Statins as immunomodulators
Comparison with interferon-β1b in MS

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Abstract—Background: Recent data suggest that statins may be potent immunomodulatory agents. In order to evaluate the potential role of statins as immunomodulators in MS, the authors studied their immunologic effects in vitro and compared them to interferon (IFN)β-1b. Methods: Peripheral blood mononuclear cells (PBMC) obtained from untreated or IFNβ-1–treated patients with relapsing-remitting MS or from healthy donors (HD) and T cells were stimulated with concanavalin A, phytohemagglutinin, or antibody to CD3 in the presence of lovastatin, simvastatin, mevastatin, IFNβ-1b, or statins plus IFNβ-1b. The authors analyzed proliferative activity of T cells and B cells, cytokine production and release, activity of matrix metalloproteinases (MMP), and surface expression of activation markers, adhesion molecules, and chemokine receptors on both T and B cells. Results: All three statins inhibited proliferation of stimulated PBMC in a dose-dependent manner, with simvastatin being the most potent, followed by lovastatin and mevastatin. IFNβ-1b showed a similar effect; statins and IFNβ-1b together added their inhibitory potentials. Furthermore, statins reduced the expression of activation-induced adhesion molecules on T cells, modified the T helper 1/T helper 2 cytokine balance, reduced MMP-9, and downregulated chemokine receptors on both B and T cells. Besides strong anti-inflammatory properties, statins also exhibited some proinflammatory effects. Conclusions: Statins are effective immunomodulators in vitro that merit evaluation as treatment for MS.

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Statins are inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and are widely used as lipid-lowering agents.1,2 These drugs greatly reduce arteriosclerosis and cardiovascular morbidity, which, in the past, was mainly attributed to their cholesterol- and low-density lipoprotein–lowering properties.3 However, statins may also be potent immunomodulators with therapeutic potential for a variety of immune-mediated disorders such as MS, rheumatoid arthritis, type I diabetes mellitus, and graft rejection in organ transplantation.4 Statins inhibit interferon (IFN)γ-induced upregulation of major histocompatibility complex (MHC) class II molecules on human endothelial cells in vitro,4 reduce leukocyte endothelial adhesion and extravasation in a rat model,5 inhibit human natural killer cell activity in vitro,6 and notably, block lymphocyte function-associated antigen-1 (LFA-1)–mediated costimulation, a crucial process in T-cell activation.7 In addition, statins reduce the stimulation-induced nitric oxide synthase and the release of proinflammatory cytokines in astrocytes, microglia, and macrophages in vitro8 and ameliorate myelin basic protein–induced experimental autoimmune encephalomyelitis (EAE) in a rat model.9 Currently, a phase II clinical trial is evaluating the drug safety of simvastatin in MS. To further explore the potential of statins for therapeutic immunomodulation in MS, we investigated immune responses in MS and compared the effects with those induced by IFNβ-1b as an established disease-modifying therapy in MS.

Patients and methods. Patients. Blood was drawn after informed consent from 74 patients with MS and from 25 healthy donors (HD). All patients had clinically definite MS according to the Poser criteria,10 and had remitting-relapsing MS (RRMS, n = 60) or secondary progressive MS (SPMS, n = 14).11 Sixteen of the patients with MS were without treatment, 39 were treated with IFNβ-1a, and 19 with IFNβ-1b. Owing to the limited blood volume drawn from each patient (25 mL) not all assays could be performed on all patients. Proliferation assays and cytometric
analyses of surface molecule expression were performed on cells from HD, untreated, and IFNβ-1–treated patients. Cytokine release, T-cell apoptosis, and activity of matrix metalloproteinases (MMP) were analyzed on HD and untreated patients with MS.

Treatment of peripheral blood mononuclear cells (PBMC) in vitro. Simvastatin, lovastatin, and mevastatin (Calbiochem, Bad Soden, Germany) were activated as described,12 diluted in RPMI 1640, and stored frozen at −20 °C. IFNβ-1b (specific activity 3.2 × 10^7 IU/mg) was obtained from Schering, Berlin, Germany. Statins and IFNβ-1b were applied at different final concentrations as indicated in Results. Mevalonic acid lactone (Sigma, Munich, Germany) is the downstream product of the HMG-CoA reductase and, hence, antagonizes statin effects. It was used in tenfold molar concentrations (100 μM) to prove reversibility of inhibition by statins at concentrations of up to 10 μM.

Proliferation assay. PBMC were isolated on a discontinuous density gradient (Lymphoprep, Nycomed, Oslo, Norway). Cells were plated in 96-well microtiter plates at a density of 2 × 10^6 cells/mL at 100 μL/well in culture medium (RPMI 1640 supplemented with 5% fetal calf serum and 1% glutamine) and stimulated with either concanavalin A (conA, 2.5 μg/mL, Pharmacia, Freiburg, Germany), antibody to CD3 (0.1 μg/mL, Immunotech, Marseille, France), phytohemagglutinin (PHA, 5 μg/mL, Sigma), or medium alone as a negative control. For selective analysis of B-cell proliferation, B cells were enriched from PBMC by negative selection using paramagnetic beads directed against non-B cells (Dynal, Hamburg, Germany) and activated with pokeweed mitogen (PWM, 2 μg/mL, Sigma). Statins, IFNβ-1b, or medium alone as a control, were added immediately after cell plating. After 48 hours cells were labeled with tritiated thymidine (Amersham, Braunschweig, Germany) for 16 hours (0.2 μCi/well), harvested, and thymidine incorporation was measured with a liquid scintillation counter (Packard, Meriden, CT). Data are given as mean counts per minute (cpm) derived from triplicate cultures. Treatment-induced changes in proliferation are given as percent of the stimulated but untreated control cells.

Secretion of cytokines by PBMC in vitro. For cytokine measurements cells were plated in serum-free defined medium (Aim V, Life Technologies, Karlsruhe, Germany) and stimulated with conA or antibody to CD3. Cells were treated with simvastatin or IFNβ-1b or medium alone as a control. Cells were culture supernatants were collected at 48 hours, immediately frozen in liquid nitrogen, and stored at −80 °C. IFNγ, tumor necrosis factor (TNF)-α, interleukin (IL)-4, IL-10, and IL-12 were measured in the supernatants by specific ELISA (R&D Systems, Wiesbaden, Germany).

Flow cytometry analysis. Surface expression of immune molecules was quantified by flow cytometry on a fluorescence activated cell sorter (FACSCalibur, Becton Dickinson, Heidelberg, Germany) by standard methods. Data were analyzed using Cellquest software (Becton-Dickinson). Antibody conjugates (all from Becton Dickinson) against the following surface molecules were used: CD3, a pan-T-cell marker; CD19, a B-cell marker; chemokine receptors CXCR2, CXCR3, CXCR4, CXCR5, CCR2, CCR5, and CCR6; human leukocyte antigen (HLA)-DR;
adhesion molecules CD11a (αL-chain of LFA-1), CD49d
(α4-chain of very late antigen-4, VLA-4), CD54 (intercellular
cell adhesion molecule-1, ICAM-1), CD62L (t-selectin);
costimulatory molecules CD80 (B7-1), CD86 (B7-2); CD95
(Fas/Apo1); CD45RA, a marker for naive, and CD45RO, a
marker for memory T cells; and corresponding isotype con-
trols. Expression of intracellular cytokines IL-4 and IFNγ
was measured as described previously. Cell viability was
assessed by propidium iodide uptake. A total of 10,000
events per antibody were evaluated.

Quantification of T-cell apoptosis. In five patients apo-
pptotic cells were visualized by flow cytometry using an-
nexin V-conjugates (Trevigen, Gaithersburg, MD). These
conjugates bind to phosphatidylserine, which is exposed on
the cell surface of early apoptotic cells. Necrosis was me-
sured by propidium iodide uptake.

Figure 2. Synergistic antiproliferative effect of simvasta-
in and interferon (IFN)-β1b on T cells. Peripheral blood
mononuclear cells (PBMC) were prepared and assessed for
cell proliferation as described in figure 1. T cells were
stimulated with antibody to CD3 (0.1
μg/mL) and incu-
bated with different concentrations of IFNβ-1b and either
with medium alone (squares) or with simvastatin 50 nM
(triangles), 250 nM (diamonds), or 1,000 nM (open cir-
cles). Proliferation is given as percent of the anti-CD3-in-
duced proliferation without treatment. IFNβ-1b-induced
inhibition of T-cell proliferation was considerably en-
hanced by the addition of simvastatin at relatively low
concentrations. Each point represents the mean ± SEM of
nine healthy donors. Similar results were obtained with
lovastatin and with simvastatin in untreated patients
with MS (n = 6).

MMP and zymography. Cells from five patients were
plated at a density of 2 × 10^6 cells/mL at 100 μL/well in
serum-free defined medium and stimulated with antibody
to CD3 as indicated above. Treatment consisted of medium
alone as a control or of simvastatin (10 μM) or IFNβ-1b
(10,000 U/mL). After 24 hours cell-free supernatants were
shock-frozen in liquid nitrogen and stored at −80 °C. Gela-
tinase activity of MMP-2 and -9 was quantified by gelatin
zymography as recently described in detail.

Statistical analysis. Significance of treatment effects
compared to control values was analyzed by a paired t-test.
Effects of different treatment modalities were compared
using the Wilcoxon signed-rank test.

Results. Inhibition of stimulation-induced T-cell prolif-
eration. Statins inhibited conA-, PHA-, and anti-CD3-
duced T-cell proliferation in a dose-dependent manner
(figure 1). The lipophilic simvastatin was significantly
more effective than the hydrophilic mevastatin, with lova-
statin having an intermediate effect (see figure 1). Prolifer-
ation of resting T cells was not significantly affected by
statins. The degree of inhibition of T-cell proliferation was
not statistically different between patients with MS and
HD. Established immunomodulatory treatment with
IFNβ-1a/b did not significantly influence statin effects on
PBMC from patients with MS. After stimulation the ratio
of memory to naive T cells, as determined by flow cytometry,
was 83.3:16.7 without treatment and was signifi-
cantly shifted up to 51.9:48.1 with simvastatin treatment.
Resting T cells did not show such a shift and had a similar
memory to naive T-cell ratio of 50.2:49.8 without treatment
and 52.4:47.6 after simvastatin treatment (n = 6, each).
This indicates a preferential antiproliferative effect
of statins on memory T cells. By contrast, after enrichment
of B cells and stimulation with PWM, the antiproliferative
effect with lovastatin was low (24.8% inhibition at 1 μM
versus 72.4% inhibition on whole PBMC), which indicates
a predominant effect on T-cell proliferation. The antiprolif-

<table>
<thead>
<tr>
<th>Table 1 Chemokine receptors are downregulated on T and B cells by interferon-β1b and simvastatin</th>
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<tbody>
<tr>
<td>Cells</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>T cells (CD3+)</td>
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<tr>
<td>CXCR3+</td>
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<td>CXCR4+</td>
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<td>CXCR5+</td>
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<td>CCR5+</td>
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<td>CCR6+</td>
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Peripheral blood mononuclear cells were isolated from patients
with MS and T cells were stimulated by antibody to CD3 (0.1
μg/mL) and treated with medium alone as a control or with
interferon-β1b (10,000 U/mL) or simvastatin (10 μM). The per-
cent of chemokine receptor positive cells is given as a mean ±
SD of eight untreated patients with MS (*p < 0.05).
Statins induced a significant modulation of the cytokine release profile of stimulated T cells of HD and patients with MS. Pretreatment with simvastatin induced a dose-dependent increase of the proinflammatory cytokines IFN-γ and IL-12 in the supernatant of anti-CD3–stimulated T cells. In addition, IL-6 secretion was markedly augmented while TNFα and IL-10 levels were significantly decreased by the treatment with simvastatin (figure 4). By contrast, IFNβ-1b significantly diminished the secretion of Th1 cytokines and enhanced the secretion of IL-4 and IL-10 (see figure 4). Thus, simvastatin modulated the Th1/Th2 balance with a relative shift toward the Th1 profile responses, and IFNβ-1b toward the Th2 profile of cytokine release.

Staining of intracellular cytokines corroborated the above results. The number of T cells expressing IL-4 was increased by the treatment with simvastatin (figure 4). By contrast, IFNβ-1b significantly diminished the secretion of Th1 cytokines and enhanced the secretion of IL-4 and IL-10 (see figure 4). Thus, simvastatin modulated the Th1/Th2 balance with a relative shift toward the Th1 profile responses, and IFNβ-1b toward the Th2 profile of cytokine release.
Table 3 Comparison of the immunomodulatory profile of simvastatin and interferon (IFN)-β1b

<table>
<thead>
<tr>
<th>Immune mechanism</th>
<th>IFN-β1b</th>
<th>Simvastatin</th>
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<tr>
<td></td>
<td>Effect</td>
<td>Potency</td>
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<tr>
<td>Proliferation of T cells</td>
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<td>+++</td>
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<tr>
<td>Costimulatory molecules*</td>
<td>↓</td>
<td>+</td>
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<tr>
<td>Adhesion molecules</td>
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<tr>
<td>ICAM-1 (CD54)</td>
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<td>↓</td>
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<tr>
<td>α4-chain of VLA-4 (CD49a)</td>
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<tr>
<td>αL-chain of LFA-1 (CD11a)</td>
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<td>L-selectin (CD62L)</td>
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<tr>
<td>Chemokine receptors†</td>
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<td>+</td>
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<tr>
<td>HLA-DR</td>
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<td>↓</td>
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<tr>
<td>Cytokine release</td>
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</tr>
<tr>
<td>IFN-γ</td>
<td>↓</td>
<td>↑</td>
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<td>TNF-α</td>
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<td>IL-10</td>
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<tr>
<td>Activity of MMP-9</td>
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<tr>
<td>Apoptosis</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Fas (CD95)</td>
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Semiquantitative estimation of the immunomodulatory effects of simvastatin and IFN-β1b showed two distinct patterns. Interferon-β1b has a clear anti-inflammatory profile. By contrast, simvastatin exhibits predominant anti- but also proinflammatory properties.

* CD80 (B7-1), CD86 (B7-2).
† CXCR3, CXCR4, CXCR5, CCR2, CCR5, and CCR6.

++ = strong effect; ++ = moderate effect; + = mild effect; – = no effect; ↑ = increase; ↓ = decrease; ICAM = intercellular cell adhesion molecule; VLA = very late antigen; LFA = lymphocyte function-associated antigen; HLA = human leukocyte antigen; TNF = tumor necrosis factor; IL = interleukin; MMP = matrix metalloproteinase.

Intracellular IFN-γ was increased to 212 ± 49% of the control by pretreatment with simvastatin (n = 5).

Discussion. The current data provide evidence for a potent immunomodulatory action of statins on peripheral blood T and B cells in patients with MS. Potentially important immune mechanisms in the pathogenesis of MS have been delineated in the past few years. These are activation of T cells by antigen presentation and their transendothelial migration, which are essentially dependent on MHC class II antigens, on costimulatory and adhesion molecules, as well as on chemokine receptors and on MMP, among others; termination and control of a local immune response by apoptosis; and T helper–type dominance of the immune response.15-20 We show that statins modify the expression of several molecules crucially implicated in these processes and have a particular pattern of immunomodulation, with similarities and marked differences to that observed with IFN-β1b (see table 3). The in vitro immunomodulatory effects of IFN-β1b described here are in agreement with previous published work,21-23 and add the downregulation of a variety of chemokine receptors on T cells as a new putative mechanism of IFN-β1b in MS.24

The effects of statins on immune cells are remarkable. In particular, the preferential action on activated but not resting T cells, and on Th1 type chemokine receptors, is of potential therapeutic relevance. CCR5 and CXCR3, which are expressed preferentially on activated Th1 cells but also on a subpopulation of circulating B cells, were significantly downregulated by statins. In the active MS lesion, CXCR3 is the predominant chemokine receptor expressed on T cells in nearly all perivascular inflammatory infiltrates,25 whereas CCR5 is present only on a minority of T cells. CSF of patients with MS was found enriched for CCR5+ and CXCR3+ T cells and CXCR3+ B cells particularly during relapses.26-28

Interestingly, statins stimulate and inhibit cytokine release of both the Th1 and Th2 type. Secretion of potentially harmful cytokines such as IFN-γ and IL-12 was markedly increased by statins. Conversely, type Th2 cytokine IL-4 secretion was strongly upregulated and IL-10 downregulated. The net direction of this Th1/Th2 balance shift is difficult to determine. However, overall there seems to be an overweight of Th1 cytokine release under statin treatment in vitro.

Treatment of PBMC with statins also reduced the secretion of MMP-9, although this effect was less prominent than that observed with IFN-β1b. Previous work had shown that statins reduce MMP-9 secretion by human macrophages.30

MMP-9 is involved in several steps of the inflammatory reaction in the CNS, such as transendothelial migration of T cells at the blood–brain barrier (BBB), disruption of the BBB, degradation of extracellular matrix and myelin, and processing of the proinflammatory molecule TNFα, among others.13,16,31 In MS, MMP-9 is upregulated in the MS lesion32,33 and elevated concentrations are found in the CSF.34 Hence, in vivo inhibition of MMP-9 by immunomodulators could be an important step in downregulating the inflammation in the MS lesion.

The precise mechanism responsible for the anti-
inflammatory effects of statins are currently unknown. The best-known mechanism of statin action is the inhibition of the HMG-CoA reductase during the endogenous biosynthesis of cholesterol. There, a variety of intermediate substances called isoprenoids are formed, which have been shown to activate inflammation via intracellular second messenger systems. Very recent data suggest that statins may act on the immune system by at least two additional pathways. First, they can bind to and block the function of the integrin LFA-1, which is crucial for lymphocyte recirculation, antigen-specific T-cell activation, and transendothelial migration of T cells in experimental inflammation of the CNS. Secondly, they may act through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway—a second messenger system involved in a variety of T-cell functions.

On a molar basis, statins were significantly less potent than IFN-β. Strong effects of statins on most parameters were seen at concentrations in the range of 1 to 10 μM, which corresponds approximately to the 25- to 250-fold steady state plasma concentration of statins after oral intake. By contrast, most effects of IFNβ-1b were observed at 1,000 to 10,000 U/mL corresponding to 1.5 to 15 nM or to the 20 to 200-fold steady state concentration of IFNβ-1b, which is usually between 40 and 80 U/mL in patients receiving 8 × 10^6 U subcutaneously three times a week. Hence, if we take the corresponding blood levels as a basis, statins were comparably potent to IFNβ at a similar n-fold plasma level. Currently, it is unknown why doses of IFNβ-1b required to see effects on immune parameters in vitro are considerably higher than measured steady state levels. A necessity of accumulation of IFNβ in immune cells or in the CNS has been invoked, but this is speculative. Therefore, comparable low steady-state levels of statins in the blood do not preclude their therapeutic potential in MS, even if in vitro considerably higher concentrations are required.

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**Figure 4.** Statins modulate the cytokine release profile of stimulated peripheral blood mononuclear cells (PBMC). PBMC were isolated and plated in defined serum-free medium and T cells were stimulated with antibody to CD3. Cells were treated with medium alone as a control or with (A) simvastatin (10 μM) or (B) interferon (IFN)β-1b (10,000 U/mL). After 48 hours proliferation ([3H]) was determined and cytokines were measured in cell-free supernatants by specific ELISA. Each box represents the interquartile range (IQR) with the median (line) of the values of 14 patients with MS. The error bars are ≤ 1.5 times IQR. Data are given as percent of the stimulated but untreated control values. The corresponding absolute values given as the control value (100%) are as follows: proliferation: 15,286 counts per minute; IFN: 14,242 pg/mL; tumor necrosis factor-α: 1,610 pg/mL; interleukin (IL)-12: 19 pg/mL; IL-4: 4 pg/mL; IL-10: 492 pg/mL.

**Figure 5.** Inhibition of matrix metalloproteinase (MMP)-9 gelatinolytic activity by simvastatin. Peripheral blood mononuclear cells were isolated from untreated patients with MS and T cells were stimulated with antibody to CD3. Cells were treated with medium alone or with simvastatin (5 μM and 10 μM) or interferon (IFN)β-1b (10,000 U/mL) After 24 hours cell-free supernatants were collected and assayed for the presence of MMP-9 activity. Compared to the control (left), IFNβ-1b clearly reduced MMP-9 gelatinase activity in the culture supernatant (right). The effect of simvastatin was less pronounced at 10 μM (middle) and not detectable at concentrations below 5 μM.
Obviously, the potent immunomodulatory profile makes statins hopeful therapeutic candidates for clinical trials in MS. Nevertheless, caution is advisable. We observed a markedly increased stimulation-induced secretion of potent proinflammatory cytokines by T cells. Another group described proinflammatory actions of statins on endothelial cells in vitro. In vivo studies with statins in two experimental models of local inflammation in mice yielded conflicting results. In one report, statins significantly inhibited local leukocyte accumulation, whereas a conflicting result was reported. In another study, statins significantly inhibited local leukocyte accumulation, whereas a conflicting result was reported. In addition, cholesterol is essentially required for myelinogenesis, and synapticogenesis, and axonal regeneration. Theoretically, inhibition of cholesterol synthesis by statins could hamper remyelination and neural repair in the MS lesion. First studies on immune cells obtained from lovastatin-treated patients did not reveal significant effects on T-cell numbers and NK-cell function. However, in this study only one dosage was applied, which may result in too low blood concentrations to be effective on the immune system.

In our experiments established immunomodulatory treatment of patients with MS with IFNβ-1 did not significantly modify the action of statins compared to untreated patients or to HD. This fact together with the observation of additive effects of statins and IFNβ-1 in vitro nurture the hope that these well-established therapeutic agents may be a useful add-on therapy. A multicenter phase II clinical trial for the evaluation of simvastatin drug safety in MS is ongoing. Our in vitro data encourage clinical trials with statins as complementary therapy to interferons in MS but further careful evaluation of statin action is necessary. There is a large variety of statins that differ in their pharmacodynamic characteristics and, as our and others’ data show, also in their immunomodulatory potency. Certain properties of candidate statins such as lipophilicity, accumulation in the normal and in inflamed CNS, individual immunologic profile, short elimination half-life, and side effects at possibly required higher steady state levels could be relevant for the therapeutic efficacy of statins in chronic neuroinflammation. These issues need to be addressed soon in EAE and in concomitant immunologic studies on patients with MS enrolled in clinical trials.

Acknowledgment

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References

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