Suppression of Autoimmune Retinal Disease by Lovastatin Does Not Require Th2 Cytokine Induction

Matthew E. Gegg, Rachel Harry, Deborah Hankey, Hadi Zambarakji, Gareth Pryce, David Baker, Peter Adamson, Virginia Calder and John Greenwood

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Intraocular inflammatory diseases are a major cause of severe visual impairment accounting for ~10% of blind registrations in the working population (1). These uveitic syndromes may occur as organ-specific (auto)immune diseases, such as pars planitis, sympathetic ophthalmia, and idiopathic posterior uveitis, or as part of a systemic immune-mediated disease such as Behçet’s disease and sarcoidosis.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, generically termed statins, are widely prescribed for their cholesterol-lowering properties, but they also possess immunomodulatory activity. Indeed, substantive evidence has recently been presented demonstrating their efficacy in the treatment of autoimmune disease, notably in a model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE; Refs. 2–10). How statins exert their anti-inflammatory therapeutic effect is poorly understood, primarily because of their pleiotropic nature where they have been shown to affect T cell activation and proliferation (4), adhesion molecule interactions (11, 12), production of chemokines (13), matrix metalloproteinase production (14, 15), NO production (2), MHC class II-restricted Ag presentation (4, 16), and leukocyte migration through the vascular wall (6, 10). Foremost, however, has been their ability to shift the T cell cytokine response from a proinflammatory Th1 to an anti-inflammatory Th2 profile (4, 7, 8). Despite these many mechanisms of action, a common feature of statin treatment of neuroinflammatory disease is a reduction in the accumulation of mononuclear cell infiltrates into the target tissue (2–8, 10).

Statins, which inhibit the enzymatic conversion of HMG-CoA to L-mevalonate and hence cholesterol biosynthesis, results not only in the depletion of cholesterol but also the downstream metabolites farnesylpyrophosphate and geranylgeranylnylpyrophosphate. These isoprenoid pyrophosphates are precursors required for the post-translational prenylation and functional activation of certain proteins, including the small GTP binding protein Rho. Activation of endothelial cell monolayers in vitro leads to inhibition of lymphocyte transmigration, which may, in part, account for drug efficacy. Unlike lovastatin, atorvastatin treatment showed little efficacy in retinal inflammatory disease despite showing significant clinical benefit in experimental autoimmune encephalomyelitis. These data highlight the potential differential activity of statins in different inflammatory conditions and their possible therapeutic use for the treatment of human posterior uveitis.

**Suppression of Autoimmune Retinal Disease by Lovastatin Does Not Require Th2 Cytokine Induction**

Matthew E. Gegg, Rachel Harry, Deborah Hankey, Hadi Zambarakji, Gareth Pryce, David Baker, Peter Adamson, Virginia Calder, and John Greenwood

Intraocular inflammatory diseases are a common cause of severe visual impairment and blindness. In an acute mouse model of autoimmune retinal disease, we demonstrate that treatment with the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, lovastatin, suppresses clinical ocular pathology, retinal vascular leakage, and leukocytic infiltration into the retina. Efficacy was reversed by coadministration of mevalonolactone, the downstream product of 3-hydroxy-3-methylglutaryl coenzyme A reductase, but not by squalene, which is distal to isoprenoid pyrophosphate metabolites within the cholesterol biosynthetic pathway. Lovastatin treatment (20 mg/kg/day i.p.) over 7 days, which resulted in plasma lovastatin hydroxyacid concentrations of 0.098 ± 0.03 μM, did not induce splenocyte Th2 cytokine production but did cause a small reduction in Ag-induced T cell proliferation and a decrease in the production of IFN-γ and IL-10. Thus, it is possible to dissociate the therapeutic effect of statins in experimental autoimmune uveitic mice from their activity on the Th1/Th2 balance. Statins inhibit isoprenoid pyrophosphate synthesis, precursors required for the prenylation and posttranslational activation of Rho GTPase, a key molecule in the endothelial ICAM-1-mediated pathway that facilitates lymphocyte migration. Consistent with inhibition of leukocyte infiltration in vivo, lovastatin treatment of retinal endothelial cell monolayers in vitro leads to inhibition of lymphocyte transmigration, which may, in part, account for drug efficacy. Unlike lovastatin, atorvastatin treatment showed little efficacy in retinal inflammatory disease despite showing significant clinical benefit in experimental autoimmune encephalomyelitis. These data highlight the potential differential activity of statins in different inflammatory conditions and their possible therapeutic use for the treatment of human posterior uveitis.

posterior uveitis in which there is a strong Th1 bias (23), we investigated the efficacy of statin treatment on disease progression. We demonstrate that parenteral administration of lovastatin attenuates EAU in the absence of Th2 cytokine production and can block lymphocyte migration across the blood-retinal barrier in vitro. This study shows that it is possible to dissociate the therapeutic effect of statins in EAU from their activity on the Th1/Th2 balance.

Materials and Methods

Lymphocyte adhesion and migration in vitro

Lymphocyte adhesion and migration assays on Lewis rat retinal endothelial cell (REC) (JG/21) and retinal pigment epithelium (RPE) (LD7.4) cell lines were conducted as previously described (20, 25, 26). Briefly, for adhesion assays, peripheral lymph node cells were harvested from Lewis rats (Harlan Olac) and stimulated for at least 24 h with 5 μg/ml Con A (Sigma-Aldrich). The T cells were fluorescently labeled with 1 μM calcein-AM (Molecular Probes), and 1 × 10^6 labeled cells/well were added to 96-well plates containing REC or RPE monolayers, and incubated for 90 min at 37°C. Each well was then washed with HBSS, and bound T cells measured by a fluorescent plate reader (excitation, 494 nm; emission, 517 nm). For migration assays, uveitogenic retinal S-Ag peptide 273–289-specific CD4+ T cell lines were established from peptide-primed Lewis rat lymph nodes and maintained as previously reported (27, 28). T cells (2 × 10^5/well) were added to 96-well plates containing REC or RPE monolayers, and incubated for 4 h at 37°C to allow T cells to settle and migrate. A minimum of 12 wells per assay was performed.

Induction and assessment of EAU

Male B10.RII (7INS) mice (5–7 wk old; Harlan Olac) were injected s.c. with 25 μg of human interphotoreceptor retinoid binding protein (IRBP)_161–180_ peptide emulsified in IFA supplemented with 60 μg/ml Mycobacterium tuberculosis H37Ra (4, 31). On the day of immunization and 48 h later, mice were also injected with 100 ng of Bordetella pertussis toxin (Sigma-Aldrich). Mice were examined daily for clinical signs of EAE and were scored as follows: 0, no disease; 1, limp tail; 2, impaired righting reflex; 3, partial hindlimb paralysis; 4, complete hindlimb paralysis; 5, moribund or dead (32). Mice were administered vehicle (PBS) or 10 mg/kg/day atorvastatin (prescription formulation) by oral gavage from day 10 postinduction onwards, within 1 day of clinical signs being observed (4).

Table I. Grading system for mouse EAU: clinical signs

<table>
<thead>
<tr>
<th>Grade</th>
<th>Clinical Signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal. Clear medium with good red reflex.</td>
</tr>
<tr>
<td>1</td>
<td>Minimal but clearly positive signs of inflammation with occasional cells in AC. Dilation of iris vessels.</td>
</tr>
<tr>
<td>2</td>
<td>Presence of AC cells with minimal hypopyon.</td>
</tr>
<tr>
<td>3</td>
<td>Diffuse exudates within AC and vitreous plus moderate hypopyon. Dull red reflex. Synchiae.</td>
</tr>
<tr>
<td>4</td>
<td>Presence of large cellular and fibrinous exudates (AC very cloudy). Large hypopyon in AC. Loss of red reflex.</td>
</tr>
<tr>
<td>5</td>
<td>Presence of grade 4 with gross orbital edema and proptosis of globe.</td>
</tr>
</tbody>
</table>

a AC, Anterior chamber.

Splenocyte proliferation and cytokine production

Splenocytes (5 × 10^6 cells/ml) were prepared from vehicle and statin-treated mice on day 12 (EAU) and day 17 (EAE) postinduction, and grown in supplemented RPMI 1640 medium (33). Cells were unstimulated or stimulated with 5 or 20 μg/ml IRBP 161–180 (EAU) or 20 μg/ml MOG 35–55 (EAE) peptide for 72 h. For the final 18 h, 1 μCi of [methyl-^3^H]thymidine (Amersham Biosciences) was added to each well. The cells were harvested, and [^3^H]thymidine uptake was determined by beta-scintillation spectrometry. Cell culture supernatants were collected at 48 h for production of IL-2 and IL-12, at 72 h for production of IFN-γ, IL-10, and TNF-α, and up to 120 h for the production of IL-4 and IL-5. Cytokine concentration in supernatants was determined by cytokine-specific ELISA (R&D Systems). For intracellular cytokine measurement, 2 × 10^6 peripheral lymph node cells/ml were cultured in the absence or presence of either the IRBP 161–180 or MOG 35–55 peptides for 96 h. Brefeldin A (10 μg/ml) was added to the culture 18 h before harvesting. Cells were fixed in Cytofix/Cytoperm (BD Biosciences) and stained with FITC-conjugated rat anti-mouse IFN-γ and PE-conjugated rat anti-mouse IL-4 mAbs (BD Biosciences) and analyzed by flow cytometry.

Table II. Grading system for mouse EAU: fluorescence angiographic signs

<table>
<thead>
<tr>
<th>Grade</th>
<th>Fluorescein Angiographic Signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal. No leakage.</td>
</tr>
<tr>
<td>1</td>
<td>Mild leakage: hazy but reasonable view of optic disc, optic cup and retinal vessels entering the optic disc.</td>
</tr>
<tr>
<td>2</td>
<td>Moderate leakage: unable to identify optic disc and retinal vessels entering the optic disc clearly.</td>
</tr>
<tr>
<td>3</td>
<td>Severe leakage: no view of optic disc, optic cup or retinal vessels entering the optic disc.</td>
</tr>
</tbody>
</table>

**Statin and cholesterol analysis**

Plasma and serum was prepared from EAU and EAE mice on days 12 and 17 postinduction, respectively. For the measurement of atorvastatin acid and lovastatin hydroxyacid in plasma and tissue culture medium, a liquid-chromatographic tandem mass spectrometry bioanalytical assay was established over the concentration range of 0.5–500 ng/ml for atorvastatin acid and 1–1000 ng/ml for lovastatin hydroxyacid. These assays were developed and conducted by HFL Contract Research (Fordham; www.hfl.co.uk). Briefly, atorvastatin acid or lovastatin hydroxyacid, was extracted using a liquid-liquid extraction procedure followed by chromatographic separation using a reversed-phase Phenomenex (Luna C18; 50 × 2.0 mm; 5 μm) analytical column. The analyte was ionized using an electrospray interface operating in negative ion mode and detection was via tandem mass spectrometry in the multiple reaction monitoring mode. Simvastatin hydroxyacid was used as internal standard for both analytes.
but not squalene (2 mg/kg, i.p.; coadministration of mevalonolactone [twice daily, 2 mg/kg, i.p.] (Fig. 1, 26 vehicle-treated mice exhibited inflammatory changes to the eye treated animals was observed on day 9 postimmunization, which clinical disease in vehicle-with no clouding of the eye (Fig. 1 a) and vehicle-treated control EAU animals at the peak of induction, did not reverse the effect of lovastatin (Fig. 2 g). We next compared the histological appearance of the retinae of normal animals with those induced for EAU. Retinal examination of normal (Fig. 3 a) and vehicle-treated control EAU animals at the peak of clinical disease (day 12) revealed in the latter characteristic lesions in 25 of 26 animals studied that consisted of leukocytic infiltration, optic disc edema, subretinal exudates, and retinal folding (b). Treatment of EAU-induced mice with lovastatin caused a significant reduction in both the number of animals in the group showing pathological changes (p < 0.01) and in the mean pathological grade (p < 0.001) (Fig. 3, c and e). As observed with clinical disease, supplementation with mevalonolactone reversed significantly the severity of retinal pathology (Fig. 3, d and e), whereas supplementation with squalene did not (e). Due to mevalonolactone being a labile compound, we were unable to achieve complete reversal of the effect of lovastatin at the doses used. Plasma concentrations of lovastatin hydroxyacid, the primary active metabolite of lovastatin, were measured in a sample of animals after 7 days of lovastatin administration. No statin was detected in plasma from vehicle-treated control animals (n = 5). In those animals

<table>
<thead>
<tr>
<th>Grade</th>
<th>Histological Signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>Minimal signs of inflammation. Occasional inflammatory cells in the vitreous base or scattered cells in the neuroretina or adjacent to the ciliary body. Very minor focal retinal detachment generally around optic nerve and occasional focal retinal folding.</td>
</tr>
<tr>
<td>2</td>
<td>Uniform inflammatory cell infiltration of the PR, ONL, and INL of the retina and infiltration of the vitreous cortex. Some perivascular cuffing. Retinal damage &lt; 20%.</td>
</tr>
<tr>
<td>3</td>
<td>Infiltration of the PR layer, ONL, and INL of the retina with some retinal detachment (&gt;=20, &lt;50%). Moderate retinal folding, diffuse cellular infiltration of the vitreous, and moderate uveal involvement.</td>
</tr>
<tr>
<td>4</td>
<td>Full thickness involvement of the retina, serious retinal detachment and folding. Subretinal exudate. Necrosis and glossis, substantial uveal involvement.</td>
</tr>
<tr>
<td>5</td>
<td>Severe retinal damage with destruction of the photoreceptor layer. Large-scale retinal folding and large subretinal exudate. Massive cellular infiltrate and loss of anatomical structure.</td>
</tr>
</tbody>
</table>

Total cholesterol concentration in serum was determined spectrophotometrically using the Infinity cholesterol liquid stable reagent (ThermoTrace).

Statistical analyses

Data are presented as mean ± SEM. For angiography, clinical and histology scores, significance between groups was determined by the Mann-Whitney U test. All other statistics were analyzed by the Student’s t test. A value of p < 0.05 was considered significant.

Results

Parenteral lovastatin treatment attenuates EAU

Following induction of EAU, animals were monitored daily for clinical signs of disease. Normal animals exhibited good red reflex with no clouding of the eye (Fig. 1a). Clinical disease in vehicle-treated animals was observed on day 9 postimmunization, which increased in severity up to day 12 postinduction (29), where 23 of 26 vehicle-treated mice exhibited inflammatory changes to the eye (Fig. 1, b and c). Lovastatin treatment (20 mg/kg, i.p., from day 5 onwards) resulted in a significant reduction in clinical disease (Fig. 1, c and d), an effect we were able to reverse significantly by coadministration of mevalonolactone (twice daily, 2 mg/kg, i.p.) but not squalene (2 mg/kg, i.p.; c and d). Using FA, we evaluated retinal vascular leakage in both normal mice and at day 10 post-EAU induction. No vascular leakage of fluorescein was observed in control animals (Fig. 2a) but in vehicle-treated EAU animals, significant hyperfluorescence, particularly at the optic disc, was observed (b and c), indicating significant breakdown of the anterior (vascular) blood-retinal barrier (g). The majority (9 of 10) of lovastatin-treated mice exhibited no vascular leakage (Fig. 2, d and e), which was significantly less (p < 0.001) than in the vehicle-treated group (g). When we supplemented lovastatin with mevalonolactone, we observed a significant reversal (p < 0.01) in the number of animals (5 of 6) exhibiting vascular leakage (Fig. 2f) compared with lovastatin-treated animals (g). Additional vascular abnormalities included dilatation and tortuosity of the retinal veins in vehicle-treated EAU animals and in EAU-induced animals treated with lovastatin and mevalonolactone compared with lov-
treated with lovastatin, plasma lovastatin hydroxyacid levels reached a mean value of 0.098 ± 0.03 μM (n = 5), which was not significantly altered upon coadministration with squalene (0.101 ± 0.02 μM; n = 5). It should also be noted that lovastatin treatment for 7 days had no effect on total cholesterol serum levels (vehicle-treated EAU mice, 156.6 ± 6.1 mg/dL; lovastatin-treated EAU mice, 177 ± 11.5 mg/dL).

**Lovastatin results in a weak inhibition of T cell proliferation during EAU in B10.RIII mice**

Although it is clear that statins inhibit mononuclear infiltration into the eye (Figs. 1–3), statins have also been reported to be antiproliferative in vitro (4). Therefore, we examined the anti-T cell-proliferative effect of lovastatin treatment, initiated 5 days after the induction of EAU. We found that the in vitro T cell recall response to IRBP161–180 peptide of splenocytes harvested on day 12 postinduction from lovastatin-treated mice was reduced compared with that observed in cells from untreated EAU mice (Fig. 4a). This inhibition of proliferation was significant in cells stimulated with either 5 or 20 μg/ml IRBP161–180 peptide (Fig. 4a). The T cell recall response to 20 μg/ml IRBP161–180 peptide in both vehicle- and lovastatin-treated EAU mice was significantly inhibited (p < 0.001) when the splenocytes were stimulated in the presence of an anti-mouse MHC class II Ab (0.5 μg/million cells).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. EAU/total</th>
<th>Mean Group Clinical Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal animals</td>
<td>0/3</td>
<td>0</td>
</tr>
<tr>
<td>Vehicle treated (i.p.)</td>
<td>9/13</td>
<td>1.38 ± 0.33</td>
</tr>
<tr>
<td>Lovastatin 20 mg/kg/day</td>
<td>1/10**</td>
<td>0.10 ± 0.10***</td>
</tr>
<tr>
<td>Lovastatin + mevalonolactone</td>
<td>5/6††</td>
<td>1.33 ± 0.42††</td>
</tr>
<tr>
<td>Lovastatin + squalene</td>
<td>2/6</td>
<td>0.33 ± 0.21*</td>
</tr>
</tbody>
</table>

**FIGURE 3.** Lovastatin treatment of EAU attenuates retinal pathology. a and b, Histological appearance of normal mouse retina (grade 0) (a) and following induction of EAU at day 12 postimmunization showing moderate retinal folding and detachment, leukocytic infiltration of the retina, and vitreous and perivascular leukocytic cuffing (arrows) (grade 3) (b). c, Lovastatin-treated (20 mg/kg/day) EAU retina (grade 1) showing normal retina with mild inflammation around the optic nerve head. d, Lovastatin (20 mg/kg/day)- and mevalonolactone (2 × 2 mg/kg/day)-treated EAU retina (grade 2) demonstrating reversal of therapeutic effect of lovastatin. Retina shows signs of perivascular leukocytic cuffing (arrows) and inflammation of the optic nerve head. e, Table showing significant reduction in EAU retinal pathology at day 12 following treatment with lovastatin. Coadministration of mevalonolactone, but not squalene, caused partial reversal of retinal pathology. Scale bar, 500 μm. *, p < 0.05; **, p < 0.01; ††, p < 0.001 compared with vehicle control; and ††, p < 0.01 compared with lovastatin treated.

**Lovastatin treatment for 7 days failed to induce a Th2 cytokine profile in B10.RIII mice**

Because therapeutic effects of statins have been attributed to Th1 to Th2 deviations (4, 7, 8), we examined cytokine production by splenocytes harvested from vehicle- and lovastatin-treated EAU mice at the peak of disease (day 12). Treatment of EAU with lovastatin for 7 days did not result in any change in the splenocyte production of IL-2 (Fig. 4b). We observed a significant reduction (p < 0.05) in the production of IFN-γ by IRBP161–180-activated splenocytes from lovastatin-treated animals compared with those treated with vehicle (Fig. 4c). Both IL-4 and IL-5 production was below the assay detection limit (<5 pg/ml), and there was no corresponding increase in IL-10 indicative of a Th2 response (Fig. 4d). On the contrary, we found IL-10 significantly reduced following lovastatin treatment and interestingly found a consistent increase in TNF-α (Fig. 4e). We next investigated whether there was a correlation between the ameliorating effect ofLovastatin on disease and the regulatory T cell (Treg) population. No differences were observed in the phenotypically assessed Treg population between normal, vehicle-treated, and lovastatin-treated mice (Fig. 4f).

**Oral administration of lovastatin failed to attenuate EAU**

Because statins are oral drugs, EAU-induced mice were also treated with 20 mg/kg lovastatin daily by oral gavage (days 5–12). In contrast to that observed with the same dose ofLovastatin administered parenterally, no significant alleviation of disease by either clinical assessment (vehicle gavage, 3.4 ± 0.45, n = 10; lovastatin gavage, 4.00 ± 0.37, n = 6) or histology (vehicle gavage, 4.25 ± 0.49; lovastatin gavage, 4.33 ± 0.33) was observed on day...
FIGURE 4. Treatment of EAU with parenteral lovastatin over 7 days does not result in an increase in Th2 cytokines. a, Proliferation of unstimulated (□) and Ag stimulated (5 μg/ml IRBP peptide ■; 20 μg/ml IRBP peptide □) splenocytes from vehicle- or lovastatin-treated EAU animals. Proliferation in the presence of 20 μg/ml IRBP peptide was MHC class II restricted, because cotreatment with class II blocking Ab inhibited proliferation (■). Splenocytes isolated from lovastatin-treated mice showed a small but significant reduction in proliferation compared with vehicle. *, p < 0.001 compared with 20 μg/ml IRBP stimulated within group. †, p < 0.05; and ††, p < 0.01 compared with corresponding vehicle-treated group. b–e, IL-2 (b), IFN-γ (c), IL-10 (d), and TNF-α (e) production from unstimulated (□) or Ag stimulated (■) isolated from normal animals and from vehicle- or lovastatin-treated EAU animals. *, p < 0.05; **, p < 0.01; ††, p < 0.001 compared with unstimulated splenocytes within group. †, p < 0.05 compared with corresponding vehicle-treated group. f, Percentage of Treg in CD4+CD25+ population.

12. Plasma concentrations of lovastatin hydroxyacid measured in these animals after 7 days of oral lovastatin administration (1.7 ± 0.8 nM; n = 6 animals) were significantly less than those concentrations recorded following parenteral administration (compared with 98 ± 3 nM; p < 0.01).

Oral atorvastatin treatment attenuates EAE but has little efficacy in EAU

Oral administration of atorvastatin (10 mg/kg/day) has previously been shown to attenuate MOG35-55-induced EAE in C57BL/6 (B6) mice (4). Using an identical protocol, we treated EAU-induced B10.RII mice with 10 mg/kg/day intragastric atorvastatin or vehicle (PBS) from days 5 to 12 postimmunization. Atorvastatin treatment resulted in a small, but significant reduction in clinical disease (Fig. 5, a and c). However, in contrast to that observed with parenteral lovastatin, FA (mean group score, 0.67 ± 0.21; n = 6) and histology (mean group score, 3.44 ± 0.32; n = 18) scores were not significantly different to EAU mice treated with vehicle alone. This poor response could not be attributed to changes in the Treg population, because there was no significant difference from the lovastatin-treated group (Fig. 4f).

Contrary to the result observed in the EAU group of animals, an identical 7-day oral delivery regimen in EAE in B6 mice resulted in a highly significant attenuation of clinical disease (Fig. 5b), in a manner similar to that reported previously (4). Following treatment of EAE with oral atorvastatin, we observed a clear and significant reduction in the number of B6 animals exhibiting clinical disease (p < 0.05) and in the group maximal clinical score (p < 0.01) (Fig. 5, b and c). To establish whether the cytokine profiles differed between the EAU and EAE disease models following oral atorvastatin, we investigated splenocyte cytokine production as described above for the lovastatin group. In the EAU group of animals, we did not observe any change in the IRBP161–180-stimulated production of either IFN-γ or TNF-α (Fig. 5, d and e) but did observe a reduction in both IL-2 and IL-10. Interestingly, the levels of IFN-γ produced in the oral vehicle group were significantly greater than those recorded in the i.p. vehicle group (see above), which might reflect an immunomodulatory effect of the DMSO vehicle. In the EAE group treated with oral atorvastatin, we observed a reduction in IFN-γ production (Fig. 5d). In addition, we also observed a significant reduction in TNF-α production (Fig. 5e) but no alteration in the levels of either IL-2 or IL-10 (f and g). In both EAU and EAE groups administered oral atorvastatin, no IL-4 or IL-5 protein was detected by ELISA in splenocyte supernatants.

We next investigated the intracellular production of the Th1 cytokine IFN-γ and the Th2 cytokine IL-4 in lymphocytes harvested from vehicle- and oral atorvastatin-treated EAU and EAE animals. In the EAU group, there was negligible IL-4 expression, but the IFN-γ-to-IL-4 ratio increased upon IRBP peptide stimulation of the splenocytes (Fig. 5h). Interestingly, in EAE animals, the intracellular IFN-γ-to-IL-4 ratio was substantially lower than in EAU animals, and in unstimulated EAE lymphocytes, atorvastatin induced an increase in the expression of IL-4 causing a significant (p < 0.05) downward shift in the intracellular IFN-γ/IL-4 ratio; suggesting a small shift toward a Th2 profile. However, these results demonstrate that the therapeutic effects of statins can be achieved without significant systemic Th2 deviation.

Plasma concentrations of atorvastatin acid, a principal active component, in both EAE and EAU animals revealed some intriguing differences. No atorvastatin was detected in plasma obtained from the vehicle-treated groups (EAE, n = 13; EAU, n = 7). In the oral atorvastatin-treated EAE group, where treatment efficacy was observed, plasma concentration reached a mean value of 0.045 ± 0.03 μM (n = 11 animals from two separate experiments). Interestingly, in the oral atorvastatin EAU group where limited efficacy was observed, despite an identical treatment regimen, plasma atorvastatin acid remained below detectable levels (<0.1 pM; n = 16 animals from two separate experiments), which is indicative of differential pharmacokinetics in the two mouse strains.

Lovastatin inhibits lymphocyte migration through REC monolayers

Following our observation that parenteral administration of lovastatin dramatically reduces intraocular leukocytic infiltration in
EAU-induced animals in the absence of Th2 cytokine production, we investigated whether lovastatin could directly inhibit lymphocyte migration across the blood-retinal barrier in vitro. Treatment of both rat REC and lymphocytes with lovastatin during a 4-h coculture had no effect on transendothelial lymphocyte migration (Fig. 6a), whereas pretreatment of REC for 24 h resulted in a significant dose-dependent inhibition of lymphocyte migration (b). The degree of inhibition was similar to that achieved following treatment of REC with C3 transferase, a toxin that specifically ribosylates and inactivates Rho proteins (20). This inhibition in migration was not due to a reduction in either T cell viability or ICAM-1 expression (data not shown). Addition of mevalonolactone, but not squalene (which is distal to isoprenoid pyrophosphate metabolites), was able to reverse lovastatin-induced inhibition of migration (Fig. 6c), demonstrating that the effect of lovastatin was due to inhibition of HMG-CoA reductase. The migration of retinal Ag-specific T cells through RPE cell monolayers that in vivo constitute the posterior blood-retinal barrier, was also found to be inhibited following pretreatment with lovastatin in a manner identical with that observed with REC (Fig. 6d). Lovastatin hydroxyacid concentrations measured in the medium from these studies demonstrated that lovastatin was successfully converted to its active metabolite. When 0, 0.1, 1, 10, 50, and 100 μM lovastatin was...
added to the culture medium, this resulted in lovastatin hydroxy-acid concentrations after 24 h of 0.07, 0.72 ± 0.01, 6.43 ± 0.24, 28.13 ± 2.01, and 51.3 ± 10.19 μM, respectively (n = 3 per group).

**Discussion**

In this study, we have shown that the HMG-CoA reductase inhibitor lovastatin delivered parenterally can inhibit disease in an animal model of posterior uveitis through mechanisms that are independent of their cholesterol-lowering effects. The data support the hypothesis that statins can elicit beneficial effects in the absence of an overt shift toward a Th2 cytokine profile and that this may, in part, be due to their inhibitory capacity on transvascular lymphocyte traffic.

Parenteral lovastatin treatment of EAU results in a substantial decrease in both the number and severity of animals showing clinical signs of EAU and an extensive reduction in leukocyte infiltration into the retina. Analysis of plasma concentrations of lovastatin hydroxyacid revealed circulating levels of 0.098 ± 0.03 μM after 7 days of treatment, which, despite the high dose, corresponded remarkably closely to that reported in human studies. Daily administration of 40 mg ofLovastatin over 4 or 28 days (34, 35) has been reported to result in mean plasma lovastatin hydroxy-acid concentrations of 0.013 μM (10 patients) and 0.028 μM (12 patients), respectively. It is important to note that the current recommended upper dose for statins in humans is currently 80 mg/day.

Treatment of EAU mice with parenteral lovastatin on day 5 postimmunization for 7 days resulted in a significant reduction in disease without a concomitant shift toward a Th2 cytokine profile. Contrary to these findings, Nath et al. (8) have recently reported that lovastatin delivered parenterally to mice attenuated EAE disease and that this correlated with a shift in the balance of cytokine production toward an anti-inflammatory Th2 pattern. Furthermore, this group was able to show up-regulation of GATA3 and down-regulation of T-bet, transcription factors associated with Th2 and Th1 differentiation, respectively. These contradictory findings may result from differences in the onset of statin treatment (see below), the duration of treatment, or differences in disease model. However, a consistent finding across these models is a robust reduction in splenocyte IFN-γ production following statin treatment (4, 7, 8, 33). In addition to a reduction in IFN-γ, we also recorded a reduction in the production of IL-10 from Ag-stimulated splenocytes harvested from statin-treated EAU mice. Although this finding is at odds with previous reports of statin treatment of EAE, it is consistent with the cytokine profile reported following simvastatin treatment of human PBMC from MS patients (36) where neither a Th1 nor Th2 bias was detected. Indeed, the predominant effect of lovastatin treatment of EAU over 7 days is to suppress Ag-stimulated cytokine release rather than to promote a compensatory Th2 response, a finding also reported in simvastatin amelioration of inflammatory arthritis in mice (33). In support of our findings, it has also recently been reported that simvastatin reduces the number and volume of brain lesions in MS without any observed alteration in the Th1/Th2 cytokine balance (37). Furthermore, i.p. but not intragastric administration of simvastatin in a Th2-mediated animal model of allergic asthma, resulted in a reduction in both IL-4 and IL-5 (38), suggesting that statins can also modify a Th2 response. Clearly, although statins are capable of altering the T cell profile and hence cytokine production, the mechanisms by which statins can alter lymphocyte fate are complex and dependent on many factors.

Because we, and others, have used splenocytes for cytokine determination, it was also not possible to establish whether the reduction in IL-10 was due to immunomodulatory effects on the T cell or macrophage population. Nevertheless, IL-10 is considered a marker for Treg, and the observed decrease in both cytokines following treatment does not support a role for this T cell subpopulation in lovastatin-induced amelioration of EAU.

Because parenterallovastatin treatment of EAU results in a significant attenuation of disease in the absence of an increase in Th2 cytokines, we subsequently evaluated whether efficacy could be the consequence of a direct inhibition of lymphocyte migration across the blood-retinal barrier. Our observation that T cell migration through REC monolayers can be inhibited withlovastatin is consistent with a previous report (6), where it was shown that this agent inhibited the prenylation, and hence activity, of the small GTPase Rho in brain endothelial cells. Rho is an essential element in the endothelial cell ICAM-1 (CD54)-mediated signaling pathway that permits lymphocyte migration through the specialized blood-brain barrier (19–21) and represents a prime target for statin activity. This ICAM-1/Rho-mediated pathway is likely to be more important in leukocyte migration into the CNS than into non-CNS tissues where the vascular beds do not form such a tight barrier and are less restrictive to leukocyte migration. Because REC ICAM-1 is also essential for lymphocyte migration across the tight blood-retinal barrier (39, 40), and because the Rho toxin C3 transferase also blocks lymphocyte migration across REC, it is highly likely that a similar ICAM-1/Rho-dependent mechanism operates at both the blood-brain and blood-retinal barriers. The inhibitory effect of lovastatin on lymphocyte migration was clearly due to inhibition of HMG-CoA reductase, because we were able to reverse significantly the outcome with mevalonolactone. Conversely, the inability to rescueLovastatin-mediated inhibition of lymphocyte migration with squalene, suggests that the mechanism of action is not related to cholesterol synthesis per se but more likely to the depletion of intermediate metabolites such as the isoprenoids. Comparison between the plasma level of lovastatin hydroxyacid measured in vivo and that measured in the culture medium showed that the circulating levels corresponded to those in vitro experiments in which 1 μM lovastatin was added (equivalent to 0.071 μM lovastatin hydroxyacid), resulting in a reduction of transendothelial lymphocyte migration of ~40%. However, it is likely that the extended exposure of REC toLovastatin in vivo (compared with the 24 h conducted in vitro) will result in a more extensive depletion of prenylated Rho and hence a greater degree of inhibition of lymphocyte migration. Given the widely reported anti-inflammatory effects of statins both in vitro and in vivo, other mechanisms in addition to the inhibition of the ICAM-1/Rho-mediated pathway could be involved in ameliorating EAU. These could include the inhibition of NO production (2) and chemokine synthesis (13), and the blockade of NF-κB activation (17, 18).

An interesting finding of this study was the failure of either lovastatin or atorvastatin to elicit a robust therapeutic effect in EAU when administered by oral gavage. The failure of atorvastatin was clearly not due to the dosing regime used, which had previously been reported to effectively treat EAE (4), because we were able to demonstrate efficacy in EAE using the same delivery route, dose, and duration as in the EAU group. However, as revealed by the plasma analysis of atorvastatin acid concentration, there was a clear correlation between circulating atorvastatin and its efficacy in alleviating disease. Thus, in B6 mice induced for EAE, intragastric atorvastatin administration resulted in a mean atorvastatin acid plasma level of 0.045 ± 0.028 μM (n = 6) and therapeutic efficacy, whereas in B10.RIII mice induced for EAU, no efficacy was observed (n = 18) and plasma levels were below the limits of detection. This interesting result suggests that the strain of mouse used may impact on the pharmacokinetics of statins. Undoubtedly other factors may also affect efficacy, such as the more aggressive...
and rapidly destructive nature of the inflammatory response in the eye in B10.RIII mice (Refs. 29 and 41; our unpublished data). Plasma levels of lovastatin hydroxyacid in EAU mice following oral gavage were, as with the oral atorvastatin, also below the limits of detection. This suggests that for the effective treatment of EAU by oral administration in B10.RIII mice, the statin dosage needs to be increased. Previously, i.p. administration of simvastatin has been shown to be more effective at ameliorating an animal model of allergic asthma when compared with intragastric administration of the same dose (38).

The data obtained with atorvastatin raises a crucial issue that EAE can be alleviated without a concomitant increase in production of peripheral Th2 cytokines by splenic T cells, although we were able to detect a Th2 shift in intracellular cytokine expression by draining lymph node-derived T cells. Although this finding would appear to contradict previous reports where an unambiguous shift toward a Th2 cytokine profile has been reported to account for therapeutic activity (4, 7, 8), in all other cases, the duration of treatment in vivo before cytokine analysis was in excess of 10 days. Thus, it is a strong possibility that statin treatment exceeding the 7-day duration used in this EAE study is required to elicit a greater Th2 response. The time at which statin treatment is started might also affect T cell differentiation. Whereas lovastatin treatment in the present EAE study commenced on day 5 postimmunization, in a number of studies where an overt Th2 switch was observed, statin treatment started before immunization (MBP Ac–I–11-specific TCR transgenic mice) (4), or at the time of immunization (SJL/J mice) (4, 7, 8). This would result in autoantigen priming of T cells in the presence of statins, which may have a major impact on subsequent Th2 cytokine profiles.

This study highlights the pleiotropic nature of statins and suggests that the therapeutic relevance of different effector mechanisms induced by statins may change over the period of treatment. It is important to bear in mind that, in the context of treating human autoimmune disease, we are still unclear about the relative significance of these diverse and complex effects of statins on the immune system. Whatever multiple effectsLovastatin may exert on the immune system, the data provided by our in vitro assay provide compelling evidence that it will also impinge on a critical stage of lesion formation, namely, lymphocyte diapedesis at the blood-retinal barrier.

We conclude that the influence of particular downstream therapeutic effector mechanisms is likely to depend on the dose and pharmacokinetics of the statin, the duration and timing of treatment, and the particular disease to be treated. Such considerations aside, statins may prove to be of significant therapeutic potential in the treatment of early posterior uveitis.

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References

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