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Suppression of autoimmune retinal disease by lovastatin does not require Th2 cytokine induction

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Abstract

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 HMG-CoA reductase inhibitor, lovastatin, suppresses clinical ocular pathology, retinal vascular leakage and leukocytic infiltration into the retina. Efficacy was reversed by co-administration of mevalonolactone, the downstream product of HMG-CoA reductase, but not by squalene which is distal to isoprenoid pyrophosphate metabolites within the cholesterol biosynthetic pathway. Lovastatin treatment over 7 days, which resulted in plasma lovastatin hydroxyacid concentrations of 0.098 ± 0.03μM, did not result in splenocyte production of Th2 cytokines but did cause a small reduction in antigen-induced T cell proliferation and a decrease in the production of IFN-γ and IL-10. Thus, contrary to expected outcome we demonstrate that it is possible to dissociate the therapeutic effect of statins 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 failed to attenuate 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.

Keywords

Autoimmunity; Endothelial Cells; Inflammation; Th1/Th2 Cells

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INTRODUCTION

Intraocular inflammatory diseases are a major cause of severe visual impairment accounting for approximately 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 systemic lupus erythematosis.

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 2-9). 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 (10,11), production of chemokines (12), MMP production (13,14), nitric oxide production (2), MHC class II restricted antigen presentation (4,15) and leukocyte migration through the vascular wall (6,9). Foremost however, has been their ability to shift the T cell cytokine response from a pro-inflammatory Th1 to an anti-inflammatory Th2 profile (4,5,7). 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 (2-7,9).

Statins, which inhibit the enzymatic conversion of HMGCoA to L-mevalonate and hence cholesterol biosynthesis, results not only in the depletion of cholesterol but also the intermediate metabolites farnesylpyrophosphate and geranylgeranylpyrophosphate. 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 Rho is known to be essential for facilitating lymphocyte transvascular migration into the CNS (6, 16-18), while lovastatin, by inhibiting the supply of isoprenoids, has been shown to inhibit Rho function and subsequent lymphocyte migration across the blood-brain barrier (6).

Infiltration of $CD4^+$ T cells into the retina is a critical stage in the development of uveitic inflammatory disorders (19-21). It is currently unknown, however, whether statins prevent the development of retinal inflammatory disease and inhibit lymphocyte migration across the blood-retinal barrier. Using experimental autoimmune uveitis (EAU) in C57BL/10.RIII (B10.RIII) mice, an animal model of posterior uveitis in which there is a strong Th1 bias (20), we investigated the efficacy of statin treatment on disease progression. We demonstrate that 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 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 REC (JG2/1), and RPE (LD7.4) cell lines were conducted as previously described (17,22,23). Briefly, for adhesion assays peripheral lymph node cells were harvested from Lewis rats (Harlan Olac, Bicester, UK) and stimulated for at least 24h with 5μg/ml concanavalin A (Sigma Aldrich, Dorset U.K.). The T cells were then fluorescently labelled with 1 μM Calcein-AM (Molecular Probes, Oregon, U.S.A.). 1×10^5 labelled cells/well were then added to 96-well plates containing REC or RPE

monolayers, and incubated for 90 minutes at 37 °C. Each well was then washed with HBSS, and bound T cells measured by a flurorescent plate reader (Excitation, 494 nm; Emission, 517 nm). For migration assays uveitogenic retinal S-antigen (S-Ag) peptide$^{273-289}$ specific CD4^{+} T-cell lines were established from peptide-primed Lewis rat lymph nodes and maintained as previously reported (24,25). T cells (2×10^{4}/well) were added to 96-well plates containing REC or RPE monolayers, and incubated for 4h at 37 °C to allow T cells to settle and migrate. A minimum of twelve wells per assay was performed.

Induction and assessment of EAU

Male B10.RIII (7INS) mice (5-7 weeks old; Harlan Olac, Oxon, UK) were injected subcutaneously with 25 μg of human interphotoreceptor retinoid binding protein (IRBP)$^{161-180}$ peptide emulsified in incomplete Freund's adjuvant supplemented with 60 μg/ml *mycobacterium* (26). Eyes were scored daily for clinical signs of EAU (Table 1A). Fluorescein angiography (FA) was performed on EAU animals at day 10 post-immunisation. Mice were injected intraperitoneally with 2% sodium fluorescein and photographs of the retina were captured at 3 minutes and 5–8 minutes later (27). The fluorescein angiograms were scored blind as indicated in Table 1B. Eyes from all mice were enucleated on day 12, immersion-fixed in half strength Karnovsky's fixative, embedded in araldite and semi-thin (0.8 μm) sections cut on an ultramicrotome. Sections were stained with toluene blue and scored blind by light microscopy using the grading system shown in Table 1C.

Treatment of EAU

Vehicle alone or lovastatin (Calbiochem, Notts, UK) dissolved immediately before use in DMSO:PBS (1:1) was administered daily by interperitoneal injection at a dose of 20 mg/kg per mouse (6). Alternatively, following a loading dose of 100mg/kg lovastatin a further group of animals were given twice daily injections of 20 mg/kg lovastatin. Vehicle or lovastatin was first administered on day 5 post immunisation, and then daily until day 12 when maximal cellular infiltration and structural damage has been reported to occur (26). In two separate groups of animals, lovastatin treatment (20 mg/kg) was supplemented with a daily injection of squalene (2 mg/kg) or a twice-daily injection of mevalanolactone (2 mg/kg).

Induction and treatment of EAE

Female C57BL/6 mice (8-12 weeks old. Harlan Olac, UK) were immunised with 100 μg myelin oligodendrocye glycoprotein$^{35-55}$ (MOG$^{35-55}$) peptide emulsified in complete Freund's adjuvant supplemented with 4 mg/ml Mycobacterium tuberculosis H37Ra (4,28). On the day of immunisation and 48 h later mice were also injected with 100 ng *Bordetella pertussis* toxin (Sigma, UK). 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 (29). Mice were administered vehicle (PBS) or 10 mg/kg/day atorvastatin by oral gavage from day 10 post-induction onwards, within one day of clinical signs being observed (4).

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) post-induction, and grown in supplemented RMPI-1640 media (30). Cells were unstimulated or stimulated with 5 μg/ml 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, 1μCi [methyl ^{3}H]-Thymidine (Amersham International, Bucks, UK) was added to each well. The cells were harvested and [^{3}H]-thymidine uptake determined by β-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, Oxon, UK). For intracellular cytokine measurement, 2×10^6 cells/ml were cultured in the absence or presence of either the $IRBP^{161-180}$ or MOG^{35-55} peptides for 96 hours. 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 monoclonal antibodies (BD Biosciences) and analysed by flow cytometry.

Statin and cholesterol serum levels

Plasma and serum was prepared from EAU and EAE mice on day 12 and day 17 post induction respectively. For the measurement of atorvastatin acid and lovastatin hydroxyacid in plasma and tissue culture media a liquid-chromatographic tandem mass spectrometric (LC/MS/MS) bioanalytical assay was established over the concentration range 0.5 – 500 ng/ml for atorvastatin acid and 1 – 1000 ng/ml for lovastatin hydroxyacid. These assays were developed and carried out by HFL Contract Research (Fordham, Cambridgeshire, UK; http://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 × 2.0mm, 5μ) analytical column. The analyte was ionised using an electrospray interface operating in negative ion mode and detection was via tandem mass spectrometry (MS/MS) in the multiple reaction monitoring (MRM) mode. Simvastatin hydroxy acid was used as internal standard for both analytes.

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

Statistical Analyses

Data are presented as mean ± s.e.m. For angiography, clinical and histology scores, significance between groups was determined by the Mann-Whitney U test. All other statistics were analysed by the Students' t-test. $P < 0.05$ was considered significant.

RESULTS

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). We first observed clinical disease in vehicle-treated animals on day 9 post immunization which increased in severity up to day 12 post-induction (26) where 22/26 vehicle-treated mice exhibited inflammatory changes to the eye (Fig 1b and 1c). Lovastatin treatment (20mg/kg i.p. from day 5 onwards) resulted in a significant reduction in clinical disease (Fig 1c and 1d), an effect we were able to reverse by co-administration of mevalonolactone (twice daily 2mg/kg i.p.) but not squalene (2mg/kg i.p. Fig 1c and 1d). We employed fluorescence angiography (FA) to evaluate retinal vascular leakage in 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 at the optic disc was observed (Fig 2b and 2c) indicating significant breakdown of the anterior (vascular) blood-retinal barrier (Fig 2g). The majority (9/10) of lovastatin-treated mice exhibited no vascular leakage (Fig 2d and 2e) which was significantly less ($P < 0.001$) than in the vehicle treated group (Fig 2g). When we supplemented lovastatin with mevalonolactone we observed a significant reversal ($P < 0.01$) in the number of animals (5/6) exhibiting vascular leakage (Fig 2f) compared to lovastatin treated animals (Fig 2g). 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 to lovastatin treated animals. As predicted co-administration of squalene, which is downstream of isoprenoid pyrophosphate production, did not reverse the effect of lovastatin (Fig 2g). We next compared the histological appearance of the retinae of normal animals with EAU animals. Retinal examination of normal (Fig 3a) and vehicle-treated control EAU animals at the peak of clinical disease (day 12) revealed in the latter characteristic lesions in 25/26 animals studied that consisted of leucocytic infiltration, optic disc oedema, subretinal exudates and retinal folding (Fig 3b). 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 3c and 3e). As observed with clinical disease, supplementation with mevalonolactone reversed the severity of retinal pathology (Fig 3d and 3e), whilst supplementation with squalene did not (Fig 3e).

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 treated with lovastatin, plasma lovastatin hydroxyacid levels reached a mean value of 0.098 ± 0.03μM (n = 5) which was not significantly altered upon co-administration with squalene (0.101 ± 0.02μM; 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 anti-proliferative *in vitro* (4). We therefore 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 $IRBP^{161-180}$ peptide of splenocytes harvested on day 12 post-induction from lovastatin-treated mice was reduced compared to that observed in cells from untreated EAU mice (Fig 4a). This inhibition of proliferation was significant in cells stimulated with either 5μg/ml or 20μg/ml $IRBP^{161-180}$ peptide (Fig 4a). The T cell recall response to 20 μg/ml $IRBP^{161-180}$ 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 antibody (0.5 μg/million cells).

Lovastatin treatment for 7 days does not induce a Th2 cytokine profile in B10.RIII mice

As therapeutic effects of statins have been attributed to Th1 to Th2 deviations (4,5,7) 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 $IRBP^{161-180}$-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 of lovastatin on disease and the regulatory T cell (T_{reg}) population. No differences were observed in the phenotypically-assessed T_{reg} population between normal, vehicle treated and lovastatin treated mice (Fig 4f).

Atorvastatin treatment attenuates EAE in C57BL/6 mice but not EAU in B10.RIII mice

To investigate whether atorvastatin administration, which attenuated MOG^{35-55} induced EAE in C57BL/6 (B6) mice (4), we treated EAU-induced mice with 10 mg/kg/day intragastric atorvastatin or vehicle (PBS) from days 5 to 12 post immunization. In contrast to that observed with parenteral lovastatin, we observed no significant reduction in disease by clinical assessment (Fig 5a and 5c), FA (mean group score 0.67 ± 0.21; n = 6) or histology (mean group score 3.44 ± 0.32; n = 18). The T_{reg} population from the atorvastatin treated animals was 40.7 ± 9.8 and did not differ significantly from the lovastatin-treated group (Fig 4f). However, the same 7 day oral delivery regimen resulted in the attenuation of clinical EAE in C57BL/6 mice (Fig 5b) in a manner similar to that reported previously (4). Following treatment with 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 5b and 5c). In order to establish whether the cytokine profiles differed between the two disease models 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 $IRBP^{161-180}$ -stimulated production of both IFN-γ and TNF-α (Fig 5d and 5e) but observed 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 intraperitoneal vehicle group, which might reflect an immunomodulatory effect of the DMSO vehicle. In the EAE group of B6 animals, we observed less IFN-γ, which was significantly reduced in MOG^{35-55} peptide-stimulated splenocytes following atorvastatin treatment 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 (Fig 5f and 5g). In both EAU and EAE groups no IL-4 or IL-5 protein was detected by ELISA in splenocyte supernatant. We therefore investigated the intracellular production of the Th1 cytokine IFN-γ and the Th2 cytokine IL-4 in lymphocytes harvested from vehicle and 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 IFN-γ to IL-4 ratio was substantially lower than in EAU animals and in unstimulated EAE splenocytes atorvastatin induced an increase in the expression of IL-4 causing a significant ($P < 0.05$) downward shift in the IFN-γ/IL-4 ratio. These results suggest that the B10.RIII mouse is more Th1 biased than the C57BL/6 strain and that therapeutic effects of statins can be achieved without significant systemic Th2 deviation.

Plasma concentrations of atorvastatin acid, a principle 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 = 6; EAU n = 5). In the atorvastatin treated EAE group, where treatment efficacy was observed, plasma concentration reached a mean value of 0.045 ± 0.03μM (n = 6). Interestingly, in the EAU group where no atorvastatin efficacy was observed and despite an identical treatment regimen plasma atorvastatin acid remained below detectable levels (<0.1pM; n = 16).

Lovastatin inhibits lymphocyte migration through retinal EC monolayers

Following our observation that 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 endothelial cells (REC) and lymphocytes with lovastatin during a 4 h co-culture had no effect on transendothelial lymphocyte migration (Fig 6a) whilst pre-treatment of REC for 24 h resulted in a significant dose-dependent inhibition of lymphocyte migration (Fig 6b). 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 (17). 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 antigen-specific T cells through RPE cell monolayers that *in vivo* constitute the posterior blood-retinal barrier, was also found to be inhibited following pre-treatment with lovastatin in a manner identical to that observed with REC (Fig 6d). Lovastatin hydroxy acid concentrations measured in the media from these studies demonstrated that lovastatin was successfully converted to its active metabolite. When 0, 0.1, 1, 10, 50 and 100μM lovastatin was added to the culture media this resulted in lovastatin hydroxy acid concentrations after 24h of 0, 0.07, 0.72 ± 0.01, 6.43 ± 0.24, 28.13 ± 2.01 and 51.3 ± 10.19μM respectively (n = 3 per group).

DISCUSSION

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

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 leucocyte infiltration into the retina. Analysis of plasma concentrations of lovastatin hydroxyacid revealed circulating levels of 0.098 ± 0.03μM after 7 days of treatment which, despite the high dose, corresponded remarkably closely to that reported in human studies. Thus, administration of 5mg/kg lovastatin over 8 days (31) or 40mg/day over 28 days (32) has been reported to result in mean plasma lovastatin hydroxyacid concentrations of 0.59μM (3 patients) and 0.013 μM (12 patients) respectively. It is important to note that the current recommended upper dose for statins in humans is currently 80mg/day.

Contrary to recent studies in which successful statin treatment of EAE correlated with in a shift in the balance of cytokine production towards an anti-inflammatory Th2 pattern (4,5,7,33), we observed a predominantly pro-inflammatory cytokine profile albeit with a reduced production of IFN-γ. We also recorded a reduction in the production of IL-10 from antigen-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 peripheral blood mononuclear cells from MS patients (34) where neither a Th1 nor Th2 bias was detected. Indeed, the predominant effect of lovastatin treatment of EAU over 7 days is to suppress antigen-stimulated cytokine release rather than to promote a compensatory Th2 response, a finding also reported in atorvastatin amelioration of inflammatory arthritis in mice (30). 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 (35). Furthermore, intraperitoneal 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 (36), suggesting that statins can also modify a Th2 response. Clearly, the mechanisms by which statins can alter lymphocyte fate are complex and dependent on many factors.

Since 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

regulatory T cells (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.

As lovastatin treatment of EAU results in 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 with lovastatin 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 signalling pathway that permits lymphocyte migration through the specialised blood-brain barrier (16-18) 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. Since REC ICAM-1 is also essential for lymphocyte migration across the tight blood-retinal barrier (37,38), and as 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 HMGCoA reductase as we were able to reverse the outcome with mevalonolactone. Furthermore, as we were unable to rescue lovastatin mediated inhibition of lymphocyte migration with squalene, the mechanism of action is not related to cholesterol synthesis *per se* but more likely to the production of 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 1μM lovastatin was added (equivalent to 0.071μM lovastatin hydroxyacid), resulting in a reduction of transendothelial lymphocyte migration of approximately 40%. It is likely, however, that the extended exposure of retinal endothelial cells to lovastatin in vivo (compared to the 24h carried out in vitro) will result in a more extensive depletion of prenylated Rho and hence a greater degree of inhibition of lymphocyte migration.

An interesting finding of this study was the failure of atorvastatin to elicit a therapeutic effect in EAU. This failure was clearly not due to the dosing regime employed which had previously been reported to effectively treat EAE (4), and we were also 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 ± 0.028μM (n = 6) and therapeutic efficacy whereas in B10.RIII mice induced for EAU no efficacy was observed (n =− 16) and plasma levels were below the limits of detection. This interesting result suggests that even the strain of mouse used may impact on the pharmacokinetics of statins. Undoubtedly other factors may also affect efficacy such the more aggressive and rapidly destructive nature of the inflammatory response in the eye in B10.RIIII mice (refs. 26,39; unpublished data). Furthermore, different statins have been found to exhibit different efficacies *in vitro*, and it is therefore not surprising that different statins also possess differential potencies *in* vivo (15). This is most likely due to the relative pharmacokinetic properties of the agents as well as their potencies but also, as seen in this study, it may also be attributed to the delivery protocol and dosage. Nonetheless, the data obtained with atorvastatin raises a crucial issue that EAE can be alleviated without a concomitant increase in peripheral Th2 cytokines. Although this finding would appear to contradict previous reports where an unambiguous shift towards a Th2 cytokine profile has been reported to account for therapeutic activity

(4,5), in all other cases the duration of treatment *in vivo* prior to 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 study is required to elicit a Th2 response. Indeed, when we looked at intracellular cytokine production there was evidence that in EAE induced in B6 mice, which are arguably less Th1 biased than B10.RIII mice (see IFN-γ response to antigen and IFN-γ/IL-4 ratios), it appears that there is the beginning of a shift towards Th2 cytokine production. Nevertheless, what this demonstrates is that prior to any such change, reduction in disease severity can occur in the absence of overt anti-inflammatory cytokine production.

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 effects lovastatin may exert on the immune system, the data provided by our *in vitro* assay provides 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 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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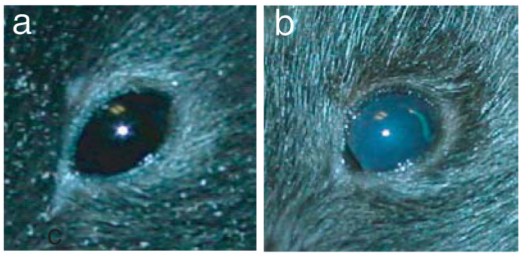
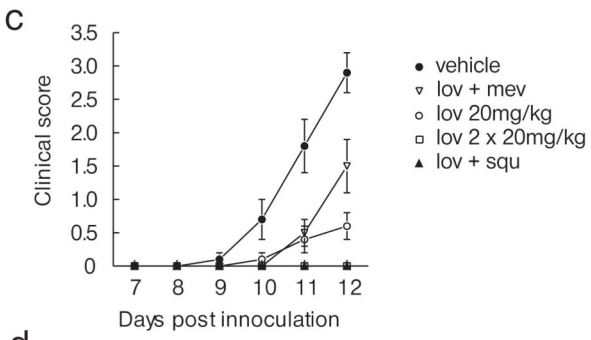


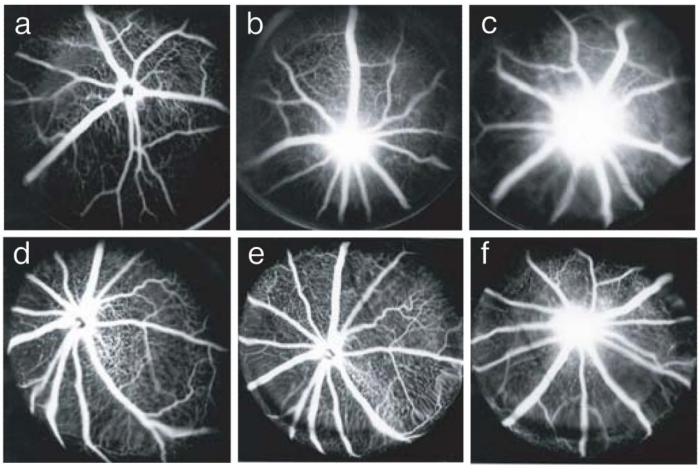


d

Treatment	No. EAU/total	Mean Group clinical score
Vehicle treated (i.p.)	23/26	2.88 ± 0.31
Lovastatin 20 mg/kg/day	8/27***	0.59 ± 0.20***
Lovastatin 2 x 20 mg/kg/day	0/5***	0***
Lovastatin + mevalonolactone	11/13††	1.54 ± 0.35* ††
Lovastatin + squalene	0/6***	0 ***

Figure 1. Lovastatin treatment of EAU attenuates retinal vascular leakage

Fluorescein retinal angiograms at day 10 post immunization of **(a)** normal retina (grade 0) 3 min post fluorescein injection (PFI), **(b)** untreated EAU retina (grade 3) 3 min PFI, **(c)** untreated EAU retina (grade 3) 6 min PFI, **(d)** lovastatin treated (20mg/kg/day) EAU retina (grade 3) 3min PFI, **(e)** same retina as (d) 7 min PFI, **(f)** lovastatin (20mg/kg/day) and mevalonolactone (2 × 2mg/kg/day) treated EAU retina (grade 3) 3 min PFI. **(g)** No vascular leakage was observed in normal animals, while significant vascular leakage was observed in angiograms of EAU animals particularly around the optic disc. Lovastatin treatment of EAU significantly reduced vascular leakage compared with diseased animals and co-administration of mevalonolactone reversed this effect. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ cf. vehicle control and †† $P < 0.01$ cf. lovastatin treated.



g

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

Figure 2. Lovastatin treatment of EAU attenuates ocular clinical pathology

(a) Clinical appearance of normal mouse eye and **(b)** following induction of EAU at day 12 post immunization (vehicle treated) showing exudate within anterior chamber and vitreous (grade 3). **(c)** Time course of clinical disease up to day 12 post immunization (peak disease) following different treatments and **(d)** showing significant reduction in clinical signs at day 12 following treatment with lovastatin. Co-administration of mevalonolactone, but not squalene, caused partial reversal of clinical disease. *** $P < 0.001$ cf. vehicle control and †† $P < 0.01$ cf. lovastatin treated.

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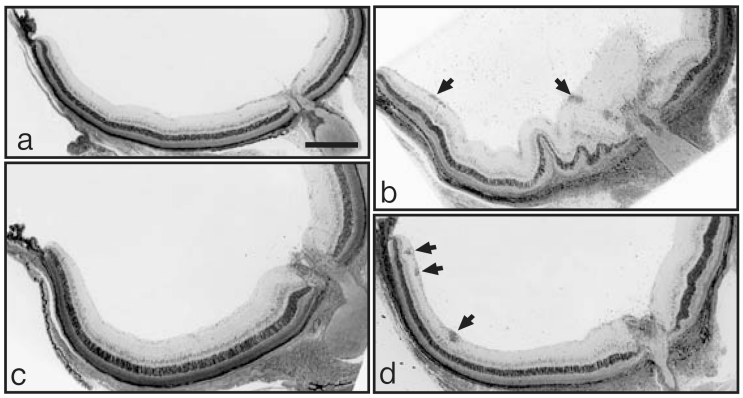

e

Treatment	No. EAU/total	Mean Group clinical score
Vehicle treated (i.p.)	25/26	3.42 ± 0.31
Lovastatin 20 mg/kg/day	15/27**	1.44 ± 0.31***
Lovastatin 2 x 20 mg/kg/day	2/5*	0.40 ± 0.24***
Lovastatin + mevalonolactone	13/13 ††	2.54 ± 0.39* †
Lovastatin + squalene	1/6**	0.33 ± 0.33 ***

Figure 3. Lovastatin treatment of EAU attenuates retinal pathology

(a) Histological appearance of normal mouse retina (grade 0) and **(b)** following induction of EAU at day 12 post immunization showing moderate retinal folding and detachment, leucocytic infiltration of the retina and vitreous and perivascular leucocytic cuffing (arrows) (grade 3). **(c)** Lovastatin treated (20mg/kg/day) EAU retina (grade 1) showing normal retina with mild inflammation around the optic nerve head. **(d)** Lovastatin (20mg/kg/day) and mevalonolactone (2 × 2mg/kg/day) treated EAU retina (grade 2) demonstrating reversal of therapeutic effect of lovastatin. Retina shows signs of perivascular leucocytic 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. Co-administration of mevalonolactone, but not squalene, caused partial reversal of retinal pathology. Scale bar = 500μm. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ cf. vehicle control and † $P < 0.05$, †† $P < 0.01$ cf. lovastatin treated.

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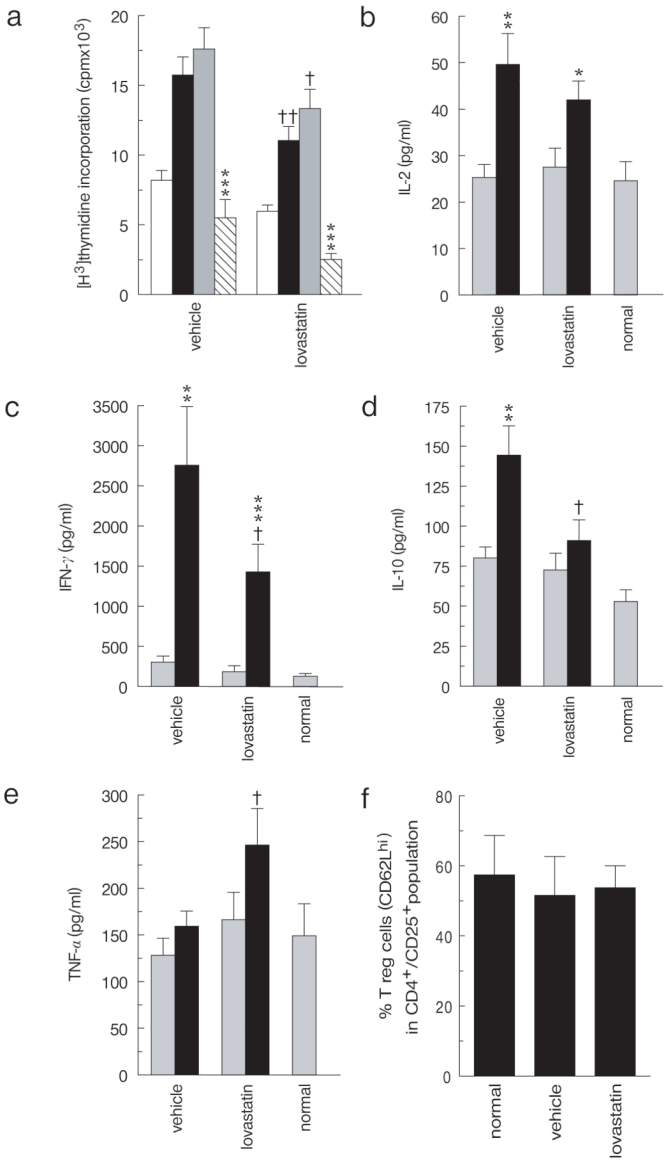

Figure 4. Treatment of EAU with lovastatin over 7 days does not result in an increase in Th2 cytokines

(a) Proliferation of unstimulated (open bars) and antigen stimulated (5μg/ml IRBP peptide, black bars; 20μg/ml IRBP peptide, grey bars) splenocytes from vehicle or lovastatin treated EAU animals. Proliferation in the presence of 20μg/ml IRBP peptide was MHC class II restricted as co-treatment with class II blocking antibody inhibited proliferation (hatched bar). Splenocytes isolated from lovastatin treated mice showed a small but significant reduction in proliferation compared with vehicle. *** $P < 0.001$ cf. 20μg/ml IRBP stimulated within group. † $P < 0.05$ and †† $P < 0.01$ cf. corresponding vehicle treated group. **(b)** IL-2 **(c)** IFN-γ **(d)** IL-10 and **(e)** TNF-α production from unstimulated (shaded bars) or antigen stimulated (black bars) isolated from normal animals and from vehicle or lovastatin treated EAU animals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ cf. unstimulated splenocytes

within group. † $P < 0.05$ cf. corresponding vehicle treated group. **(f)** Percentage of regulatory T cells (Treg) in $CD4^+/CD25^+$ population.

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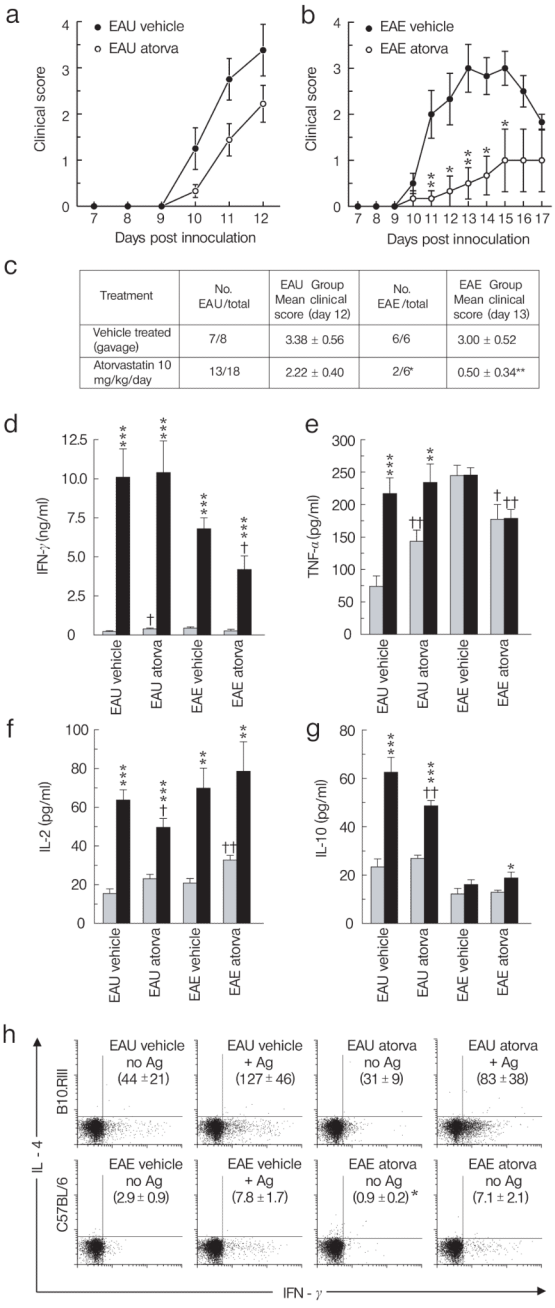

Treatment	No. EAU/total	EAU Group Mean clinical score (day 12)	No. EAE/total	EAE Group Mean clinical score (day 13)
Vehicle treated (gavage)	7/8	3.38 ± 0.56	6/6	3.00 ± 0.52
Atorvastatin 10 mg/kg/day	13/18	2.22 ± 0.40	2/6*	0.50 ± 0.34**

Figure 5. Atorvastatin treatment for 7 days attenuates EAE but not EAU

Time course of clinical disease in **(a)** B10.RIII EAU mice and **(b)** C57Bl/6 EAE mice. EAU mice were treated from day 5 to 12 and EAE mice from day 10 to 17. **(c)** Atorvastatin treatment induces a significant attenuation of clinical disease in EAE but not EAU. * $P <$ 0.05, ** $P <$0.01 cf. vehicle control. **(d)** IFN-γ **(e)** TNF-α **(f)** IL-2 and **(g)** IL-10 production from unstimulated (shaded bars) or antigen stimulated (black bars) splenocytes isolated from vehicle or atorvastatin treated EAU and EAE animals. **(h)** Representative flow cytometric dot plots for intracellular IFN-γ and IL-4 production. Figures in brackets show the mean

IFN-γ/IL-4 ratio for that group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ cf. unstimulated splenocyte group and † $P < 0.05$, †† $P < 0.01$ cf. corresponding vehicle treated group.

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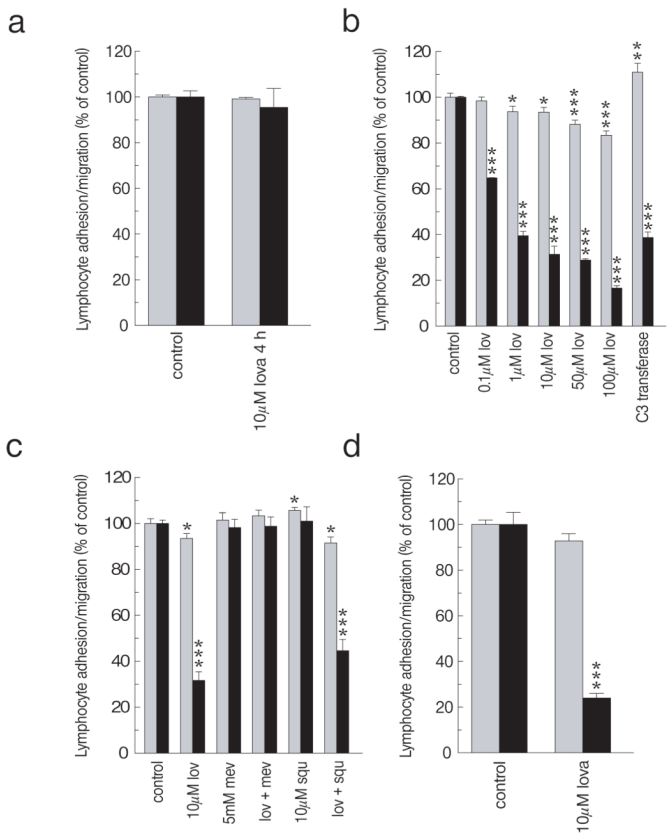

Figure 6. Lovastatin inhibits lymphocyte migration through retinal endothelial cell monolayers

(a) The presence of lovastatin (10μM) during the 4 h EC/lymphocyte co-culture does not affect lymphocyte adhesion (shaded bars) or transendothelial migration (solid bars). **(b)** Pre-treatment of retinal EC for 24 h with lovastatin (0.1-100μM) or 16h with C3 transferase (10μg/ml) significantly inhibits transendothelial lymphocyte migration. **(c)** Mevalonolactone, but not squalene, reverses lovastatin-induced inhibition of lymphocyte migration. **(d)** Pre-treatment of retinal pigment epithelium for 24 h with lovastatin (10μM) significantly inhibits transepithelial lymphocyte migration. All groups consist of a minimum of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ cf control values.

Table 1

Grading system for mouse EAU.

A.	
Grade	**Clinical signs**
0	Normal. Clear medium with good red reflex.
1	Minimal but clearly positive signs of inflammation with occasional cells in Anterior chamber. Dilation of iris vessels.
2	Presence of anterior chamber (AC) cells with minimal hypopyon.
3	Diffuse exudates within AC and vitreous plus moderate hypopyon. Dull red reflex. Synechiae.
4	Presence of large cellular and fibrinous exudates (AC very cloudy). Large hypopyon in AC. Loss of red reflex.
5	Presence of grade 4 with gross orbital oedema and proptosis of globe.

B.	
Grade	**Fluorescein angiographic signs**
0	Normal. No leakage.
1	Mild leakage: hazy but reasonable view of optic disc, optic cup and retinal vessels entering the optic disc.
2	Moderate leakage: unable to identify optic disc and retinal vessels entering the optic disc clearly.
3	Severe leakage: no view of optic disc, optic cup or retinal vessels entering the optic disc.

C.	
Grade	**Histological signs**
0	Normal
1	Minimal signs of inflammation. Occasional inflammatory cells in the vitreous base or scattered cells in the neuroretina or adjacent to the ciliary body. V. minor focal retinal detachment generally around optic nerve and occasional focal retinal folding.
2	Uniform inflammatory cell infiltration of the photoreceptor cell layer (PR), outer nuclear layer (ONL) and inner nuclear layers (INL) of the retina and infiltration of the vitreous cortex. Some perivascular cuffing. Retinal damage < 20%
3	Infiltration of the PR layer, ONL and INL of the retina with some retinal detachment (>20<50%). Moderate retinal folding, diffuse cellular infiltration of the vitreous, and moderate uveal involvement.
4	Full thickness involvement of the retina, serious retinal detachment and folding. Subretinal exudate. Necrosis and gliosis, substantial uveal involvement
5	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.