studies reported a decrease in the number and volume of Gd-enhancing lesions ($p = 0.02$) [85]. The PRISMS study in 1998 tested the subcutaneous IFN- β 1a (Rebif) in 560 patients with clinically active RRMS and an EDSS score of 0–5 and reported that IFN- β 1a treatment reduced the relapse rate at 2 years by 33%. MRI results revealed a significant decrease in T2 lesion load ($p < 0.001$) [86]. Several studies (Pivotal Betaseron study, PRISMS and EVIDENCE) have provided evidence that there is a dose effect, with higher IFN- β doses being more effective with respect to the majority of the outcome measures. The results from three studies on patients with a clinically isolated syndrome (CIS) suggestive of MS (CHAMPS, ETOMS and BENEFIT) determine that early treatment delays or prevents conversion to clinically definitive MS.

IFN- β has been demonstrated to have multiple anti-inflammatory effects (Table 1). While its initial use in MS was supported by its antiviral effect, multiple other mechanisms of action were demonstrated over the years as more relevant for achieving the immunomodulatory effect. IFN- β has a strong antiproliferative effect on T cells and other inflammatory cells, as well as the inhibitory effects against IFN- γ . IFN- β decreases MHC class II and costimulatory molecule B71 and B72 expression on APCs within the peripheral circulation (DCs and B cells) and microglia within the CNS, which overall inhibits antigen presentation and the initiation and perpetuation of the inflammatory response. More specifically, IFN- β was shown to inhibit IL-12 production, the cytokine critical for the Th1 differentiation, and to induce IL-10 immunoregulatory cytokine [87]. Multiple studies have reported on the IFN- β -induced decrease of Th1 cytokines IFN- γ , IL-12 and TNF- α , however these results have not been uniformly confirmed [88]. Perhaps the best established IFN- β 1a mechanism of action is the inhibition of T-cell trafficking [27]. IFN- β increases shedding of adhesion molecules (VCAM-1 and ICAM-1), decreases chemokine (MIP-1 α and RANTES) and chemokine receptor (CCR)5 expression and inhibits production of MMP-2, -7 and -9, enzymes that are involved in disintegration of the extracellular matrix, which facilitates cell migration across the endothelial barriers [88].

Glatiramer acetate is a mixture of short peptides containing four amino acids: glutamic acid, alanine, lysine and tyrosine, which are over-represented in the myelin basic protein immunodominant epitope. It has been serendipitously discovered as a compound that suppresses EAE. Following two decades of laboratory studies, in 1995 GA was tested in a clinical trial involving 251 RRMS patients randomized into treatment versus placebo groups over 2 years. The relapse rate was decreased in the GA-treated patients by 29%, and there was a difference in the disability measures at the end of the study [89]. A subsequent MRI study [90] that involved 239 patients and analyzed monthly imaging studies over a 9-month treatment period, reported a 29% reduction in the number of Gd-enhancing lesions. The effect was first apparent after 6 months of treatment, and there was no difference in the accumulation of T2 lesions. A subsequent publication [91] reported that GA reduced the percentage of new Gd-enhancing lesions that develop into persistent T1 hypointense ('black hole') lesions by 50%. These reports suggest that GA has a less prominent and slower effect on the MRI markers of inflammatory changes than IFN- β , but may favorably affect the proportion of MRI lesions that develop significant axonal loss.

Regarding GA's mechanisms of action, human *in vitro* studies have demonstrated that GA peptides bind to MHC class II DR molecules and may competitively inhibit myelin peptide presentation to the autoreactive cells. While this mechanism probably does not play a role in vivo due to rapid GA degradation and no measurable concentrations in the peripheral circulation, in vitro studies have revealed that GA inhibits the activation, proliferation and IFN- γ secretion of myelin basic protein-reactive T cells in a dose-dependent manner [92]. In addition, GA induces anergy or unresponsiveness in myelin basic protein-reactive T cells. Most importantly, GA generates GA-specific T cells that predominantly secrete Th2 cytokines [88]. GA induces a strong proliferative response, since it is a mixture of multiple altered peptide ligands, which are well presented by heterogeneous MHC class II molecules and stimulate multiple T-cell receptors. Studies in patients treated with GA report that GA-reactive cells exhibit a shift from a Th1 to a Th2 phenotype, leading to the decline of the proliferative response to GA [87]. Multiple researchers have proposed that there is cross-recognition of myelin antigens and bystander suppression of the myelin response by Th2-cytokine-secreting GA-reactive cells, however, the proof in humans is still not available. Finally, GA-reactive T cells have been reported to have neuroprotective effects in animal models of CNS injury [88]. GA-reactive T cells produce brain-derived neurotrophic factor (BDNF), a potent neuroprotective neurotrophin, which may play a role in neuroprotection or neurorepair within the MS lesions.

Mitoxantrone (Novantrone[®]) is an anti-neoplastic agent that inhibits DNA and RNA synthesis and suppresses T- and B-cell proliferation. In 2002, Hartung *et al.* conducted a 2-year pivotal double-blinded, placebo-controlled study in 194 patients with either worsening RR or secondary progressive MS [93]. The study compared the effect of mitoxantrone intravenous infusions every 3 months with placebo. At 24 months, a benefit was reported in all outcome measures, including change in the EDSS, the ambulation index, standardized neurological status, number of relapses and time to first relapse. Mitoxantrone reduced T2 lesion number ($p = 0.03$) and the number of new contrast-enhancing lesions ($p = 0.02$). It is important to note that mitoxantrone has significant toxicity, including bone marrow suppression, increased risk for infections and cardiomyopathy. Blood counts and cardiac monitoring are required prior to the infusions within the recommended safe cumulative dose of 100 mg/m².

Natalizumab (Tysabri[®]) is a humanized anti-VLA4 monoclonal antibody that blocks the $\alpha 4$ integrin on the lymphocytes and monocytes and inhibits their binding to the endothelial VCAM-1, subsequent cell adhesion and transendothelial migration. Early studies in EAE and MS demonstrated that natalizumab inhibited inflammatory cell migration across the BBB and cellular infiltration of the CNS. In a large Phase III trial, 942 patients with clinically active RRMS were randomized to monthly natalizumab or placebo infusions for 28 months.

Natalizumab reduced the risk of sustained disability progression by 42%. It reduced the clinical relapse rate by 68%, and was associated with a significantly higher proportion of relapse-free patients than placebo ($p < 0.001$). Natalizumab reduced the accumulation of new or enlarging T2 hyperintense lesions by 83%, and reduced the number of Gd-enhancing lesions by 92% [94]. Upon interim analysis at the median treatment duration of 13 months, natalizumab was approved for use in RRMS. However, it was subsequently withdrawn from the market due to two lethal cases of progressive multifocal leukoencephalopathy (PML) in MS patients treated with natalizumab and IFN- β 1a over 2 years. Later, the third case of lethal PML was reported in a patient with Crohn's disease who received eight infusions of natalizumab and multiple prior immunosuppressive therapies. While the estimated incidence of PML is one per 1000 patients, the medication has now returned to the market with specific recommendations regarding patient selection and extensive monitoring for early detection of PML and other opportunistic infections.

In conclusion, while IFN- β and GA are widely used for treatment of RRMS, they reduce relapse rates by approximately 30% and have only moderate long-term benefits. More effective therapies, including mitoxantrone and natalizumab, are associated with significant toxicity and are typically reserved for the patients with a more aggressive disease course. Therefore, the need for an effective and safe therapy for RRMS is still unmet. All the available therapies target only the inflammatory aspect of the disease, while there are no effective neuroprotective or reparative therapies. The immunomodulatory and neuroprotective mechanisms of statins have been recognized over the past several years. However, statins' clinical testing in inflammatory diseases is still in its infancy. Sena *et al.* reported an open-label 1-year long study testing 40 mg oral lovastatin therapy in seven patients with clinically active RRMS [95]. The treatment was associated with the stabilization of disability progression. A subsequent open-label study of high-dose oral simvastatin (80 mg daily) [46] in 28 patients with active RRMS reported a 44% decrease in the number and a 41% decrease in the volume of Gd-enhancing lesions over the 6-month treatment period. Owing to the short treatment period, there was no significant change in the clinical outcome or disability measures. In addition to monotherapy, *in vitro* studies indicate that statins may have synergistic effects with other immunomodulatory therapies, including IFN- β 1a [43] and GA [96]. Surprisingly, in 2007, Birnbaum *et al.* reported that adding atorvastatin (40 or 80 mg daily) to high-dose subcutaneous IFN- β 1a in a cohort of 24 stable RRMS patients was associated with an increase in enhancing MRI lesions and/or clinical relapses after 6 months of treatment ($p = 0.004$) [97]. While this study raised concerns regarding statins' ability to block some of the IFN- β signaling pathways, these results have not been replicated in other studies [98,99]. We have recently reported the results from a pilot study in ten chronic ischemic syndrome patients in which simvastatin 80 mg daily was added to a IFN- β 1a weekly intramuscular therapy in a placebo-controlled fashion. The combination therapy was safe and well tolerated. All patients remained clinically stable, with a significant decrease in T2 lesion load over the 12 months of treatment [100]. Currently, six additional studies are testing statins as a monotherapy or in combination with IFN- β 1a or GA in patients with RRMS.

Future perspective

Multiple sclerosis is a CNS demyelinating disease with a chronic inflammatory response directed against the CNS myelin antigens. The clinical disease activity correlates with inflammatory demyelination and axonal damage that can be observed early in the development of MS lesions. Therefore, for therapies to be effective against MS, they need to be immunomodulatory as well as neuroprotective. Currently approved treatments for MS (IFN- β and GA) are immunomodulatory, but they do not have neuroprotective and neuroreparative effects. In addition, these medications are administered parenterally and have significant side effects. Statins represent an attractive new treatment option for MS because they seem to have

neuroprotective and neuroreparative activities in addition to immunomodulatory activity. Their immunoregulatory activity in MS models is well established, but information regarding their neuroprotective and neuroreparative activities is limited. Even though studies of animal models of spinal cord and brain injuries provide significant evidence for the possible role of statins in neuroprotection and neurorepair, detailed mechanistic studies of statins' activities in neurodegeneration and regeneration may provide the basis for future research targeting the pathobiology of MS and other neuroinflammatory diseases. Combination therapy is gaining momentum over monotherapy for improving the treatment of MS. The initial studies of the combination of statins with IFN- β or GA have provided encouraging results [101]. However, to minimize adverse effects, the medications need to be tested at lower doses. It is possible that medications with different mechanisms of action will have a synergistic/additive effect and will, thus, provide a rationale for the use of combination therapy in complex diseases, such as MS. Large-scale, placebo-controlled, clinical studies of statins as monotherapy and in combination with current or future therapies will be required in the next 5 years to evaluate the therapeutic potential of statins in MS and in other neuroinflammatory diseases.

Executive summary

- Statins are being investigated as possible therapeutics for multiple sclerosis (MS) by various laboratories around the world because of their oral bioavailability and favorable safety record.
- Using tissue cultures and experimental autoimmune encephalomyelitis (EAE) animal disease models, our laboratory was the first to report that statins have anti-inflammatory effects, attenuate the infiltration of immune cells into the CNS and prevent and ameliorate disease activity in EAE. Subsequent studies from other laboratories have reported that statins shift the Th response to a Th2 anti-inflammatory response, block the expression of metalloproteinases and disrupt the BBB and, thus, transmigration of peripheral blood immune cells into the CNS.
- Recent studies have also reported that statins are neuroprotective: lovastatin inhibited the loss of oligodendrocyte progenitors and myelin damage in the spinal cords of animals with EAE and provided a higher degree of remyelination in comparison with untreated animals.
- Recent studies from animal models of traumatic spinal cord and brain injury provide further evidence that statins protect against traumatic and inflammatory neurodegeneration, and that statin treatment leads to clinical improvement.
- Moreover, the observed higher efficacy of statins in combination with glatiramer acetate and IFN- β 1a, suggests that combinations of these therapies with statins could improve the therapeutic effect in MS.
- Studies using cultured cells, animal models, and the initial human clinical studies have established the anti-inflammatory effects of statins, which are independent of their cholesterol-lowering effects and may be effective in neuroinflammatory diseases such as MS, stroke and Alzheimer's disease, as well as in traumatic spinal cord and brain injuries. However, prospective randomized trials will be required to evaluate the effectiveness of statins for MS and other neuroinflammatory diseases.

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Figure 1. Cholesterol biosynthesis pathway highlighting the biologically active metabolites.
HMG-CoA: 3-hydroxy-3-methyl glutaryl coenzyme A.

Figure 2.

Participation of various cell types & inflammatory mediators in immune mechanisms of multiple sclerosis.

APC: Antigen-presenting cell; CRP: C-reactive protein; LFA: Lymphocyte function-associated antigen;

mDC: Myeloid dendritic cell; MMP: Matrix metalloproteinase; NO: Nitric oxide; ROS: Reactive oxygen species; TCR: T-cell receptor; TLR: Toll-like receptor.

Figure 3.

Mechanism of action of lovastatin-mediated oligodendrocyte progenitor survival and their differentiation into mature oligodendrocytes in the CNS.

Lovastatin inhibits the synthesis of isoprenoids required for isoprenylation of small G-proteins (Rho family GTPases, specifically, RhoA, cdc42 and Rac1).

AS: Astrocyte; CM: Cytokine mixture; HMG-CoA: 3-hydroxy-3-methyl glutaryl coenzyme A; iNOS: Inducible nitric oxide synthase; MC: Microglia; NO: Nitric oxide; OP: Oligodendrocyte progenitor.

Adapted from [82].

Table 1

Similarities & differences in effects of statins as compared with these of glatiramer acetate & interferon in multiple sclerosis.

Site of action	IFN- β	Glatiramer acetate	Statins
<i>Effects on the peripheral blood inflammatory cells</i>			
Shift in Th1 to Th2 cytokine production	Yes	Yes	Yes
Inhibition of Th17 cell differentiation	Not known	Not known	Yes
Decreased inflammation	Yes	Yes	Yes
Inhibition of macrophage activation	No	No	Yes
<i>Effects on the blood–brain barrier</i>			
Expression of extracellular proteins	Yes	No	Yes
Blocking the activity of metalloproteinases	Yes	Not known	Yes
Blocking the transmigration of inflammatory cells	Yes	No	Yes
<i>Effects in the CNS</i>			
Blocking the inflammation	Not known	Not known	Yes
Protection of CNS resident cells	Not known	Not known	Yes
Protection of axons	Not known	Not known	Yes
Promoting remyelination	Not known	Not known	Yes