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Atorvastatin Inhibits Autoreactive B Cell Activation and Delays Lupus Development in New Zealand Black/White F1 Mice

Sarah Lawman,* Claudia Mauri,* Elizabeth C. Jury,* H. Terrence Cook,† and Michael R. Ehrenstein2*

Systemic lupus erythematosus is a multisystem autoimmune disease characterized by a wide range of immunological abnormalities that underlie the loss of tolerance. In this study we show that administration of atorvastatin to lupus-prone NZB/W F1 mice resulted in a significant reduction in serum IgG anti-dsDNA Abs and decreased proteinuria. Histologically, the treatment was associated with reduced glomerular Ig deposition and less glomerular injury. Disease improvement was paralleled by decreased expression of MHC class II on monocytes and B lymphocytes and reduced expression of CD80 and CD86 on B lymphocytes. Consequent upon this inhibition of Ag presentation, T cell proliferation was strongly impaired by atorvastatin in vitro and in vivo. A significant decrease in MHC class II expression was also observed in the target organ of lupus disease (i.e., the glomerulus). Serum cholesterol in atorvastatin-treated lupus mice fell to the level found in young NZB/W mice before disease onset. This is the first demonstration that atorvastatin can delay the progression of a spontaneous autoimmune disease and may specifically benefit patients with systemic lupus erythematosus. The Journal of Immunology, 2004, 173: 7641–7646.

Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, statins, have been shown to reduce cardiovascular mortality and stroke. This effect is mediated in part through beneficial effects on lipid profile, but it has also been demonstrated that statins have immunomodulatory properties that reduce the inflammatory components of the atherosclerotic plaque (1). The original observation that statins reduce IFN-γ-induced MHC class II expression on APCs in vitro through regulation of CIITA (2) suggested that statins may play an immunomodulatory role in Th1-driven autoimmune disease. The therapeutic potential of statins has been demonstrated in experimental allergic encephalomyelitis (3–5) and in collagen-induced arthritis (6). In both animal models administration of atorvastatin resulted in reduced MHC class II (MHC-II) expression and a shift from the pathogenic Th1 response to a protective Th2 response (3, 5, 6) together with impaired lymphocyte trafficking (4).

We hypothesized that statins could also be beneficial in a spontaneous multisystem autoimmune disease such as systemic lupus erythematosus (SLE) for two reasons: 1) the increased production of anti-nuclear Abs and a lupus-like syndrome has been reported in female mice with established disease. Serum cholesterol levels were in-creased compared with those detected in the control mice. The retarded progression of disease was mirrored by a significantly reduced expression of MHCII and CD86/CD80 on B lymphocytes. As a consequence of impaired APC activation, autoreactive T cell proliferation was profoundly impaired in the treated group compared with the control mice.

Materials and Methods

Mice

Female NZB/W F1 mice (Harlan, Longborough, U.K.) were kept in a specific pathogen-free facility. Mice, aged 5 mo, were injected i.p. with 30 mg/kg/day with atorvastatin or PBS/5% ethanol for 14 consecutive wk. Proteinuria was assessed using a Cobas Integra 700 (Roche, Indianapolis, IN). Serum cholesterol was assayed using a Roche Integra.

Flow cytometric analyses

Flow cytometric analyses were performed on single-cell suspensions and were analyzed by FACScan using CellQuest software (BD Biosciences,
San Jose, CA). The Abs used were FITC-conjugated anti-CD4 (GK1.5) and anti-MHC-II 1-A^d (39-10-8), and PE-conjugated anti-CD45R/B220 (RA3-6B2), anti-CD28 (37.51), biotin-conjugated anti-CD80 (B7-1, 16-10A1), and anti-CD86 (B7-2, GL-1). All Abs were purchased from BD Pharmaningen Europe (Oxford, U.K.). Biotinylated Abs were revealed with RPE-Cy5-conjugated streptavidin (DakoCytomation, Glostrup, Denmark).

**Cell proliferation assays**

Single cell suspensions of splenocytes from 5-mo-old NZB/W F$_1$ mice were prepared and cultured at 5 $\times$ 10$^6$ cells/ml in medium for 72 h with or without 10 $\mu$m atorvastatin. Thy1.2+ T lymphocytes were purified by positive selection (MACS; Miltenyi Biotec, Auburn, CA) and labeled with 5 $\mu$m CFSE for 15 min at 37°C in PBS, followed by extensive washing. CFSE-labeled T cells were then remixed with the Thy1.2+ fraction and cultured at 5 $\times$ 10$^6$ cells/ml in medium alone for 5 days. The percentage of T cells that had completed zero, one, two, three, or four cell cycles was determined by CFSE intensity (based upon sequential halving of CFSE intensity after each cell cycle) by FACs analysis.

**Measurement of CIITA promoter mRNA expression**

Total RNA was isolated from splenocytes from TRIzol (Invitrogen Life Technologies Gaithersburg, MD). Double-stranded cDNA was made using the cDNA Synthesis System (Roche, Mannheim, Germany). Relative quantitation with real-time RT-PCR was performed using SYBR Green PCR reagents, the ABI PRISM 7000 sequence detection system, and SDS software (all from Applied Biosystems, Warrington, U.K.) according to the manufacturer’s instructions. The mRNA levels were normalized to those of $\beta$-actin. The PCR primers were: for pCommon, 5'-CTTCGTCAGACTGGCCTTGTA-3' and 5'-TGAAACGTGTCAGACAGCT-3'; for pIII, 5'-AAGGAGAAGGCTACACTGAGC-3' and 5'-CAGCCTTGCACT CATCCAAA-3'; for pHII, 5'-GACTGAGCTGAGTCACAGCT-3' and 5'-GCTTCTTATGTTAATTTCAGTTC-3'; for pHIII, 5'-AAGGAGAAGGCTACACTGAGC-3' and 5'-CAGCCTTGCACT CATCCAAA-3'; for pHIV, 5'-GACTGAGCTGAGTCACAGCT-3' and 5'-GCTTCTTATGTTAATTTCAGTTC-3'; for pHV, 5'-TCTCTGGAAAGCAGTGACACGC-3' and 5'-CATCCAAAACG-3'; and for $\beta$-actin, 5'-CTGTGAAAGATGACAGCCATCA-3' and 5'-ACA GGCTGGATGCTACGT-3'.

**ELISA**

Serum titers of IgG anti-dsDNA (S1 nuclease-treated) Abs were measured as previously described using alkaline phosphatase-conjugated goat anti-mouse IgG (BD Pharmingen Europe) (15). The assays were calibrated using serum from an untreated 5-mo-old NZB/W mouse, which was arbitrarily assigned a titer of 0.5 U/ml; all readings were related to that using appropriate 2-fold serial dilutions. Total IgG was assayed as previously described (16).

**Analysis of tissue sections**

Immunofluorescence microscopy was performed on cryostat sections of kidneys that had been snap-frozen in an N-hexane/dry ice bath using goat anti-mouse, IgG-biotinylated Abs (Southern Biotechnology Associates, Birmingham, AL), followed by fluorescein-conjugated streptavidin (DakoCytomation). The intensity of fluorescence was graded on a semiquantitative scale from 0–4 on coded sections. Frozen spleen and kidney sections were stained with FITC-conjugated anti-mouse MHC-II (BD Pharmingen, San Diego, CA). Sections were viewed using a UV light microscope at $\times$20 magnification (Axioskop 2; Zeiss, Oberkochen, Germany). H&E stains were performed on paraffin-embedded kidney sections, which were then analyzed blindly by an experienced renal pathologist (H.T.C.). Glomerular hypercellularity and glomerular matrix were each scored on a semiquantitative scale from 0–4.

**Results**

**Atorvastatin delays lupus development in NZB/W mice**

The long term effect of atorvastatin administration was evaluated on established lupus nephritis in NZB/W mice. Atorvastatin was administered at a dose of 30 mg/kg/day i.p. for 14 wk to 4-mo-old NZB/W mice (10 mice/group). IgG anti-dsDNA Ab production was significantly reduced throughout the treatment period ($p < 0.001$, by two-way ANOVA; Fig. 1A), whereas total IgG levels were not affected (Fig. 1B). Atorvastatin also retarded the progression of lupus nephritis, as shown by significantly less proteinuria ($p < 0.01$; Fig. 1C) and lower serum urea levels (Fig. 1D; $p < 0.05$) in the treated mice compared with the control group. Histological examination of the kidneys revealed an overall improvement compared with the control group, as demonstrated by a significantly reduced glomerular hypercellularity, interstitial infiltrates, and a smaller increase in glomerular matrix (Fig. 2, A and B). After 14 wk of treatment, 3 of 10 treated mice died compared with 5 of 10 control mice. Because the results in Fig. 1A demonstrated a significant difference in anti-dsDNA Ab production after only 2 wk of treatment, a separate experiment was performed to examine the short term effects of atorvastatin. Thus, 5-mo-old NZB/W mice were treated with atorvastatin for 2 wk using the same dosage regimen as that used for the longer term experiment. As well as a lowering of anti-dsDNA Ab titers in the atorvastatin-treated mice (data not shown), a reduction in glomerular Ig deposition was found in the treated group compared with the control group (mean IgG deposition was 1.2 in the treatment group compared with 2.9 in controls; $p = 0.002$), suggesting that atorvastatin might already exert a significant effect after 2 wk of treatment (Fig. 2C).

**Atorvastatin reduced MHC-II expression in spleen and kidney**

In view of the reduced anti-DNA Abs, proteinuria, and renal injury in atorvastatin-treated mice, we analyzed whether the amelioration in disease was mirrored by inhibition of the expression of costimulatory molecules required for T-B cell activation. MHC-II is critical to the activation and expansion of autoreactive T cells as well as autoantibody production in experimental lupus-like disease (17), and it has been shown to be a key target of atorvastatin (2). As shown in Fig. 3A, 2 wk of atorvastatin treatment significantly reduced MHC-II expression on splenic B lymphocytes as well as monocytes compared with the levels in the control group. The inhibitory effect of atorvastatin on splenic MHC-II expression was also confirmed on histology (Fig. 3B). It has been previously postulated that an increase in renal MHC-II expression is not only a...
feature of the renal lesions, but may play a role in their pathogenesis (18). In this study we demonstrate a specific reduction in MHC-II expression in the target organ of lupus disease, namely, the renal glomeruli (Fig. 3B).

Previous in vitro data have suggested that statins reduce MHC-II expression through regulation of CIITA. As assayed by quantitative PCR (TaqMan), we found that pIV CIITA transcription (in whole spleen) was reduced by 55% in the treated group (Fig. 3C), mirroring a reduction in splenic MHC-II expression. Levels of pIII CIITA transcription and pCommon were unaffected. Splenic expression levels of pI were not detectable, consistent with previous reports (19). To gain more insight into the possible immunological mechanisms by which atorvastatin delays the development of lupus, we next assessed whether the activated phenotype of splenic lymphocytes was altered by the treatment. A reduction in the number of CD4+ T cells occurred after 2 wk of atorvastatin treatment, whereas the number of B cells and the total number of splenic cells remained unchanged (Fig. 3D). A significant reduction in B cell expression of CD80/CD86 occurred in the treated mice (Fig. 3E). The levels of CD28 were also significantly reduced on CD4+ T cells (Fig. 3E), whereas other markers of T lymphocyte activation, such as CD69 and CD25, remained unaffected (data not shown).

**FIGURE 2.** Atorvastatin delayed glomerular injury in NZB/W mice. A, H&E-stained kidney sections (magnification, ×40) from mice treated with atorvastatin (14 wk) showed less glomerular hypercellularity and less glomerular matrix increase than those from PBS-treated mice. Arrows indicate the glomerulus (G) and the tubule (T). B, Kidney sections from treated and control mice (14 wk) were scored by a blind observer for indices of renal injury. C, Mice treated with atorvastatin for 2 wk had less IgG deposited in their glomeruli compared with control treated mice (magnification, ×10).

**FIGURE 3.** Atorvastatin decreased MHC class II expression and lymphocyte activation. A, Atorvastatin decreased MHC-II expression by 56% on monocytes (19.5 ± 4% expressed MHC-II in control mice; 8.48 ± 3% in statin-treated mice; cells defined as positive to the right of the line) and by 32% on B cells (48.2 ± 2.3% in control mice; 28.0 ± 4.5% in statin-treated mice; p < 0.01), as measured by FACS. B, Immunostaining of spleen and kidney demonstrates a reduction in MHC-II expression after atorvastatin treatment. The arrow identifies the germinal center that stains positively for MHC-II. The most pronounced MHC-II expression in the kidney occurred in the glomerulus (arrow; G) of untreated mice, whereas atorvastatin abolished this staining. Magnification, ×20. C, Atorvastatin reduced pIV CIITA transcription by 55% (3.59 ± 0.59 pIV in control spleen; 1.6 ± 0.47 copy units), as assayed by quantitative real-time RT-PCR (p < 0.001), but pII CIITA transcription was unaffected. Data are expressed as the mean ± SE relative to GAPDH. D, Flow cytometric analysis of spleen cells revealed a significant reduction in the percentage of CD4+ T cells after 2 wk of treatment (p < 0.005), whereas the percentage of B cells remained unchanged. E, Atorvastatin decreased the expression of CD80 by 40% (32.1 ± 2.7% expressed CD80 in controls; 19.3 ± 2% in statin-treated mice) and that of CD86 by 54% (61.6 ± 5% in controls; 28.34 ± 5.1% in statin-treated mice) on B lymphocytes (p < 0.05). The percentage of CD4+ T cells expressing CD28 was reduced by 38% (65.67 ± 1.6% in controls; 41.65 ± 1.2% in statin-treated mice; p < 0.05; n = 9 mice/group).
Atorvastatin inhibits T cell proliferation in NZB/W mice predominantly through its effects on the splenic T cell-negative fraction

The reduced levels of MHC-II and the down-regulation of CD86/CD28 and CD80/CD28 expression suggest a possible alteration in the APC-T cell axis. To determine whether T cell proliferation was directly affected by atorvastatin, splenocytes were isolated from 5-mo-old NZB/W mice and cultured in the presence of 10 μM atorvastatin (or PBS) for 3 days. T cells (from both groups) were then enriched by positive selection, stained with CFSE, and cultured in medium alone for an additional 5 days, with the depleted fraction (APC) isolated from either control or atorvastatin-treated splenocytes. The results depicted in Fig. 4A show that although T cells actively proliferate in the presence of APC isolated from PBS-treated splenocytes, when T cells were primed in the presence of APC isolated from splenocytes exposed in vitro to atorvastatin, their response was markedly suppressed. Moreover, the exposure of T cells to atorvastatin cultured with PBS-treated APC (Fig. 4Ai) had little effect on proliferation, suggesting that atorvastatin primarily affects T cell proliferation via inhibition of APC activation, rather than through a direct effect on T cells, as has been shown in other diseases. We next investigated whether a similar effect was observed in vivo. T cells were isolated from splenocytes of statin- or PBS-treated animals (30 mg/kg atorvastatin i.p. daily for 2 wk), stained with CFSE, and primed for 5 days with the T cell-negative fraction (APC), isolated from either the atorvastatin- or PBS-treated group. Similar to the results obtained in vitro, the response of T cells isolated from statin-treated mice was largely unaffected when they were mixed with splenic APC derived from PBS-treated mice (Fig. 4Aii). However, when splenic APC were isolated from statin-treated mice and used to prime splenic T cells isolated from PBS-treated mice, the spontaneous proliferative response was strongly inhibited (Fig. 4Aiii) compared with the T cell response primed by control APC (Fig. 4Aii), consistent with the in vitro data (Fig. 4A). These results suggest that atorvastatin exerts its immunomodulatory effect mainly on APC. Moreover, splenic T cells isolated from mice treated with atorvastatin recovered their proliferative capacity when mixed with untreated APC, suggesting that the inhibitory effect of atorvastatin on T cell proliferation is reversible.

Atorvastatin suppresses serum cholesterol in NZB/W mice

Importantly, serum cholesterol levels in untreated NZB/W mice were significantly higher than those in nonautoimmune NZW mice (Fig. 5). After 2- or 14-wk treatment with atorvastatin, serum cholesterol levels were significantly lowered (2-wk treatment, 2.5 mmol/L compared with 4.8 mmol/L in PBS-treated mice ($p < 0.01$); 14-wk treatment, 3.9 mmol/L compared with 5.9 mmol/L in PBS-treated mice ($p < 0.05$)).
lupus mice (20), suggesting that B lymphocytes, rather than any other cell type, play an important role as APC. Secondly, MRL/lpr mice, which lack MHC-II expression, prevent autoreactive T cell activation, autoantibody production, and disease progression (7). NZB/W mice treated with mAbs to MHC-II also exhibit delayed onset of disease (17). Important to the proposal that atorvastatin acts primarily through its effects on B cells, we show a significant impairment in the T cell proliferative response only when APC were isolated from atorvastatin-treated animals, of which B cells would represent a significant fraction, although when T cells were isolated from statin-treated mice and challenged with PBS-treated APC, they exhibited a much smaller reduction in proliferation. Atorvastatin treatment led to a reduction in MHC-II expression on monocytes, which may also be responsible for the reduction in T cell proliferation in NZB/W mice.

Statins have previously been used to treat autoimmune disease in models that are primarily T cell driven and are characterized by a pathogenic Th1 response (3, 6). In the disease model examined in this study, where both Th1 and Th2 inflammatory cytokines are up-regulated (21), statins did not alter T cell cytokine production or markers of T cell activation (data not shown). In the context of lupus disease, it has been suggested that the validity of the Th1/Th2 dichotomy in explaining the pathogenesis of lupus appears too simplistic (22). This suggests that the effect of statins might depend on the immunopathological environment. Although it is difficult to compare the effects of statin on diseases that have a different pathogenesis, the effect of atorvastatin has been more impressive on induced models of disease that are Th1 driven compared with this report on lupus, which is a spontaneous disease with a more complex etiology. In particular, although we observed a significant reduction in anti-DNA Abs and less glomerular injury, the effects on mortality were not as marked. This dissociation thereby reducing T cell proliferation. However, the reduction in serum cholesterol may also have directly affected the progression of disease. Hyperlipidemia-induced atherosclerosis is an important cause of morbidity and mortality in patients with SLE; consequently, the effect of giving patients a statin may yield a 2-fold benefit, with reductions in their lupus disease activity and in the incidence of atheroma.

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References


