

SCIENTIFIC REPORT

The effects of atorvastatin in experimental autoimmune uveitis

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Aim: To investigate the effect of atorvastatin (Lipitor), a commonly used drug for dyslipidaemia in experimental autoimmune uveitis (EAU).

Methods: 48 B10-RIII mice were immunised with human interphotoreceptor retinoid binding protein (IRBP) peptide p161–180. They were divided into three groups of 16 each and treated orally once daily for 14 days; group one received phosphate buffered saline (control group), group two received 1 mg/kg of atorvastatin (low dose group), and group three received 10 mg/kg (high dose). On day 14 lymph nodes, spleens, and right eyes were harvested. RNA was extracted from lymph nodes for RNase protection assay (RPA) to determine proinflammatory (IL-1 α and IL-1 β), Th1 (TNF- α , IL-2, IL-12), and Th2 (IL-4, IL-5, and IL-10) cytokine levels. Protein was extracted from spleens for western blot to detect the expression of phosphorylated signal transducer and activator of transcription (STAT) 4 and STAT6. The severity of inflammation in enucleated eyes was graded by a masked observer. Paired *t* test was performed for the mean difference in histological scoring between treated groups and the immunised control group.

Results: Surprisingly, atorvastatin did not modulate the immune response. The proinflammatory cytokines, IL-1 α and IL-1 β , and Th1 cytokines, TNF- α and IL-2, were upregulated equally in control and atorvastatin treated groups. IL-12 and Th2 cytokines were not upregulated in all three groups. Western blot analysis showed high levels of phosphorylated STAT4, but not STAT6 protein in the control and atorvastatin treated groups. Mean differences in histological scoring between treated groups and the immunised control group were not statistically significant.

Conclusions: Atorvastatin treatment had no effect on Th1 and Th2 cytokine transcription. Although histological grading suggested mildly decreased inflammation in the high dose treated group, the equivalence of cytokine expression in all groups suggests that the statins may not modulate IRBP induced uveoretinitis.

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) inhibitors or statins are widely used as hypolipidaemic drugs. Atorvastatin is the most widely used statin and is comparably well tolerated in patients. The major outcome in the use of these drugs is the decreased rate of coronary events.^{1,2} Recent studies have revealed the immunomodulatory effect of statins in mouse models of experimental autoimmune encephalomyelitis (EAE), a Th1 mediated autoimmune disorder.^{3–5} In this model oral administration of atorvastatin at a dose of 1 mg/kg and 10 mg/kg profoundly modulated the Th1 to a Th2 response, which was associated with inhibition and reversal of chronic and relapsing encephalomyelitis.⁴ In addition to upregulating Th2 cytokines

(IL-4, IL-5, and IL-10), atorvastatin induced signal transducer and activator of transcription (STAT) 6 tyrosine phosphorylation, which is associated with IL-4 upregulation. Conversely, STAT4 phosphorylation, associated with IL-12 induction, was inhibited and transcription of Th1 cytokines (IL-2, IL-12, interferon (IFN)- γ and TNF- α) was suppressed.

Although experimental autoimmune uveoretinitis (EAU) is also considered to be predominantly Th1 mediated,^{6,7} it is known that Th2 mediated autoimmunity in EAU can also lead to significant retinal pathology in EAU.⁷ For this reason, in spite of many similarities between EAE and EAU, it was unclear what effect oral administration of atorvastatin would have in modulation of EAU. In the present study, we investigated the effects of atorvastatin on the Th1 and Th2 cytokine profiles and severity of uveitis in B10-RIII mice with EAU.

MATERIALS AND METHODS

EAU induction and histology

Fifty eight 6–8 week old B10-RIII mice (Jackson Laboratories, Bar Harbor, ME, USA) were used. Forty eight animals were immunised with 25 μ g of IRBP peptide 161–180 (SGIPYIISYLHPGNTILHVD) in phosphate buffered saline (PBS) emulsified 1:1 vol/vol in complete Freund's adjuvant that had been supplemented with *Mycobacterium tuberculosis* to 2.5 mg/ml, as previously described.^{8,9} The experiment was performed identically twice: two groups of 24 animals were immunised on different days. The remaining 10 animals remained non-immunised and provided baseline levels of cytokine expression as described below. Animals were maintained and used in compliance with the institutional guidelines.

The 48 immunised animals were divided into three groups of 16 each. The mice were fed orally using 20 mm feeding needles (Popper and Sons Inc) with 0.5 ml of atorvastatin (Lipitor, Pfizer Inc, prescription formulation) suspension in phosphate buffered saline (PBS) or PBS alone once daily for 14 days starting on the day of immunisation, including the day they were killed. One group received atorvastatin at 1 mg/kg body weight (low dose group), the second group received atorvastatin at 10 mg/kg body weight (high dose group); and the third group received PBS alone (control group). The mice were killed on day 14. The right eyes were enucleated, fixed in 4% formaldehyde solution, and paraffin embedded sections were stained with haematoxylin and eosin. The sections were evaluated by a masked observer and the intraocular inflammation was graded as follows: absence of inflammation was graded as 0; presence of inflammatory cells only in the pars plana, vitreous, or focally in the

Abbreviations: EAE, experimental autoimmune encephalomyelitis; EAU, experimental autoimmune uveitis; IFN, interferon; IRBP, interphotoreceptor retinoid binding protein; RPA, RNase protection assay; STAT, signal transducer and activator of transcription

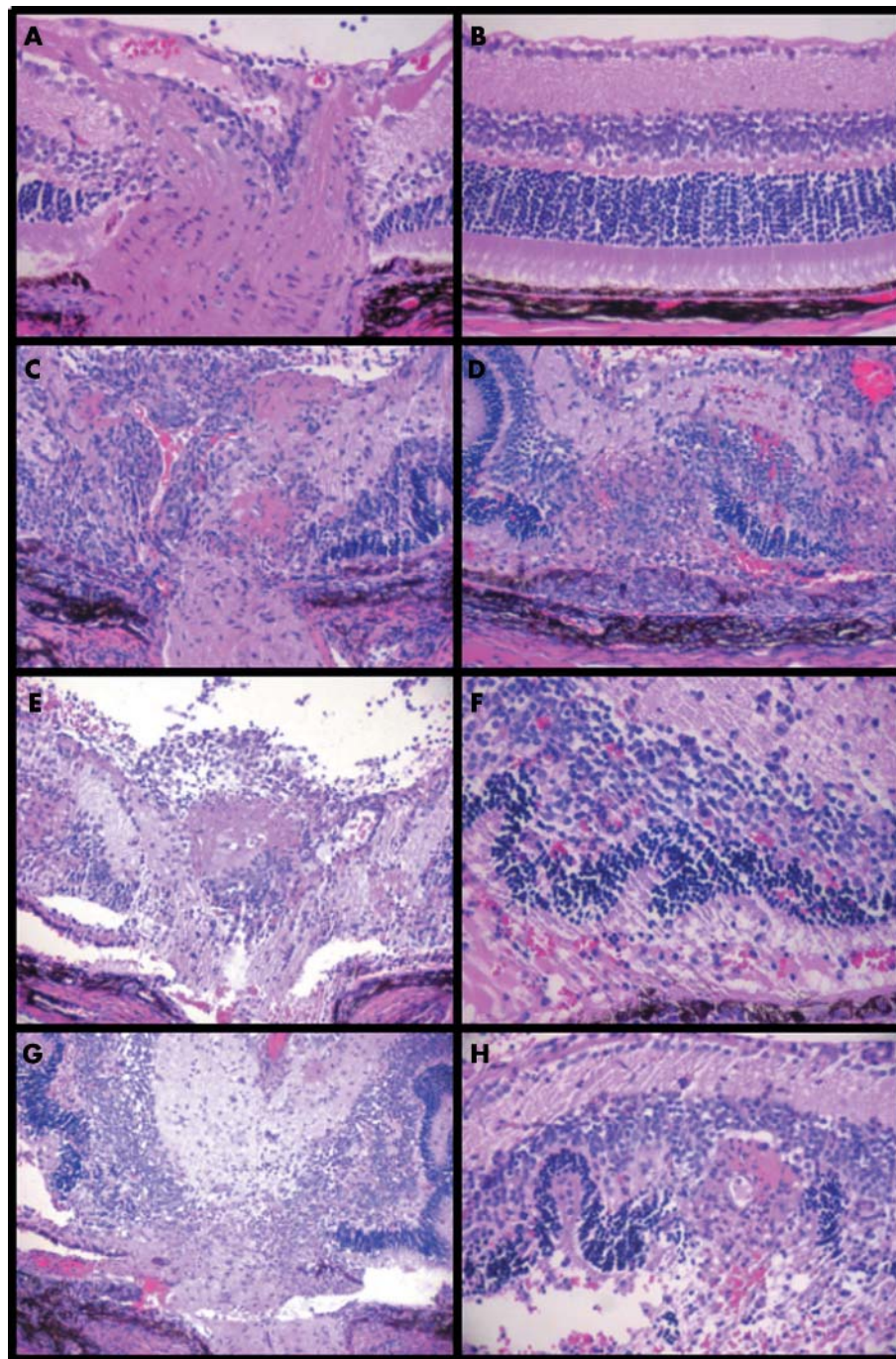


Figure 1 Representative histology. Haematoxylin and eosin stained sections from enucleated eyes 14 days after immunisation (100 \times). (A) Optic nerve and (B) retina from animal in 10 mg/kg group. There is a mild vitritis, graded as 1+. (C) Optic nerve and (D) retina from animal in 10 mg/kg group. (E) Optic nerve and (F) retina from animal in 1 mg/kg group. (G) Optic nerve and (H) retina from animal in control group. (C-H) There is a heavy vitritis, choroiditis, and full thickness retinitis, graded as 4+.

juxtapapillary choroid was defined as grade 1+; presence of findings noted in grade 1+ infiltration of inflammatory cells diffusely in the choroid was graded as 2+; the findings noted in grade 2+ and the presence of inflammatory cells in the inner retina were graded as 3+; and findings noted in 3+ associated with inflammatory cells noted in the outer retina and sclera were graded as 4+. Paired *t* test was performed using the standard deviations and differences in mean histological grading comparing the low dose group or high dose group with the immunised control group.

RNA isolation and RNase protection assay

Lymph nodes were harvested from control and atorvastatin treated animals (nine animals each from the control and two atorvastatin treatment groups). In addition, lymph nodes harvested from 10 non-immunised B10-R111 mice provided normal baseline expression of RNA. RNA was isolated from lymph nodes using Triazol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Quality of the RNA was determined by denaturing gel electrophoresis and RNA quantitated by spectrophotometer at A_{260} . RNase

protection assays (RPA) were performed according to the protocol described elsewhere.¹⁰ Briefly, RPA used total RNA extracted from lymph nodes from immunised and non-immunised mice using custom-made multiprobe templates for proinflammatory cytokines (IL-1 α and β), Th1 cytokines (TNF- α , IL-2, p40 subunit of IL12) and Th2 cytokines (IL4, IL5, IL10) and the housekeeping genes L-32 and GAPDH (BD PharMingen, San Diego, CA, USA). Templates were labelled with [α -³²P] UTP using T7 RNA polymerase according to manufacturer's protocol. Ten μ g of total RNA were hybridised with ³²P labelled probe (8×10^5 cpm) for 12–16 hours at 56°C. The contents were treated with RNase mixture (BD PharMingen), followed by extraction with phenol-chloroform. Protected mRNA hybrids were resolved on a 6% denaturing polyacrylamide sequencing gel and exposed to x ray film for 24 hours.

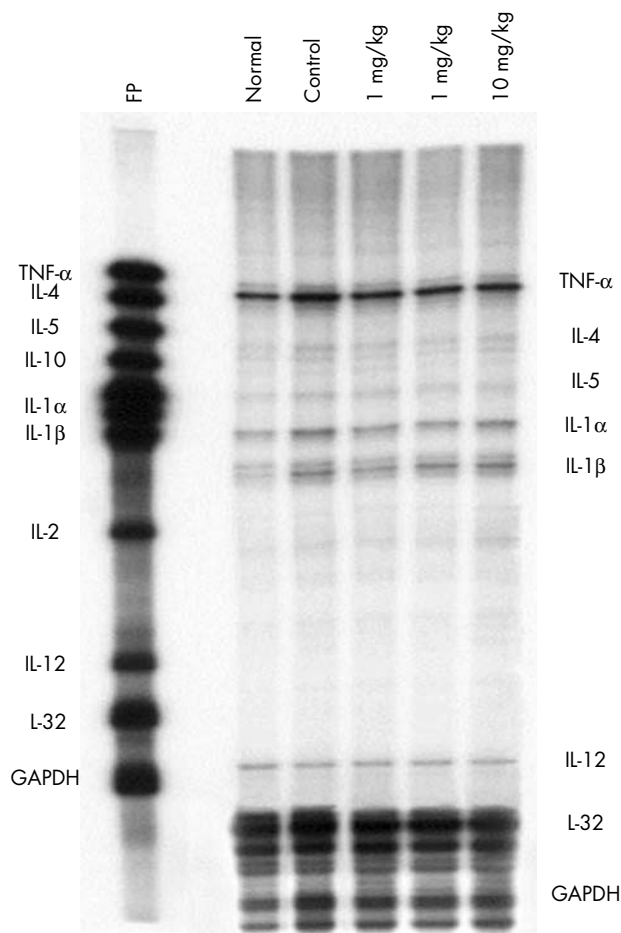


Figure 2 Cytokine mRNA quantification by RNase protection assay. The first column (FP, free probe) shows the size of the undigested ³²P labelled RNA probe, which is slightly larger than the protected probe because of non-homologous sequences. Second column (Normal) shows the protected band of each labelled RNA sequence for RNA isolated from non-immunised animals. The third column (Control) shows the protected bands for RNA derived from immunised control animals treated with PBS. The third and fourth columns (1 mg/kg) show the protected bands from RNA derived from 1 mg/kg atorvastatin treated animals. The fifth column (10 mg/kg) shows the protected bands for RNA derived from 10 mg/kg atorvastatin treated animals. TNF- α , IL-1 α , IL-1 β , and IL-2 are upregulated in the control and treated groups compared to the non-immunised group. There is no difference in expression between control and treated groups. IL-4, IL-5, IL-10, and IL-12 are expressed equally in all groups.

Western blot analysis

Spleens isolated from control and atorvastatin treated mice were homogenised in lysis buffer (50 mM TRIS-HCl, pH 7.4; 1% Nonidet P-40; 0.25% Na-deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 μ g/ml of each: aprotinin, leupeptin, pepstatin; 1 mM Na₃VO₄; 1 mM NaF). Following determination of protein concentration (DCC assay from BioRad, Hercules, CA, USA) identical amounts of protein extract (100 μ g) were separated on 7% polyacrylamide SDS gels and electroblotted onto polyvinylidenedifluoride (PVDF) membrane (PALL Life Sciences, Ann Arbor, MI, USA). The transfer was monitored by prestained See Blue molecular weight marker (Invitrogen, Carlsbad, CA, USA).

Following the transfer, blots were probed with polyclonal rabbit anti-phosphorylated STAT4 antibody (Zymed laboratories Inc, San Francisco, CA, USA) or anti-phosphorylated STAT6 antibody (Cell signalling technology, Beverly, MA, USA). Immunodetection was done by chromogenic visualisation with ABC kit (Vector laboratories Inc, Burlingame, CA, USA) and with DAB/NiCl₂.

RESULTS

Histology

These data are pooled from two separate identical experiments as described in the methods section above. Mean histological grading of the inflammation revealed a mild decrease in the group treated with the higher dose of atorvastatin compared to the control group. In the group treated with 10 mg/kg of atorvastatin, 12/16 eyes showed evidence of uveitis. All 16 eyes in the 1 mg/kg group as well as 15/16 eyes in the control group developed uveitis. Ten of 16 controls and 13/16 1 mg/kg treated group developed 4+ disease in both of these groups. Among the 10 mg/kg treated group 9/16 developed 4+ disease with a median score of 3+. Representative histopathology is shown in figure 1.

The mean histological grading for immunised control animals was 3.2 with standard deviation (SD) of 1.2; for low dose treated animals 3.8, with an SD of 0.4; for high dose treated animals 2.3 with an SD of 1.7. The difference from immunised control in mean histological grading was 0.6 for the low dose group and the distribution of the difference was non-normal; signed rank test yielded a p value of 0.13. The difference from immunised control in mean histological

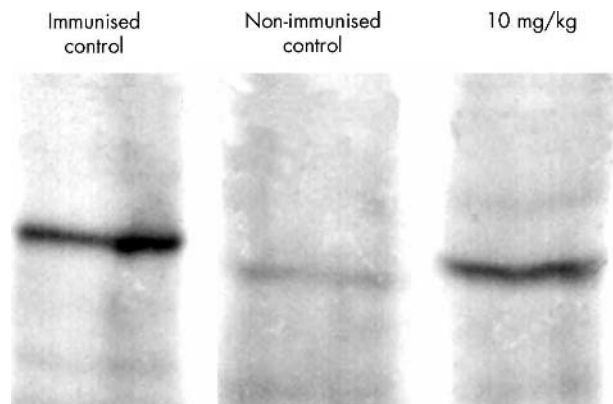


Figure 3 Western blot for phosphorylated STAT4. Controls and treated animals show upregulation of phosphorylated STAT4 compared to non-immunised animals. Spleens isolated from control and treated mice were homogenised in lysis buffer western blot was probed with polyclonal rabbit anti-phosphorylated STAT4 antibody (Zymed Laboratories Inc, San Francisco, CA, USA). Immunodetection was done by chromogenic visualisation with ABC kit (Vector laboratories Inc, Burlingame, CA, USA) and with DAB/NiCl₂.

grading was -0.9 for the high dose group and the distribution of the difference was not detected to be non-normal; paired t test yielded a p value of 0.14 . Tests for normality were performed to assess the deviation from normality. In addition, histograms were evaluated to look for skewness. The distribution of the 1 mg/kg change from control only slightly deviated from normality and the distribution did not look skewed. Even though small deviations from normality do not severely affect the validity of analysis of variance tests, a signed rank test p value was used for the 1 mg/kg change. The distribution of the 10 mg/kg change from control was not detected to be non-normal, so the paired t test was used. A comparison of the parametric and non-parametric p values do not give conflicting conclusions.

RNase protection assay

Proinflammatory cytokines (IL1 α and IL1 β) and Th1 cytokines (TNF α , and IL2) were upregulated in treatment and control groups compared to baseline expression in non-immunised animals. The Th1 cytokine, IL-12 (p40 subunit), and Th2 cytokines (IL4, IL5 and IL10) were not upregulated in treatment or control groups (fig 2).

Western blot analysis

Western blot analysis showed that phosphorylated STAT6 was not upregulated in the control or the treatment groups compared to non-immunised animals. The immunoreactive band of ~ 84 kDa indicates that phosphorylated STAT4 protein is upregulated equally in the control and the 10 /kg of atorvastatin treated group (fig 3).

DISCUSSION

Statins reduce cholesterol levels through competitive inhibition of HMG-CoA reductase, the key enzyme that regulates cholesterol synthesis. However, mevalonate, the immediate product of HMG-CoA reductase, is the precursor of isoprenoids known to regulate diverse cellular functions via post-translational modification (isoprenylation) of cell signalling molecules, and allowing statins to exert pleiotropic effects.^{11–12} Multiple immunomodulatory effects of statins have been elucidated. Statins decrease natural killer cell cytotoxicity *in vitro*,¹³ inhibit ICAM binding to LFA-1,¹⁴ inhibit iNOS as well as LPS induced expression of TNF- α , IL-1 β , and IL-6 in rat astrocytes, microglia, and macrophages,¹⁵ and inhibit IFN γ inducible MHC class II upregulation in antigen presenting cells.¹⁶ Clinically, statins have been shown to decrease rates of cardiac allograft rejection independently of their cholesterol lowering effects.¹⁷ Preliminary evidence suggests their usefulness in reducing number and volume of CNS lesions in patients with relapsing remitting multiple sclerosis.¹⁸

The present study was done to explore the immunomodulatory effect of atorvastatin in an EAU model. We assayed for cytokine expression in cells of the lymphatic system of immunised animals because differential cytokine expression was seen in these cells in EAE models after atorvastatin treatment. We have not excluded the possibility that ocular cytokine levels would have been altered by atorvastatin. However, we feel measurement of cytokine levels is more reliable from tissues with large numbers of lymphocytes than from recovered mouse retina. In this study, there was no evidence of Th1 or proinflammatory (IL1 α and β) cytokine suppression in treated animals, nor was there any evidence of Th2 cytokine induction in treated animals (fig 2). It is well documented that statins have multifaceted effects in EAE.^{3–5} Although atorvastatin has been found to suppress differentiation of Th0 CD4+ cells to Th1 cells *in vivo* and *in vitro* in two murine models of EAE,^{3, 4} we found no evidence of this

phenomenon in our model. Statins are thought to exert their immunomodulatory effects via downstream products of HMG-CoA reductase, primarily through the mevalonate pathway. It is unlikely that this pathway is any less important in immune competent cells in the eye than it is in the same cells in the CNS. Statins are also known to directly inhibit T cell migration and activation by binding LFA-1 and inhibiting its interaction with ICAM-1. It also seems unlikely that this mechanism would be any less important in the eye compared to the central nervous system (CNS). The striking difference in the atorvastatin effect on Th0 differentiation in murine EAE versus EAU may be explained in at least four ways. Firstly, there may be lower penetration of the orally administered drug in ocular tissues compared to the CNS. Secondly, there may be a strain dependent relative affinity to Th1 differentiation that cannot be overcome with atorvastatin treatment in B10-RIII mice, compared to SJL/J, B10.PL, and C57BL/6 mice used in murine EAE models. Thirdly, antigen presentation at the site of immunisation with peptide or in the uvea may provide a stronger drive for Th1 differentiation in EAU compared to EAE. Finally, since the immunomodulatory effects of statins are varied and sometimes proinflammatory—for example, treatment of human monocytes with atorvastatin enhances the generation of reactive oxygen molecules,¹⁹ there may be a difference in the relative effect of the different mechanisms by which statins can modulate inflammation. Reactive oxygen species have been shown to be important in the development of EAU.²⁰

In addition to altering Th0 differentiation, the statins alter the immune response by inhibiting IFN- γ induced expression of MHC class II on endothelial and smooth muscle cells, while not altering MHC class II expression on professional antigen presentation cells.¹⁶ IFN- γ induced MHC class II expression on endothelial cells in addition to constitutively expressed MHC class II on professional antigen presenting cells may be relatively more important in the genesis of EAE than EAU. The relative roles of multiple pathways by which statins modulate the immune response still need to be further worked out *in vitro* and *in vivo*.²¹ This study suggests that neither suppression of Th1 nor enhancement of Th2 responses is found in atorvastatin treatment in this murine model of EAU.

We did not find any evidence of IL-12 upregulation in either control or treated animals compared to non-immunised animals. This was surprising since all the other Th1 cytokines were upregulated in both these groups. IL-12 is expressed by antigen presenting cells and is important in the early determination of Th1 phenotype in autoimmunity.²² The absence of IL-12 upregulation on day 14 is consistent with the notion that IL-12 is upregulated at the time of initial antigen presentation with decreasing transcription by day 14. Alternatively, it is possible that IL-12 does not play as critical a part in the development of Th1 mediated autoimmunity as previously thought.²³ Further study is needed to define the pattern of IL-12 expression in EAU.

Upregulation of the Th2 cytokine, IL-4 leads to an array of cellular responses which causes tyrosine phosphorylation of STAT6. Tyrosine phosphorylation of STAT6 is necessary for the induction of IL-4 responsive gene transcription. Similarly, upregulation of the Th1 cytokine, IL-12, is associated with phosphorylation of STAT4. There is some evidence that the Th1 cytokine IL-2 can upregulate STAT4 as well.²⁴ In this experiment we examined whether the atorvastatin treatment induced STAT6 phosphorylation or suppressed STAT4 phosphorylation, as this would serve as a marker of immunomodulation towards a Th2 response. We did not find STAT6 phosphorylation in the treatment groups; however, phosphorylated STAT4 was not suppressed in

10 mg/kg atorvastatin treated animals (fig 3). These results were consistent with the cytokine expression profile.

Consistent with the lack of atorvastatin mediated alteration of Th0 differentiation, we did not observe as pronounced suppression of inflammation in treated animals as observed EAE. Our study does demonstrate a small statistically insignificant reduction in the extent of uveoretinitis in animals treated with high dose atorvastatin compared to controls. In light of the equivalence in the measured strength of the Th1 cytokine profile in these animals, we do not think that the small qualitative difference in the extent of inflammation represents a significant inhibition of the immune response. Nevertheless, the significance of this histological finding needs to be revisited as the multitude of mechanisms by which HMG-CoA reductase inhibitors alter inflammation in EAU are elucidated.

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