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Potential Targets of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitor for Multiple Sclerosis Therapy

Narender Nath,* Shailendra Giri,* Ratna Prasad,* Avtar K. Singh,† and Inderjit Singh2*.

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors or statins are newly identified immunomodulators. In vivo treatment of SJL/J mice withLovastatin reduced the duration and clinical severity of active and passive experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis. Lovastatin induced the expression of GATA3 and the phosphorylation of STAT6, whereas it inhibited tyrosine phosphorylation of Janus kinase 2, tyrosine kinase 2, and STAT4. Inhibition of the Janus kinase-STAT4 pathway byLovastatin modulated T0 to Th1 differentiation and reduced cytokine (IFN-γ and TNF-α) production, thus inducing Th2 cytokines (IL-4, IL-5, and IL-10). It inhibited T-box transcription factor and NF-κB in activated T cells and significantly reduced infiltration of CD4+ and MHC class II-positive cells to CNS. Further, it stabilized IL-4 production and GATA-3 expression in differentiated Th2 cells, whereas in differentiated Th1 cells it inhibited the expression of T-box transcription factor and reduced the production of IFN-γ. Moreover, lovastatin-exposed macrophage and BV2 (microglia) in allogeneic MLRs induced the production of the anti-inflammatory cytokine IL-10. These observations indicate that the anti-inflammatory effects of Lovastatin are mediated via T cells as well as APCs, because it modulates the polarization patterns of naive T cell activation in an APC-independent system. Together, these findings reveal that lovastatin may have possible therapeutic value involving new targets (in both APCs and T cells) for the treatment of multiple sclerosis and other inflammatory diseases. *The Journal of Immunology, 2004, 172: 1273–1286.

Multiple sclerosis (MS) is an inflammatory disease limited to CNS white matter. The CNS inflammation consists of a variable degree of T lymphocytes, macrophage, B lymphocytes, and Abs at the leading edge of the white matter destruction (1–4). A substantial percentage of MS patients develop clinical paralysis, and there is no curative therapy for MS (1–4). Experimental autoimmune encephalomyelitis (EAE) shares many of the clinical and histopathological features of MS and thus serves as a useful animal model (5). Although the disease is mediated by Th1 cells secreting the proinflammatory cytokines IFN-γ and TNF-α, genetic targeting of these cytokines in many cases aggravates clinical disease (6–9). Neutralization of lymphotixin and TNF-α prevents transfer of EAE, but they still have T cell infiltration (10, 11). Current literature describing the anti-inflammatory cytokines, such as IL-10 and TGF-β, suggest a range of divergent and sometimes paradoxical effects on EAE (12–15). EAE can be induced in susceptible strains of rodents by immunizing the animals with whole brain homogenate or purified neur Ags, such as myelin basic protein, proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein. Transfer of T cells specific to antigenic epitopes of neural Ags are sufficient to induce the disease (16–18).

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, are potent inhibitors of cholesterol biosynthesis, with benefits in the primary and secondary prevention of coronary heart disease (19, 20). Recent experimental and clinical evidence indicates that some of the cholesterol-independent, or so-called pleiotropic, effects of statins involve improving or restoring endothelial function, enhancing the stability of atherosclerotic plaques, and decreasing oxidative stress and inflammation (21–23). The therapeutic potential of Lovastatin has been well recognized with its antioxidant, antitumor, and anti-inflammatory activities and is under clinical trial for the treatment of cancer and some inflammatory diseases (21, 24–28). Previously (29–31) we have shown that anti-inflammatory activity of Lovastatin was associated with inhibition of proinflammatory cytokine production, such as IFN-γ, TNF-α, IL-1, IL-6, and inducible NO synthase. Other studies have shown that statins inhibit the expression of MHC-II on APCs, and thereby inhibit the recognition of Ag by T cells in vitro (32). Statins change the molecular confirmation of LFAs, facilitating binding to ICAM-1 (33). Oral administration of atorvastatin prevented paralysis in mice by inducing Th2-biased immune responses (34). The exact mechanisms involved in the anti-inflammatory activity of statins are yet to be defined. In this study we elucidated the effect of Lovastatin on T cells in vitro as well in vivo involving the transcription factors T-box transcription factor, and NF-κB in attenuation of EAE. T-box transcription factor has been master switch for IFN-γ production (35). Lovastatin inhibited T-box transcription factor in Th1 cells, thus inhibiting the generation of IFN-γ. Lovastatin inhibited NF-κB translocation to nucleus, which regulates the translation of various proinflammatory cytokines. It inhibited the phosphorylation of STAT4 by inhibiting the upstream kinases of the Janus kinase family (Jak2) and tyrosine kinase 2 (Tyk2). Lovastatin induced the expression of the GATA3 (master controller for IL-4) transcription factor of Th2 cells (36). Moreover, it stabilized its

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expression of GATA-3 and production of IL-4 in previously differentiated Th2 cells, whereas T-bet was inhibited and IFN-γ generation was reduced in differentiated Th1 cells. Further, we showed that lovastatin exerted its anti-inflammatory effects on APCs (peritoneal macrophage and BV2, microglia cells) by potently inhibiting the production of TNF-α in allogeneic MLR, whereas the expression of IL-10 was induced.

These observations strongly support the idea that lovastatin shifts T0 cells to Th2 via inhibition of T-bet and up-regulation of GATA3 and thus promotes attenuation of EAE. Further, these results emphasize that statins act on APCs as well as T cells of the immune system and therefore may prove to be of therapeutic value in MS and other Th1 cell-mediated inflammatory diseases.

Materials and Methods

Mice

Female SJL/J mice, 4- to 5-week-old, were purchased from The Jackson Laboratory (Bar Harbor, ME) and Harlan Laboratories (Indianapolis, IN). Mice were housed in the Medical University of South Carolina animal care facility and received standard laboratory food and water ad libitum. Six- to 10-week-old mice were used for all experiments. Paralyzed mice required alternate food sources and were fed with surgical Transpel (Charles River Laboratories, Wilmington, MA) and water.

Peptide, reagents, and cell line

Myelin PLP<sub>39-151</sub> was purchased from Peptide International (Louisville, KY). Lovastatin was purchased from Calbiochem (La Jolla, CA). Recombinant murine IL-2, IL-4, and IL-12 were purchased from BD Pharmingen (San Diego, CA). The purified (NAE) anti-CD3e (145-2C11) and CD28 (37.51) Abs were purchased from BD Pharmingen. For FACS, anti-mouse CD4 (FITC; H129.19), IA<sup>a</sup> (FITC; 7-16.17), and CD16/CD32 (FcIII/II, 2.4G2) were used. The anti-pSTAT4/STAT4 and pSTAT6/STAT6 Abs were purchased from Zymed Laboratories (San Francisco, CA), and T-bet, GATA3, Jak2, Tyk2, and pIκBα/β Abs were obtained from (Santa Cruz Biotechnology, Santa Cruz, CA). Mouse BV-2 microglial cells were a gift from V. Bocchini (University of Perugia, Perugia, Italy).

Clinical evaluation of peptide-induced EAE

For PLP<sub>39-151</sub>-induced EAE, 6- to 8-week-old female mice were immunized with an emulsion (200 μl s.c.) containing 200 μg of M. tuberculosis H37Ra (Difco, Detroit, MI) and 100 μg of myelin PLP<sub>39-151</sub> distributed over two spots on the flank with a booster given on day 7. Each mouse additionally received pertussis toxin (200 ng i.v.; Sigma-Aldrich, St. Louis, MO) in 200 μl of PBS on days 0 and 7 postimmunization. Individual animals were observed daily, and clinical scores were assessed as described above.

Initiation of EAE by adoptive transfer

Female donor mice (6–10 wk old) were immunized with an emulsion (200 μl s.c.) containing 200 μg of Mycobacterium tuberculosis H37Ra and 100 μg of myelin PLP<sub>39-151</sub> distributed over two spots on the flank on days 0 and 7. Draining lymph node cells (DLN) were harvested from donor mice on day 10 for in vitro stimulation. DLN cells were cultured (5 x 10<sup>6</sup> cells/ml) in RPMI-complete (containing RPMI 1640 (Life Technologies, Gaithersburg, MD), 10% FBS, and 100 μg/ml streptomycin and penicillin (Atlanta Biologicals Norcross, GA), 1 mM glutamine, 1 mM nonessential amino acids and 5 x 10<sup>-5</sup> M 2-ME (Sigma-Aldrich), and 10 μg/ml mouse rIL-12 (rmIL-12; BD Pharmingen)). After 96-h incubation, cells were harvested, washed, counted, and resuspended (3 x 10<sup>5</sup> DLN cells/0.3 ml) in buffered salt solution. On day 0, 6- to 8-week-old female naive SJL/J mice were injected i.p. with 30 x 10<sup>6</sup> T cells/mouse (in 300 μl). Recipient mice received 200 ng of pertussis toxin in 200 μl of PBS i.p. on days 0 and 2 (37, 38). Individual animals were observed daily, and clinical scores were assessed as described above.

Treatment of EAE with lovastatin

The mice were treated with lovastatin (2 and 5 mg/kg body weight i.p.; formulation volume: 200 μl/mouse daily from 0–60 days after induction of active or passive EAE. Lovastatin was dissolved in distilled, deionized water, and 1 N NaOH was added to create the open-ring structure of lovastatin (29, 30). Mice in the control group received 200 μl of PBS. The clinical score in active and passive EAE was graded as previously described.

In vivo and in vitro T cell proliferation assays

The DLN cells were removed on day 10 from the PLP<sub>39-151</sub>-immunized and lovastatin-treated (2 and 5 mg/kg body weight) mice, stimulated with 1, 2, 5, and 10 μg/ml of PLP<sub>39-151</sub>, and incubated for 72 h. The effect ofLovastatin on neural Ag-induced T cell proliferation was measured by [%H]TdT incorporation assay. Myelin PLP<sub>39-151</sub>-immune DLN cells (2 x 10<sup>6</sup>/100 μl/well) were cultured in 96-well, round-bottom microculture plates (Falcon Labware, Oxnard, CA) in 0.1 ml of RPMI-complete in the presence of 1–10 μg/ml PLP<sub>39-151</sub> peptide and lovastatin (10–50 μM). [%H]TdT incorporation (H129.19) was added at 48 h, and the uptake of radioactivity was measured after 72 h with a Top Count Microplate Scintillation Counter (Packard Bioscience, Mississauga, Canada). The naive T cells were isolated from SJL/J mice and purified by T cell enrichment columns (R&D Systems, Minneapolis, MN), and their purity was assessed by FACS. The anti-CD3e and -CD28 Abs were coated at 2 μg/ml in 96-well microtiter plates at 4°C overnight in RPMI 1640. After washing the plates with PBS, T cells were added to the wells (2 x 10<sup>5</sup> cells/well) and cultured in RPMI-complete. The naive T cells were pretreated with different concentrations ofLovastatin (10 and 20 μM) before being added to the plates. For proliferation, [%H]TdT incorporation was measured after 72 h using a Top Count Microplate Scintillation Counter (Packard Bioscience). The results are expressed as counts per minute (= mean counts per minute).

Long term T cell lines

Long term PLP<sub>39-151</sub>-T cell lines were established from the DLN of SJL/J mice primed 10 days earlier with 100 μg of the myelin PLP<sub>39-151</sub> peptide emulsified in CFA supplemented with 200 μg of M. tuberculosis H37Ra. Lines were propagated by culturing in vitro stimulation of 1 x 10<sup>6</sup> T cells with 5 x 10<sup>6</sup> irradiated syngeneic splenic APCs and 5 μg/ml of the peptide. T cells were restimulated every 3–4 wk with fresh spleen cells and Ag. T cell lines were maintained in RPMI-complete and rmIL-2 (10 ng/ml). Peptide-specific restimulation was repeated every 15–30 days (15).

Effect of lovastatin on PLP<sub>139–151</sub>-specific T cells and APCs

Splines were collected from naive, SJL/J mice as indicated. RBCs were removed by Pharmalyse (1 x), and the remaining cells were used as APCs. The APCs were irradiated (3000 rad), washed, and cultured in 96-well microtiter plates at a density of 5 x 10<sup>5</sup> cells/well. Different concentrations of PLP<sub>139–151</sub> peptide were added to the APCs. A PLP<sub>139–151</sub>-specific T cell line (10<sup>5</sup> cells/well) was cocultured with the APCs and Ags in a total volume of 100 μl with 10 and 20 μM lovastatin. For the effect on APCs, the peritoneal macrophage (5 x 10<sup>5</sup> cells/well) were pretreated with 10 and 20 μM lovastatin, PLP<sub>139–151</sub> (5 μg/ml) was added, and cells were further incubated for 2 h, after that PLP<sub>139–151</sub>-specific T cells (10<sup>5</sup> cells/well) were added to them. The cells were incubated for 96 h, and were pulsed with 1 μCi/well [%H]TdT for the final 24 h of the 48-h incubation period. [%H]TdT uptake was detected as given above, and results are expressed as the mean of triplicate cultures ± SD (37). The long term, PLP<sub>139–151</sub>-specific T cell line was derived from a SJL/J mice primed with PLP<sub>139–151</sub> as described above.

In vivo and in vitro Th1 and Th2 cytokine generation and effect of lovastatin

For in vivo cytokine analysis, the DLN cells from PLP<sub>139–151</sub>-immunized and lovastatin-treated (2 and 5 mg/kg body weight) mice were cultured for 48 h (Th1, IFN-γ) and 96 h (Th2, IL-4). For in vitro analysis, the myelin PLP<sub>139–151</sub>-immunized DLN cells (5 x 10<sup>5</sup> cells/ml) were cultured in RPMI-complete in 24-well plates with 5 μg/ml PLP<sub>139–151</sub> in the presence of lovastatin (10 and 20 μM). Naïve T cells were isolated from SJL/J mice and purified by T cell enrichment columns, and the purity of cells was assessed by FACS. The anti-CD3e and -CD28 Abs were coated in 24-well plates at 2 μg/ml in RPMI 1640 at 4°C overnight. After washing the plates with PBS, T cells were added (1 x 10<sup>5</sup> cells/well) and cultured in RPMI-complete. The naïve T cells were pretreated with different concentrations of lovastatin (10 and 20 μM) before being added to the plates. The culture
Effect of lovastatin on differentiated Th1 and Th2 cells

Naive T cells were isolated from SJL/J mice. CD4+ T cells were purified by CD4+ enrichment columns, and the purity of the cells was assayed by FACS (≥95%). The anti-CD3ε and -CD28 Abs were coated at 2 μg/ml in 24-well plates at 4°C overnight in RPMI 1640. After washing the plates with PBS, CD4+ T cells were suspended in RPMI 1640-complete and added to 24-well plates (1 × 10^6 cells/well), and the cytokine and Ab mixture was added for Th1 (rmIL-12, 10 ng/ml; anti-IL-4, 10 μg/ml) and Th2 (rmIL-4, 10 ng/ml; anti-IFN-γ and IL-12, 5 μg/ml) cell generation. After 96 h Th1 and Th2 cells (ascertained by cytokine analysis; data not shown) were harvested, and then these cells were restimulated with anti-CD3ε and -CD28 for Th1 (IFN-γ) and Th2 (IL-4) cytokines and T-bet and GATA-3 analysis. To determine the effect of lovastatin, the cells were pretreated with different concentrations of lovastatin (10 and 20 μM) before being added to the plates. The culture supernatants were collected at 48 h and checked for cytokine levels as mentioned above, and the cells were analyzed for T-bet and GATA-3 by Western blot.

APC-dependent priming of cytokine production

APCs (peritoneal macrophage from C57BL/6 mice and BV2 cells, a microglial cell line) were used to generate an allogeneic T cell stimulatory response. T cells were isolated from the lymph nodes of BALB/c mice. APCs were pretreated with lovastatin for 2 h at 10 μM, and then T cells were added at a 1/1 ratio (2 × 10^6 cells of each T cell vs APC) in 96-well plates. Cells were labeled with tritiated thymidine (1 Ci/ml) for 16 h before harvesting, and [3H]Tdr uptake was detected as in the proliferation assay. Supernatant was obtained from cell cultures before the addition of tritiated thymidine and analyzed for cytokines (IL-10 and TNF-α) by ELISA (BD PharMingen) (39).

Western blot analysis

Cells were incubated in the presence or the absence of different stimuli, harvested, washed with Hanks’ buffer, and sonicated in 50 mM Tris-HCl (pH 7.4) containing protease inhibitors (1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml antipain, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin). Proteins were resolved by 8–16% gradient SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were then blocked for 1 h in 5% nonfat dry milk/TTBS (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20, pH 7.4) for 1 h at room temperature. The membranes were then incubated on plates at a concentration of 10 μg/ml anti-CD4 and -CD28 Abs were preincubated with water or competitor DNA for 5 min at room temperature, and the samples were processed for EMSA.

TransSignal protein/DNA arrays

Purified naive T cells were stimulated with anti-CD3ε and CD28 (2 μg/ml) coated on 100-mm tissue culture plates at 4°C overnight. The cells were incubated on plates at a concentration of 10 × 10^6 cells/ml at 37°C for 4 h and then harvested from lovastatin-treated and untreated samples to determine various transcription factors by protein/DNA arrays according to the manufacturer’s instructions (Panomics, Redwood, CA). Briefly, a set of biotin-labeled DNA binding oligonucleotides (TransSignal probe mix) were preincubated with nuclear extract to allow the formation of protein/DNA (or transcription factor/DNA) complexes. The protein/DNA complexes were then separated from the free probes. The probes in the complexes were extracted and hybridized to the TransSignal Array. Signals were detected using a chemiluminescent imaging system.

Histological analysis

To assess the degree of CNS inflammation and demyelination, SJL/J mice treated with lovastatin after induction of active EAE were euthanized on day 13 (at the peak of the disease) by CO2 asphyxiation and perfused by intracardiac injection of PBS containing 4% paraformaldehyde and 1% glutaraldehyde. Four-micrometer-thick transverse sections were taken from lumbar regions of the spinal cord (six sections per mouse). The sections were stained with H&E to assess leukocyte infiltration and inflammation (30, 31).

Isolation and FACS of cells from spinal cord

For phenotypic characterization of cells from the CNS, mice were anesthetized after disease transfer. The spinal cords were isolated and mashed on a 200-mesh screen, resuspended in 30% Percoll, and overlaid on 70% Percoll. Cells were then spun for 15 min at 400 × g, and cells at the 30/70% interface were collected and washed twice with PBS (38). The cells were then stained for FITC- or PE-conjugated Abs anti-mouse CD4 and anti-mouse IAα. Nonspecific staining was blocked by anti-CD16/CD32. The cells were acquired by FACs and analyzed by CellQuest (BD PharMingen).

Statistical analysis

Data are presented as the mean ± SEM or the mean ± SD. All statistics were analyzed with a one-way multiple-range ANOVA test. Significant (p value) between groups were determined using the Newman-Keuls test. Values of p < 0.05 and above (p < 0.01, very significant; p < 0.001, highly significant) were considered significant.

Results

Prophylactic and therapeutic efficacy of lovastatin in EAE

To test the protective efficacy of lovastatin in MS, we examined effect of lovastatin on the pathogenesis of active EAE. SJL/J mice were treated with lovastatin i.p. from days 0–60 after induction of EAE by immunization with myelin PLP139–151. All mice in the EAE-ununtreated group developed clinical paralysis from day ~13 and reached a maximum mean clinical score (MMCS) of 3.5 on day 18 (Fig. 1a). Fifty percent of the mice treated withLovastatin (2 and 5 mg/kg) developed paralysis starting on day 14, and by day 20 all mice had developed paralysis, which lasted up to day 23 (2 mg) and day 28 (5 mg). EAE was delayed in lovastatin groups with a MMCS of 2.2, which was significantly less (p < 0.001) that in the EAE group. After the first relapse, disease was observed only in the EAE group, and there were no signs of relapse in treated groups. We observed significant (p < 0.01) weight loss in the EAE mice as disease progressed compared with healthy controls (Fig. 1b). In the 2 mg/kg Lovastatin-treated group, weight loss was observed, but it was significantly less (p < 0.01) than that in the EAE group. In 5 mg/kg Lovastatin-treated mice, weight loss was similar to that observed in the EAE group.
FIGURE 1. Lovastatin inhibits the clinical symptoms of EAE. The mean clinical scores of the diseased animals are given in a, c, and e, and weight measurements are given in b, d, and f. a. Active EAE was induced in SJL/J mice by immunization with myelin PLP_{139-151} peptide in CFA. c and e, Passive EAE was induced by adoptive transfer of myelin-PLP_{139-151}-sensitized T cells into recipient SJL/J mice. The mice (six per group) were treated i.p. with 2 or 5 mg/kg lovastatin every day from days 0–60 after induction of EAE; e, lovastatin started on day 10. a, c, and e, Lovastatin-treated mice developed significantly less (p < 0.001) severe disease. b, d, and f, Weights measured biweekly for active and passive EAE mice. Data are representative of three independent experiments with consistent results. g and h, The histopathology of spinal cord sections from adoptive EAE and lovastatin-treated SJL/J mice prepared from the lumbar regions (six sections per mouse) and fixed in 10% buffered formalin. The tissues were stained with H&E and are shown at ×100 magnification. h, The cells from spinal cord were isolated and stained for CD4^+ and MHC class II cells, acquired by FACS, and analyzed by CellQuest (see Materials and Methods).
Next, we examined the in vivo effect of lovastatin on adoptive EAE. SJL/J mice were treated with lovastatin (in the same manner as for active EAE) after induction of EAE by adoptive transfer of myelin PLP139–151-specific T cells. All 18 mice in the EAE-unpretreated group developed clinical paralysis starting on day 8, with a MMCS of 3.2 on day 13 (Fig. 1c). Treatment with 2 and 5 mg/kg lovastatin delayed disease onset by 3 days and decreased the development of paralysis (MMCS of 2 and 2.2) on day 13 during the first peak of EAE. Relapse was observed in lovastatin-treated groups on day 38 and 44, with MMCS of 0.67 and 1.61 in 2 and 5 mg/kg groups, respectively. In the EAE group, MMCS was 2 and peaked at 3.5 during the course of the study (60 days). In the adoptive EAE model, untreated animals lost weight continuously during the study, whereas the 2 mg/kg lovastatin-treated group did not show any weight loss compared with healthy controls (Fig. 1d). Mice treated with 5 mg/kg lovastatin lost weight during the first peak of disease; subsequently, mice regained the weight and maintained it similar to healthy controls.

To examine the therapeutic efficacy of lovastatin in ongoing EAE, SJL/J mice were treated withLovastatin (2 mg/kg i.p.) daily, and in another group it was given on alternate days from days 10–60 after induction of passive EAE (as above). Day 10 was chosen for lovastatin therapy because in adoptive EAE the first peak started on approximately days 8 and 10. The EAE-unpretreated group developed clinical paralysis starting on day 8, which progressed to a MMCS of 2.4 on day 18 (Fig. 1e). Treatment of mice withlovastatin starting on day 10 decreased the clinical severity of adoptive transfer EAE in the relapsing stage, but was unable to stop the first peak of disease. Lovastatin treatment on alternate days was unable to stop both the first peak and relapsing and remitting EAE. In the group treated daily withLovastatin, EAE peaked, and then relapsing and remitting disease was observed, but the clinical severity never reached an MMCS >2, which was significantly less (p < 0.001) than that in EAE. However, in the alternate dayLovastatin-treated group, MMCS reached 3.5, similar to that in the EAE group. We observed that mice inLovastatin-treated and -untreated groups both lost weight compared with the healthy controls (Fig. 1f).

**Lovastatin decreased infiltration of cells to CNS**

We examined the effect ofLovastatin on the pathogenesis of inflammation and demyelination in the CNS of mice with EAE. Spinal cord sections from mice treated withLovastatin after induction of passive EAE were analyzed for the infiltration of mononuclear cells (inflammation) and myelin loss (demyelination). As shown in Fig. 1g, the untreated EAE mice showed profound inflammation in the CNS. Treatment of EAE mice withLovastatin significantly reduced the number of inflammatory cells in both the artery and border region (Fig. 1g). We observed fewer demyelinated regions inLovastatin-treated animals compared with EAE mice (data not shown). Further, we examined the phenotype of infiltrating cells to CNS by FACS and found that the untreated EAE group had increased infiltration of CD4+ T cells along with MHC class II-positive cells compared with controls. However, Lovastatin treatment significantly reduced (p < 0.001) the infiltration of CD4+ and MHC class II-positive cells (Fig. 1h).

**Lovastatin inhibited neural Ag-specific and naive T cell proliferation**

To define the mechanisms involved in the regulation of CNS demyelination byLovastatin, we examined its effect on neural Ag-specific T cell responses in vitro and in vivo. As shown in Fig. 2a, stimulation of the myelin PLP139–151-immune T cell response in the presence of PLP139–151 (1–10 μg/ml) increased [3H]TdR uptake with a high proliferation index, which was significantly decreased (p < 0.001) after treatment with 10 μM lovastatin. We examined the effect ofLovastatin on naive T cell (>98% purified) proliferation. Preincubation with 10 and 20 μM lovastatin significantly reduced (p < 0.001) CD3ε and CD28-induced proliferation in a dose-dependent manner (Fig. 2b). Further, we examined the in vivo effect oflovastatin on T cell activation. As shown in Fig. 2c, compared with the untreated mice, in vivo treatment with lovastatin (2 and 5 mg/kg body weight) suppressed the memory responses to encephalitogenic PLP139–151 peptide. In vivo the production ofTh2 cytokine (IL-4) was increased (p < 0.001) with 2 and 5 mg/kg lovastatin, whereas theTh1 cytokine IFN-γ was significantly inhibited (Fig. 2d). Next, we determined the effect oflovastatin on PLP139–151-specific T cells in an APC-dependent system. Irradiated APCs (spleen cells) from syngenic mice loaded with PLP139–151 were incubated with aLovastatin-pretreated PLP139–151-specific T cell line. As shown in Fig. 2e, Lovastatin significantly inhibited (p < 0.001) T cell proliferation, emphasizing its direct effect on T cells. Further, we observed its effect on APCs for that autologous monocytes were incubated withLovastatin-pretreated peritoneal macrophage and PLP139–151-specific T cells (Fig. 2f) in which Ag presentation was significantly inhibited (p < 0.001).

**Lovastatin inhibited Th1 and induced Th2 cytokines in Ag-specific and naive T cells**

We examined the Ag-induced secretion of Th1 and Th2 cytokines in PLP139–151-immunized and in vitro-stimulated DLN cells. Culture supernatants were examined for Th1 (IFN-γ and TNF-α) andTh2 (IL-10, IL-5, and IL-4) cytokines. As shown in Fig. 3, a and c, Lovastatin treatment reduced the secretion of Th1; in contrast, the secretion ofTh2 cytokines was increased (Fig. 3, e, g, and i). Thus, Lovastatin inducedTh2-biased immune responses in vivo. To determine whetherLovastatin-induced Th2 bias only reflected a manipulation of APC function or was due to a direct immunomodulatory effect on T cells, we examined the effect ofLovastatin on purified (98% ascertained by FACS) naive T cells (CD3+). Th cells (CD3+) were obtained from SJL/J mice, pretreated withLovastatin (10 and 20 μM), and stimulated with anti-CD3ε and -CD28 Abs, and the secretion of Th1 and Th2 cytokines was measured. The production ofTh1 cytokines was significantly reduced (p < 0.001; Fig. 3, b and d); however, that ofTh2 cytokines was significantly increased (p < 0.001; Fig. 3, f, h, and j).

**Effect of lovastatin on expression of the transcription factors GATA3 and T-bet**

GATA3, a regulator of IL-4 inTh2 cells, acts both in a STAT6-dependent and -independent fashion (42). Expression of T-bet correlates with IFN-γ production inTh1 and NK cells, a proinflammatory cytokine inEAE and MS pathology (35, 43). BecauseLovastatin inhibited the production ofTh1 cytokines and inducedTh2, we examined its effect on transcription factors T-bet andGATA3, which are potentially involved inTh1 andTh2 cell development. As shown in Fig. 4a, in vivo treatment withlovastatin (2 and 5 mg/kg body weight) suppressed the expression ofT-bet and significantly induced (p < 0.01) the expression ofGATA3 (Fig. 4b). Next, we examined the expression ofT-bet andGATA3 in a myelin PLP139–151-specific T cell line treated with rmIL-12 and rmIL-4 for 48 h in the presence oflovastatin (10 and 20 μM); subsequently, T-bet andGATA3 expression were determined by immunoblot. Lovastatin (both 10 and 20 μM) inhibited T-bet expression (Fig. 4c), whereas the expression ofGATA3 was induced (p < 0.05; Fig. 4d).
results were further examined in naive T cells stimulated with anti-CD3ε and -CD28 Abs (Fig. 4, e and f). Naive T cells expressed T-bet and GATA3 at basal levels, whereas stimulation with CD3ε and CD28 under polarized conditions with rmIL-12 and rmIL-4 induced their expression significantly. Lovastatin (10 and 20 μM) completely abolished the expression of T-bet, whereas GATA3 was further enhanced (Fig. 4, e and f).

Effect of lovastatin on Th1/Th2 cytokines and transcription factors (T-bet/GATA3) in differentiated Th1 and Th2 cells

As we have seen, lovastatin inhibited the production of Th1 cytokines and induced a Th2 response. Next, we examined its effect on differentiated Th1 and Th2 cells by examining their cytokine profiles and the transcription factors T-bet and GATA3. As shown in Fig. 5, a and b, in vitro treatment with lovastatin (10 and 20 μM) on differentiated Th1/Th2 cells inhibited IFN-γ and stabilized IL-4 production. It suppressed the expression of T-bet significantly in Th1 cells (Fig. 5c); however, GATA3 expression was stabilized (Fig. 5d). Nonpolarized T cells were pretreated with lovastatin (10 and 20 μM) and stimulated with anti-CD3ε and -CD28. As shown in Fig. 5, e and f, T-bet was completely inhibited, whereas GATA3 expression was intact. GATA3 expression was intact in both polarized and unpolarized conditions with lovastatin, which supports our hypothesis that it induces Th2 cytokines involving GATA3.
Lovastatin inhibited phosphorylation of Jak2, Tyk2, and STAT4 and induced phosphorylation of STAT6 in PLP<sub>139-151</sub>-specific and naive T cells

As an activated (tyrosine-phosphorylated) STAT, STAT4 has a key role in IL-12-dependent Th1 lineage commitment (44). STAT6 is also required for IL-4-dependent Th2 lineage commitment (42). Thus, we examined whether lovastatin treatment suppressed the phosphorylation of STAT4 or induced the phosphorylation of STAT6 in PLP<sub>139-151</sub>-specific T cells. As shown in Fig. 6, a and b, the PLP<sub>139-151</sub>-specific T cell line was stimulated with rmIL-12 and rmIL-4 in the presence of lovastatin, which reduced the tyrosine phosphorylation of STAT4 in 15 min and induced STAT6 phosphorylation in 30 min (p < 0.001).

To define the mechanisms involved in the lovastatin-mediated regulation of T cell responses, we examined the effect of lovastatin on nuclear translocation of STAT4 and STAT6 translocation factors in naive T cells that were stimulated with anti-CD3ε and -CD28. Pretreatment of naive T cells with 10 μM lovastatin inhibited the CD3ε- and CD28-mediated nuclear translocation of STAT4 (Fig. 6c) and potentiated STAT6 nuclear translocation (Fig. 6d). To determine whether the inhibition of STAT4 proteins by lovastatin was a direct effect or a consequence of inhibition of upstream Jak, we examined the effect of lovastatin on spleen cells from PLP<sub>139-151</sub>-immunized mice. The cells were stimulated in vitro with rmIL-12 (10 ng/ml) for tyrosine phosphorylation of Jak2 and Tyk2. Immunoprecipitation and in vitro kinase assay showed that the stimulation of T cells with IL-12 induced the activity of Jak2 and Tyk2 in 15 min (Fig. 6, e and f). However, pretreatment of cells with lovastatin inhibited IL-12 and induced the activity of Jak2 (p < 0.01) and Tyk2 (p < 0.001).

Lovastatin inhibited NF-κB pathway in activated T cells

Activation of NF-κB is necessary for cell survival and for induction of IFN-γ, TNF-α, IL-2, and MHC classes I and II (45, 46). To understand the basis of inhibition of these cytokines in lovastatin-treated cells, we examined its effect on CD3ε- and CD28-induced activation of NF-κB in naive T cells. In naive T cells, NF-κB (p65/p50 heterodimer) is retained in the cytoplasm by its association with IκBα. After stimulation of naive cells with various agents, the cytosolic NF-κB/IκBα complex dissociates, and free NF-κB translocates to the nucleus and regulates the transcription of various genes. Phosphorylation of IκBα by upstream kinase IKK is essential for the dissociation of IκBα from NF-κB and its degradation (46, 47). Stimulation of naive T cells with CD3ε and CD28 significantly induced the nuclear translocation of NF-κB, as measured by gel-shift assay; however, 2-h pretreatment of T cells with lovastatin (10 μM) significantly inhibited NF-κB (p < 0.001; Fig. 7a). Inhibition of NF-κB by lovastatin appears to be dose dependent (Fig. 7b). The gel-shift detected a specific band in response to CD3ε and CD28 stimulation that was competed off by an unlabeled probe (data not shown). These results were further supported by the observation obtained from TransSignal protein/DNA arrays (Fig. 7c). Because lovastatin inhibits nuclear translocation of NF-κB, we examined the effect of lovastatin on phosphorylation and degradation of IκBα. Stimulation of naive T cells with CD3ε and CD28 induced phosphorylation and degradation of IκBα, which was inhibited by lovastatin (p < 0.001; Fig. 7d).

Lovastatin-pretreated APCs induce Th2 cells accompanied by generation of IL-10

We have shown earlier that lovastatin inhibits proinflammatory cytokines in glial cells in response to LPS activation (29). In this study we found that exposure of APCs (peritoneal macrophage and
microglia) to lovastatin hampers the early commitment of naive T cell to become Th1 cell. For this, peritoneal macrophage from SJL/J mice and BV2, a microglia cell line were used to stimulate allogeneic naive T cells from BALB/c mice. The polarization of naive T cells was evaluated by determining the production of IL-12p70, TNF-α, and IL-10. Fig. 8, a and b, shows that lovastatin inhibited the generation of TNF-α in allogeneic MLR; however, IL-12p70 was undetectable (data not shown). We subsequently evaluated whether this inhibition of proinflammatory cytokine (TNF-α) and potent Th1-favoring cytokine (IL-12p70) accompanied the induction of IL-10, and, as shown in Fig. 8, c and d, we found that it was significantly increased.

**Discussion**

In the present study we used the HMG-CoA reductase inhibitor lovastatin for in vivo studies with mice in an attempt to decrease the clinical and pathological symptoms of active and passive EAE in a relapsing and remitting mouse (SJL/J) model. Lovastatin significantly attenuated active and adoptive EAE. We observed that lovastatin induced the production of Th2 cytokines (IL-4, IL-5, and IL-10) by inducing the expression of GATA3 and the phosphorylation of STAT6 transcription factors, which aid in the remission of Th1 cell-mediated CNS autoimmune disease (EAE). The attenuation of EAE by lovastatin was associated with the inhibition of T-bet, NF-κB, and STAT4 signaling pathway and a decrease in the differentiation of neural Ag-specific Th1 cells in vivo in addition to the generation of proinflammatory cytokines (IFN-γ and TNF-α). We showed that lovastatin induced STAT6 nuclear translocation, thereby inducing Th2 responses. However, inhibiting the nuclear translocation of STAT4 inhibits the differentiation of T0 to Th1 cells and subsequent proinflammatory cytokine generation. Lovastatin inhibited tyrosine phosphorylation and activation of Jak2 and Tyk2, which are upstream kinases of STAT4; this might be an underlying mechanism for inhibition of STAT4 nuclear translocation in T cells. Further, we noted that lovastatin inhibits PLP139–151-specific T cell proliferation and Th1 cytokine generation as well as CD3ε- and CD28-stimulated naive T cell functions by blocking their proliferation and cytokine production. Lovastatin inhibited CD3ε- and CD28-induced activation and nuclear translocation of NF-κB, suggesting that inhibition of Th1
cytokine expression by lovastatin is possibly due to inhibition of NF-κB nuclear translocation. Lovastatin increased the phosphorylation of STAT6 in myelin PLP 139–151-specific T cells, which suggests that it induces Th2-biased immune modulation involving STAT6. Lovastatin potentiated the Th2-specific transcription factor GATA3 in PLP 139–151-specific T cells, which is known as a master switch for IL-4 production from Th2 cells (36). This study shows that lovastatin induces anti-inflammatory Th cell responses by modulating APC function as well as by directly affecting T cells, as it was observed that TNF-α and IL-12p70 were inhibited in the allogeneic MLR, whereas IL-10 was enhanced. It is noteworthy that lovastatin induces a Th1 to Th2 shift accompanied by the production of anti-inflammatory cytokine IL-10 in both APC-dependent and APC-independent systems.

The pathogenesis of EAE and MS is a complex process involving the activation of macrophage, microglia, and dendritic cells and the differentiation of neural Ag-specific Th1 cells (48, 49). Previously we (29–31, 50) showed that in vivo treatment with lovastatin inhibits the production of inducible NO synthase and proinflammatory cytokines (IFN-γ, TNF-α, and IL-6) in macrophage and brain glial cells and ameliorated EAE in the Lewis rat. In this study we have used the relapsing and remitting mouse model of EAE to test the potential therapeutic efficacy of lovastatin in the treatment of active and passive EAE, because MS pathology can be acute or relapsing/remitting. Further, we investigated the molecular mechanism of action of lovastatin to identify its possible in vivo target for disease remission. The inhibition of clinical paralysis by lovastatin was associated with a decrease in infiltration of T cells and demyelination in the CNS. The decrease in inflammation caused by lovastatin is potentially useful in the treatment of inflammatory diseases due to its direct effect on T cells by blocking both their differentiation from T0 to Th1 cells and subsequent cytokine generation. Earlier studies have attributed the blockade of macrophage activation and secretion of proinflammatory cytokines and chemokines as the mechanism by which statins inhibit inflammatory diseases (32, 33, 51). However, to our knowledge no study has systematically examined the exact molecular mechanism of action of statins on T cells, which are chief initiators of the disease. We identified putative targets of lovastatin in T cells that might be key in MS therapy.
This is the first study with lovastatin to demonstrate that inhibition of T-bet expression in vivo and in vitro from Th1 cells might be the underlying mechanism for disease attenuation (35). T-bet-deficient mice showed normal lymphoid development, but exhibited profound defects in mounting a Th1 immune response in response to IL-12 (43, 52, 53). CD4+ T and NK cells of T-bet-deficient mice produced smaller amounts of IFN-γ, whereas CD8+ T cells showed normal cytokine production (43, 52, 53). These studies suggest that T-bet controls Th cell differentiation and effector function in vivo. We observed that lovastatin inhibited T-bet in vivo and in vitro in T cells, indicating its direct correlation with IFN-γ inhibition and disease remission. The Th1 cytokines, IFN-γ and TNF-α, have been linked to more severe disease forms in EAE; however, IFN-γ−/− and IFN-γ receptor−/− mice develop severe disease (6–8), and treatment of mice with anti-IFN-γ Ab worsened the disease (8). Similarly, TNF-α-deficient mice develop more severe EAE than wild-type controls, indicating that during some phase of the disease, TNF-α and IFN-γ may play dual roles in disease initiation and remission (7, 9). It was shown that Abs to lymphotoxin and TNF-α prevent transfer of EAE, but T cell infiltration still occurs (10, 11). These results suggest that T-bet is a potential target of statins in autoimmune disease therapy.

The STAT (cytoplasmic proteins) are activated after phosphorylation via the Jak family of tyrosine kinases, which, in turn, are

![Figure 6](http://www.jimmunol.org/DownloadedFrom/1282_LOVASTATIN_THERAPY_IN_T_CELL-MEDIATED_AUTOIMMUNE_DISEASE)
activated by interaction of a cytokine and its receptor. STAT4 plays a pivotal role in Th1 immune responses (54, 55). STAT4 is activated after IL-12 interacts with the IL-12R, inducing transcription of IFN-γ (56). Mice deficient in STAT4 lack IL-12-induced IFN-γ production and Th1 differentiation and display a predominant Th2 phenotype (44, 57). It was shown that mice deficient in STAT4 are resistant to EAE, with minimal inflammatory infiltrates in the CNS. However, STAT6-deficient mice had a more severe clinical course of EAE than wild-type or STAT4 knockout mice (58). Lovastatin inhibited STAT4 transcription factors in APC-primed, myelin-PLP139–151 T cells and in naive T cells (induced with CD3ε and CD28). These observations strongly suggest that inhibition of Th1 cytokines by lovastatin involves the STAT4 signaling pathway. We report in this study for the first time that lovastatin blocked tyrosine phosphorylation of Jak2 and Tyk2 in T cells, suggesting that the inhibition of STAT4 may be consequent to the blockade of the upstream kinases Jak2 and Tyk2. The blockade of the Jak-STAT pathway by lovastatin resulted in decreased proliferation and Th1 differentiation in T cells, suggesting that Jak-STAT signaling pathway molecules might play a key role in MS therapy.

This report discusses the role of lovastatin in enhancing Th2-related transcription factors. It has been shown that STAT6 is essential for maximal Th2 differentiation in vitro and that STAT6+/− cells produce small amounts of Th2 cytokines (42, 59, 60). In vivo Th2 immune responses, however, can be elicited in STAT6+/− mice, and Th2 cells derived from these mice express normal levels of GATA3 and produce normal levels of Th2 cytokines (59). The increased expression and phosphorylation of GATA3 and STAT6 suggest that lovastatin may act on both independently and can induce Th2 responses in vivo as well as in vivo. These results suggest that induction of IL-4 with lovastatin might be regulated by both STAT6 and GATA3. It was shown that in STAT6−/− mice, IL-4 secretion is restored by GATA3 (42, 60, 61). Further, the present study shows that lovastatin not only induces the Th2 profile from T0, but also stabilizes the expression of GATA-3 and the production of IL-4 in differentiated Th2 cells. The expression of GATA3 is slightly down-regulated in differentiated Th2 cells and in naive T cells in unpolarized conditions. However, the production of IL-4 was not inhibited in either of these conditions (see Fig. 5, b and j), which further strengthens our hypothesis that lovastatin acts on more than one transcription factor to induce Th2-biased immune responses. Moreover, GATA3 is more important in vivo, because in STAT6−/− mice it restore IL-4 levels to normal (42, 60), and our data demonstrate that lovastatin increased in vivo GATA3 expression in treated mice (Fig. 4b). Lovastatin potentiated GATA3 in ex vivo-generated, Ag-specific T cells, suggesting that statins play an important role in vivo in the differentiation of T cells to Th1/Th2. The observed induction of GATA3 by lovastatin in undifferentiated T0 cells and its stabilization in differentiated Th2 cells strongly suggest its therapeutic value for MS.

The transcription factor NF-κB is expressed by both the immune and nervous systems and may therefore regulate autoimmune inflammation in the CNS through two mechanisms. First, NF-κB regulates the expression of both TCR and costimulatory molecules that are required for activation and differentiation of myelin-specific precursor T cells (46, 62). Second, NF-κB regulates the expression of genes that are required for migration (chemokines, adhesion molecules) and effector functions of inflammatory cells (cytokines, inflammatory enzymes) (63). Alternatively, NF-κB may indirectly regulate T cell activation through modulating the expression of molecules such as cytokines chemokines, MHC, and costimulatory and adhesion molecules, which are required for T
cell activation (62–64). Lovastatin significantly inhibited CD3ɛ- and CD28-induced NF-κB nuclear translocation by inhibiting phosphorylation and degradation of IκBα in T cells. Inhibition of IκBα degradation may be due to inhibition of proteasome activity, because lovastatin previously has been shown to inhibit their activity (62–64). Induction of NF-κB directly correlates with IFN-γ generation, and it was observed that c-Rel deficiency led to a defect in Th1 immune responses both in vitro and in vivo and resistance to EAE (45, 65). It is intriguing that a marked defect in IFN-γ production by c-Rel-deficient Th1 cells results in normal levels of T-bet and STAT4 activation, as both are important for optimal production of IFN-γ (45, 65). The correlation between NF-κB and T-bet regulation is another area that needs investigation. Identifying the upstream target of the NF-κB pathway, such as IκB kinase (IKK) α/β and NF-κB-inducing kinase, could be critical, because lovastatin inhibits phosphorylation of IκBα (47). Akt has been shown to be an upstream kinase of IKKα/β, which regulates its activity via phosphorylation (47). Akt might be a potential target for lovastatin, because it inhibits CD3ɛ- and CD28-induced Akt activity in naive T cells (data not shown).

This study reports that statins can directly affect the TCR-induced cytokine profile for the induction of IL-10 (Fig. 3f). As observed in allogeneic MLR, the induction of IL-10 by lovastatin favors a shift of Th1 to Th2 with its anti-inflammatory properties.

We have documented that lovastatin is pivotal in the transmission of protective Th2/IL-10 responses, a mechanism not described previously to explain the anti-inflammatory effects of statins. Lovastatin reduced the infiltration of mononuclear cells to CNS in EAE mice, and infiltrated cells were enriched with CD4+ T cells and MHC class II-positive cells. We also observed less demyelination in the lovastatin-treated mice (data not shown). These results suggest that lovastatin inhibits CNS inflammation and demyelination in EAE, additional support for our previous studies (29–31, 50). Our future studies are focused on the involvement of lovastatin in the inhibition of infiltration of mononuclear cells and demyelination and whether it may promote remyelination.

In conclusion, this study provides evidence that lovastatin inhibits active and passive EAE by modulating T cell as well as APC responses. Lovastatin inhibited the early events of T cell activation when given during the first peak of disease. However, lovastatin treatment was unable to attenuate the disease process when given on day 10 (when the disease process became manifest); however, we observed attenuation compared with the untreated group in subsequent relapses. Lovastatin-primed peritoneal macrophage/microglia drive the development of anti-inflammatory effector Th cells from naive T cells responding to any Ag presented by these APCs. The effects of lovastatin on macrophage/microglia in an Ag-nonspecific system suggest that lovastatin is not only effective
in EAE, but may also prove to be effective in other inflammatory diseases. Further, the possible mechanism involved in disease progression is the expression of T-bet, STAT4, and NF-κB, which contribute to the induction of Th1 cytokines. Lovastatin inhibited these transcription factors (T-bet, NF-κB, and STAT4), which are responsible for CNS inflammation via induction of Th1 cell differentiation and cytokine (IFN-γ and TNF-α) production. We have demonstrated that lovastatin induced the expression of GATA3 and STAT6 in Th2 cells, which promoted the remission of the disease by enhancing T0 to Th2 cell differentiation and cytokine (IL-4, IL-5, and IL-10) generation by affecting both APCs and T cells. The stabilization of IL-4/GATA3 in differentiated Th2 cells and the concomitant down-regulation of IFN-γ/T-bet in Th1 cells by lovastatin identified these molecules as novel targets for MS therapy.

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