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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 Singh[2]*

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors or statins are newly identified immunomodulators. In vivo treatment of SJL/J mice with lovastatin 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 by lovastatin 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-bet (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-bet 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.**

M ultiple sclerosis (MS)[3] 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 lymphotoxin 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 neural 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-bet (T box transcription factor), and NF-κB in attenuation of EAE. T-bet a newly identified molecule in Th1 cells, is a master switch for IFN-γ production (35). Lovastatin inhibited T-bet expression 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

*Department of Pediatrics, Medical University of South Carolina, and †Department of Pathology, Ralph H. Johnson Veterans Affairs Medical Center, Charleston, SC 29425

[2] Address correspondence and reprint requests to Dr. Inderjit Singh, Medical University of South Carolina, 96 Jonathan Lucas Street, Clinical Science Building, Suite 316, Charleston, SC 29425. E-mail address: singhi@musc.edu

[3] Abbreviations used in this paper: MS, multiple sclerosis; DLN, draining lymph node; EAE, experimental autoimmune encephalomyelitis; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; Jak, Janus kinase; m, mouse; MMCS, maximum mean clinical score; PLP, proteolipid protein; T-bet, T box transcription factor; Tyk, tyrosine kinase; IKK, IκB kinase.

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-wk-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-wk-old mice were used for all experiments. Paralyzed mice required alternate food sources and were fed with surgical Transgel (Charles River Laboratories, Wilmington, MA) and water.

Peptide, reagents, and cell line

Myelin PLP$_{139-151}$ 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 (NA/LE) anti-CD3ϵ (145-2C11) and CD28 (37.51) Abs were purchased from BD PharMingen. For FACS, anti-mouse CD4 (FITC; H129.19), IA^p (FITC; 7-16.17), and CD16/CD32 (FcγIII/IIR, 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 pIκB/IκB 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$_{(139-151)}$-induced EAE, 6- to 8-wk-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$_{139-151}$, 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 in a blinded fashion on a 0–5 scale as follows: 0 = no abnormality, 1 = limp tail, 2 = limp tail, 2.5 = hind limb weakness (legs slip through cage top), 3 = hindlimb paralysis, 4 = hind- and forelimb paralysis, and 5 = moribund (37). The data are reported as the mean daily clinical score ± SEM for all animals in a particular group and/or as the mean peak clinical score ± SEM, i.e., the mean clinical score for all animals at the peak of disease. All mice were age- and sex-matched for each experiment.

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$_{139-151}$ distributed over two spots on the flank on days 0 and 7. Draining lymph nodes (DLN) were harvested from donor mice on day 10 for in vitro stimulation. DLN cells were cultured (5 × 10^6 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 × 10^{-5} M 2-ME (Sigma-Aldrich), and 10 ng/ml mouse rIL-12 (rmIL-12; BD PharMingen)). After 96-h incubation, cells were harvested, washed, counted, and resuspended (30 × 10^6 DLN cells/0.3 ml) in buffered salt solution. On day 0, 6- to 8-wk-old female naive SJL/J mice were injected i.p. with 30 × 10^6 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.; injection 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$_{139-151}$-immunized and lovastatin-treated (2 and 5 mg/kg body weight) mice, stimulated with 1, 2, 5, and 10 μg/ml of PLP$_{139-151}$, and incubated for 72 h. The effect of lovastatin on neural Ag-induced T cell proliferation was measured by [^{3}H]TdR incorporation assay. Myelin PLP$_{139-151}$-immune DLN cells (2 × 10^5/100 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$_{139-151}$ peptide and lovastatin (10–50 μM). [^{3}H]TdR (1 μCi/well) 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-CD3ϵ 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 × 10^5 cells/well) and cultured in RPMI-complete. The naive T cells were pretreated with different concentrations of lovastatin (10 and 20 μM) before being added to the plates. For proliferation, [^{3}H]TdR (1 μCi/well) was added at 48 h, and the uptake of radioactivity 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$_{139-51}$ T cell lines were established from the DLN of SJL/J mice primed 10 days earlier with 100 μg of the myelin PLP$_{139-51}$ peptide emulsified in CFA supplemented with 200 μg of M.tuberculosis H37Ra. Lines were propagated by repeated in vitro stimulation of 1 × 10^6 T cells with 5 × 10^6 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$_{139-151}$-specific T cells and APCs

Spleens were collected from naive, SJL/J mice as indicated. RBCs were removed by Pharmalyse (1×), 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 × 10^5 cells/well. Different concentrations of PLP$_{139-151}$ peptide were added to the APCs. A PLP$_{139-151}$-specific T cell line (10^5 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 × 10^5 cells/well) were pretreated with 10 and 20 μM lovastatin, PLP$_{139-151}$ (5 μg/ml) was added, and cells were further incubated for 2 h, after that PLP$_{139-151}$-specific T cells (10^5 cells/well) were added to them. The cells were incubated for 96 h, and were pulsed with 1 μCi/well [^{3}H]TdR for the final 24 h of the 96-h incubation period. [^{3}H]TdR uptake was detected as given above, and results are expressed as the mean of triplicate cultures ± SD (37). The long term, PLP$_{139-151}$-specific T cell line was derived from a SJL/J mice primed with PLP$_{139-151}$ 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$_{139-151}$-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$_{139-151}$-immunized DLN cells (5 × 10^6/ml) were cultured in RPMI-complete in 24-well plates with 5 μg/ml PLP$_{139-151}$ in the presence of lovastatin (10 and 20 μM). Naive 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-CD3ϵ 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 × 10^6 cells/well) and cultured in RPMI-complete. The naive T cells were pretreated with different concentrations of lovastatin (10 and 20 μM) before being adding to the plates. The culture

supernatants were collected at different time points (48 and 120 h) and checked for cytokine production. The levels of Th1 (IFN-γ and TNF-α) and Th2 (IL-4, IL-5, and IL-10) cytokines in culture supernatants were measured by the OPTEIA system and cytometric bead array (BD PharMingen) according to the manufacturer's instructions. Briefly, in the cytometric bead array system, cell supernatants were mixed with the beads for each cytokine labeled with PE and then acquired by FACS and analyzed on CellQuest program (BD PharMingen).

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 assessed 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 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 microglia 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 × 10^4 cells of each T cell vs APC) in 96-well plates. Cells were labeled with tritiated thymidine (1 μCi/well) for ~18 h before harvesting, and [^{3}H]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 7.5) and incubated for 1.5 h in primary antisera (pSTAT4/STAT4, pSTAT6/STAT6, GATA3, and T-bet) containing 5% nonfat dry milk. Blots were washed with TTBS (four times, 5 min/wash) and incubated for 45 min at room temperature. HRP-conjugated anti-rabbit or anti-mouse secondary Ab was added at a dilution of 1/5000. The blots were washed three times in TTBS and developed with an ECL detection system (40).

Immunoprecipitation and in vitro kinase assay

Briefly, T cells (10^7/lane) were pretreated with different concentrations of lovastatin for 2 h and then stimulated with anti-CD3ϵ and -CD28 at 37°C for 15 min. The cells were washed in PBS and solubilized in 25 mM HEPES, 2 mM Na$_3$VO$_4$, 0.1% Triton X-100, 0.5 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin, pH 7.4. The soluble proteins (200 μg) were incubated with 5 μg of rabbit anti-Jak2, and Tyk2 Ab at 4°C for 4 h. Protein G-Sepharose was added and incubated for an additional hour. Jak2/Tyk2 immune complexes on protein G-Sepharose were washed three times with 50 mM HEPES, 150 mM NaCl, 0.1% Triton X-100, and 0.5 mM DTT, pH 7.6, and then once with 50 mM HEPES, 100 mM NaCl, 6.25 mM MnCl$_2$, 0.1% Triton X-100, and 0.5 mM DTT, pH 7.6 (HNHT). The Jak2/Tyk2 protein immobilized on protein G-Sepharose beads was mixed with 25 μl of phosphorylation buffer (HNHT containing 20 μg/ml aprotinin and 20 μg/ml leupeptin); 20 μCi of [γ-^{32}P]ATP was then added to 10 μM ATP and 5 mM MnCl$_2$. After 15 min at 30°C, the reaction was stopped with the addition of 1× SDS sample buffer and resolved on 8–16% gradient SDS-PAGE. The gels were dried and exposed to autoradiograph (41).

EMSA

Nuclear extracts from stimulated or unstimulated T cells were prepared as described previously (40). EMSAs were performed with the NF-κB consensus sequence end-labeled with [^{32}P]ATP. The gels were dried and autoradiographed at −70°C. For competition analysis, nuclear extracts were preincubated with water or competitor DNA for 5 min at room temperature, and the samples were processed for EMSA.

TranSignal 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 (TranSignal probe mix) was preincubated with nuclear extract to allow the formation of protein/DNA (or transcription factor/DNA) complexes. The protein/DNA complexes were than separated from the free probes. The probes in the complexes were extracted and hybridized to the TranSignal 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 CO$_2$ 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 asses 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 IA^p. 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. Significances (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 PLP$_{139-151}$. All mice in the EAE-untreated 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 with lovastatin (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 ($p < 0.001$) than 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.
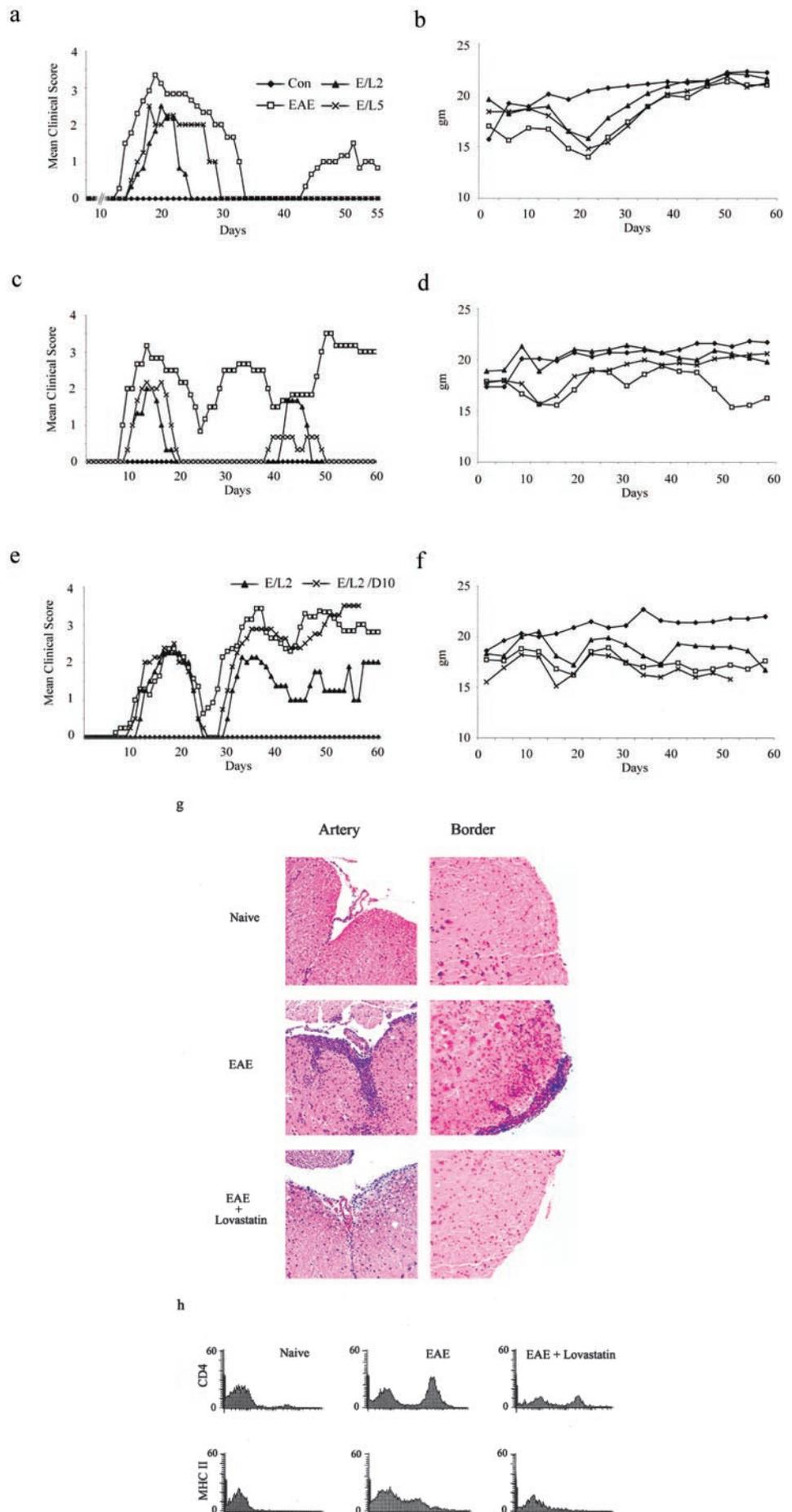
FIGURE 1. Lovastatin inhibits the clinical symptoms of EAE. The mean clinical scores of the diseased animals are given in *a*, *c*, and *e*, and weight measurements are given in *b*, *d*, and *f*. *a*, Active EAE was induced in SJL/J mice by immunization with myelin $PLP_{139–151}$ peptide in CFA. *c* and *e*, Passive EAE was induced by adoptive transfer of myelin-$PLP_{139–151}$-sensitized T cells into recipient SJL/J mice. *a* and *c*, 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 embedded in paraffin and sectioned at 5-μm thickness. *g*, 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 PLP$_{139-151}$-specific T cells. All 18 mice in the EAE-untreated 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 with lovastatin (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-untreated group developed clinical paralysis starting on day 8, which progressed to a MMCS of 2.4 on day 18 (Fig. 1e). Treatment of mice with lovastatin 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 with lovastatin, 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 day lovastatin-treated group, MMCS reached 3.5, similar to that in the EAE group. We observed that mice in lovastatin-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 of lovastatin on the pathogenesis of inflammation and demyelination in the CNS of mice with EAE. Spinal cord sections from mice treated with lovastatin 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 with lovastatin significantly reduced the number of inflammatory cells in both the artery and border region (Fig. 1g). We observed fewer demyelinated regions in lovastatin-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 by lovastatin, 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 PLP$_{139-151}$-immune T cell response in the presence of PLP$_{139-151}$ (1–10 μg/ml) increased [^{3}H]TdR up-

take with a high proliferation index, which was significantly decreased ($p < 0.001$) after treatment with 10 μM lovastatin. We examined the effect of lovastatin 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 of lovastatin 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 PLP$_{139-151}$ peptide. In vivo the production of Th2 cytokine (IL-4) was increased ($p < 0.001$) with 2 and 5 mg/kg lovastatin, whereas the Th1 cytokine IFN-γ was significantly inhibited (Fig. 2d). Next, we determined the effect of lovastatin on PLP$_{139-151}$-specific T cells in an APC-dependent system. Irradiated APCs (spleen cells) from syngenic mice loaded with PLP$_{139-151}$ were incubated with a lovastatin-pretreated PLP$_{139-151}$-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 MLR was carried out with lovastatin-pretreated peritoneal macrophage and PLP$_{139-151}$-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 PLP$_{139-151}$-immunized and in vitro-stimulated DLN cells. Culture supernatants were examined for Th1 (IFN-γ and TNF-α) and Th2 (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 of Th2 cytokines was increased (Fig. 3, e, g, and i). Thus, lovastatin induced Th2-biased immune responses in vivo. To determine whether lovastatin-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 of lovastatin on purified (98% ascertained by FACS) naive T cells (CD3$^+$). T cells (CD3$^+$) were obtained from SJL/J mice, pretreated with lovastatin (10 and 20 μM), and stimulated with anti-CD3ε and -CD28 Abs, and the secretion of Th1 and Th2 cytokines was measured. The production of Th1 cytokines was significantly reduced ($p < 0.001$; Fig. 3, b and d); however, that of Th2 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 in Th2 cells, acts both in a STAT6-dependent and -independent fashion (42). Expression of T-bet correlates with IFN-γ production in Th1 and NK cells, a proinflammatory cytokine in EAE and MS pathology (35, 43). Because lovastatin inhibited the production of Th1 cytokines and induced Th2, we examined its effect on transcription factors T-bet and GATA3, which are potentially involved in Th1 and Th2 cell development. As shown in Fig. 4a, in vivo treatment with lovastatin (2 and 5 mg/kg body weight) suppressed the expression of T-bet and significantly induced ($p < 0.01$) the expression of GATA3 (Fig. 4b). Next, we examined the expression of T-bet and GATA3 in a myelin PLP$_{139-151}$-specific T cell line treated with rmIL-12 and rmIL-4 for 48 h in the presence of lovastatin (10 and 20 μM); subsequently, T-bet and GATA3 expression were determined by immunoblot. Lovastatin (both 10 and 20 μM) inhibited T-bet expression (Fig. 4c), whereas the expression of GATA3 was induced ($p < 0.05$; Fig. 4d). These
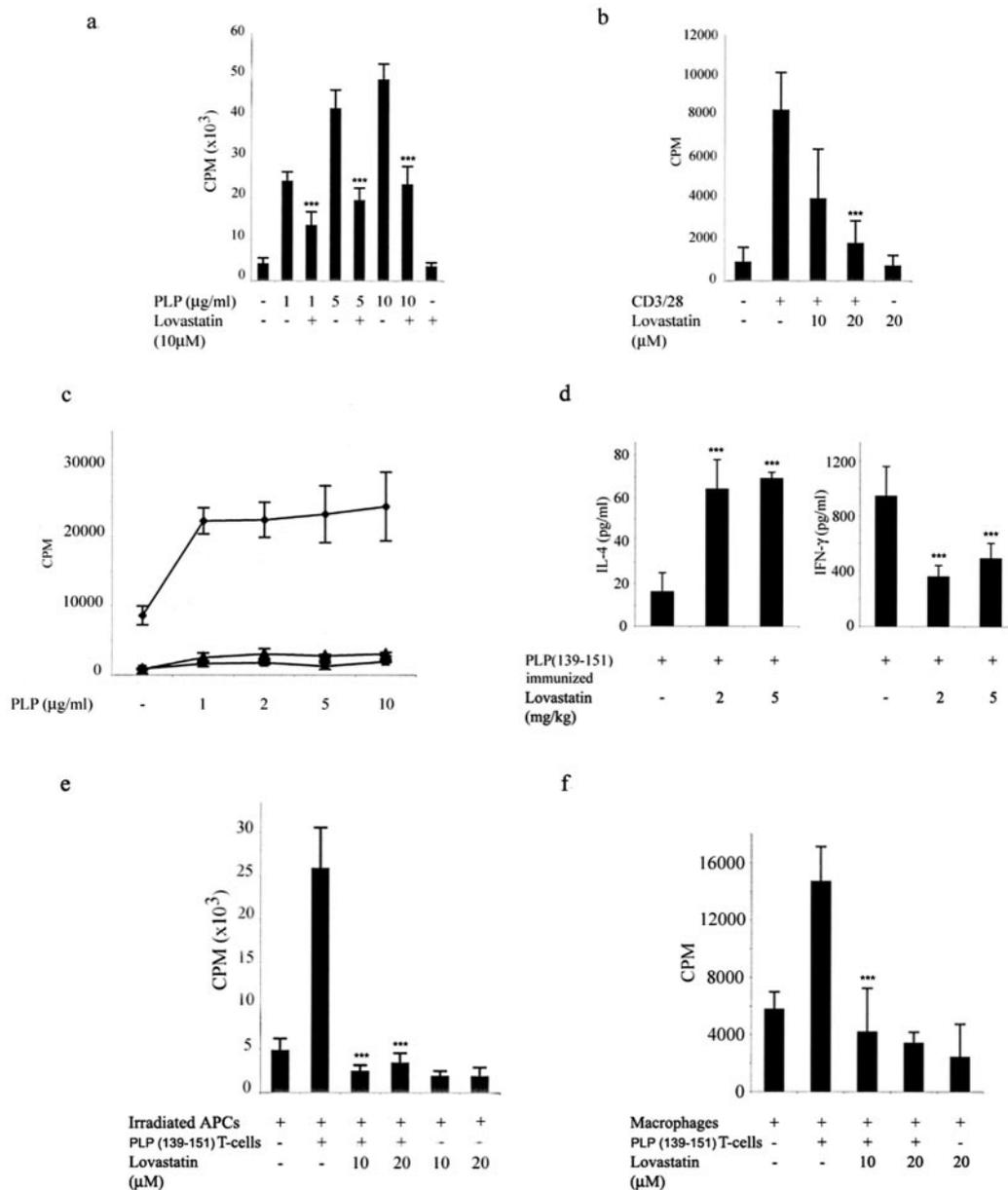
FIGURE 2. Lovastatin inhibits neural Ag-specific and naive T cell proliferation. a, PLP$_{139-151}$-immunized DLN cells were restimulated in vitro with Ag PLP$_{139-151}$ (5 μg/ml) in the presence of lovastatin (10 μM). b, Naive T cells were stimulated with anti-CD3ϵ and -CD28 for 48 h and with different concentrations of lovastatin (10 and 20 μM). c and d, DLN cells from PLP$_{139-151}$-immunized and lovastatin-treated mice were stimulated in vitro with Ag PLP$_{139-151}$ (1–10 μg/ml) for 48 and 96 h to determine proliferation and the Th1/Th2 cytokine pattern. e, PLP$_{139-151}$-specific T cells (10^5 cells/well) pretreated with lovastatin were cocultured with irradiated syngenic APCs, loaded with PLP$_{139-151}$ (5 μg/ml) for 96 h. f, Peritoneal macrophage pretreated with lovastatin were cocultured with PLP$_{139-151}$-specific T cells for 48 h. a–c, [^{3}H]TdR (1 μCi/well) was added at 48 h; e and f, TdR was added at 72 h. Uptake of radioactivity was measured after 24 h. Data are representative of four independent experiments with consistent results.

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.
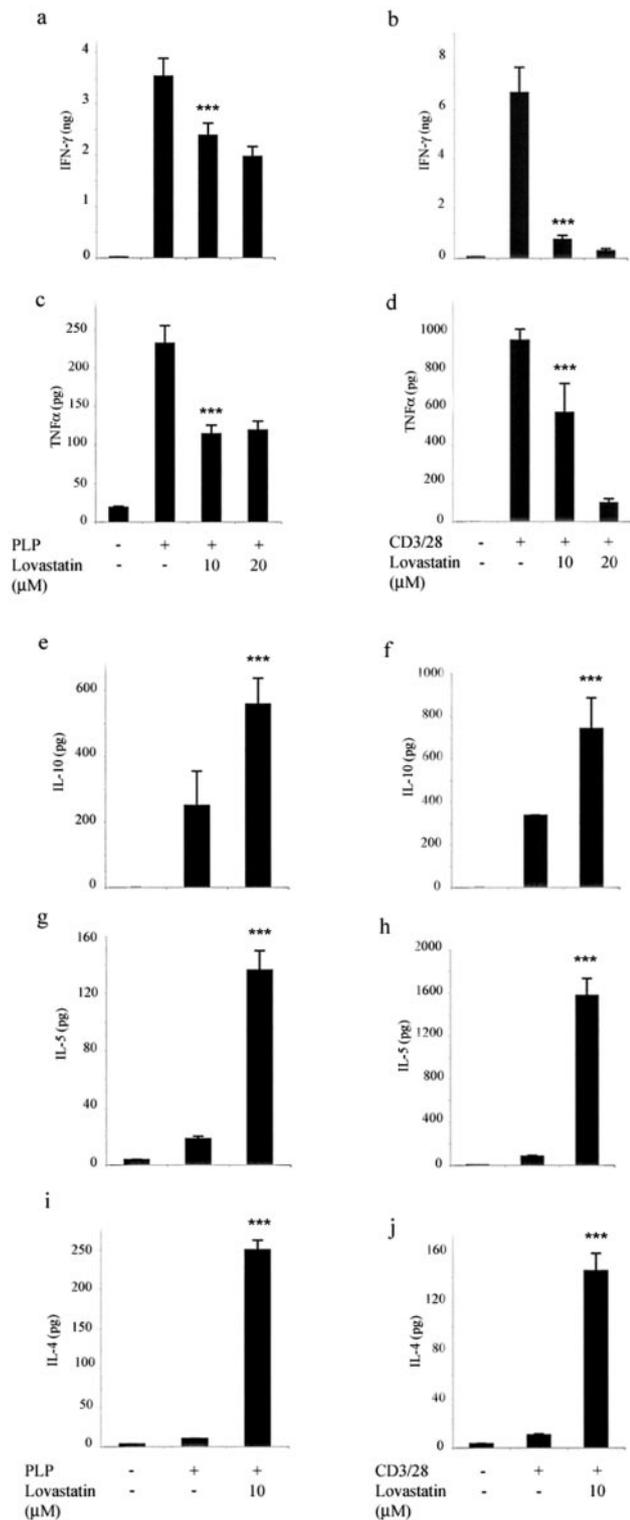
FIGURE 3. Induction of Th2 cytokines with lovastatin. *a*, *c*, *e*, *g*, and *i*, DLN cells were isolated on day 10 from $PLP_{139-151}$-immunized SJL/J mice and cultured in vitro at 5×10^6 cells/ml in the presence of $PLP_{139-151}$ (5 μg/ml) and lovastatin (10 and 20 μM). *b*, *d*, *f*, *h*, and *j*, Naive T cells (98% purified) isolated from lymph nodes were cultured in anti-CD3ϵ- and anti-CD28-precoated plates at 1×10^6 cells/ml with lovastatin (10 and 20 μM). The supernatants were collected at 48 h (for IFN-γ and TNF-α) and 120 h (for IL-4, IL-5, and IL-10) for cytokine measurements. *a*–*d*, IFN-γ and TNF-α are significantly reduced ($p < 0.001$) in both PLP-primed and naive T cells; *e*–*j*, IL-10, IL-5, and IL-4 are significantly increased ($p < 0.001$). The values are the means of triplicate determinations at each point, and the error bars represent $\pm$SD. Data are representative of four different experiments with consistent results.

Lovastatin inhibited phosphorylation of Jak2, Tyk2, and STAT4 and induced phosphorylation of STAT6 in $PLP_{139-151}$-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 $PLP_{139-151}$-specific T cells. As shown in Fig. 6, *a* and *b*, the $PLP_{139-151}$-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 transcription 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-induced nuclear translocation of STAT4 (Fig. 6*c*) and potentiated STAT6 nuclear translocation (Fig. 6*d*). 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 $PLP_{139-151}$-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. 7*a*). Inhibition of NF-κB by lovastatin appears to be dose dependent (Fig. 7*b*). 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. 7*c*). 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. 7*d*).

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
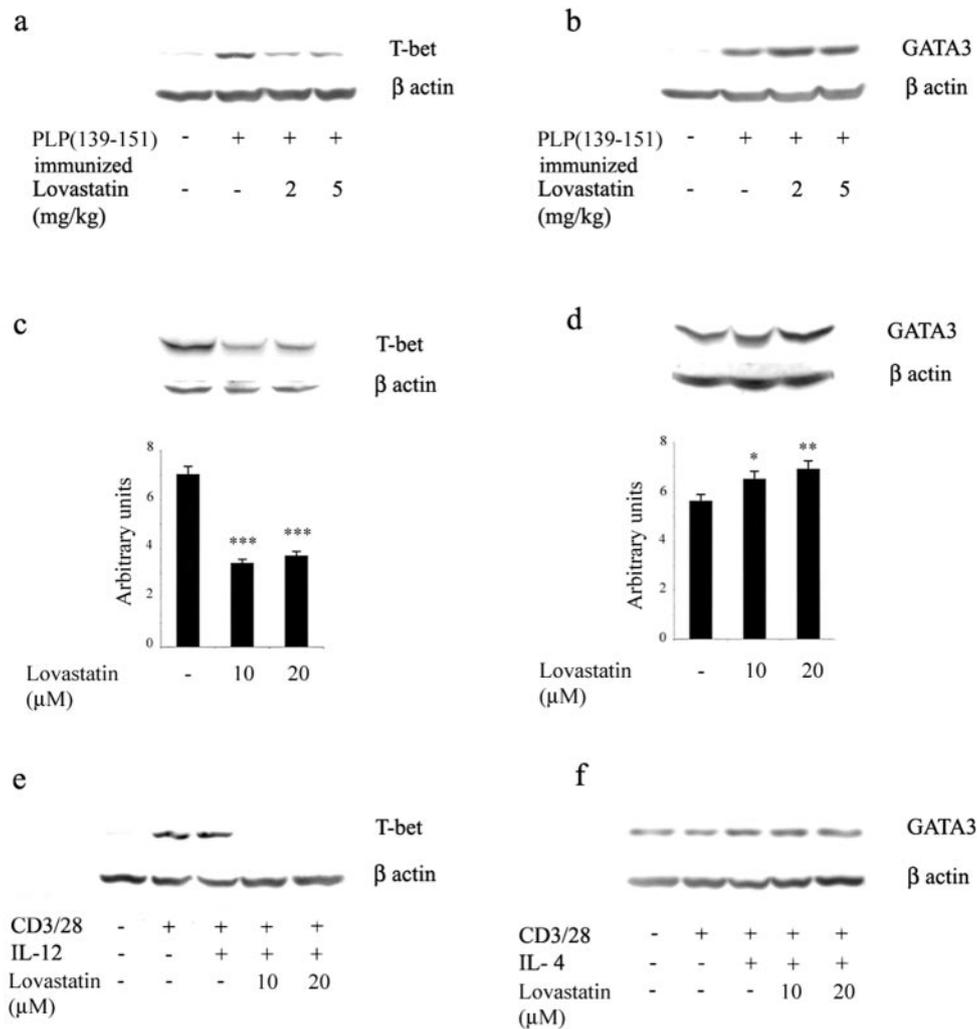
FIGURE 4. Effect of lovastatin on the expression of GATA-3 and T-bet in Th1 and Th2 cells. GATA3 and T-bet were analyzed in vivo in $PLP_{139-151}$-immunized (100 μg/mouse) and lovastatin-treated (2 and 5 mg/kg) mice and in vitro in $PLP_{139-151}$-specific and naive T cells. The DLN from immunized and lovastatin-treated mice were harvested on day 10 and analyzed by Western blot for T-bet (a) and GATA3 (b). $PLP_{139-151}$-specific T cells were incubated with 10 and 20 μM lovastatin for 48 h; c and d, T-bet (c) and GATA3 (d) were analyzed by Western blot, bands were scanned with a densitometer, and arbitrary units were plotted. Naive T cells were stimulated with anti-CD3ϵ and -CD28 for 48 h in the presence of rmIL-12 or rmIL-4 (10 ng/ml) and lovastatin (10 and 20 μM). Cells were harvested and lysed, and 50 μg protein was resolved, blotted onto a membrane, and probed with anti-T-bet (e) and anti-GATA3 (f) Abs. Data are representative of three independent experiments with consistent results.

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 inflammatory 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 inflammatory 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 $PLP_{139-151}$-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
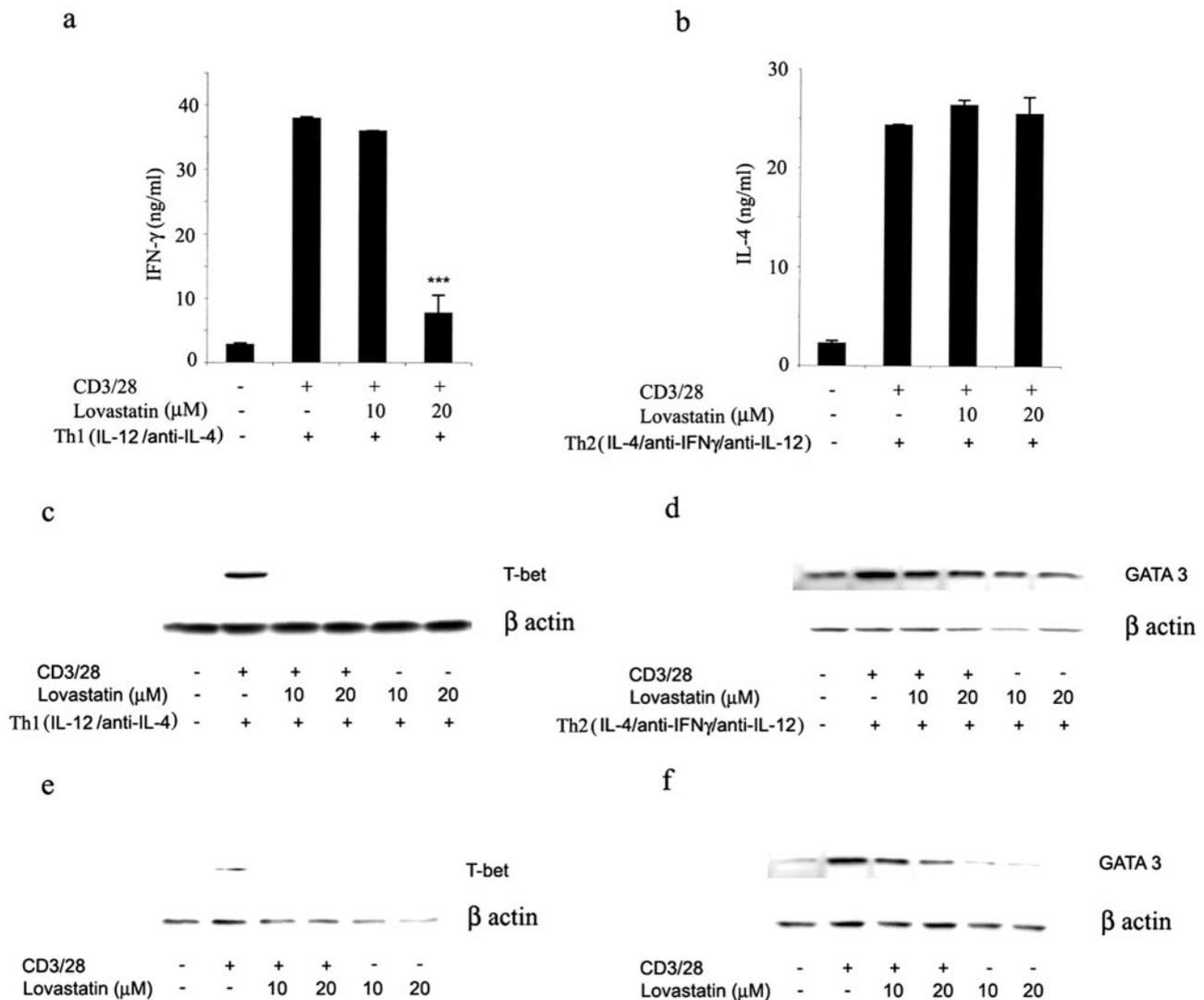
FIGURE 5. Effect of lovastatin on previously differentiated Th1/Th2 cells. Naive T cells stimulated with anti-CD3ϵ and CD28 in Th1 (rmIL-12, 10 ng/ml; anti-IL-4, 10 μg/ml) and Th2 (rmIL-4, 10 ng/ml; anti-IFN-γ and IL-12, 5 μg/ml) conditions and without Th1/Th2. After 96 h the cells were harvested and restimulated with anti-CD3ϵ and -CD28 in the presence of lovastatin (10 and 20 μM). At 48 h supernatants were collected for IFN-γ (*a*) and IL-4 (*b*) analysis, and cells were harvested and analyzed for T-bet (*c*) and GATA (*d*). Naive T cells pretreated with lovastatin stimulated with anti-CD3ϵ and CD28 in nonpolarized conditions were analyzed for T-bet (*e*) and GATA-3 (*f*). Data are representative of two independent experiments with consistent results.

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 mac-

rophage 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.
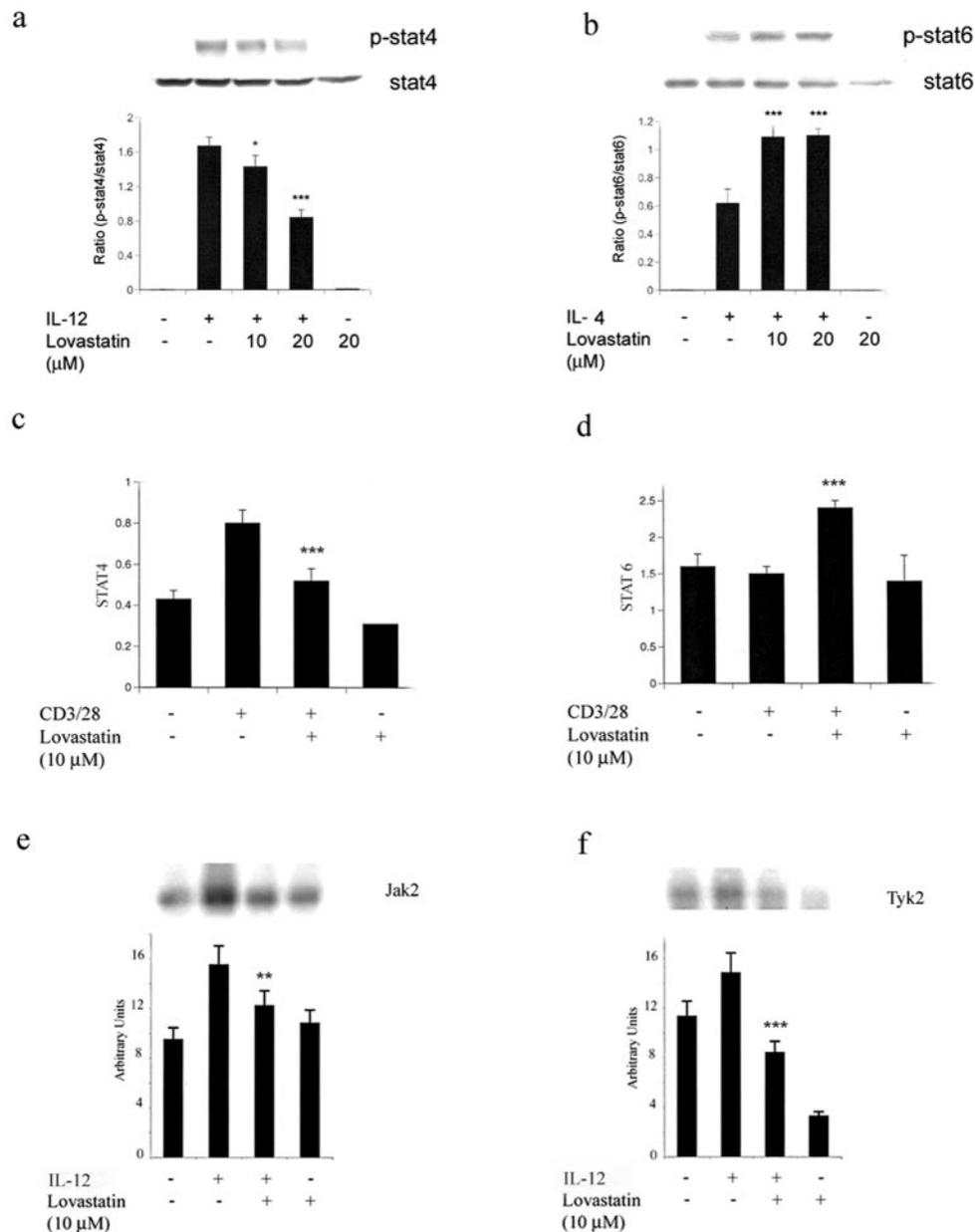
FIGURE 6. Effects of lovastatin on STAT6, STAT4, Jak2, and Tyk2. PLP$_{139-151}$-specific T cells were generated as described in *Materials and Methods*, stimulated with rmIL-12 or rmIL-4 (10 ng/ml) with lovastatin (10 and 20 μM) for 48 h, and analyzed for p-STAT4 (*a*) and p-STAT6 (*b*) by Western blot analysis. The blots were stripped and reprobed with anti-STAT4 (*a*) and anti-STAT6 (*b*) Abs for equal loading of protein. Naive T cells, isolated as previously described, were stimulated with anti-CD3ϵ and -CD28 Abs in the presence of lovastatin (10 μM) and incubated at 37°C for 4 h. The nuclear extract was prepared, and nuclear translocation of STAT4 (*c*) and STAT6 (*d*) was analyzed by TranSignal array. The spleen cells from PLP-immunized mice were stimulated with rmIL-12 (10 ng/ml) in the presence of lovastatin (10 μM) for 15 min, and Jak2 (*e*) and Tyk2 (*f*) were immunoprecipitated. An in vitro kinase assay was performed as described in *Materials and Methods*. Band intensities were plotted after scanning with a densitometer. Data are representative of two independent experiments with consistent results.

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-$\gamma^{-/-}$ 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 of 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 7. Lovastatin inhibits nuclear translocation of NF-κB in stimulated T cells. Naive T cells (98% purified) were pretreated for 2 h with different concentrations of lovastatin and stimulated with plate-bound anti-CD3ε and -CD28 (2 μg/ml) for 4 h (for NF-κB) and 30 min (for IκBα). *a*, The expression of NF-κB was analyzed by gel-shift; *b*, further inhibition was observed in a dose-dependent manner (5–50 μM). *c*, The nuclear extract was prepared, and nuclear translocation of NF-κB was analyzed by TranSignal array. *d*, For determination of pIκBα and IκBα, T cells stimulated as described above were harvested, and cytosolic fractions were used for detection of pIκBα and IκBα. The bands were scanned by densitometer, and the ratio of pIκBα/IκBα was plotted. Data are representative of two independent experiments with consistent results.
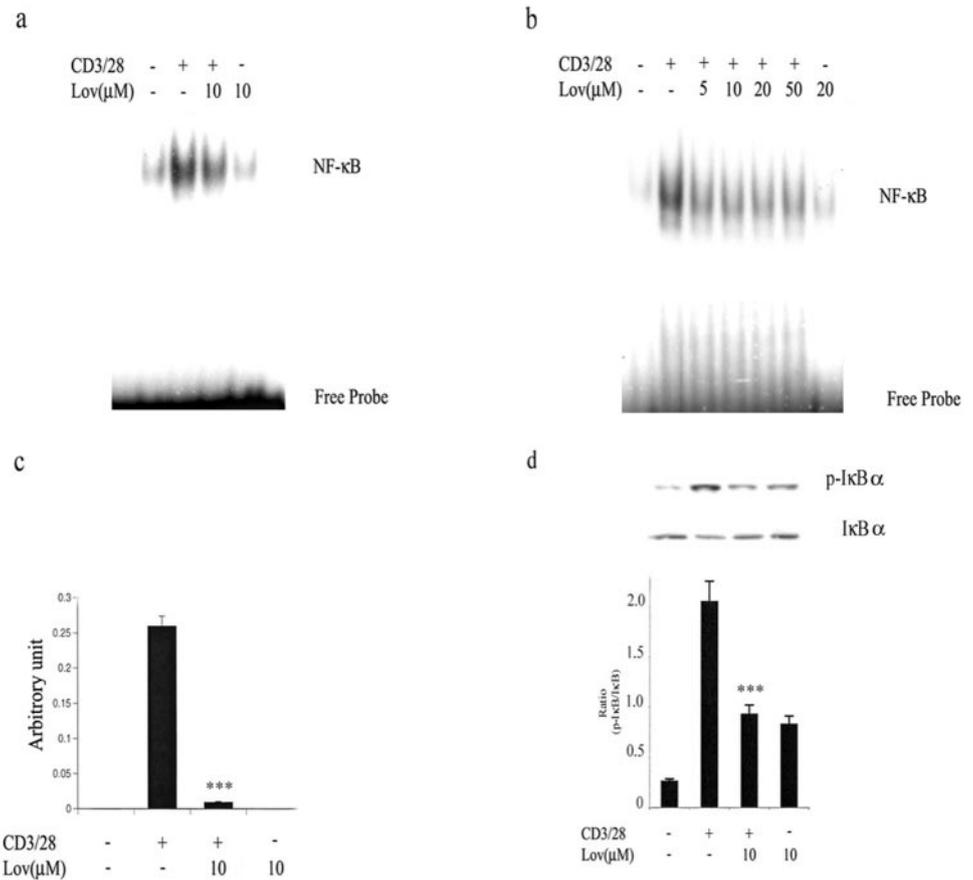
activated by interaction of a cytokine and its receptor. STAT4 plays a pivotal role in Th1 immune responses (54, 55). STAT4 is activated after IL-12 interacts with the IL-12R, inducing transcription of IFN-γ (56). Mice deficient in STAT4 lack IL-12-induced IFN-γ production and Th1 differentiation and 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-PLP$_{139-151}$ 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 high 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 vitro 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. 4*b*). 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
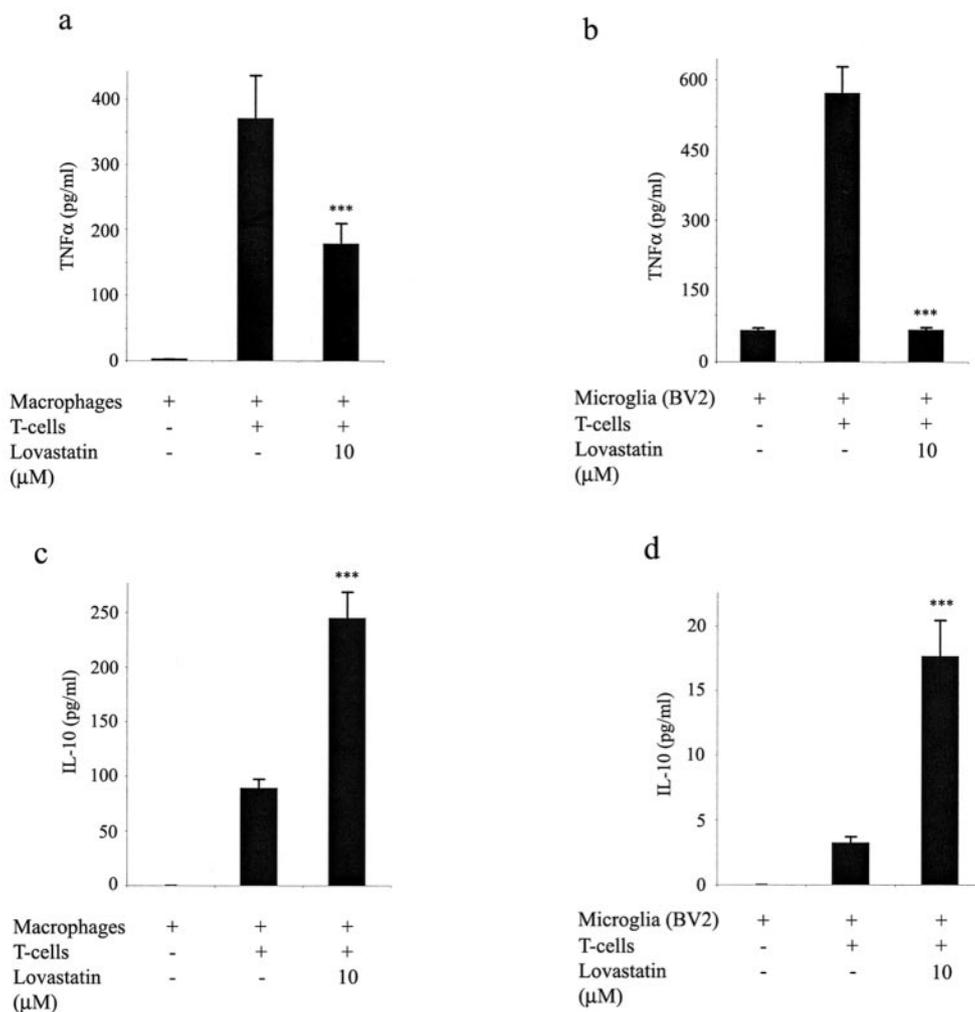
FIGURE 8. Lovastatin potentiates IL-10 allogeneic MLR. Peritoneal macrophage (C57BL/6 mice) and microglia (BV2) cells were pretreated with lovastatin (10 μM), mixed with naive T cells (BALB/c mice) at a 1/1 ratio, and incubated for 48 h. Supernatants were collected and analyzed for TNF-α (*a* and *b*) and IL-10 (*c* and *d*). MLR data are representative of two independent experiments with consistent results.

cell activation (62–64). Lovastatin significantly inhibited CD3ϵ- 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 IKK$\alpha\beta$, which regulates its activity via phosphorylation (47). Akt might be a potential target for lovastatin, because it inhibits CD3ϵ- 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. 3*f*). 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.

Acknowledgments

References

1. Ozawa, K., G. Suchanek, H. Breitschopf, W. Bruck, H. Budka, K. Jellinger, and H. Lassmann. 1994. Patterns of oligodendroglia pathology in multiple sclerosis. *Brain 117:1311.*

2. Lassmann, H., C. S. Raine, J. Antel, and J. W. Prineas. 1998. Immunopathology of multiple sclerosis: report on an international meeting held at the Institute of Neurology of the University of Vienna. *J. Neuroimmunol. 86:213.*

3. Lucchinetti, C. F., W. Bruck, M. Rodriguez, and H. Lassmann. 1996. Distinct patterns of multiple sclerosis pathology indicate heterogeneity on pathogenesis. *Brain Pathol. 6:259.*

4. Weiner, H. L., and D. J. Selkoe. 2002. Inflammation and therapeutic vaccination in CNS diseases. *Nature 420:879.*

5. Swanborg, R. H. 1995. Experimental autoimmune encephalomyelitis in rodents as a model for human demyelinating disease. *Clin. Immunol. Immunopathol. 77:4.*

6. Ferber, I. A., S. Brocke, C. Taylor-Edwards, W. Ridgway, C. Dinisco, L. Steinman, D. Dalton, and C. G. Fathman. 1996. Mice with a disrupted IFN-γ gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *J. Immunol. 156:5.*

7. Frei, K., H. P. Eugster, M. Bopst, C. S. Constantinescu, E. Lavi, and A. Fontana. 1997. Tumor necrosis factor α and lymphotoxin α are not required for induction of acute experimental autoimmune encephalomyelitis. *J. Exp. Med. 185:2177.*

8. Lublin, F. D., R. L. Knobler, B. Kalman, M. Goldhaber, J. Marini, M. Perrault, C. D'Imperio, J. Joseph, S. S. Alkan, and R. Korngold. 1993. Monoclonal anti-gamma interferon antibodies enhance experimental allergic encephalomyelitis. *Autoimmunity 16:267.*

9. Liu, J., M. W. Marino, G. Wong, D. Grail, A. Dunn, J. Bettadapura, A. J. Slavin, L. Old, and C. C. Bernard. 1998. TNF is a potent anti-inflammatory cytokine in autoimmune-mediated demyelination. *Nat. Med. 4:78.*

10. Ruddle, N. H., C. M. Bergman, K. M. McGrath, E. G. Lingenheld, M. L. Grunnet, S. J. Padula, and R. B. Clark. 1990. An antibody to lymphotoxin and tumor necrosis factor prevents transfer of experimental allergic encephalomyelitis. *J. Exp. Med. 172:1193.*

11. Korner, H., F. A. Lemckert, G. Chaudhri, S. Etteldorf, and J. D. Sedgwick. 1997. Tumor necrosis factor blockade in actively induced experimental autoimmune encephalomyelitis prevents clinical disease despite activated T cell infiltration to the central nervous system. *Eur. J. Immunol. 27:1973.*

12. Young, D. A., L. D. Lowe, S. S. Booth, M. J. Whitters, L. Nicholson, V. K. Kuchroo, and M. Collins. 2000. IL-4, IL-10, IL-13, and TGF-β from an altered peptide ligand-specific Th2 cell clone down-regulate adoptive transfer of experimental autoimmune encephalomyelitis. *J. Immunol. 164:3563.*

13. Croxford, J. L., M. Feldmann, Y. Chernajovsky, and D. Baker. 2001. Different therapeutic outcomes in experimental allergic encephalomyelitis dependent upon the mode of delivery of IL-10: a comparison of the effects of protein, adenoviral or retroviral IL-10 delivery into the central nervous system. *J. Immunol. 166:4124.*

14. Santambrogio, L., G. M. Hochwald, B. Saxena, C. H. Leu, J. E. Martz, J. A. Carlino, N. H. Ruddle, M. A. Palladino, L. I. Gold, and G. J. Thorbecke. 1993. Studies on the mechanisms whereby transforming growth factor-β (TGF-β) protects against allergic encephalomyelitis: antagonism between TGF-β and tumor necrosis factor. *J. Immunol. 151:1116.*

15. Legge, K. L., B. Min, J. J. Bell, J. C. Caprio, L. Li, R. K. Gregg, and H. Zaghouani. 2000. Coupling of peripheral tolerance to endogenous interleukin 10 promotes effective modulation of myelin-activated T cells and ameliorates experimental allergic encephalomyelitis. *J. Exp. Med. 191:2039.*

16. Ando, D. G., J. Clayton, D. Kono, J. L. Urban, and E. E. Sercarz. 1989. Encephalitogenic T cells in the B10. PL model of experimental allergic encephalomyelitis (EAE) are of the Th-1 lymphokine subtype. *Cell. Immunol. 124:132.*

17. Zamvil, S., P. Nelson, J. Trotter, D. Mitchell, R. Knobler, R. Fritz, and L. Steinman. 1985. T-cell clones specific for myelin basic protein induce chronic relapsing paralysis and demyelination. *Nature 317:355.*

18. Zamvil, S. S., and L. Steinman. 1990. The T lymphocyte in experimental allergic encephalomyelitis. *Annu. Rev. Immunol. 8:579.*

19. Laufs, U., K. Gertz, U. Dirnagl, M. Bohm, G. Nickenig, and M. Endres. 2002. Rosuvastatin, a new HMG-CoA reductase inhibitor, upregulates endothelial nitric oxide synthase and protects from ischemic stroke in mice. *Brain Res. 942:23.*

20. Zamvil, S. S., and L. Steinman. 2002. Cholesterol-lowering statins possess anti-inflammatory activity that might be useful for treatment of MS. *Neurology 59:970.*

21. Weitz-Schmidt, G. 2002. Statins as anti-inflammatory agents. *Trends Pharmacol. Sci. 23:482.*

22. Sadeghi, M. M., M. Collinge, R. Pardi, and J. R. Bender. 2000. Simvastatin modulates cytokine-mediated endothelial cell adhesion molecule induction: involvement of an inhibitory G protein. *J. Immunol. 165:2712.*

23. Kobashigawa, J. A., S. Katznelson, H. Laks, J. A. Johnson, L. Yeatman, X. M. Wang, D. Chia, P. I. Terasaki, A. Sabad, G. A. Cogert, et al. 1995. Effect of pravastatin on outcomes after cardiac transplantation. *N. Engl. J. Med. 333: 621.*

24. Dimitroulakos, J., L. Y. Ye, M. Benzaquen, M. J. Moore, S. Kamel-Reid, M. H. Freedman, H. Yeger, and L. Z. Penn. 2001. Differential sensitivity of various pediatric cancers and squamous cell carcinomas to lovastatin-induced apoptosis: therapeutic implications. *Clin. Cancer Res. 7:158.*

25. Mueck, A. O., H. Seeger, and D. Wallwiener. 2001. Further evidence for direct vascular actions of statins: effect on endothelial nitric oxide synthase and adhesion molecules. *Exp. Clin. Endocrinol. Diabetes 109:181.*

26. Denoyelle, C., P. Albanese, G. Uzan, L. Hong, J. P. Vannier, J. Soria, and C. Soria. 2003. Molecular mechanism of the anti-cancer activity of cerivastatin, an inhibitor of HMG-CoA reductase, on aggressive human breast cancer cells. *Cell. Signalling 15:327.*

27. Leung, B. P., N. Sattar, A. Crilly, M. Prach, D. W. McCarey, H. Payne, R. Madhok, C. Campbell, J. A. Gracie, F. Y. Liew, et al. 2003. A novel anti-inflammatory role for simvastatin in inflammatory arthritis. *J. Immunol. 170:1524.*

28. Feleszko, W., I. Mlynarczuk, E. Z. Balkowiec-Iskra, A. Czajka, T. Switaj, T. Stoklosa, A. Giermasz, and M. Jakobisiak. 2000. Lovastatin potentiates antitumor activity and attenuates cardiotoxicity of doxorubicin in three tumor models in mice. *Clin. Cancer Res. 6:2044.*

29. Pahan, K., F. G. Sheikh, A. M. Namboodiri, and I. Singh. 1997. Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia, and macrophages. *J. Clin. Invest. 100:2671.*

30. Stanislaus, R., K. Pahan, A. K. Singh, and I. Singh. 1999. Amelioration of experimental allergic encephalomyelitis in Lewis rats by lovastatin. *Neurosci. Lett. 269:71.*

31. Stanislaus, R., A. K. Singh, and I. Singh. 2001. Lovastatin treatment decreases mononuclear cell infiltration into the CNS of Lewis rats with experimental allergic encephalomyelitis. *J. Neurosci. Res. 66:155.*

32. Kwak, B., F. Mulhaupt, S. Myit, and F. Mach. 2000. Statins as a newly recognized type of immunomodulator. *Nat. Med. 6:1399.*

33. Weitz-Schmidt, G., K. Welzenbach, V. Brinkmann, T. Kamata, J. Kallen, C. Bruns, S. Cottens, Y. Takada, and U. Hommel. 2001. Statins selectively inhibit leukocyte function antigen-1 by binding to a novel regulatory integrin site. *Nat. Med. 7:687.*

34. Youssef, S., O. Stuve, J. C. Patarroyo, P. J. Ruiz, J. L. Radosevich, E. M. Hur, M. Bravo, D. J. Mitchell, R. A. Sobel, L. Steinman, et al. 2002. The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. *Nature 420:78.*

35. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell 100:655.*

36. Zheng, W., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell 89:587.*

37. Tompkins, S. M., J. Padilla, M. C. Dal Canto, J. P. Ting, L. Van Kaer, and S. D. Miller. 2002. De novo central nervous system processing of myelin antigen is required for the initiation of experimental autoimmune encephalomyelitis. *J. Immunol. 168:4173.*

38. Howard, L. M., and S. D. Miller. 2001. Autoimmune intervention by CD154 blockade prevents T cell retention and effector function in the target organ. *J. Immunol. 166:1547.*

39. Weigel, B. J., N. Nath, P. A. Taylor, A. Panoskaltsis-Mortari, W. Chen, A. M. Krieg, K. Brasel, and B. R. Blazar. 2002. Comparative analysis of murine marrow-derived dendritic cells generated by Flt3L or GM-CSF/IL-4 and matured with immune stimulatory agents on the in vivo induction of antileukemia responses. *Blood 100:4169.*

40. Giri, S., M. Jatana, R. Rattan, J. S. Won, I. Singh, and A. K. Singh. 2002. Galactosylsphingosine (psychosine)-induced expression of cytokine-mediated inducible nitric oxide synthases via AP-1 and C/EBP: implications for Krabbe disease. *FASEB J. 16:661.*

41. Bright, J. J., L. D. Kerr, and S. Sriram. 1997. TGF-β inhibits IL-2-induced tyrosine phosphorylation and activation of Jak1 and Stat 5 in T lymphocytes. *J. Immunol. 159:175.*

42. Ouyang, W., M. Lohning, Z. Gao, M. Assenmacher, S. Ranganath, A. Radbruch, and K. M. Murphy. 2000. Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. *Immunity 12:27.*

43. Szabo, S. J., B. M. Sullivan, C. Stemmann, A. R. Satoskar, B. P. Sleckman, and L. H. Glimcher. 2002. Distinct effects of T-bet in TH1 lineage commitment and IFN-γ production in CD4 and CD8 T cells. *Science 295:338.*

44. Thierfelder, W. E., J. M. van Deursen, K. Yamamoto, R. A. Tripp, S. R. Sarawar, R. T. Carson, M. Y. Sangster, D. A. Vignali, P. C. Doherty, G. C. Grosveld, et al. 1996. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature 382:171.*

45. Hilliard, B., E. B. Samoilova, T. S. Liu, A. Rostami, and Y. Chen. 1999. Experimental autoimmune encephalomyelitis in NF-κB-deficient mice: roles of NF-κB in the activation and differentiation of autoreactive T cells. *J. Immunol. 163:2937.*

46. Li, Q., and I. M. Verma. 2002. NF-κB regulation in the immune system. *Nat. Rev. Immunol. 2:725.*

47. Karin, M., and Y. Ben-Neriah. 2000. Phosphorylation meets ubiquitination: the control of NF-κB activity. *Annu. Rev. Immunol. 18:621.*

48. Fischer, H. G., and G. Reichmann. 2001. Brain dendritic cells and macrophages/microglia in central nervous system inflammation. *J. Immunol. 166:2717.*

49. Aloisi, F., F. Ria, S. Columba-Cabezas, H. Hess, G. Penna, and L. Adorini. 1999. Relative efficiency of microglia, astrocytes, dendritic cells and B cells in naive CD4$^+$ T cell priming and Th1/Th2 cell restimulation. *Eur. J. Immunol. 29:2705.*

50. Stanislaus, R., A. G. Gilg, A. K. Singh, and I. Singh. 2002. Immunomodulation of experimental autoimmune encephalomyelitis in the Lewis rats by Lovastatin. *Neurosci. Lett. 333:167.*

51. Romano, M., L. Diomede, M. Sironi, L. Massimiliano, M. Sottocorno, N. Polentarutti, A. Guglielmotti, D. Albani, A. Bruno, P. Fruscella, et al. 2000. Inhibition of monocyte chemotactic protein-1 synthesis by statins. *Lab. Invest. 80:1095.*

52. Finotto, S., M. F. Neurath, J. N. Glickman, S. Qin, H. A. Lehr, F. H. Green, K. Ackerman, K. Haley, P. R. Galle, S. J. Szabo, et al. 2002. Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science 295:336.*

53. Mullen, A. C., F. A. High, A. S. Hutchins, H. W. Lee, A. V. Villarino, D. M. Livingston, A. L. Kung, N. Cereb, T. P. Yao, S. Y. Yang, et al. 2001. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science 292:1907.*

54. Pernis, A. B., and P. B. Rothman. 2002. JAK-STAT signaling in asthma. *J. Clin. Invest. 109:1279.*

55. Ohashi, P. S. 2002. T-cell signalling and autoimmunity: molecular mechanisms of disease. *Nat. Rev. Immunol. 2:427.*

56. Jacobson, N. G., S. J. Szabo, R. M. Weber-Nordt, Z. Zhong, R. D. Schreiber, J. E. Darnell, Jr., and K. M. Murphy. 1995. Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4. *J. Exp. Med. 181:1755.*

57. Kaplan, M. H., Y. L. Sun, T. Hoey, and M. J. Grusby. 1996. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature 382:174.*

58. Chitnis, T., N. Najafian, C. Benou, A. D. Salama, M. J. Grusby, M. H. Sayegh, and S. J. Khoury. 2001. Effect of targeted disruption of STAT4 and STAT6 on the induction of experimental autoimmune encephalomyelitis. *J. Clin. Invest. 108:739.*

59. Finkelman, F. D., S. C. Morris, T. Orekhova, M. Mori, D. Donaldson, S. L. Reiner, N. L. Reilly, L. Schopf, and J. F. Urban, Jr. 2000. Stat6 regulation of in vivo IL-4 responses. *J. Immunol. 164:2303.*

60. Jankovic, D., M. C. Kullberg, N. Noben-Trauth, P. Caspar, W. E. Paul, and A. Sher. 2000. Single cell analysis reveals that IL-4 receptor/Stat6 signaling is not required for the in vivo or in vitro development of CD4$^+$ lymphocytes with a Th2 cytokine profile. *J. Immunol. 164:3047.*

61. Ho, I. C., and L. H. Glimcher. 2002. Transcription: tantalizing times for T cells. *Cell 109(Suppl.):S109.*

62. Barnes, P. J., and M. Karin. 1997. Nuclear factor-κB: a pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med. 336:1066.*

63. Beg, A. A., and D. Baltimore. 1996. An essential role for NF-κB in preventing TNF-α-induced cell death. *Science 274:782.*

64. Ting, A. T., F. X. Pimentel-Muinos, and B. Seed. 1996. RIP mediates tumor necrosis factor receptor 1 activation of NF-κB but not Fas/APO-1-initiated apoptosis. *EMBO J. 15:6189.*

65. Hilliard, B. A., N. Mason, L. Xu, J. Sun, S. E. Lamhamedi-Cherradi, H. C. Liou, C. Hunter, and Y. H. Chen. 2002. Critical roles of c-Rel in autoimmune inflammation and helper T cell differentiation. *J. Clin. Invest. 110:843.*