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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 F1 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 statin 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)3 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 IFN-γ and up-regulation of MHC-II expression, which are known to contribute to disease pathogenesis in SLE in both patients and murine models of the disease (7–10); and 2) the established effect that statins have on lipid metabolism coupled with accumulating evidence that the presence of immune complexes and systemic inflammation in SLE contribute to the atherosclerosis-mediated vascular pathology (11). However, the development of anti-nuclear Abs and a lupus-like syndrome has been reported in patients receiving statin therapy (12, 13). Thus, to examine the potential immunomodulatory effect of atorvastatin in lupus we have used NZB/W F1 female mice. These mice begin to develop a lupus-like disease characterized by production of IgG anti-dsDNA Abs at ~4 mo of age, which results in rapidly progressive glomerulonephritis (14).

In this study we show that atorvastatin is effective in reducing serum urea levels, proteinuria, autoantibody production, glomerular Ig deposition, and glomerular hypercellularity in NZB/W F1 female mice with established disease. Serum cholesterol levels were also significantly reduced in the atorvastatin-treated group 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 atorvastatin (Pfizer, Groton, CT) made up with PBS/5% ethanol; controls received PBS/5% ethanol via the same route. In a separate experiment, 4-mo-old female NZB/W F1 mice were injected i.p. with 30 mg/kg/day with atorvastatin or PBS/5% ethanol for 14 consecutive wk. Proteinuria was assessed using Albustix (Baxter, Deerfield, IL), and urea was measured 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 (NK1.1.5) and anti-MHC-II 1-A4 (39-10-8), and PE-conjugated anti-CD45R/B220 (RA3-6B2), anti-CD24 (37.51), biotin-conjugated anti-CD80 (B7-1, 16-10A1), and anti-CD86 (B7-2, GL-1). All Abs were purchased from BD Pharmingen 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 F1 mice were prepared and cultured at 5 × 10⁶ cells/ml in medium for 72 h with or without 10 μM atorvastatin. Thy1.2+ T lymphocytes were purified by positive selection (MACS; Miltenyi Biotec, Auburn, CA) and labeled with 5 μM 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 × 10⁶ 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 β-actin. The PCR primers were: for pCommon, 5′-CTCTGTCAGACTG GCGTTGA-3′ and 5′-TGAAACTGGTGACACAGCTGAGT-3′; for pl, 5′-AAGGAAGAGCTACCTGACAAACA-3′ and 5′-CAGGACTGA CAGATC TCAA-3′; for pH1, 5′-GACTGAGCTGAGTGCAGCTT-3′ and 5′-GTCTCTTATGTAATTATTCGTC-3′; for pHV, 5′-TCTCTCG GAAAGACCTCACTGACAAACA-3′ and 5′-ATATCCACCACGTGCTTTCTCA-3′; and for β-actin, 5′-CGTGTGAAAAGATGACCCAGATCA and 5′-ACA GCTTGGATGCGTACG-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 Ab (Southern Biotechnology Associates, Birmingham, AL), followed by fluorescein-conjugated streptavidin (DakoCytomation). The intensity of fluorescence was graded on a semiquantitative scale from 0–4. All slides were coded and scored independently by two experienced renal pathologists (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-DNA 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-DNA 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 control mice; 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 costimulatory molecules required for T-B cell activation. MHC-II expression 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 expression in spleen and kidney sections was stained with FITC-conjugated anti-mouse MHC-II (BD Pharmingen, San Diego, CA). Sections were viewed using a UV light microscope at ×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.
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 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. 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 pIII 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; 28.3 ± 5.1% in statin-treated mice; 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 negative 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 to atorvastatin, their response was markedly suppressed. Moreover, the exposure of T cells to atorvastatin cultured with PBS-treated APC (Fig. 4Aii) 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. 4Biii). 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. 4Biiiii) compared with the T cell response primed by control APC (Fig. 4Biii), 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)).

Discussion

The data presented in this study demonstrate that atorvastatin can delay the progression of established autoimmune disease in the NZB/W spontaneous murine model of SLE. Atorvastatin treatment significantly reduced serum anti-dsDNA Ab titers, glomerular Ig deposition, glomerular hypertrophy, proteinuria, and serum urea levels. Taken together with recent studies from other groups showing that atorvastatin statins can prevent or ameliorate disease in induced models of autoimmunity (3–6), this work supports the idea that statins have a broad immunoregulatory effect in a variety of different autoimmune diseases.

The immunological results reported in this study suggest that atorvastatin might reduce disease in NZB/W mice through down-modulation of MHC-II expression on monocytes and B lymphocytes. We propose that MHC-II expression on B cells is particularly important in the pathogenesis of lupus not only through the production of Abs, but also in autoantigen presentation to T cells. Two lines of evidence have supported the role of B cells as APC in the generation of lupus autoimmunity. Firstly, autoreactive T cell activation is markedly impaired in B cell-deficient MRL/lpr mice.
PBS-treated NZB/W mice. Data are expressed as the mean ± SE.

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 in 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 of autoantibody production, renal Ig deposition, and mortality has been observed previously in statin- and statin-deficient mice (22), the former of which is a target of atorvastatin (5).

We are the first to show that after atorvastatin treatment, MHC-II expression was reduced not only in the spleen but also in the target organ, i.e., the glomerulus, in NZB/W mice. Glomerular MHC-II expression is increased in lupus patients, and this increase correlated with disease activity, whereas prednisolone therapy reduced MHC-II expression (10), suggesting that targeting glomerular MHC-II expression may be a useful approach in lupus patients. There are a number of other possible mechanisms by which atorvastatin might reduce glomerular injury, including a reduction in cellular proliferation via modulation of the Rho GTPase signaling pathway (23) and a reduction in trafficking of macrophages and lymphocytes to the glomerulus (4, 24). Indeed, the reduced glomerular proliferation observed in the atorvastatin-treated mice may reflect a reduced influx of inflammatory cells. In accordance with recent in vitro observations that atorvastatin down-regulates inducible MHC-II expression through inhibition of transcription of the CIITA promoter pIV (2), we observed a similar effect in vivo, with the splenic reduction in pIV CIITA mirroring the MHC-II reduction.

Considering the established effects of statins on lipid metabolism, it is not surprising that serum cholesterol decreased significantly after treatment with atorvastatin. However, atorvastatin has been used in other murine autoimmune diseases without any fall in serum cholesterol (6). This difference may be due to the elevated serum cholesterol levels found in NZB/W mice compared with other strains, which could therefore be more sensitive to the effects of atorvastatin. An alternative explanation is that the decrease in cholesterol is secondary to the amelioration of the autoimmune disease itself. Equally, the reduced serum cholesterol levels in NZB/W mice may have a beneficial effect on lupus disease, because both dietary restriction and a diet rich in fish oil have been shown to improve disease in NZB/W mice (25, 26). Increased serum cholesterol is also associated with increased accumulation of lipid in the kidney, which contributes to tissue inflammation and worsens the progression of glomerulonephritis in NZB/W mice (27).

This is the first report of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors reducing disease in a spontaneous model of autoimmunity. The mechanisms by which atorvastatin could affect autoimmunity appear to be multifactorial, but we favor the possibility that atorvastatin affects autoreactive B cell activation, 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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