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Simvastatin Treatment Ameliorates Autoimmune Disease Associated with Accelerated Atherosclerosis in a Murine Lupus Model

Tamar Aprahamian,* Ramon Bonegio,† Jennifer Rizzo,* Harris Perlman,‡ David J. Lefer,§ Ian R. Rifkin,† and Kenneth Walsh2*

Patients with systemic lupus erythematosus develop accelerated atherosclerosis independent of traditional risk factors. The 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors are widely prescribed for hyperlipidemia, but they also exhibit anti-inflammatory actions that appear to be independent of their suppressive actions on plasma cholesterol levels. In this study, we analyzed the effect of the HMG-CoA reductase inhibitor simvastatin on disease manifestations in gld apoE--/− mice that lack functional Fas ligand and apolipoprotein E and exhibit accelerated atherosclerosis and aggravated lupus-like features. Wild-type, gld, apoE--/−, and gld apoE--/− mice were maintained on a high cholesterol Western diet and received daily simvastatin (0.125 mg/kg) or saline for 12 wk. Serum cholesterol levels were unaffected by simvastatin treatment, but atherosclerotic lesion area was reduced in both apoE--/− and gld apoE--/− mice treated with simvastatin. Simvastatin also reduced the lymphadenopathy, renal disease, and proinflammatory cytokine production seen in gld apoE--/−, but not gld mice. The immunomodulatory effects in gld apoE--/− mice were associated with enhanced STAT6 and decreased STAT4 induction in submandibular lymph node cells. Along with reductions in serum TNF-α and IFN-γ levels, there was also an increase in IL-4 and IL-10 transcript levels in lymph nodes. These data indicate that HMG-CoA reductase inhibitors ameliorate atherosclerosis and lupus-like autoimmune disease independent of their cholesterol-lowering effects via a shift from a Th1 to a Th2 phenotype in the gld apoE--/− model. Thus, the anti-inflammatory activities of statins may have utility for the treatment of both autoimmunity and atherosclerosis in patients with systemic lupus erythematosus. The Journal of Immunology, 2006, 177: 3028–3034.

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ystemic lupus erythematosus (SLE) is a complex autoimmune disease involving multiple organs that is characterized by autoantibody production (1). In recent years, much attention has been given to the rising incidence of accelerated atherosclerosis in SLE, which precedes more advanced cardiovascular diseases in these patients (2–5). Accelerated atherosclerosis has also been shown to occur in other autoimmune diseases, including rheumatoid arthritis and systemic sclerosis (3, 6, 7). One factor that contributes to the initiation and progression of atherosclerosis is hypercholesterolemia, in particular increased low-density lipoprotein (LDL) (8). In this regard, it has been shown that both pediatric and adult SLE patients have abnormal lipid profiles, including decreased levels of high-density lipoprotein, and increased triglycerides and vLDL (9, 10).

Hypercholesterolemia is widely treated with statins, a class of drugs that inhibit 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, the first committed step of cholesterol synthesis. By decreasing plasma lipid levels, statin treatment decreases the risk for cardiovascular disease and stroke in hypercholesterolemic patients (11–13). Other studies have shown that in addition to lowering plasma cholesterol levels, HMG-CoA reductase inhibitors have immunomodulatory properties that are independent of their serum lipid-lowering properties (14). In this regard, pilot studies indicate that SLE patients treated with simvastatin exhibit decreased proteinuria, and rheumatoid arthritis patients treated with atorvastatin have reduced C-reactive protein levels as well as a clinical improvement of the disease (15). Other studies demonstrate that atorvastatin reduces disease activity in rheumatoid arthritis patients (16), and simvastatin inhibits the inflammatory components of multiple sclerosis (17).

Interestingly, lipid levels in rodents remain unaffected by statin treatment due to robust feedback regulation of hepatic HMG-CoA reductase (14, 18, 19). For this reason, mice and rats are good models to study the anti-inflammatory and immunomodulatory properties of these drugs in the absence of confounding metabolic effects. For example, mice with experimental autoimmune encephalomyelitis treated with lovastatin exhibit a decrease in duration and clinical severity of the disease (20). In addition, fluvastatin treatment in a model of acute peritoneal inflammation in rats inhibits adhesion and extravasation of leukocytes (21).

The goal of the current study was to examine clinically relevant doses of statin on disease in the apoE--/−, gld, and gld apoE--/− mouse strains that are models of atherosclerosis, autoimmunity, and accelerated atherosclerosis associated with autoimmunity, respectively (22). Previous experiments with gld apoE--/− mice have

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3 Abbreviations used in this paper: SLE, systemic lupus erythematosus; LDL, low-density lipoprotein; ANA, anti-nuclear Ab; HMG-CoA, 3-hydroxy-3-methyl-glutaryl coenzyme A; wt, wild type.
shown that this strain exhibits more atherosclerosis than apoE<sup>−/−</sup> mice and more autoimmunity and lymphoproliferation than gld mice, indicating positive feedback interactions between these disease processes (22). The current data suggest that statin treatment in gld/apoE<sup>−/−</sup> mice may lessen not only the severity of atherosclerosis, but also of SLE when it is associated with accelerated atherosclerosis.

Materials and Methods

Study protocol

The Jackson Laboratory B6Smn.C3-* (stock 00102) is considered fully congenic on C57BL/6 background, as is the apoE<sup>−/−</sup> mouse. Therefore, the gld/apoE<sup>−/−</sup> mouse has a C57 background that complements the gld and apoE<sup>−/−</sup> single mutants, both on a C57 background. Mice on a C57BL/6 background with the genotypes apoE<sup>−/−</sup>, gld, gld/apoE<sup>−/−</sup>, and gld<sup>+/+</sup> (wild type; wt) were maintained on a Purina ProLab 3000 mouse diet (normal diet), and at 7 wk of age, mice from each genotype received Teklad (Harlan Teklad) adjusted calories Western diet: 21% (w/w) fat, 0.15% (w/w) cholesterol, and 19.5% (w/w) casein, without sodium cholate. At 7 wk of age, mice from each genotype received either saline vehicle (200 μl) or activated simvastatin (0.125 mg/kg) administered by daily i.p. injection for 12 wk. Because simvastatin has little or no inherent activity in the absence of lactone ring hydrolysis, an alkaline hydrolysis procedure was performed before administration to mice (23). All mouse experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee of Boston University School of Medicine.

Quantitative analyses of atherosclerosis, hyperlipidemia, splenomegaly, and lymphadenopathy

After 12 wk on a high cholesterol Western diet, food was removed for an 8-h fast. Following the fast, the mice were weighed and sacrificed; aorta was drawn by cardiac puncture for determination of total plasma cholesterol, LDL, HDL, triglyceride levels, which was determined by cardiac puncture for determination of total plasma cholesterol, LDL, HDL, and triglyceride levels in the four groups of mice (Table I). Regardless of treatment, LDL levels were elevated in both apoE<sup>−/−</sup> and gld/apoE<sup>−/−</sup> mice, which is a size distribution coefficient (stock 001021) is considered fully congenic on C57BL/6 background with the genotypes apoE<sup>−/−</sup>, gld, gld/apoE<sup>−/−</sup>, and apoE<sup>+/+</sup> (wild type; wt) were maintained on a Purina ProLab 3000 mouse diet (normal diet), and at 7 wk of age, mice from each genotype received Teklad (Harlan Teklad) adjusted calories Western diet: 21% (w/w) fat, 0.15% (w/w) cholesterol, and 19.5% (w/w) casein, without sodium cholate. At 7 wk of age, mice from each genotype received either saline vehicle (200 μl) or activated simvastatin (0.125 mg/kg) administered by daily i.p. injection for 12 wk. Because simvastatin has little or no inherent activity in the absence of lactone ring hydrolysis, an alkaline hydrolysis procedure was performed before administration to mice (23). All mouse experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee of Boston University School of Medicine.

**Results**

Simvastatin treatment does not alter cholesterol levels, but decreases atherosclerotic lesion area in apoE<sup>−/−</sup> and gld/apoE<sup>−/−</sup> mice

At 7 wk of age, wt, apoE<sup>−/−</sup>, gld, and gld/apoE<sup>−/−</sup> mice were put on Western diet and administered simvastatin (0.125 mg/kg/day) or saline by i.p. injection for 12 wk. Consistent with previous studies in rodents (14, 18, 19), simvastatin did not affect plasma cholesterol levels in the four groups of mice (Table I). Regardless of treatment, LDL levels were elevated in both apoE<sup>−/−</sup> and gld/apoE<sup>−/−</sup> mice. Consistent with previously published data (22), LDL levels were lower in the gld/apoE<sup>−/−</sup> mice than apoE<sup>−/−</sup> mice, which may result from a suppression of hepatic cholesterol synthesis by inflammatory cytokines (25). In addition, the ratio of LDL to high-density lipoprotein was markedly higher in apoE<sup>−/−</sup> mice than apoE<sup>−/−</sup> mice that were harvested and homogenized. After determination of concentration with a protein assay kit (Pierce), 1 mg of protein was immunoprecipitated for STAT4 or STAT6 overnight with anti-STAT4 Ab or anti-STAT6 Ab (Zymed Laboratories) at 1/1000 dilutions. The secondary Abs were anti-mouse or anti-rabbit IgG/HRP conjugate (Cell Signaling Technology) diluted 1/5000 in TBS containing 5% skim milk for detection. The primary Abs were used for phospho-STAT4 or phospho-STAT6 overnight with anti-STAT4 Ab or anti-STAT6 Ab (Zymed Laboratories) at 1/1000 dilutions. The secondary Abs were anti-mouse or anti-rabbit IgG/HRP conjugate (Cell Signaling Technology) diluted 1/5000 in TBS containing 5% skim milk.

**Statistical analysis**

Results are shown as the mean ± SEM or SD. Differences between groups were determined by ANOVA and Student’s <i>t</i> test using SigmaPlot, and were considered statistically significant for <i>p</i> < 0.05.

<table>
<thead>
<tr>
<th>Total Cholesterol (mg/dl)</th>
<th>Vehicle treated</th>
<th>Statin treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>158.1 ± 14.3</td>
<td>147.5 ± 8.4</td>
</tr>
<tr>
<td>apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>1450.3 ± 64.6</td>
<td>1290.3 ± 57.2</td>
</tr>
<tr>
<td>gld</td>
<td>149.5 ± 15.9</td>
<td>124.2 ± 12.5</td>
</tr>
<tr>
<td>gld/apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>757.7 ± 76.5*</td>
<td>743.1 ± 75.6*</td>
</tr>
</tbody>
</table>

*<i>p</i> < 0.001 vs wt, gld, and apoE<sup>−/−</sup>.
mice treated with statin was decreased ~25% compared with vehicle-treated mice: from 28.4 ± 2.1 to 21.6 ± 1.9 mm², respectively. Similarly, statin treatment led to a 21% reduction in atherosclerotic lesion area in apoE−/− mice, from 9.1 ± 0.9 to 5.9 ± 0.8 mm² (Fig. 1B), consistent with findings from previous studies (14, 26). No atherosclerotic lesions were observed in wt or gld mice (data not shown). Thus, statin treatment reduces atherosclerosis independent of detectable effects on serum cholesterol.

Simvastatin treatment decreases lymphoproliferation and autoantibody production in gld.apoE−/− mice

Enlargement of spleen and lymph nodes is a feature of gld mice, and is more pronounced in gld.apoE−/− mice (22). After 12 wk of simvastatin treatment, submandibular lymph node sizes in the gld.apoE−/− mice decreased from 1.05 ± 0.09 to 0.41 ± 0.05 g (Fig. 2A). In addition, splenomegaly was significantly decreased in simvastatin-treated gld.apoE−/− mice compared with vehicle-treated controls (Fig. 2B). In contrast, gld mice did not show any response to statin treatment with regard to lymphadenopathy and splenomegaly. Simvastatin also had no effect on spleen or lymph node weight in wt or apoE−/− mice. Treatment with simvastatin significantly decreased ANA titer in gld.apoE−/− mice, from 822.9 ± 118 to 352 ± 78.4 (Fig. 2C). In contrast, simvastatin had no effect on ANA titers in gld mice.

Renal disease in gld.apoE−/− mice is improved with simvastatin treatment

In earlier studies, we observed that the gld.apoE−/− mice exhibited proteinuria, enlarged glomeruli, and tubular vacuolization (22), whereas these features are not seen in the gld mice on the C57BL/6 genetic background (27). Simvastatin treatment in gld.apoE−/− mice reduced proteinuria (Fig. 3A), and histological analyses of kidney revealed that glomerular tuft volume was significantly smaller when gld.apoE−/− mice were treated with simvastatin (Fig. 3, B and C). In contrast, simvastatin had no effects on normal urine protein levels and kidney glomeruli size in wt, apoE−/−, and gld mice. Thus, simvastatin treatment ameliorates several components of the pathological renal phenotype observed in gld.apoE−/− mice.

Simvastatin decreases apoptotic debris in lymph nodes of gld.apoE−/− mice

Failure to clear apoptotic cells has been implicated in the development and progression of autoimmune diseases in several mouse models (28–31), and we previously observed impaired apoptotic cell clearance in the gld.apoE−/− mice (22). Thus, we examined whether simvastatin treatment would affect the levels of apoptotic cells in lymph nodes. Simvastatin treatment significantly decreased the amount of apoptotic cells within the submandibular lymph nodes of the gld.apoE−/− mice, whereas this treatment had little or
no effect on the accumulation of apoptotic cells in gld mice (Fig. 4). Very few apoptotic cells could be detected in the lymph nodes of wt or apoE−/− mice, and statin treatment had no detectable effect on these levels (data not shown).

**Simvastatin up-regulates the Th2 immune response in gld apoE−/− mice**

The STAT6 transcription factor promotes Th2 immune responses and anti-inflammatory cytokine production (32, 33), whereas the STAT4 transcription factor promotes Th1 immune responses and the production of proinflammatory cytokines (34–36). Because murine SLE is associated with Th1 proinflammatory cytokine production, we examined whether simvastatin differentially regulates STAT4 and STAT6 phosphorylation in this model. Western blot analysis of STAT4 and STAT6 immunoprecipitated from submandibular lymph nodes of gld apoE−/− mice revealed that simvastatin treatment increased STAT6 phosphorylation levels, whereas phosphorylated STAT4 was down-regulated (Fig. 5A), indicating a shift toward a Th2 response. Consistent with a modulation of the Th1/Th2 response in gld apoE−/− mice, serum levels of proinflammatory cytokines TNF-α and IFN-γ were lower in the gld apoE−/− mice treated with simvastatin (Fig. 5B). In addition, treatment with simvastatin led to an increase in mRNA levels of anti-inflammatory cytokines IL-4 and IL-10 in the submandibular lymph nodes of gld apoE−/− mice (Fig. 5C). Therefore, simvastatin modulates the immune response in gld apoE−/− mice, at least in part, by altering the Th1/Th2 cytokine response.

**Discussion**

Premature atherosclerosis associated with SLE is a major cause of morbidity and mortality (37, 38). However, the pathogenesis of atherosclerosis is poorly understood in this patient population. Recently, we described the gld apoE−/− mouse model of premature atherosclerosis in SLE (22). In the current study, we show that statin treatment substantially reduces both atherosclerosis and autoimmune disease in this model independent of effects on serum cholesterol levels. Simvastatin has a modest (21%) inhibitory effect on atherosclerosis, consistent with the findings of others in hyperlipidemic mice (14, 26). However, the effects of statins on autoimmune disease were more striking in the gld apoE−/− model. Simvastatin treatment reduced ANA titers by 57%, lymphadenopathy by 66%, and splenomegaly by 48%. Furthermore, simvastatin treatment normalized kidney glomeruli size and protein levels in urine.

Although there may be several mechanisms by which simvastatin exerts its anti-inflammatory and immunomodulatory properties,
we provide evidence that simvastatin influences the expression of proinflammatory cytokines in gld.a apoE−/− mice. Statins have been shown to ameliorate signs of experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis, via effects on the Th1/Th2 balance of cytokines (20, 39). Several studies have implicated Th1 cytokines and their upstream transcription factors in the pathogenesis of lupus in mouse models (39–42). It has been shown that retinoic acid treatment to New Zealand Black/New Zealand White F1 mice resulted in decrease in proinflammatory cytokines IFN-γ and IL-2 as well as reduced renal dysfunction, contributing to increased survival (43). Furthermore, treatment with a peptide that inhibits SLE progression was associated with a down-regulation of IFN-γ and IL-2 and up-regulation of anti-inflammatory cytokine TGF-β (44). Proinflammatory cytokines have also been implicated in the pathology of lupus nephritis in human patients (45, 46). These studies provide evidence that
proinflammatory cytokine signaling contributes to the progression of autoimmune diseases. In our study, simvastatin therapy promoted a decrease in the serum levels of the Th1 cytokines TNF-α and IFN-γ and an increase in the lymph node mRNA levels of the Th2 cytokines of IL-4 and IL-10 in gld apoE<sup>−/−</sup> mice, indicating a shift toward a more anti-inflammatory immune response. Consistent with this interpretation, our immunoprecipitation experiments detected a statin-mediated increase in phosphorylated STAT6 and a decrease in STAT4 phosphorylation in submandibular lymph nodes. Collectively, these findings suggest that statin therapies down-regulate Th1 cytokine signaling, and promote a shift toward a Th2 response.

Although atherosclerosis is associated with a Th1 response, it is paradoxical that severe hypercholesterolemia conditions in mice will modulate the T cell response toward a Th2 type (47, 48). This Th2 shift is seen with diets containing a 1.25% cholesterol and more so with diets containing a 1.25% cholesterol and 0.5% cholic acid, but little or no Th2 shift is observed with the 0.15% cholesterol diet that is typically used to assess the anti-atherosclerotic effects of statins in apoE<sup>−/−</sup> mice (49). The diet used in the current study contains 0.15% cholesterol and no cholic acid. Thus, it is unlikely that the Th2 shift seen with severe hypercholesterolemia and dietary supplementation of cholic acid would confound our observations of a statin-induced Th2 shift in gld apoE<sup>−/−</sup> mice under the conditions of our assays. Of interest, simvastatin treatment did not affect inflammation in gld mice. We speculate that there is no detectable effect of statins because the Th1 inflammatory response is minimal in gld compared with gld apoE<sup>−/−</sup> mice. Consistent with this hypothesis, TNF-α levels are 58% lower in gld than gld apoE<sup>−/−</sup> mice (p < 0.05) and simvastatin treatment had little or no effect on the level of this cytokine in gld mice or on the relative ratio of STAT4 to STAT6 phosphorylation levels in lymph nodes (data not shown).

The utility of statin therapy for the treatment of autoimmune disease is currently being investigated in the clinic (16, 17) and animal models (20, 39). Recently, Lawman et al. (50) reported that atorvastatin at a dose of 30 mg/kg/day diminished autoimmune disease in New Zealand Black/New Zealand White F1 mice. This level of statin approaches the lethal dose for this class of drugs (51), and it is much higher than the clinical dose (typically in the range of 0.1–0.5 mg/kg/day). High doses of statins can be toxic through their ability to interfere with synthesis of nonsterol products that are involved in cellular upkeep (52). Whereas it is well established that statins typically do not affect serum lipid levels in mice (14, 18, 19), Lawman et al. (50) reported a 50% decrease in serum cholesterol levels, indicative of high dosing in this model. Thus, an aim of our study was to evaluate a physiological level of simvastatin (0.125 mg/kg/day). Despite no detectable effect on serum lipid levels, statin treatment led to a marked reduction in the autoimmune and lymphoproliferative phenotypes in the gld apoE<sup>−/−</sup> mice. These data suggest that statins can act to limit both atherosclerotic and autoimmune phenotypes via anti-inflammatory and immunomodulatory actions that are independent of serum cholesterol levels.

It is becoming increasingly evident that chronic inflammation is a significant component of atherosclerotic disease progression, and there are several possibilities by which the statins may be impacting the development of atherosclerosis in the setting of autoimmune. First, it is reasonable to consider that statins decrease the severity of autoimmunity, and, consequently, the severity of atherosclerosis. However, statins are widely recognized to reduce atherosclerosis. In murine models that display no autoimmune phenotype, there is a decrease in the serum levels of the Th1 cytokines TNF-α that promotes SLE (53), and it has been shown that apoE<sup>−/−</sup> mice lacking TNF-α do not develop extensive atherosclerosis (54, 55). Therefore, the statin-mediated reduction in TNF-α and other Th1 cytokines could account for the atheroprotective and immunomodulatory properties of this drug.

Another issue of interest is that simvastatin treatment decreased the amount of apoptotic cells within lymph nodes of gld apoE<sup>−/−</sup> compared with vehicle treatment. We have shown previously that accelerated atherosclerosis increases the number of apoptotic bodies within the lymph nodes of gld apoE<sup>−/−</sup> mice, and this apoptotic material does not colocalize with macrophages, indicating an impaired recognition and clearance (22). Dysregulated apoptosis has been associated both with SLE pathogenesis (56) and atherosclerosis pathogenesis (57). Thus, statin therapy may decrease the accumulation of apoptotic debris by reducing inflammation in atherosclerotic lesions; however, further studies will be required to elucidate how simvastatin reduces the level of TUNEL-positive apoptotic material in the gld apoE<sup>−/−</sup> mice.

The model presented in this study is the first to look at the effects of simvastatin on the inflammatory feedback mechanisms that are observed in a mouse model of accelerated atherosclerosis and autoimmune disease. Our results indicate that simvastatin promotes a shift from Th1 cytokine production toward a more anti-inflammatory phenotype. We propose that these immunomodulatory effects of statins, rather than their lipid-lowering actions, mediate their beneficial effects on accelerated atherosclerosis and autoimmunity in this model.

Disclosures
The authors have no financial conflict of interest.

References