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The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease

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Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, which are approved for cholesterol reduction, may also be beneficial in the treatment of inflammatory diseases[1–3]. Atorvastatin (Lipitor) was tested in chronic and relapsing experimental autoimmune encephalomyelitis, a CD4$^+$ Th1-mediated central nervous system (CNS) demyelinating disease model of multiple sclerosis[4,5]. Here we show that oral atorvastatin prevented or reversed chronic and relapsing paralysis. Atorvastatin induced STAT6 phosphorylation and secretion of Th2 cytokines (interleukin (IL)-4, IL-5 and IL-10) and transforming growth factor (TGF)-β. Conversely, STAT4 phosphorylation was inhibited and secretion of Th1 cytokines (IL-2, IL-12, interferon (IFN)-γ and tumour necrosis factor (TNF)-α) was suppressed. Atorvastatin promoted differentiation of Th0 cells into Th2 cells. In adoptive transfer, these Th2 cells protected recipient mice from EAE induction. Atorvastatin reduced CNS infiltration and major histocompatibility complex (MHC) class II expression. Treatment of microglia inhibited IFN-γ-inducible transcription at multiple MHC class II transactivator (CIITA) promoters and suppressed class II upregulation. Atorvastatin suppressed IFN-γ-inducible expression of CD40, CD80 and CD86 co-stimulatory molecules. L-Mevalonate, the product of HMG-CoA reductase, reversed atorvastatin's effects on antigen-presenting cells (APC) and T cells. Atorvastatin treatment of either APC or T cells suppressed antigen-specific T-cell activation. Thus, atorvastatin has pleiotropic immunomodulatory effects involving both APC and T-cell compartments. Statins may be beneficial for multiple sclerosis and other Th1-mediated autoimmune diseases.

In 1995, it was reported that pravastatin treatment of cardiac transplant recipients was associated with a reduction in haemodynamically significant rejection episodes and increased survival, independent of its cholesterol-lowering effects[1]. Subsequent studies demonstrated that certain statins could inhibit production of specific pro-inflammatory molecules[2,3,6]. Lovastatin inhibited production of TNF-α and inducible nitric oxide synthetase (iNOS) by microglia and astrocytes[2]. MHC class II expression is central to immune regulation in T-cell-mediated autoimmune disease[5,7,8]. Statins prevented IFN-γ-inducible MHC class II expression on non-professional APC[9], suggesting that statins might inhibit antigen presentation to pro-inflammatory Th1 cells. In that study, it was observed that statins inhibited IFN-γ-inducible expression of the MHC class II transactivator (CIITA), the master regulator for MHC class II expression[10]. In endothelial cells, IFN-γ-inducible CIITA transcription was inhibited at CIITA promoter (p) IV[11], which

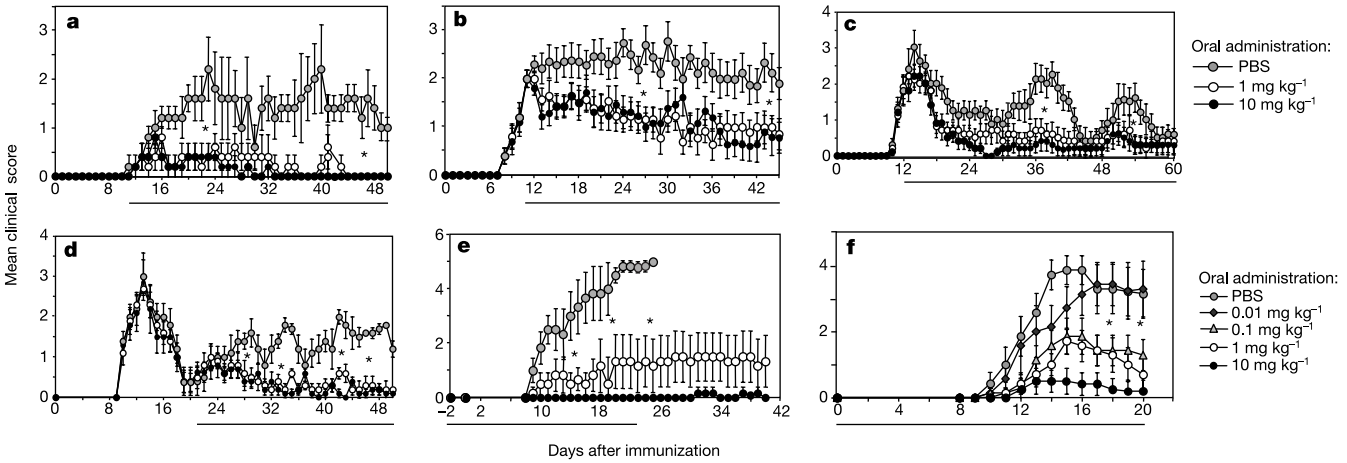

Figure 1 Atorvastatin treatment inhibits or reverses chronic and relapsing EAE. **a**, Oral atorvastatin ameliorated MOG p35–55-induced EAE in C57BL/6 mice when administered at EAE onset (within one day of initial symptoms), or **b**, after acute EAE was established. **c**, Oral atorvastatin prevented exacerbations of relapsing EAE induced by immunization of SJL/J mice with PLP p139–151. **d**, Relapsing EAE in SJL/J mice was reversed when atorvastatin treatment began at the onset of the first relapse. **e**, Limited EAE development in MBP Ac1–11-specific TCR transgenic mice after atorvastatin treatment was discontinued. **f**, PLP p139–151-induced EAE in SJL/J mice was significantly reduced by 0.1 mg kg^{-1}, but not 0.01 mg kg^{-1} atorvastatin. Number of mice per group in each experiment: **a** (7), **b** (14), **c** (10), **d** (10), **e** (7) and **f** (7). In **a**–**d** mice were scored and randomized immediately before first treatment. Open circle, 1 mg kg^{-1} atorvastatin; filled circle, 10 mg kg^{-1} atorvastatin; shaded circle, vehicle only (PBS). Solid bars beneath each panel indicate atorvastatin treatment. *P value < 0.001, comparison of either atorvastatin-treated group with vehicle-only (PBS)-treated group.

suggested to those authors that statins selectively inhibited CIITA transcription at pIV. Statins inhibited lymphocyte secretion of matrix metalloprotease-9 (MMP-9) (ref. 3), an enzyme involved in basement membrane degradation[12]. These observations suggest that statins may be beneficial in multiple sclerosis (MS). Currently approved MS treatments, which are administered parenterally, are only partly effective[13] and are often limited by side-effects or toxicities. Statins are administered orally, are well tolerated and, in general, are considered safe.

Here we examine the immunomodulatory effects of atorvastatin in chronic and relapsing experimental autoimmune encephalomyelitis (EAE). We chose atorvastatin, the most widely prescribed statin[14], with one of the most favourable safety profiles of any statin available[15], because it inhibited CIITA transcription and class II expression more potently than other statins tested *in vitro*[9]. In humans 80 mg d^{-1} of atorvastatin is the highest recommended dose for treatment of hypercholesterolaemia[15]. For an individual who weighs 70 kg this dose equals 1.1 mg kg^{-1}. Pharmacokinetic data in rodents indicate that larger atorvastatin doses are required to achieve similarly effective concentrations[16,17]. Therefore, for most experiments, we used two atorvastatin doses: 1 mg kg^{-1} and 10 mg kg^{-1}. When atorvastatin treatment was started at clinical onset in MOG p35–55-induced EAE in C57BL/6 mice, clinical symptoms were significantly reduced in both treatment groups (Fig. 1a). When treatment was started during the acute phase, chronic EAE was ameliorated (Fig. 1b). In contrast with MOG p35–55, which causes chronic EAE, PLP p139–151 induces relapsing–remitting EAE in SJL/J mice. Atorvastatin administration prevented relapses when treatment began during the acute EAE phase (Fig. 1c) and reversed relapsing paralysis when administration was initiated at the onset of the first relapse (Fig. 1d). Atorvastatin also suppressed EAE induction in MBP Ac1-11-specific T-cell antigen receptor (TCR) transgenic mice (Fig. 1e), which develop fulminant EAE[18]. After atorvastatin was discontinued these did not develop fulminant EAE, whereas mice in the vehicle-only (PBS)-treated group succumbed to severe EAE. Thus, atorvastatin was effective in treatment at separate stages in three different murine EAE models. Although 1 mg kg^{-1} and 10 mg kg^{-1} doses were effective, we also

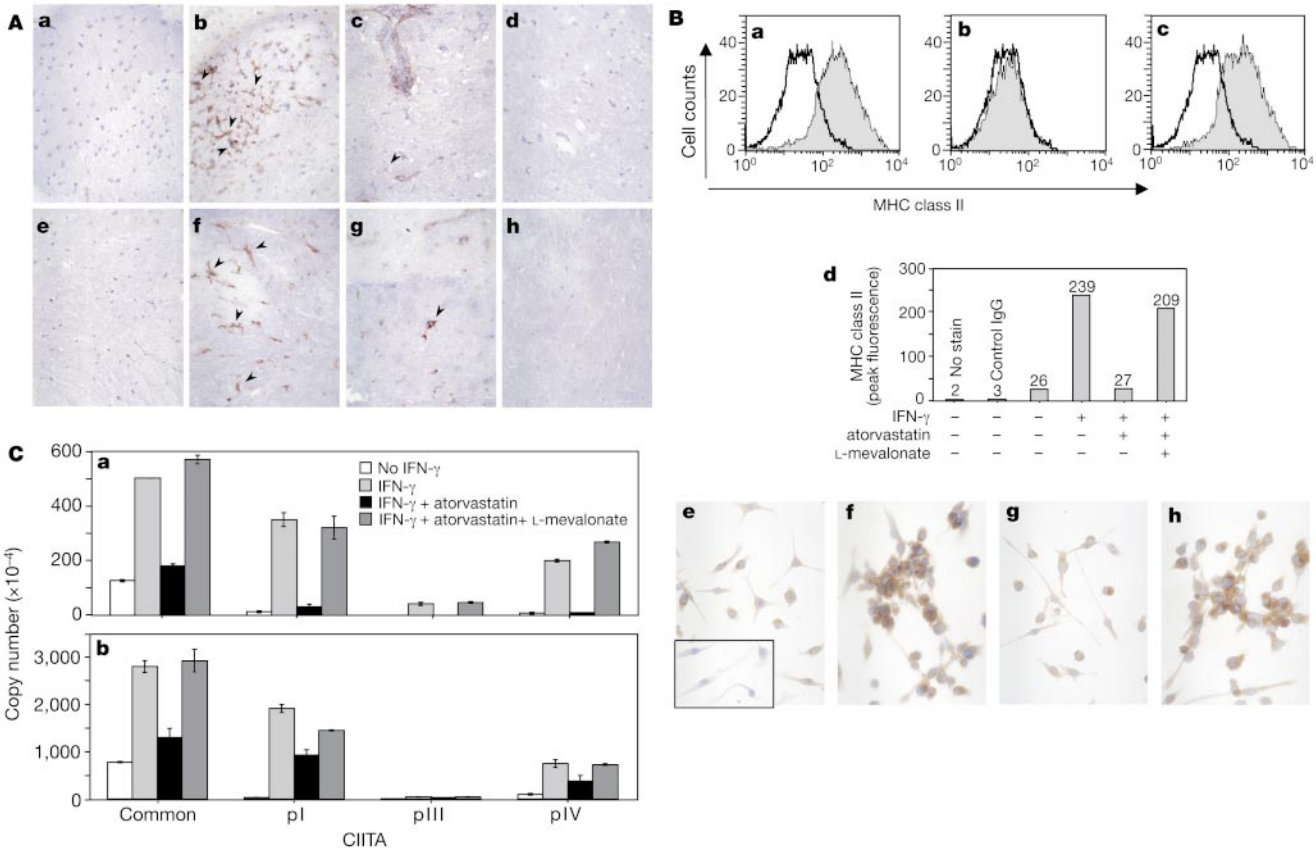

Figure 2 Atorvastatin treatment suppresses *in vivo* and *in vitro* MHC class II expression on microglia. **A**, MHC class II expression was reduced in the CNS white matter of SJL/J mice (**a**–**d**) and C57BL/6 mice (**e**–**h**) treated for EAE with atorvastatin. MHC class II molecules were rarely detected in naive CNS (**a** and **e**). High-level class II expression was detected on cells (microglia) within, or adjacent to, EAE lesions in PBS-treated mice (**b** and **f**). MHC class II expression was downregulated in the CNS of mice treated with either 1 mg kg^{-1} atorvastatin (**c** and **g**) or 10 mg kg^{-1} atorvastatin (**d** and **h**). Original magnification × 160. Arrowheads indicate stained cells. **B**, Atorvastatin inhibits IFN-γ-inducible MHC class II expression on microglia. Flow cytometric analysis of cell-surface class II molecules on untreated or IFN-γ-treated (100 units ml^{-1}, 48 h) EOC 20 microglia (**a**). Atorvastatin (10 μM) inhibited IFN-γ-inducible class II expression (**b**), whereas L-mevalonate (100 μM) reversed inhibition (**c**). **d**, The mean peak fluorescence intensity for MHC class II expression shown in **a**–**c** and for unstained and isotype-matched control IgG stained samples. Immunohistochemical staining for MHC class II molecules on untreated (**e**) or IFN-γ-treated (**f**) microglia. Suppression of IFN-γ-inducible class II expression on cultured microglia by atorvastatin (**g**) was reversed by addition of L-mevalonate (**h**). Inset shows isotype-matched control IgG staining. Original magnification × 160. **C**, Atorvastatin inhibits multiple CIITA promoters from directing IFN-γ-inducible CIITA transcription in EOC microglia (**a**) and primary B10.PL microglia (**b**). Atorvastatin inhibited overall (common) IFN-γ-inducible CIITA transcription. IFN-γ-inducible CIITA transcription directed by pI, pIII and pIV was inhibited by atorvastatin. L-Mevalonate reversed inhibition of overall CIITA transcription and transcription initiated by each promoter. Common CIITA and promoter-specific transcripts were measured by quantitative real-time RT–PCR.

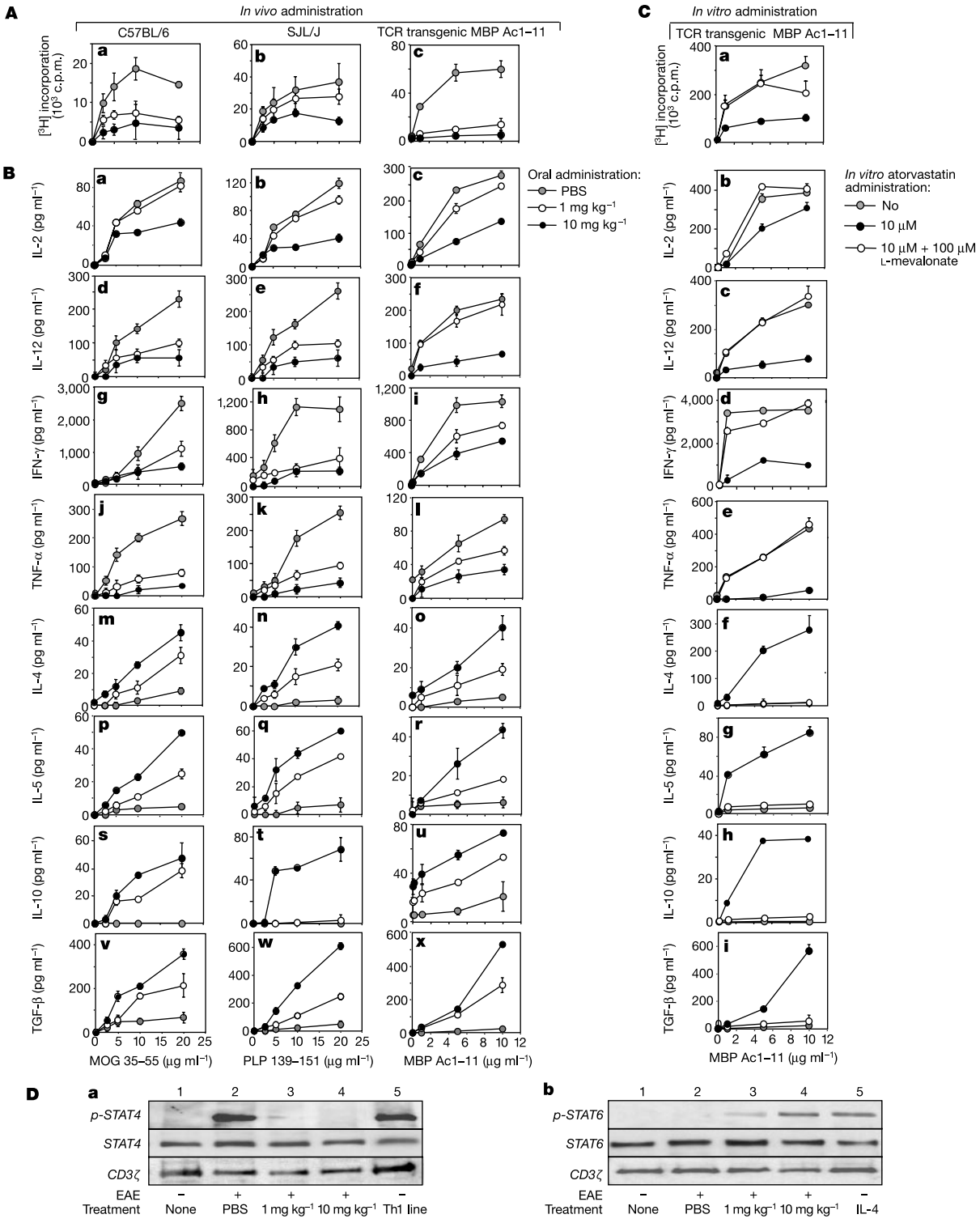

Figure 3 Atorvastatin inhibits T-cell proliferation and promotes a Th2 response. **A**, *In vivo* atorvastatin treatment of MOG p35–55-immunized C57BL/6 mice (**a**), PLP p139–151-immunized SJL/J mice (**b**) and MBP Ac1–11-immunized MBP Ac1–11-specific Tg mice (**c**) suppressed recall splenic proliferative responses. Splenocytes were isolated from mice treated *in vivo* for 12 days: vehicle-only (PBS), 1 mg kg^{-1} atorvastatin or 10 mg kg^{-1}. **B**, *In vivo* atorvastatin treatment suppressed secretion of IL-2 (**a**–**c**), IL-12 (**d**–**f**), IFN-γ (**g**–**i**) and TNF-α (**j**–**l**), whereas secretion of IL-4 (**m**–**o**), IL-5 (**p**–**r**), IL-10 (**s**–**u**) and TGF-β (**v**–**x**) was increased. **C**, Atorvastatin treatment of naive CNS autoantigen-specific Th0 cells promoted Th2 differentiation. Atorvastatin treatment (10 μM) of unprimed Th0 cells from MBP Ac1–11-specific B10.PL Tg mice suppressed proliferation (**a**) and secretion of IL-2 (**b**), IL-12 (**c**), IFN-γ (**d**) and TNF-α (**e**), whereas secretion of IL-4 (**f**), IL-5 (**g**), IL-10 (**h**) and TGF-β (**i**) was augmented. Addition of L-mevalonate (100 μM) prevented atorvastatin-induced suppression of proliferation (**a**) and induction of the Th2 'bias' (**b**–**i**). **D**, *In vivo* atorvastatin treatment suppressed STAT4 phosphorylation (**a**) and promoted STAT6 phosphorylation (**b**) by T cells. Protein extracts were isolated from lymph nodes of naive SJL/J mice (lane1), draining lymph nodes from PLP p139–151-immunized mice with EAE (PBS treated, lane 2), PLP p139–151-immunized mice treated with 1 mg kg^{-1} atorvastatin (lane 3) or with 10 mg kg^{-1} atorvastatin (lane 4). An SJL/J PLP p139–151 specific Th1 line served as a positive control for STAT4 phosphorylation (**a**, lane 5). Naive SJL/J lymph node cells treated with recombinant IL-4 *in vitro* served as a positive control for STAT6 phosphorylation (**b**, lane 5). Phosphorylated (p) STAT4 and STAT6 proteins were detected by Western blot analysis. Anti-CD3ζ served as a protein-loading control.

tested two lower doses: 0.1 mg kg^{-1} and 0.01 mg kg^{-1} in PLP p139–151-induced EAE in SJL/J mice (Fig. 1f). The dose of 0.1 mg kg^{-1} significantly suppressed clinical EAE, whereas 0.01 mg kg^{-1} was not significantly effective through most of the observation period.

Atorvastatin-treated and vehicle-only-treated mice were examined for histological EAE. There was a large reduction in the number of inflammatory lesions in brains and spinal cords from mice treated with either 1 mg kg^{-1} or 10 mg kg^{-1} atorvastatin (Table 1 and Supplementary Information), although infiltration was reduced to a greater extent in mice treated with 10 mg kg^{-1}. Thus, clinical EAE suppression correlated with a reduction in histological EAE. A significant reduction in CNS class II expression was observed in atorvastatin-treated mice (Fig. 2A). Class II expression on microglia was also reduced in atorvastatin-treated mice (Fig. 2A) and to a greater extent in mice treated with 10 mg kg^{-1} than in those treated with 1 mg kg^{-1} (Fig. 2A, c, d, g and h), suggesting that *in vivo* class II reduction on microglia occurred in a dose-dependent fashion.

CIITA is differentially regulated by non-homologous promoters[11]. A study reported that statins suppressed IFN-γ-inducible CIITA transcription by selective inhibition at CIITA pIV[9]. Although those authors observed that statins inhibited IFN-γ-inducible class II expression on different non-professional APC, they examined promoter-specific CIITA transcription using only endothelial cells, non-professional APC that use primarily pIV, and they only examined pIV. However, other CIITA promoters can be used to direct IFN-γ-inducible CIITA transcription[19–21]. INF-γ-inducible CIITA transcription in bone-marrow-derived (haematopoetic) APC, including microglia and monocytes, is directed predominantly by pI[20]. As shown in Fig. 2B, atorvastatin inhibited IFN-γ-inducible class II expression on microglia, and this effect was reversed by L-mevalonate. CIITA pI and, to a lesser extent, pIV accounted for most of the IFN-γ-inducible CIITA transcripts in untreated microglia (Fig. 2C). Atorvastatin prevented nearly all IFN-γ-inducible CIITA transcription directed by each CIITA promoter and this inhibition was reversed by exposure to L-mevalonate. These results clearly indicate that statins are not selective for pIV, but inhibit IFN-γ-inducible CIITA transcription in general.

As EAE is mediated by Th1 cells[4,5], we examined whether T-cell activation and Th regulation was altered in atorvastatin-treated mice. Compared with PBS-treated mice, *in vivo* treatment with atorvastatin suppressed the recall responses to the encephalitogenic myelin peptide used in all three models (Fig. 3A, a–c). Inhibition of proliferation appeared dose related. Culture supernatants were examined for Th1 cytokines (IL-2, IL-12, IFN-γ and TNF-α) and Th2 cytokines (IL-4, IL-5 and IL-10). As shown in Fig. 3B, atorvastatin treatment was associated with a reduction in secretion of IL-2 (panels a–c), IL-12 (d–f), IFN-γ (g–i) and TNF-α (j–l). In contrast, secretion of IL-4 (panels m–o), IL-5 (p–r), IL-10 (s–u) and transforming growth factor TGF-β (v–x), an immunoregulatory cytokine sometimes secreted concomitantly with Th2 cytokines, was increased. Thus, atorvastatin induced a Th2 bias in all three murine EAE models.

We addressed whether atorvastatin promoted Th2 differentiation of naive Th0 cells. Splenocytes from unprimed MBP Ac1–11 specific TCR transgenic mice, which contain a population of Th0 cells, were activated by MBP Ac1–11 in the presence or absence of atorvastatin. There were no significant differences in viability between treated and untreated splenocytes. As shown in Fig. 3C, atorvastatin treatment of naive Th0 cells suppressed proliferation (panel a) and secretion of Th1 cytokines (b–e), whereas secretion of Th2 cytokines (f–h) and TGF-β (i) was enhanced. Thus, atorvastatin treatment promoted Th2 differentiation. Inhibition of proliferation and Th1 cytokine secretion, as well as induction of Th2 cytokine secretion by atorvastatin, was reversed by L-mevalonate (Fig. 3C, a–i), the product of HMG-CoA reductase, indicating that these effects

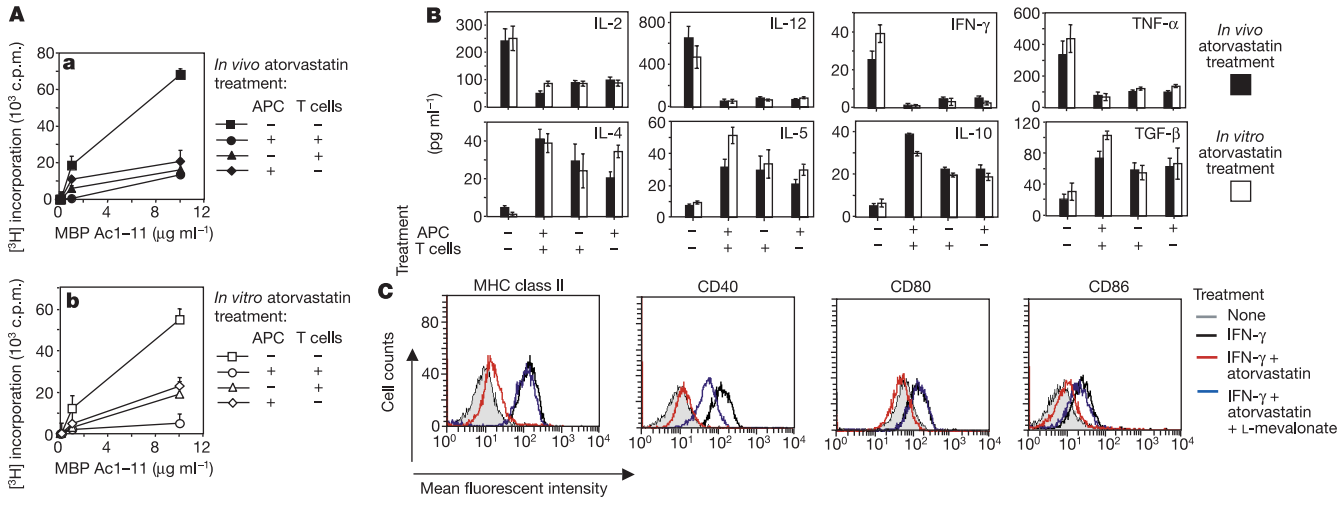

Figure 4 Atorvastatin has immunomodulatory effects on both APC and T cells. **A**, MBP Ac1–11-specific T-cell proliferation was examined using untreated or atorvastatin-treated APC with either untreated or atorvastatin-treated Th0 cells. (**a**) APC were isolated from B10.PL mice treated with either PBS or 10 mg kg^{-1} atorvastatin daily for 10 days. CD3$^+$ T cells were purified from MBP Ac1–11-specific TCR transgenic mice that were treated with either PBS or 10 mg kg^{-1} atorvastatin daily for 10 days. In a reciprocal manner, atorvastatin-treated or untreated APC (5×10^5 per well) were cultured with atorvastatin-treated or untreated CD3$^+$ purified Th0 cells (10^4 per well) and stimulated with MBP Ac1–11. Proliferative responses were reduced using APC or T cells isolated from atorvastatin-treated mice. (**b**) *In vitro* atorvastatin treatment of either APC or T cells alone also suppressed proliferation. Splenic APC (6×10^6 ml^{-1}) from B10.PL mice were cultured in the presence of 10 μM atorvastatin or media alone for 4 h at 37 °C. Separately, purified CD3$^+$ T cells (6×10^6 ml^{-1}) from MBP Ac1–11-specific TCR transgenic mice were cultured in the presence of atorvastatin (10 μM) or media for 4 h. APC and T cells were then washed three times by centrifugation. APC were irradiated and both APC and T cells were counted. No loss in viability was detected in either atorvastatin-treated APC or T cells compared with untreated cells. In a reciprocal manner, untreated or atorvastatin-treated APC were cultured with untreated or atorvastatin-treated purified CD3$^+$ and stimulated with MBP Ac1–11. Proliferative responses were reduced when either APC or CD3$^+$ T cells were treated *in vitro* with atorvastatin. **B**, Inhibition of proliferation by either *in vivo* or *in vitro* treatment of APC or T cells in **A** corresponded to a Th2 cytokine pattern of secretion (**B**). **C**, Atorvastatin treatment of primary macrophages inhibited IFN-γ-inducible expression of class II molecules and CD40, CD80 and CD86 co-stimulatory molecules as measured by flow cytometric analysis: untreated macrophages (grey area), IFN-γ-treated (100 units ml^{-1}, 48 h, black line), IFN-γ and atorvastatin (10 μM, red line). L-Mevalonate (100 μM, blue line) reversed inhibition by atorvastatin.

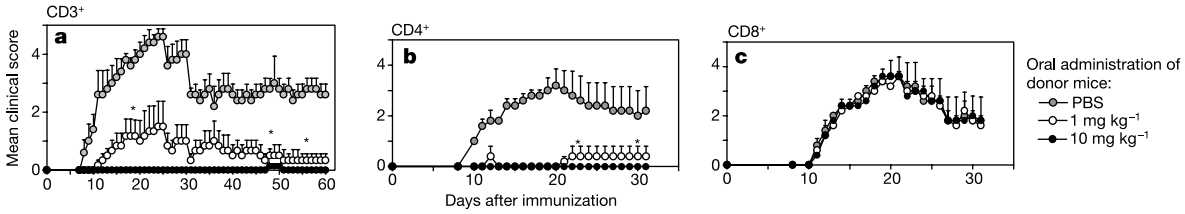

Figure 5 Adoptive transfer of atorvastatin-treated T cells prevents induction of EAE in recipient mice. Purified $CD3^+$ T cells (**a**) and $CD4^+$ T cells (**b**), but not $CD8^+$ T cells (**c**) from atorvastatin-treated donor mice protected recipient mice from EAE induction. Donor MBP Ac 1–11-specific TCR Tg B10.PL mice were treated with either PBS, 1 mg kg^{-1} atorvastatin or 10 mg kg^{-1} atorvastatin for 10 days. Splenocytes were isolated and cultured in the presence of MBP Ac1–11 for 48 h. $CD3^+$ T cells were isolated and injected intravenously into recipient B10.PL mice (10^7 T cells per mouse) (**a**). One day later, recipient mice were immunized for EAE induction. Recipient mice of PBS-treated, 1 mg kg^{-1} atorvastatin and 10 mg kg^{-1} atorvastatin were examined and scored for clinical EAE daily. In a separate experiment, $CD4^+$ (**b**) and $CD8^+$ (**c**) T cells were purified and tested in a similar manner. $^*P < 0.001$ when comparing atorvastatin-treated groups with PBS control groups.

were mediated through inhibition of the mevalonate pathway.

Activated (tyrosine-phosphorylated) signal transducer and activator of transcription (STAT) 4 has a key role in IL-12-dependent Th1 lineage commitment[22]. Conversely, STAT6 is required for IL-4-dependent Th2-dependent lineage commitment[22]. Thus, we examined whether atorvastatin treatment suppressed formation of activated STAT4 or induced activation of STAT6 in T cells from atorvastatin-treated or PBS-treated mice (Fig. 3D). *In vivo* atorvastatin treatment was associated with inhibition of STAT4 phosphorylation (Fig. 3D, a, lanes 3 and 4) and induction of STAT6 phosphorylation (b, lanes 3 and 4).

It was possible that the atorvastatin-induced Th2 bias reflected an alteration in APC function only. Alternatively, atorvastatin may have had direct immunomodulatory effects on both APC and T cells. To distinguish between these possibilities, we examined MBP-specific T-cell activation when only APC or T cells were exposed to atorvastatin. APC were isolated from atorvastatin-treated or PBS-treated mice. T cells that were positive for the CD3 cell-surface protein (referred to hereafter as $CD3^+$ cells) were purified from atorvastatin-treated or PBS-treated mice. In a reciprocal manner, APC from atorvastatin-treated or untreated mice were cultured with $CD3^+$ purified Th0 cells from atorvastatin-treated or untreated mice. Proliferation was inhibited by using either *in vivo*-treated APC or T cells (Fig. 4A, a). A Th2 cytokine pattern was also observed when using either *in vivo*-treated APC or T cells (Fig. 4B). We similarly analysed this by treating either APC or purified transgenic $CD3^+$ T cells separately *in vitro*. Again, proliferation was suppressed (Fig. 4A, b) and a Th2 bias was induced (Fig. 4B) by using either atorvastatin-treated APC or $CD3^+$ T cells. These results confirmed that atorvastatin exerts immunomodulatory effects on both APC and T cells. As atorvastatin treatment suppressed class II (signal 1) upregulation on APC (Fig. 2B) and inhibited secretion of IL-12 (Fig. 3), we examined whether atorvastatin altered the APC expression of co-stimulatory (signal 2) molecules. Atorvastatin inhibited IFN-γ-inducible expression of CD40, CD80 (B7-1) and CD86 (B7-2) co-stimulatory molecules, as well as class II molecules on primary macrophages; these effects were reversed by L-mevalonate (Fig. 4C). Atorvastatin-treated macrophages suppressed proliferation of untreated, purified $CD3^+$-syngeneic Th0 cells and promoted a Th2 bias (data not shown).

We examined directly whether atorvastatin-induced Th2 cells could serve as regulatory cells *in vivo*. By adoptive transfer we tested whether atorvastatin-induced Th2 cells could protect recipient mice from EAE induction. Splenocytes were isolated from B10.PL MBP Ac1–11-specific TCR Tg mice treated with either atorvastatin or PBS, then cultured with MBP Ac 1–11. Cytokine analysis confirmed a Th2 bias for lymphocytes from atorvastatin-treated mice and a Th1 bias for lymphocytes from PBS-treated mice (data not shown). $CD3^+$ T cells were purified. One day after adoptive transfer, recipient B10.PL mice were immunized for EAE induction. All of the recipient mice that received lymphocytes from PBS-treated mice developed severe EAE (Fig. 5a). In contrast, recipient mice of donor lymphocytes from mice treated with 10 mg kg^{-1} atorvastatin were almost entirely protected from EAE. Mild EAE developed in 50% of mice that received lymphocytes from donor mice treated with 1 mg kg^{-1} atorvastatin. In a subsequent experiment, atorvastatin-treated donor T cells were separated into $CD4^+$ and $CD8^+$ subpopulations. The $CD4^+$ T cells (Fig. 5b), but not the $CD8^+$ T cells (Fig. 5c), protected recipient mice from EAE induction. These

Table 1 **Atorvastatin suppresses clinical and histological EAE**

Atorvastatin dose per day (mg kg^{-1})	Clinical EAE			Number of inflammatory foci†		
	Incidence	Mean day of onset ± s.e.m.	Mean score ± s.e.m.	Meningeal	Parenchymal	Total
a, Prevention of relapsing EAE in SJL/J mice*			Day 14			
0	7/7	10 ± 1	3.3 ± 0.31	30 ± 9	25 ± 10	55 ± 20
1	3/7	13 ± 1	0.57 ± 0.3	10 ± 2‖	2 ± 1‖	12 ± 2‖
10	0/7	0 ± 0	0 ± 0	0.4 ± 0.44‖	0 ± 0‖	0.4 ± 0.44‖

b, Treatment of relapsing EAE in SJL/J mice‡								
	Day 16§	Day 36§	Day 53§	Day 16§	Day 36§	Day 53§		
0	10/10	7/8	5/5	2.5 ± 0.3	2.2 ± 0.4	1.5 ± 0.25	33 ± 10	28 ± 10	61 ± 20
1	9/10	4/10	2/7	2 ± 0.3	0.7 ± 0.3	0.5 ± 0.4	0.33 ± 0.4‖	0 ± 0‖	0.33 ± 0.4‖
10	10/10	2/10	1/7	2 ± 0.27	0.3 ± 0.2	0.2 ± 0.19	0.33 ± 0.4‖	0 ± 0‖	0.33 ± 0.4‖

*For EAE prevention, mice were treated daily for 16 days, starting 2 days before EAE induction.
†Brains and spinal cords from all treatment groups (**a**, prevention: five mice per group; **b**, treatment: three mice per group were examined for inflammatory foci at day 14 (**a**, prevention) or at day 36 (**b**, treatment). Results are shown as the mean number of inflammatory foci ± s.e.m.
‡Atorvastatin treatment began during acute EAE at day 12 (mean EAE score 1.78 ± 0.4) and continued for 48 days (Fig. 1c).
§Acute EAE (day 16, Fig. 1c); first relapse (day 36, Fig. 1c); second relapse (day 53, Fig. 1c).
‖*P* value < 0.001, comparison of either atorvastatin-treated group with vehicle-only (PBS)-treated group.

adoptive transfer studies provided evidence that atorvastatin treatment induced a population of regulatory T cells that suppressed clinical autoimmune disease.

We have shown that oral statin treatment suppressed MHC class II upregulation *in vivo*, inhibited APC upregulation of co-stimulatory molecules, and was effective in prevention or reversal of chronic and relapsing CNS autoimmune disease. We also demonstrated that statin treatment promoted differentiation of Th2 cells, which were biologically active in regulating Th1-mediated autoimmune disease. Thus, our results demonstrate that statins can have pleiotropic immunomodulatory effects involving APC and T-cell compartments.

Statins may have multiple targets in immune modulation[2,6,9,23]. Certain statins bind LFA-1 and inhibit its interaction with ICAM-1 (ref. 23), indicating that statins can interact with a protein involved in T-cell adhesion/co-stimulation. Other recognized effects of statins are mediated through inhibition of the mevalonate pathway[2,6]. Mevalonate is a substrate in cholesterol biosynthesis, but also participates in post-translational modification (isoprenylation) of proteins involved in cell division and maturation[24,25]. Statins inhibited the production of proinflammatory cytokines and chemokines, effects that were reversed by mevalonate[2,6]. We have shown that inhibition in expression of IFN-γ-inducible CIITA, class II and co-stimulatory molecules on APC, and Th2 differentiation induced by atorvastatin, are mediated through inhibition of the mevalonate pathway.

MS is a multiphasic disease[26]. CNS inflammation and demyelination characterize the early relapsing–remitting phase, whereas neuronal loss and atrophy occur in the chronic 'secondary progressive' phase[26]. Our results in this study support use in the inflammatory phase. Lovastatin, which partly suppressed acute EAE in rats when administered parenterally[27], inhibited production of iNOS and TNF-α[2,27], pro-inflammatory molecules that are neurotoxic, suggesting that statins might be beneficial in chronic MS. Simvastatin is being tested in a small open-label trial in relapsing–remitting MS (information available at the National Multiple Sclerosis Society, New York (http://www.nationalmssociety.org)). MS trials using atorvastatin are being planned. Statins are attractive candidates for prophylactic treatment in patients who have experienced a single demyelinating event, a 'clinically isolated syndrome', and are at risk of recurrences or conversion to clinically definite MS. As statins have different mechanisms of action from currently approved MS treatments, they may be useful in combination therapy. Our results also provide a rationale for testing atorvastatin in other organ-specific Th1-mediated autoimmune diseases, including diabetes and rheumatoid arthritis. The anti-inflammatory properties described here may also contribute to the protective effects of statins in heart disease and stroke, effects traditionally attributed to cholesterol reduction. □

Methods

Animals

Female SJL/J, B10.PL and C57BL/6 mice (8–12-weeks old) were purchased from the Jackson Laboratory (Bar Harbor). MBP Ac 1-11 TCR transgenic mice, obtained from C. Janeway Jr[18], were backcrossed into the B10.PL background. All animal protocols were approved by the Division of Comparative Medicine at Stanford University and the Committee of Animal Research at the University of California San Francisco, in accordance with the National Institutes of Health guidelines.

Peptides

MBP Ac1–11 (Ac-ASQKRPSQRHG), MOG p35–55 (MEVGWYRSPFSRVVHLYRNGK), PLP p139–151 (HCLGKWLGHPDKF) and the control peptide HSVP16 (DMTPADALDDRDLEM) were synthesized on a peptide synthesizer (model 9050; MilliGen) by standard 9-fluorenylmethoxycarbonyl chemistry, and purified by high-performance liquid chromatography (HPLC). Amino acid sequences were confirmed by amino acid analysis and mass spectroscopy. The purity of each peptide was greater than 95%.

Microglia and macrophages

Microglia EOC 20 cells, derived from C3H/HeJ CH-2k mice[28], were obtained from the American Type Culture Collection (ATCC) and were grown as recommended using DMEM media supplemented with 1 mM sodium pyruvate, 10% (v/v) fetal calf serum (FCS) and 20% (v/v) conditioned media as a source for mouse CSF-1. These microglia stained positively for CD11b. Primary microglia were isolated from 2-day-old B10.PL mice as described previously[29]. Primary microglia were 95% $CD11b^+$ by fluorescence-activated cell sorting (FACS). Primary macrophages (peritoneal exudate cells (PEC)) were harvested from B10.PL mice 24 h after intraperitoneal injection with 1 ml of 3% (w/v) thioglycollate. PEC were cultured with media alone for 72 h, then activated with IFN-γ (100 U ml^{-1}) or treated with media alone. PEC were 98% (w/v) $CD11b^+$ by FACS analysis.

Atorvastatin treatment

Atorvastatin (Pfizer Inc.) (prescription formulation) was brought into suspension in PBS. Atorvastatin was administered orally in 0.5 ml (for example, 0.04 mg ml^{-1} for 1 mg kg^{-1} dose or 0.4 mg ml^{-1} for 10 mg kg^{-1} dose) once daily using 20-mm feeding needles (Popper and Sons Inc.). PBS was administered as control. Purified atorvastatin, used for *in vitro* studies, was provided by R. Laskey (Pfizer Inc.).

Experimental autoimmune encephalomyelitis induction

EAE was induced in SJL/J mice, C57BL/6 mice or MBP Ac1-11-specific TCR transgenic mice by immunization with 100 μg of PLP p139–151, MOG p35–55 or MBP Ac1–11, respectively. All peptides were dissolved in complete Freund's adjuvant (CFA) containing 4 mg ml^{-1} of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories) as described in ref. 5. On the day of immunization and 48 h later, C57BL/6 mice and MBP Ac1–11 TCR transgenic mice were injected with 100 ng of *Bordetella pertussis* toxin (BPT) in PBS, intravenously (i.v.). Mice were examined daily for clinical signs of EAE and scored as follows: 0, no paralysis; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, hindlimb and forelimb paralysis; 5, moribund or dead.

Adoptive transfer

B10.PL MBP Ac1-11-specific TCR Tg mice (four per group) were treated daily with either 1 mg kg^{-1} atorvastatin or 10 mg kg^{-1} atorvastatin or vehicle (PBS) only. After 12 days of oral treatment, mice were killed. Splenocytes were isolated and cultured in the presence of MBP Ac1–11 (10 μg ml^{-1}) for 48 h. Viable cells were removed by Ficoll gradient. $CD3^+$, $CD4^+$ or $CD8^+$ T cells were purified by high-affinity negative selection (R and D Systems). Greater than 85% purity was obtained for each. One million T cells were injected intravenously into naive B10.PL recipient mice. Twenty-four hours after adoptive transfer, recipient mice were immunized with MBP Ac 1–11 for EAE induction as previously described and scored daily for clinical signs: Fig. 5a, PBS ($n = 5$), 1 mg kg^{-1} atorvastatin ($n = 6$), 10 mg kg^{-1} atorvastatin ($n = 7$); Fig. 5b and c, $n = 5$ for all groups.

Flow cytometry

Immunofluorescent staining was done as described in ref. 30. After incubation for 48 h, cells were washed with FACS buffer (PBS containing 0.1% (w/v) sodium azide and 2% (v/v) FCS) and preincubated with anti-mouse CD16/CD32 monoclonal antibody (clone 2.4G2, PharMingen) for 10 min at 4 °C to block non-specific binding to Fc receptors. Fluorochrome-conjugated monoclonal antibodies (rat anti-mouse Mac-1/CD11b-PE (M1/70, IgG2b), mouse anti-mouse MHC class II ($I\text{-}A^k$)-FITC (10-3.6, IgG2b), hamster anti-mouse CD40-FITC (HM40-3, IgM), hamster anti-mouse CD80-FITC (16-10A1, IgG) and rat anti-mouse CD86-FITC (GL1, IgG2a) were purchased from PharMingen. Background fluorescence was evaluated by staining the cells with isotype control antibodies: rat IgG2b-PE or-FITC and mouse IgG2b-FITC (PharMingen). After incubation, cells were washed twice with FACS buffer and analysed by FACScan using CellQuest software (Becton Dickinson). Twenty thousand events were analysed.

Antigen-specific T-cell proliferation assays

Splenocytes or lymph node cells (LNC) were isolated from atorvastatin or vehicle-only-treated mice and culture *in vitro* with the specific encephalitogenic peptide (PLP p139–151, MOG p35–55 or MBP Ac1–11) used for the immunization or with either concanavalin A (con A) (positive control) or HSVP16 (negative control). Cells were cultured in 96-well microtitre plates at a concentration of 5×10^6 cells ml^{-1}. Culture medium consisted of RPMI 1640 supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), non-essential amino acids (0.1 mM), penicillin (100 U ml^{-1}), streptomycin (0.1 mg ml^{-1}), 2-mercaptoethanol (5×10^{-5} M) and 1% (v/v) autologous normal mouse serum. Splenocytes and LNC from SJL/J or C57BL/6 mice were incubated for 72 h whereas cultures from MBPAc-1-11 Tg mice were incubated for 48 h. Cultures were then pulsed for 18 h with 1 μCi per well of [^{3}H]thymidine before harvesting. Results are shown as mean of triplicates ± s.e.m.; s.e.ms were within 10% of the mean.

Cytokine analysis

Supernatants from splenocytes and LNC cultured in parallel with those cells used in proliferation assays tested were used for cytokine analysis. Supernatants were collected at different times for measurements of cytokine levels: 48 h for IL-2 and IL-12, 72 h for IFN-γ, TNF-α and TGF-β, and 120 h for IL-4, IL-5 and IL-10. IL-2, IL-5, IL-10, IL-12 (p70), IFN-γ and TNF-α levels were determined by using specific enzyme-linked immunosorbent assay (ELISA) kits for the corresponding cytokines according to the manufacturer's protocols (anti-mouse OPTEIA Kits, PharMingen). IL-4 was measured using a cytometric bead array kit (BD Biosciences). TGF-β was measured by ELISA (R and D Systems). Results are shown as mean of triplicates ± s.e.m.; s.e.ms were within 10% of the mean.

Total RNA isolation

Mice were killed and perfused with 20 ml of cold sterile PBS. Total RNA was isolated from

brain tissues using Trizol reagent (Invitrogen) as recommended in the manufacturer's protocol. Microglia cell cultures were washed once with PBS, and RNA was isolated using Trizol reagent as described by the manufacturer's protocol. RNA concentration was measured by spectrophotometry at 260 nm.

Evaluation of CIITA promoter-specific mRNA expression

One-step polymerase chain reaction with reverse transcription (RT–PCR) was performed by using a master mix that was prepared with 400 mM dUTP and 200 mM each of dATP, dCTP and dGTP; 0.2 mM each oligonucleotide primer, 0.2 × SYBR green in DMSO (1% final concentration); 2.5% (v/v) glycerol; 1 U uracyl *N*-glycosylase; 4 mM $Mn(OAc)_2$; and 5 U rTth polymerase. RT–PCR parameters: initial incubation 10 min at 45 °C with activating uracyl *N*-glycosylase followed by reverse transcription 30 min at 60 °C; 50 cycles at 95 °C for 15 s and 57 °C for 30 s. β-Actin was amplified from all samples as a housekeeping gene to normalize expression. A control (no template) was included for each primer set. For quantification, a tenfold dilution series of a CIITA run-off transcript (10^7–10^2 initial CIITA copies) was included in each reaction plate. Data were analysed by Sequence Detection Systems software and transferred to a Microsoft Excel spreadsheet for analysis. A calibration curve was generated by plotting CIITA (run-off transcript) for each tenfold dilution against the number of cycles required for each product to exceed a preset threshold (Ct). Ct values were compared with those obtained on a standard curve. Primers for common CIITA (nucleotides 2374–2458) were: 5′-GCCCACGAGACACAGCAA and 5′-TGAGCCGGGTGCCCAGGAA. The 5′ (forward) promoter-specific primers were: pI CIITA (pI nt 259), 5′-CCTGACCCTGCTGGAGAA; pIII CIITA (pIII nt 112), 5′-GCATCACTCTGCTCTCTAA; pIV CIITA (pIV nt 43), 5′-TGCAGGCAGCACTCAGAA. CIITA (nt 265) reverse primers for promoter-specific transcripts were: 5′-GGGGTCGGCACTGTTA; β-actin: (301–538): 5′-CGACCTGGGGATCTTCTA and 5′-TCGTGCCCTCAGCTTCCAA.

Western blot analysis for STAT4 and STAT6 phosphorylation

Lymph nodes from control and atorvastatin-treated mice were isolated and homogenized in T-PER protein extraction buffer (Pierce Chemical Co.), with 20 mg ml^{-1} aprotinin, 20 mg ml^{-1} leupeptin, 1.6 mM Pefablock SC (Roche), 10 mM NaF, 1 mM Na_3VO_4 and 1 mM $Na_4P_2O_7$ (Sigma). Naive lymph-node cells cultured with mouse recombinant IL-4 (10 ng ml^{-1}) for 1 h served as a positive control for STAT6 activation. Protein concentrations were determined by bicirchoninic acid protein assay (Pierce). Lysates were added to 3× SDS loading buffer (Cell Signaling Technology) with 40 mM DTT. Products were separated by electrophoresis on a 4–15% SDS–PAGE gradient gel (Bio-Rad Laboratories Inc.). Pre-stained markers (Invitrogen) were used to determine molecular mass. Gels were blotted to PVDF membranes at 100 V in 25 mM Tris, 192 mM glycine and 20% (v/v) methanol, then blocked for 1 h at room temperature with Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20 and 5% (w/v) non-fat dry milk. After washing in TBS and 0.1% (v/v) Tween 20, membranes were hybridized overnight at 4 °C with anti-phospho-STAT4 (Zymed Laboratories Inc.) antibody or anti-phospho-STAT6 antibody (Cell Signaling Technology, Inc.) diluted 1:1,000 in TBS, 0.1% (v/v) Tween 20 and 5% (w/v) BSA. The membranes were then processed by ECL Plus protocol (Amersham BioSciences, Inc.) for visualization of the bands. Membranes were stripped in 100 mM 2-mercaptoethanol, 2% (w/v) SDS and 62.5 mM Tris (pH 7.4) for 30 min at 60 °C, then probed with anti-CD3ζ (Pharmingen), anti-STAT4 and anti-STAT6 (BD Biosciences) as a control to verify equal protein loading. Phosphorylated (p) STAT4 and STAT6 migrated at a relative molecular mass of ~100,000.

Histopathology and immunohistochemistry

Anaesthetized mice were perfused with 20 ml cold PBS. Brains and spinal cords were fixed in 4% (w/v) paraformaldehyde and embedded in paraffin. Sections were stained with haematoxylin and eosin. Selected brain, thoracic and lumbar spinal cord sections were evaluated by an examiner blinded to the treatment status of the animal. For MHC class II evaluation, frozen sections were used. Brain tissues collected on day 14 after immunization from two to five mice in each experimental group, were embedded in Tissue-Tek O.C.T. Compound (VWR Scientific Products) quick-frozen in solid CO_2 mixed with isopentane, and maintained at −80 °C until sectioning. Cryostat sections (4–6 μm) were fixed with acetone and then labelled with mouse anti-I-$A^{k,s}$ monoclonal antibody (mAb) 10-3.6 (PharMingen) or mouse anti-I-A^b mAb AF6-120.1 (PharMingen), using the avidin–biotin technique (Vector Laboratories). Staining was visualized by reaction with diaminobenzidine (DAB). Slides were counterstained with haematoxylin. Isotype-matched IgG and omission of the primary antibody served as negative controls. Microglia cells were grown on cover slides and treated with IFN-γ alone or with the addition of atorvastatin or atorvastatin and L-mevalonate for 48 h. Slides were then fixed with acetone and stained with anti I-A^k (10-3.6) using the same procedure as for frozen sections.

Statistical analysis

Data are presented as mean ± s.e.m. For clinical scores, significance between each two groups was examined by using the Mann–Whitney *U* test. A value of $P < 0.05$ was considered significant. All other statistics were analysed by using a one-way multiple-range analysis of variance test (ANOVA) for multiple comparison. A value of $P < 0.05$ was considered significant.

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