Simvastatin Inhibits Airway Hyperreactivity

Implications for the Mevalonate Pathway and Beyond

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***Rationale*: Statin use has been linked to improved lung health in asthma and chronic obstructive pulmonary disease. We hypothesize that statins inhibit allergic airway inflammation and reduce airway hyperreactivity via a mevalonate-dependent mechanism.**

***Objectives*: To determine whether simvastatin attenuates airway inflammation and improves lung physiology by mevalonate pathway inhibition.**

***Methods*: BALB/c mice were sensitized to ovalbumin over 4 weeks and exposed to 1% ovalbumin aerosol over 2 weeks. Simvastatin (40 mg/kg) or simvastatin plus mevalonate (20 mg/kg) was injected intraperitoneally before each ovalbumin exposure.**

***Measurements and Main Results*: Simvastatin reduced total lung lavage leukocytes, eosinophils, and macrophages ($P < 0.05$) in the ovalbumin-exposed mice. Cotreatment with mevalonate, in addition to simvastatin, reversed the antiinflammatory effects seen with simvastatin alone ($P < 0.05$). Lung lavage IL-4, IL-13, and tumor necrosis factor-α levels were all reduced by treatment with simvastatin ($P < 0.05$). Simvastatin treatment before methacholine bronchial challenge increased lung compliance and reduced airway hyperreactivity ($P = 0.0001$).**

***Conclusions*: Simvastatin attenuates allergic airway inflammation, inhibits key helper T cell type 1 and 2 chemokines, and improves lung physiology in a mouse model of asthma. The mevalonate pathway appears to modulate allergic airway inflammation, while the beneficial effects of simvastatin on lung compliance and airway hyperreactivity may be independent of the mevalonate pathway. Simvastatin and similar agents that modulate the mevalonate pathway may prove to be treatments for inflammatory airway diseases, such as asthma.**

Keywords: statin; asthma; physiology; HMG-CoA reductase; small G protein

Current asthma therapies do not adequately inhibit disease progression in all patients. Statins have been proposed as potential novel treatments for respiratory diseases, including bronchial asthma (1, 2). Reductions in chronic obstructive pulmonary disease morbidity and mortality, and improvements in lung function, have been attributed to statin use (1, 3–5). Statins are also associated with lower asthma exacerbation rates and reduced endotracheal intubation rates for respiratory failure (6). The mechanisms of these beneficial effects remain unknown, but may be due to the antiinflammatory effects of the statin drugs (7, 8).

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The statin drugs have pleiotropic immune modulatory properties beyond their lipid-lowering effects. Reports indicate that the statin drugs may have beneficial effects on lung health including asthma and chronic obstructive pulmonary disease. The mechanisms of this observed benefit in the lung are not yet clear.

What This Study Adds to the Field

Bronchial influx of inflammatory cells in allergic lung disease occurs via a mevalonate-dependent pathway. Improvements in lung physiology after simvastatin treatment may occur via mevalonate-independent pathways, and are likely due to effects predominantly on resident airway cells rather than inflammatory cells.

The statin drugs (or "statins") inhibit the enzyme hydroxymethylglutaryl (HMG)-CoA reductase, the rate-limiting step in the cholesterol biosynthesis pathway. These widely used lipid-lowering drugs possess pleiotropic antiinflammatory and immune-modulatory effects with potential clinical applications beyond cardiovascular disease (9, 10). The immediate product of HMG-CoA reductase is mevalonate (MA), which is metabolized into the nonsterol isoprenoids farnesyl pyrophosphate and geranylgeranyl pyrophosphate (GGPP), and cholesterol (Scheme 1). These isoprenoid pyrophosphates are necessary for the posttranslational isoprenylation and activation of intracellular monomeric small G proteins (also known as guanosine triphosphatases), which in turn control many important biological functions, including immune cell function and cell proliferation (11). The antiinflammatory effects of statins are thought to be mediated partly via inhibition of isoprenoid synthesis.

Some authors have demonstrated an antiinflammatory effect of simvastatin in murine models of asthma (12, 13), but the exact mechanism of this effect remains unclear. Furthermore, the effect of the statin drugs on lung physiology in mouse models of allergic airway inflammation remains unknown, but one report based on a rat model showed beneficial effects on airway resistance (14). Given the pleiotropic effects of the statin drugs, we decided to focus on the MA pathway as a reasonable mechanism for the beneficial effect of statins. We hypothesized that inhibition of the MA pathway can attenuate allergic inflammation and airway hyperreactivity (AHR). We used simvastatin to inhibit HMG-CoA reductase and provided MA simultaneously to reverse the drug effect, while measuring indicators of inflammation and lung function.

The present study was designed to test whether simvastatin given systemically inhibits allergic airway inflammation and improves lung physiology in an MA-dependent manner, using a mouse model of asthma. We found that simvastatin inhibits ovalbumin-induced influx of eosinophils, lymphocytes, and mac-

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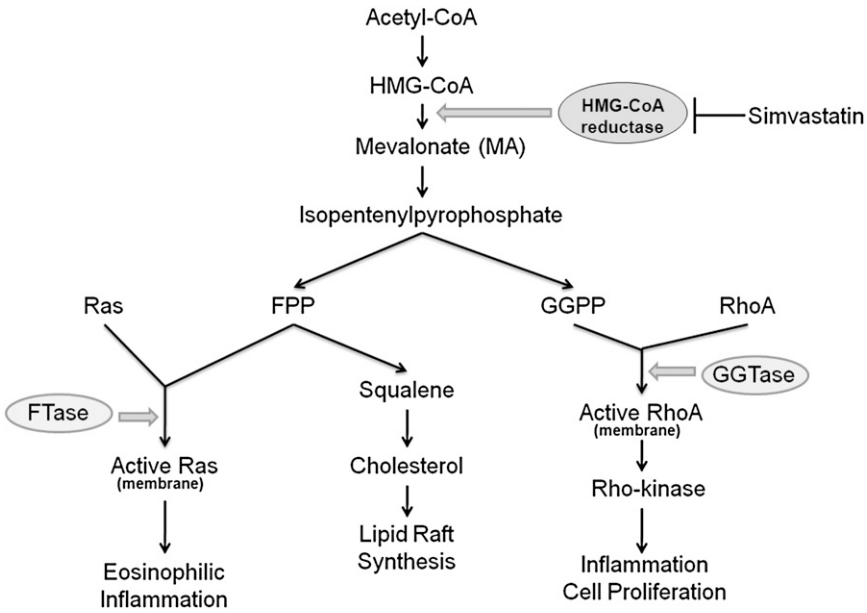


Scheme 1. The mevalonate (MA) pathway: sterol and nonsterol products. Simvastatin and other statins directly inhibit the rate-limiting enzyme hydroxymethylglutaryl (HMG)-CoA reductase to prevent conversion of HMG-CoA to mevalonate. The MA pathway leads to sterol and nonsterol products, that is, cholesterol and the isoprenoids farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), respectively. Isoprenylation describes the formation of covalent bonds between FPP and Ras, and/or between GGPP and RhoA via enzymes farnesyltransferase and geranylgeranyltransferase, respectively. This then allows inactive cytosolic small G proteins Ras and RhoA to attach to the cell membrane as the active form, to transduce signaling cascades. Adapted from Takeda and colleagues (38). FTase = farnesyltransferase; GGTase = geranylgeranyltransferase.

rophages into airways by an MA-dependent mechanism. To our knowledge, this is the first study to reveal the beneficial effects of simvastatin on lung compliance and AHR by an MA-independent mechanism, in a mouse model of allergic asthma. The improvements in lung function due to simvastatin treatment appear to be predominantly dependent on resident cells within the airway compartment. Some of the results of this study have been previously reported in abstract form at the American Thoracic Society international conference (15).

METHODS

Animals

Pathogen-free male BALB/c mice (age, 8–10 wk old; weight, 20–30 g), were purchased from Charles River Breeding Laboratories (Wilmington, MA). Animals were maintained until used in Bio-Clean facilities including a HEPA-filtered laminar flow cage rack with a 12-hour light/dark cycle and allowed free access to food (rodent diet; PMI Nutrition International, St. Louis, MO) and water. Mice were killed at the end of an experiment with an intraperitoneal overdose of Beuthanasia-D (pentobarbital sodium and phenytoin sodium). All procedures were performed under an institutional animal care and use committee–approved protocol.

Drug Solutions

Simvastatin and mevalonic acid (or mevalonate) were purchased from Sigma (St. Louis, MO). Simvastatin was made as a 4-mg/ml stock solution, dissolved in 10% ethanol as the drug vehicle. The drug vehicle was made with 100 μl of ethanol plus 150 μl of 0.1 N NaOH plus 750 μl of phosphate-buffered saline (PBS) to make 1 ml of 10% ethanol. Hydrochloric acid (HCl, 0.1 N) was added as necessary to obtain a solution pH of 7.0. Mevalonate (MA) was made as a 6.3-mg/ml stock solution dissolved in PBS (1×).

Exposure of Mice to Ovalbumin Aerosol

All mice (ovalbumin aerosol–exposed and control) were sensitized by intraperitoneal injections of ovalbumin (OVA, grade V, 98% pure; Sigma), 10 μg/0.1 ml in PBS, pH 7.4, with alum adjuvant (16), on Days 0 and 14 of the experiment (Scheme 2). After OVA sensitization, mice were divided into six treatment groups, including three OVA-sensitized/OVA-exposed groups, and three OVA-sensitized/filtered air (FA)–exposed groups (Scheme 2). There were 5 or 6 mice per group for lung physiology measurements and 9–12 mice for bronchoalveolar lavage fluid (BALF) cell counts (sample size is indicated in each figure). Starting on Day 28, mice were exposed for 30 minutes per exposure to the OVA aerosol three times per week for a period of 2 weeks (six exposures in total, or 6OVA). Mice in the OVA-sensitized/FA-exposed groups were exposed to FA only. FA-exposed mice received intraperitoneal injections of 0.3 ml of PBS (1×), 0.3 ml of 10% ethanol, or 0.3 ml of simvastatin (Sim, 40 mg/kg). OVA-exposed groups received intraperitoneal injections of 0.3 ml of 10% ethanol (simvastatin drug vehicle), 0.3 ml of Sim (40 mg/kg), or 0.3 ml of Sim plus 0.1 ml of MA (20 mg/kg). Injections were administered 30 minutes before exposure to FA or 1% OVA aerosol in PBS (10 ml of a 10-mg/ml OVA solution). Exposures to OVA aerosols were performed with chambers and generators as previously described (17). All aerosol delivery was conducted via side stream nebulizer (Invacare Corp., Elyria, OH) and air compressor (Invacare Corp., Sanford, FL). Mass concentration and aerodynamic size distributions have been determined with a Mercer-type cascade impactor (17). One to 3 hours after the sixth and final air or OVA aerosol exposure (Day 42), all mice underwent lung physiology studies with a full body plethysmograph for restrained animals. Immediately thereafter, all mice were killed with intraperitoneally administered Beuthanasia-D (0.3 ml at 1:6 dilution), followed by collection of specimens (blood draw, lung lavage, and lung isolation). *Note*: The use of "filtered air (FA)" and "air" have equivalent meaning in RESULTS and respective figures.

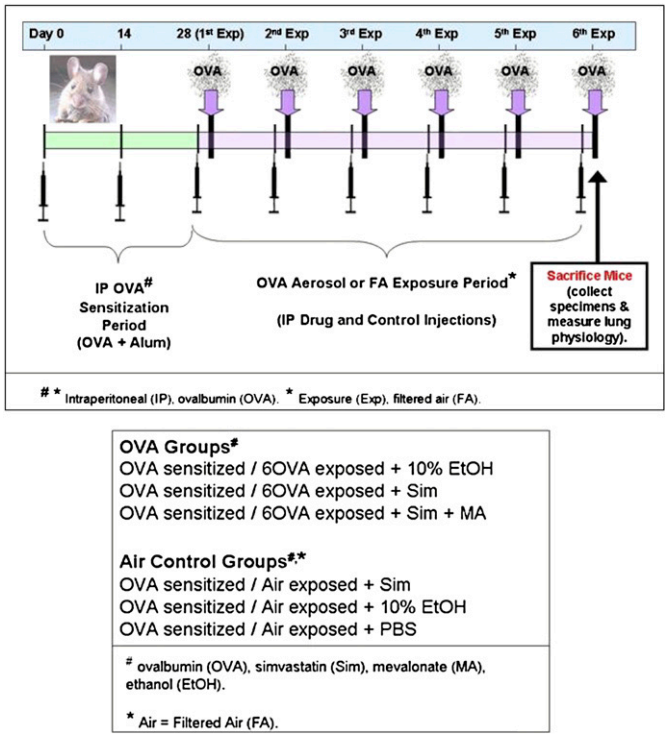
Lung Compliance and Resistance Measurements

The dynamic lung compliance (Cdyn) and resistance of the respiratory system (Rrs) were simultaneously measured with a plethysmograph for restrained animals (Buxco Inc., Troy, NY) 1–3 hours after termination of the final (or sixth) OVA exposure (Day 42). Mice were deeply anesthetized and sedated with medetomidine (Domitor, 0.5 mg/kg; Orion Pharma, Finland), and tiletamine–zolazepam (Telazol, 50 mg/kg; Fort Dodge Laboratories, Overland Park, KS). Mouse tracheas were surgically cannulated, and then the mice were ventilated at 7–8 cm^3/kg with a mouse ventilator (MiniVent; Harvard Apparatus, Cambridge, MA) for the duration of the procedure. Compliance (Cdyn, as ml/cm H_2O) and resistance (Rrs, as cm H_2O·s/ml) measurements were made at baseline and immediately after serial 3-minute nebulizations of saline and methacholine (MCh, 0.5, 1.0, and 2.0 mg/ml), with 3-minute recovery periods allowed after each exposure to MCh.

Lung Tissue Processing

See the online supplement for details on lung tissue processing.

Immediately after lung physiology measurements, mice were killed with an overdose of intraperitoneally administered Beuthanasia-D (pentobarbital sodium and phenytoin sodium). After blood collection, lungs were lavaged twice with 1 ml of PBS (pH 7.4), immediately placed on ice, and then centrifuged at 2,500 rpm on a tabletop centrifuge for



Scheme 2. *Top*: Ovalbumin (OVA) sensitization and exposure timeline, timing of drug injections, day of animal sacrifice, and specimen collections. *Bottom*: Various experimental treatment groups.

8 minutes. The total lavage live cell number and differential cell counts were determined. Mice had their lungs fixed or immediately frozen and stored at −80°C. Lungs were fixed for histological evaluation at 30 cm H_2O with 1% paraformaldehyde for 1 hour, and then immersed in fixative overnight (for at least 24 h) at 4°C. After fixation and paraffin embedding, the lungs were stained with hematoxylin and eosin to qualitatively assess peribronchiolar inflammation (at a magnification of ×100).

Cytokine and Chemokine Assay

The concentrations of selected helper T-cell type 1 (Th1) and helper T-cell type 2 (Th2) cytokines and chemokines from BALF supernatant were measured with commercially available multiplex assays (Millipore, St. Charles, MO). For cytokine/chemokine sample measurements below the lower detection limit, results were assigned a value equal to the minimal detection limit for the specific assay to facilitate statistical analysis of the data.

Statistical Analysis

Results are presented as mean values ± SEM. Means were compared by unpaired Student *t* test or analysis of variance (ANOVA, one-way or two-way), with the Tukey or Bonferroni correction for multiple comparisons applied when appropriate, using the Prism 5 software package (Graphpad, Inc., San Diego, CA). A P value of 0.05 or less was taken to indicate statistical significance. Values that differed by more than 2 standard deviations from the mean were excluded from the statistical analysis.

RESULTS

BALF Cell Counts

To determine whether HMG-CoA reductase inhibition affects allergic lung inflammation, we exposed six groups of mice to inhaled OVA or FA for 2 weeks, treated them with simvastatin or drug vehicle (with and without MA) before all exposures, and then measured lung lavage total and differential cell counts (Scheme 2).

In the OVA-exposed mice, simvastatin treatment significantly reduced BALF total leukocyte influx by 60% ($P < 0.05$) (Figure 1A). Cotreatment with MA reversed this effect to near OVA control levels ($P < 0.05$). The BALF differential cell counts showed a similar pattern (Figures 1B–1D). Simvastatin significantly reduced eosinophil influx by 67% and macrophage influx by 47% ($P < 0.05$). Although simvastatin administration reduced lymphocyte influx by 53%, this apparent reduction was not significant by one-way ANOVA. For all cell types except macrophages, MA cotreatment reversed the simvastatin inhibitory effect ($P < 0.05$). After MA cotreatment, the macrophage cell count trended in the same direction as total cell count, eosinophils, and lymphocytes, but this trend did not reach statistical significance by one-way ANOVA. There was no significant simvastatin effect on neutrophil influx (P = not significant; data not shown). Mevalonate cotreatment reversed the antiinflammatory effect of simvastatin *in vivo*, indicating that allergic inflammation is, at least partially, MA pathway dependent.

We performed an additional experiment, in which we added two more treatment groups: 6OVA plus MA and filtered air (FA) plus MA (data not shown). The administration of MA alone did not alter OVA-induced airway inflammation (i.e., 6OVA plus ethanol is not significantly different from 6OVA plus MA). The FA plus MA group was also not significantly different from any of the other three air control groups. The same pattern was seen with respect to lung lavage absolute eosinophil, lymphocyte, and macrophage counts (data not shown). Statistical analyses were performed by one-way ANOVA.

BALF Cytokine Measurements

Simvastatin affected several key Th1 and Th2 cytokines known to be important in allergic asthma. In OVA-challenged animals, simvastatin significantly decreased the lung lavage content of IL-4 by 69.5% ($P < 0.05$), IL-13 by 83% ($P < 0.05$), and tumor necrosis factor (TNF)-α by 55.5% ($P < 0.05$) (Figures 2A–2C). Mevalonate cotreatment did not reverse the simvastatin inhibitory effect on these cytokines. Simvastatin had no significant effect on BALF concentrations of eotaxin, IL-5, IL-6, IL-1α, IL-9, IL-10, IL-17, or vascular endothelial growth factor (data not shown). Although there were trends toward decreased macrophage inflammatory protein-1α, keratinocyte-derived cytokine, IP-10, RANTES (regulated upon activation normal T cell expressed and secreted), and IL-2 after simvastatin treatment, none of these decreases was statistically significant (data not shown). There was a trend of increased monocyte chemotactic protein-1 with simvastatin treatment, but this did not reach statistical significance (data not shown).

Lung Histology

Lung histology was examined to qualitatively assess the degree of peribronchiolar airway inflammation. Four different treatment groups are shown in Figures 3A–3D, including all OVA groups and the air plus PBS control group. The histological pattern parallels the inflammatory response seen in the BALF cell counts (Figures 1A–1D). In the 6OVA plus ethanol control group, extensive peribronchiolar inflammation is evident, which is greatly reduced after simvastatin treatment (6OVA plus Sim) (Figures 3A and 3B). Mevalonate cotreatment reversed the antiinflammatory simvastatin effect (6OVA plus Sim plus MA), showing inflammation similar to the 6OVA plus ethanol control group (Figures 3A and 3C). Only one air

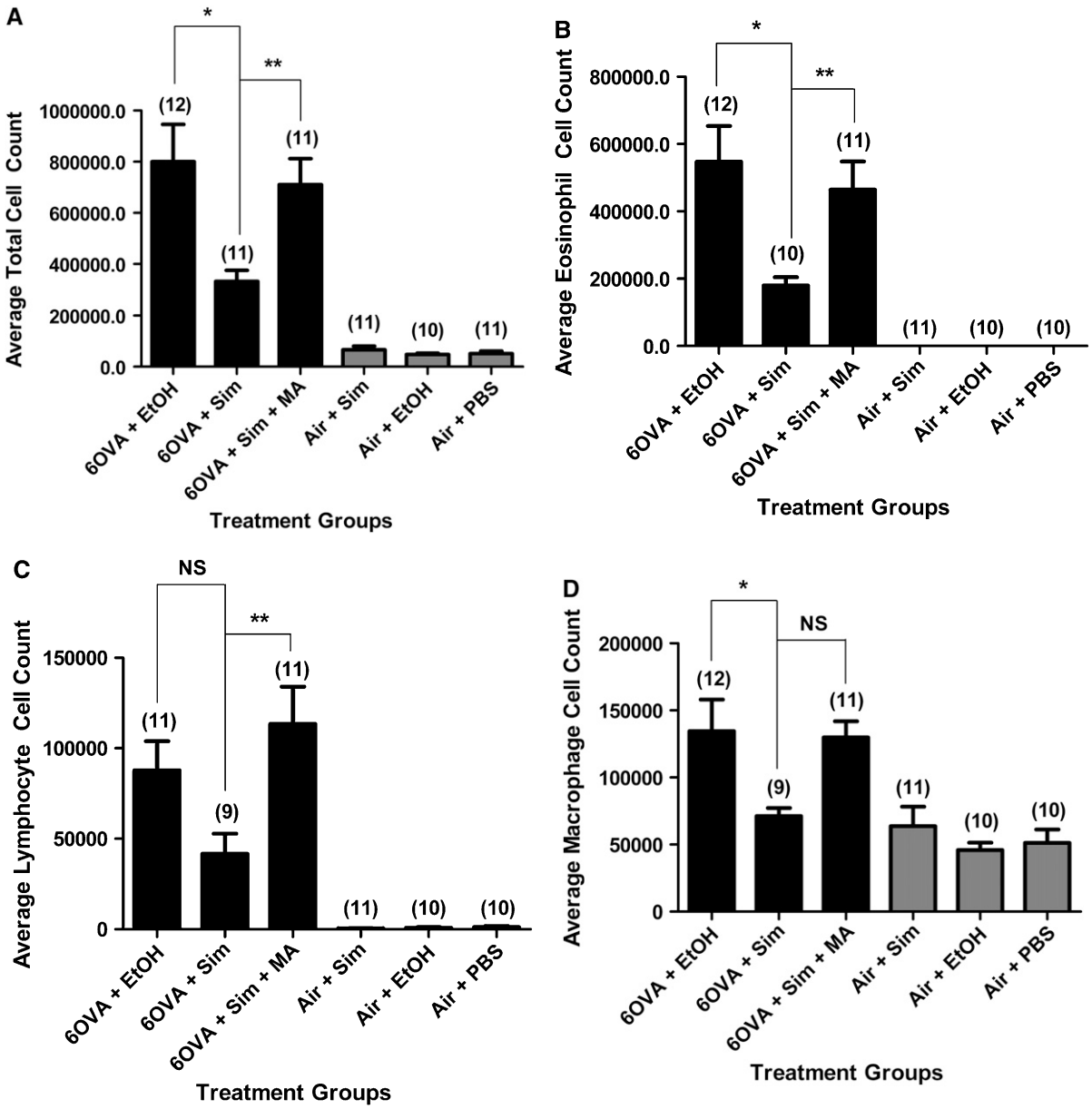


Figure 1. (*A*) Bronchoalveolar lavage fluid (BALF) total cell count. Simvastatin (40 mg/kg) reduced BALF leukocyte influx by 60%, and this antiinflammatory effect was reversed by mevalonate (MA, 20 mg/kg) cotreatment. (*, **$P < 0.05$ by one-way analysis of variance [ANOVA]). (*B*) BALF eosinophil count. Simvastatin (40 mg/kg) reduced BALF eosinophil influx by 67%, and this antiinflammatory effect was reversed by MA (20 mg/kg) cotreatment. (*, **$P < 0.05$ by one-way ANOVA). (*C*) BALF lymphocyte count. Simvastatin (40 mg/kg) reduced BALF lymphocyte influx by 53% (P = not significant [NS] by one-way ANOVA), and this antiinflammatory effect was reversed by MA (20 mg/kg) cotreatment (**$P < 0.05$ by one-way ANOVA). (*D*) BALF macrophage count. Simvastatin (40 mg/kg) reduced BALF macrophage influx by 47% (*$P < 0.05$ by one-way ANOVA), and MA (20 mg/kg) cotreatment trended toward a reversal of this effect (P = NS by one-way ANOVA). Each *column* represents mean values ± SEM. The number of mice per treatment group is listed in parentheses above each *column*. EtOH = ethanol; OVA = ovalbumin; PBS = phosphate-buffered saline; Sim = simvastatin.

control (air plus PBS) is shown because the others had a similar appearance (Figure 3D).

Lung Compliance and Airway Resistance

To determine whether simvastatin treatment ameliorates AHR, dynamic lung compliance (Cdyn) and airway resistance (Rrs) were measured in response to nebulized methacholine (Figures 4A and 4B and Figures 5A and 5B). Cdyn and Rrs of the OVA control group (i.e., 6OVA plus ethanol) were significantly different ($P < 0.05$) compared with their respective air control groups, indicating an appropriate response to OVA allergen challenge (Figures 4 and 5). Simvastatin treatment before MCh bronchial challenge increased Cdyn and reduced AHR in the OVA groups ($P = 0.0001$ for overall effect by treatment group, assessed by two-way ANOVA). In the OVA groups, simvastatin treatment increased Cdyn to air control levels at all time points (baseline, saline, and MCh at 0.5, 1, and 2 mg/ml) ($P < 0.01$ for baseline, and $P < 0.001$ for all other time points) (Figure 4B), and decreased AHR to air control levels at all time points except the baseline Rrs measurement ($P < 0.01$ for saline and MCh at 0.5 mg/ml, and $P < 0.001$ for MCh at 1 and 2 mg/ml) (Figure 5B). Mevalonate cotreatment (20 mg/kg,

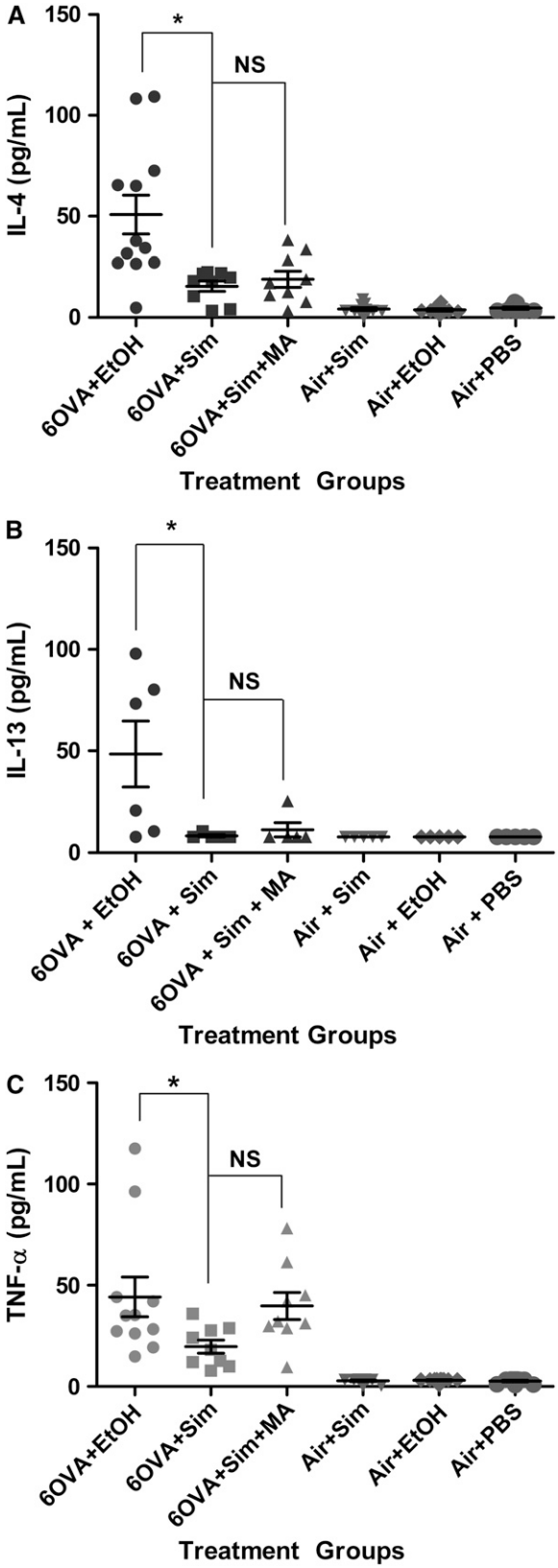


Figure 2. (*A*) Bronchoalveolar lavage fluid (BALF) IL-4 concentration. Simvastatin (40 mg/kg) reduced IL-4 by 69.5% (*$P < 0.05$ by one-way analysis of variance [ANOVA]). Mevalonate (20 mg/kg) cotreatment did not reverse the inhibitory simvastatin effect (P = not significant [NS]). (*B*) BALF IL-13 concentration. Simvastatin (40 mg/kg) reduced IL-13 by 83% down to air control levels (*$P < 0.05$ by one-way ANOVA). Mevalonate (20 mg/kg) cotreatment did not reverse the inhibitory simvastatin effect (P = NS). (*C*) BALF tumor necrosis factor (TNF)-α concentration. Simvastatin (40 mg/kg) reduced TNF-α concentration by 55.5% (*$P < 0.05$ by one-way ANOVA). Mevalonate (20 mg/kg) cotreatment did not reverse the inhibitory simvastatin effect (P = NS). Each dot represents one animal. For the nondetectable cytokine measurements we used the lower limit of detection in the calculation of means, SEM, and statistical analysis. EtOH = ethanol; MA = mevalonate; OVA = ovalbumin; PBS = phosphate-buffered saline; Sim = simvastatin.

administered intraperitoneally) did not reverse the effect of simvastatin on Cdyn or AHR (Figures 4B and 5B), despite reversing the effect of simvastatin on bronchial inflammation (Figures 1 and 3).

Simvastatin significantly improved Cdyn in the air control groups when compared with ethanol (drug vehicle) and PBS, at all time points ($P < 0.001$ and $P < 0.05$ by two-way ANOVA, respectively) (Figure 4A). Simvastatin significantly improved Rrs in the air control groups when compared with ethanol drug vehicle and PBS ($P < 0.001$ for Sim vs. ethanol, and $P < 0.01$ for Sim vs. PBS, by one-way ANOVA). The three air control treatment groups were different overall with respect to Rrs ($P < 0.0001$ by one-way and two-way ANOVA); however, there were no statistically significant simvastatin effects on Rrs at the various bronchial challenge time points by two-way ANOVA (Figure 5A).

DISCUSSION

We studied the effects of simvastatin on allergic airway inflammation and AHR, and examined the role of the MA pathway, using a mouse model of allergic asthma. Simvastatin attenuated allergic airway inflammation, inhibited key Th1 and Th2 chemokines, and improved lung physiology. Our findings suggest a role for the statin drugs not only for modulating lung inflammation, but also for attenuating AHR, a key pathologic feature of asthma. The antiinflammatory effects of statins have been previously demonstrated in models of allergic inflammation, including simvastatin (12, 13), lovastatin (14), and pravastatin (18). However, to our knowledge, the present study is the first to document the beneficial effect of simvastatin on lung physiology by an MA-independent mechanism, in the OVA mouse model.

Beyond lowering cholesterol and inhibiting the isoprenoids, the pleiotropic effects of statins may also be due to inhibition of MA-independent pathways, that is, independent of HMG-CoA reductase inhibition (19–21). Therefore, we sought to determine whether the effects of simvastatin in our experimental model of asthma are modulated by MA inhibition. We showed that simvastatin not only reduces allergic airway inflammation, but that this inflammation is in large part dependent on the MA pathway (Figures 1A–1D and 3). Although we cannot entirely exclude additional HMG-CoA reductase–independent inhibition, the data support a major role for direct inhibition of HMG-CoA reductase. Kim and colleagues showed that simvastatin inactivated small G proteins of the Rho family in BALF and lung tissue, a signaling process that occurs via the MA pathway important in inflammation and cell migration (13). Inhibition of this pathway with lovastatin has also been shown to be important in LPS-induced pulmonary inflammation in C57BL/6 mice (22). In our study, simvastatin also inhibited the

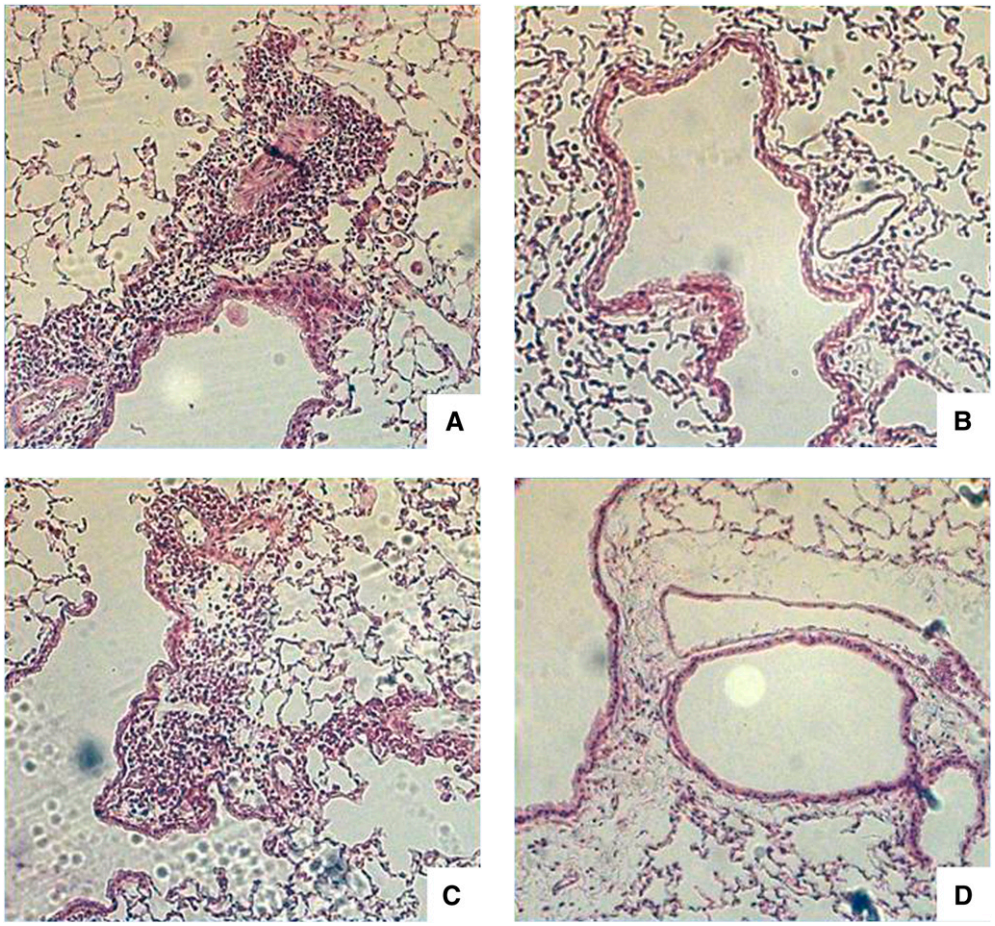


Figure 3. (*A–D*) Mouse lung histology. The four different treatment groups are seen at an original magnification of ×100 after hematoxylin–eosin staining. (*A*) 6OVA (six exposures to ovalbumin) + ethanol group: High-grade peribronchiolar inflammation surrounding one airway. (*B*) 6OVA + simvastatin (Sim) group: Minimal to no peribronchiolar inflammation. (*C*) 6OVA + Sim + mevalonate (MA) group: Peribronchiolar inflammation similar to the control group seen in (*A*). (*D*) Air + PBS control group: No inflammatory cells are apparent. The Sim-treated 6OVA group (*B*) looks similar to the air control group (*D*) with respect to inflammatory cell influx. PBS = phosphate-buffered saline.

production of important Th2 cytokines and TNF-α found in BALF. Although MA cotreatment reversed the simvastatin effect with respect to BALF leukocyte counts and peribronchiolar inflammation (Figures 1A–1C and 3), there was no reversal with respect to IL-4, IL-13, and TNF-α (Figure 2). However, trends for MA reversal were present for cytokines IL-4 and TNF-α.

Simvastatin significantly decreased IL-4, IL-13, and TNF-α concentrations in lung lavage fluid; these cytokines play a central role in Th1 and Th2 allergic inflammation and AHR (Figures 2A–2C). Both IL-4 and IL-13 are known to enhance AHR, whereas IL-4 maintains the allergic response via effects on T and B lymphocytes, and IgE (23). The proinflammatory cytokine TNF-α can induce airway hyperresponsiveness in healthy subjects, and it has been strongly linked to human asthma (24). The beneficial effects of simvastatin on AHR may be due to inhibition of these key cytokines. Chiba and colleagues showed that IL-13 up-regulates RhoA small G protein, resulting in increased bronchial smooth muscle cell hyperresponsiveness in a mouse model of allergic inflammation (25). Simvastatin may be working by multiple mechanisms: (*1*) by reducing IL-13 production (or by inhibiting the cells that make this cytokine), and/or (*2*) by depleting MA and GGPP, thereby preventing activation of RhoA signaling. Although simvastatin inhibited BALF eosinophilia (Figure 1B), it had no effect on the eosinophil chemoattractants eotaxin, IL-5, and RANTES, thereby implicating other mechanisms by which simvastatin attenuates eosinophilic influx. Imamura and colleagues showed that pravastatin inhibits lung IL-13 and IL-17, while suppressing systemic sensitization to OVA and attenuating the antigen-presenting capacity of $CD11c^+$ cells (26). In our study, the influx of eosinophils, lymphocytes, and macrophages into the airways after OVA exposure seems to be MA dependent (Figure 1). In our model, eosinophils account for approximately 75% of the BALF total cell count, with the remainder being lymphocytes, macrophages, and neutrophils. Although BALF differential cell counts all displayed the same pattern of simvastatin inhibition and reversal with MA cotreatment, we may note that the 53% reduction in lymphocytes after simvastatin treatment was not statistically significant by one-way ANOVA (Figure 1C), whereas by unpaired *t* testing (data not shown) this reduction was statistically significant. The weight of the evidence both from our experiments and those previously reported in the literature seems to support a critical role for the MA pathway in pulmonary inflammation.

The MA pathway and downstream products play a major part in immune cell responses. Statins modulate T-lymphocyte differentiation and activation, and suppress lymphocyte function by Ras and Rho small G-protein inactivation (by depleting intracellular MA) (27). T-cell Ras small G proteins, in turn, mediate Th2-dependent cytokine production, modulating eosinophilic airway inflammation, and AHR in OVA-exposed animals (28). Simvastatin can induce eosinophilic apoptosis in an MA-dependent fashion (29), while Ras regulates eosinophil chemotaxis and survival (30). Furthermore, the observed simvastatin inhibition of airway eosinophilia (Figure 1B) could also be due to broader systemic effects on T cells with subsequent inhibition of eosinophilic chemotaxis and transmigration (31, 32). Statins also modulate monocyte–macrophage function and inflammatory responses (33, 34), consistent with our finding of reduced BALF macrophage counts (Figure 1D). Peripheral blood mononuclear cells from patients with asthma display

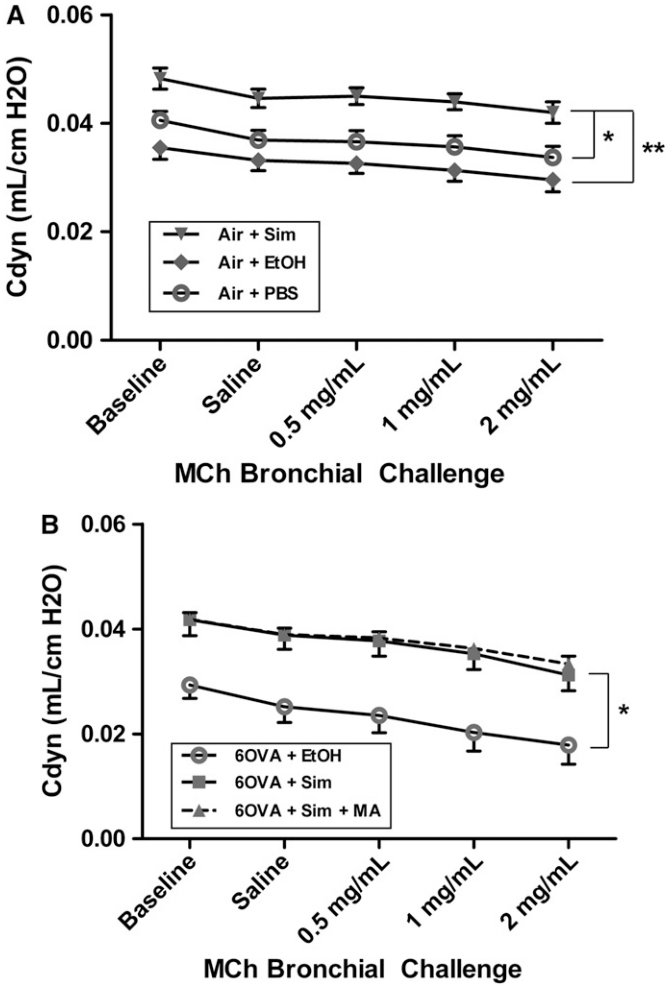


Figure 4. (*A*, *B*) Dynamic lung compliance. Effects of simvastatin (Sim, 40 mg/kg) on *in vivo* dynamic lung compliance (Cdyn). Lung compliance changes in (*A*) air control groups and (*B*) ovalbumin (OVA)-treated animals after methacholine (MCh) bronchial challenge were measured as described in METHODS. Each point represents the mean ± SEM from five or six different animals. (*A*) Simvastatin significantly improved Cdyn in the air control groups when compared with ethanol (EtOH) drug vehicle and phosphate-buffered saline (PBS), at all time points (**$P < 0.001$ and *$P < 0.05$ by two-way analysis of variance [ANOVA], respectively). The Cdyn of the 6OVA + EtOH group (*B*) is significantly lower than that of any of the air control groups (*A*) ($P < 0.05$ by two-way ANOVA), indicating an appropriate response to allergen challenge. (*B*) Simvastatin significantly increased Cdyn after OVA allergen challenge compared with its control (6OVA + Sim vs. 6OVA + EtOH) (*$P < 0.0001$ by two-way ANOVA for the different treatment groups; specifically, $P < 0.01$ for the baseline, and $P < 0.001$ for the saline and three MCh bronchial challenge time points; two-way ANOVA). Mevalonate (20 mg/kg) cotreatment did not reverse the effect of simvastatin on Cdyn. 6OVA = six exposures to ovalbumin; MA = mevalonate.

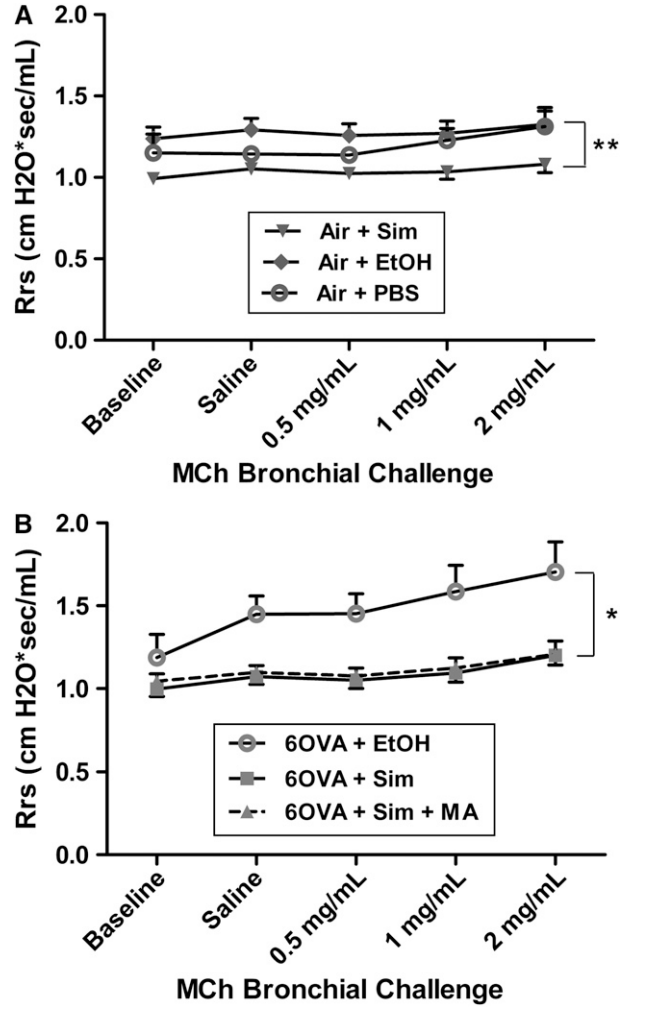
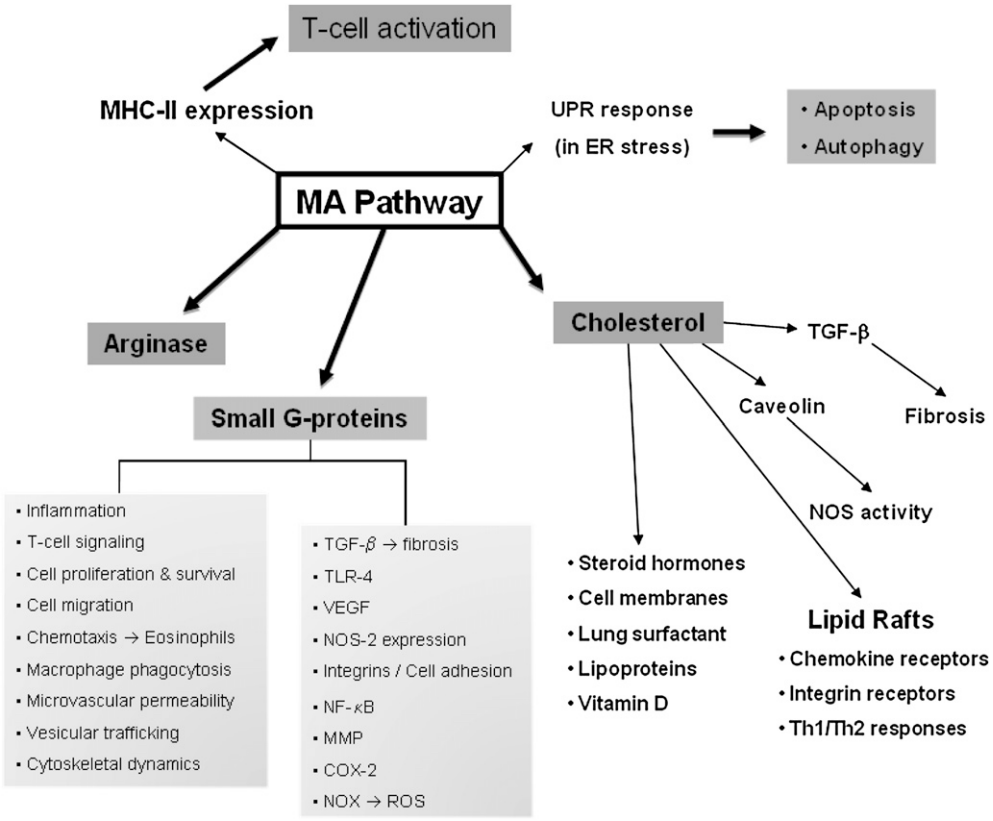


Figure 5. (*A*, *B*) Airway hyperreactivity (AHR). Effects of simvastatin (Sim, 40 mg/kg) on *in vivo* AHR induced by repeated ovalbumin (OVA) exposures. Lung resistance changes in (*A*) air control groups and (B) OVA animals after methacholine (MCh) bronchial challenge were measured as described in METHODS. Each point represents the mean ± SEM from five or six different animals. (*A*) Simvastatin significantly improved respiratory system resistance (Rrs) in the air control groups when compared with ethanol (EtOH) drug vehicle and phosphate-buffered saline (PBS) (**$P < 0.001$ for Sim vs. EtOH, and **$P < 0.01$ for Sim vs. PBS, by one-way analysis of variance [ANOVA] comparing the three different treatment groups). The Rrs of the 6OVA + EtOH group (*B*) is significantly higher than that of any of the air control groups (*A*) ($P < 0.05$ by two-way ANOVA), indicating an appropriate response to allergen challenge. (*B*) Simvastatin significantly decreased AHR after OVA allergen challenge compared with its control (6OVA + Sim vs. 6OVA + EtOH) (*$P < 0.0001$ by two-way ANOVA for the different treatment groups; specifically, P = not significant [NS] for the baseline time point, $P < 0.01$ at the saline and 0.5-mg/ml MCh time points, and $P < 0.001$ for the 1- and 2-mg/ml MCh time points; by two-way ANOVA). Mevalonate (20 mg/kg) cotreatment did not reverse the effect of simvastatin on AHR. 6OVA = six exposures to ovalbumin; MA = mevalonate.

reduced chemokine and cytokine production after fluvastatin treatment (35), antiinflammatory effects that may be mediated via GGPP depletion (Scheme 1) (36). Clearly, there is mechanistic precedence that supports the central role of the MA pathway in modulating the simvastatin inhibition of OVA-induced airway inflammation.

The improvements in lung physiology seen with simvastatin are unique benefits of such treatment, but the exact nature of this improvement and its cellular localization in our experimental model is not known. RhoA/Rho kinase signaling and small G-protein activation are increasingly recognized as key players in pulmonary disease, in particular asthma (37). Several studies have determined that the statin effects on airway smooth muscle



Scheme 3. Mevalonate (MA) and other related pathways. The MA pathway is ubiquitous in all cells and is essential for cell function and survival. The *shaded boxes* represent important downstream pathways that are likely highly pertinent to asthma pathogenesis and treatment. Beyond regulation of small G proteins, other important processes include modulation of cholesterol and its downstream metabolites (e.g., lipid rafts), arginase expression, MHC-II expression, and T-cell activation, and pathways leading to control of apoptosis/autophagy, which are pertinent to airway inflammation and remodeling. *Definition of abbreviations*: COX-2 = cyclo-oxygenase-2; ER = endoplasmic reticulum; MA = mevalonate; MHC = major histocompatibility complex; MMP = matrix metalloproteinase; NF-κB = nuclear factor-κB; NOS = nitric oxide synthase; NOX = NAD(P)H oxidase; ROS = reactive oxygen species; TGF-β = transforming growth factor-β; TLR-4 = Toll-like receptor-4; UPR = unfolded protein response; VEGF = vascular endothelial growth factor.

cell proliferation and contractility occur via inhibition of the MA pathway, specifically Rho small G-protein signaling (14, 38). Simvastatin inhibits human bronchial smooth muscle cell proliferation, a major feature of airway remodeling in asthma (38). In mice, lovastatin inhibits OVA-induced bronchial smooth muscle hyperresponsiveness as measured *ex vivo* (39). In our study, simvastatin improved lung compliance and attenuated AHR; however, we conclude that the effects of simvastatin on lung physiology were independent of bronchial inflammation because MA cotreatment abolished the drug's inhibitory effect on bronchial leukocyte influx (Figures 1 and 3), but did not do so with respect to lung compliance and AHR (Figures 4 and 5). This suggests that the main target of the beneficial effect of simvastatin on lung function is the resident airway cells, not the inflammatory cells. It also suggests that the simvastatin inhibition of AHR is MA independent. Interestingly, in the air-exposed control groups, and for both Cdyn and Rrs measurements, simvastatin improved lung function, supporting an independent effect on airway resident cells (Figures 4A and 5A). This observation raises the important possibility that even in the absence of inflammation statins affect the airway compartment in beneficial ways.

The attenuation of AHR by simvastatin implies reduced airway smooth muscle mass, function, or cell proliferation (38). For example, lovastatin treatment has been shown to significantly reduce bronchial smooth muscle contractility and AHR via Rho signaling inhibition (14). In addition to effects on smooth muscle, statins have proapoptotic effects (29, 40, 41), which could modulate AHR (42). Simvastatin inhibits connective tissue growth factor expression and inhibits profibrogenic markers in lung fibroblasts, processes that promote airway remodeling (43).

Overall, our data seem to support the use of statins to treat AHR, as is presently being tested in several National Institutes of Health clinical trials. Two clinical trials have examined the effects of statins in asthma. In both studies, the treatment duration was brief (4 and 8 wk) (44, 45) and neither study showed an improvement in clinical outcomes, including lung function. Hothersall and colleagues reported a reduction in sputum macrophage counts and leukotriene B_4 concentration after atorvastatin treatment (44). The reduction in sputum macrophages suggests a modest antiinflammatory effect in humans. The lack of effect on sputum eosinophils may be due to the relatively low baseline sputum eosinophil count (median of 1.5%) (44). We speculate that outcomes may be different in subjects with eosinophilic asthma treated for a much longer period of time.

Products of the MA pathway regulate many diverse intracellular functions, with some critical not only to inflammation but also to the chronic changes that lead to tissue remodeling (Scheme 3). Although small G-protein activation by isoprenoid intermediates has many essential intracellular functions (Scheme 1), the MA-dependent statin antiinflammatory effects observed in our experiments may not occur solely via inactivation of small G proteins. Several other important MA-dependent effects of statins have been described including modulation of T-cell activation, apoptosis and autophagy, lipid raft synthesis, and arginase expression (Scheme 3) (27, 40, 46). These and other MA-dependent processes may be important players in asthma disease pathogenesis. Our results raise important questions regarding the downstream effects of MA regulation, whether by statins or other novel agents. The opportunity to modulate these related pathways is of great interest for future research given their therapeutic potential in inflammatory airway diseases such as asthma and chronic obstructive pulmonary disease.

This study has several important limitations. First, we are limited in our ability to determine the main airway cell type responsible for our observations. Second, although reversal of the antiinflammatory effect of simvastatin by MA cotreatment confirms inhibition of HMG-CoA reductase as the underlying

mechanism, it does not distinguish between the three downstream effector products of the MA pathway: (*1*) farnesyl pyrophosphate, (*2*) GGPP, and (*3*) cholesterol (Scheme 1). Future studies are needed to determine the main mechanism of the simvastatin effect, in which specific inhibitors of isoprenylation (e.g., farnesyltransferase or geranylgeranyltransferase inhibitors) or cholesterol synthesis (e.g., squalene synthase inhibitors) may discriminate between these subpathways (47).

Third, it is not clear whether our drug doses and dosing regimen are comparable to that in humans who ingest the drug orally once daily. The simvastatin dose administered in our experiments (40 mg/kg, given intraperitoneally) is much higher than that prescribed for humans; however, the half-life ($t_{1/2}$) of simvastatin in rodent plasma is approximately 4 minutes and in humans it is almost 7 hours (48, 49). In humans, oral simvastatin at a dose of 60 mg daily reaches a peak serum concentration of almost 19 ng/ml (48). In comparing human and animal investigations, rats given oral simvastatin at 25 mg/kg achieve a serum concentration (also measured as nanograms per milliliter) that is numerically much higher (approximately $\times 10^2$) than the human serum concentration over a similar time period (48, 49). Another possibility might be that statin treatment up-regulates HMG-CoA reductase in mice, thereby requiring higher dosing to achieve adequate inhibition (50). The MA dose we chose (20 mg/kg, injected intraperitoneally) was based on similar dosing in the literature (22), on results from a preliminary pilot experiment, and on our estimates of possible serum concentrations.

In conclusion, the present study demonstrates that simvastatin attenuates allergic airway inflammation in an MA-dependent fashion, inhibits the production or release of key Th1 and Th2 chemokines, and improves lung function. These data demonstrate for the first time the MA-independent beneficial effects of simvastatin on lung compliance and AHR in the OVA mouse model. The likely main target of the beneficial effect of simvastatin on lung function is the airway resident cells, rather than inflammatory cells. The mechanisms that regulate the effects of simvastatin on inflammation and lung physiology appear different. Simvastatin or other inhibitors of the MA pathway may be beneficial for the treatment of asthma, and results of ongoing clinical trials will be of high interest.

Conflict of Interest Statement: A.A.Z. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; L.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; J.L. received more than $100,001 in sponsored grants from the Philip Morris External Research Program from 2001–2004/Interactions of ozone and PM with Ovalbumin in a mouse asthma model. None of the Philip Morris funding was used for the experiments described in the present manuscript; N.J.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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[ONLINE DATA SUPPLEMENT MATERIAL]

Simvastatin Inhibits Airway Hyperreactivity: Implications for the Mevalonate Pathway and Beyond.

Amir A. Zeki, Lisa Franzi, Jerold Last, Nicholas J. Kenyon.

[ONLINE DATA SUPPLEMENT MATERIAL]

METHODS (additional material)

Animals

Mice were certified as chronic respiratory disease free by the supplier, and were routinely screened for health status by serology and histology by our veterinary animal resources facility. There were no viral pathogens present in these animals that might cause chronic airway inflammation. Animals were housed and cared for by the veterinary staff of Animal Resource Services at University of California, Davis in AALAC-accredited facilities, in plastic cages over autoclaved bedding in HEPA-filtered cage racks.

Blood Collection

Immediately following lung physiology measurements, mice were euthanized with an overdose of IP Beuthanasia-D (pentobarbital sodium and phenytoin sodium). Approximately 1 mL of whole blood was collected in heparinized tubes by cardiac puncture. Plasma was obtained by centrifugation and stored at -80°C.

Whole Lung Bronchoalveolar Lavage

Animals were placed on a restraining board, followed by blood collection. After blood collection, lungs were lavaged twice with 1 mL of PBS (pH 7.4), immediately placed on ice, and then centrifuged at 2,500 rpm on a tabletop centrifuge for 8 minutes. The lavage supernatant was saved and stored at −80 °C for determination of BALF cytokines. The remaining cell pellet was treated with ACK/RBC lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3) to remove red blood cells that may have been present, recentrifuged at 2,500 rpm and the remaining cell pellet resuspended in 0.5

mL PBS. The total lavage live cell number was determined using a hemacytometer with trypan-blue staining to assess cell viability.

For differential cell analysis, an aliquot (100 μL) of BALF cell suspension was processed onto slides using a cytocentrifuge at 1,650 rpm for 15 minutes. Slides were air dried and stained with a Hema3 (Diff-Quick) stain set as described in the manufacturer's instructions (Biochemical Sciences, Swedesboro, NJ) and sealed using Cytoseal (Stephens Scientific, Kalamazoo, MI). Cell percent differentials were calculated by counting 10 random fields under 40x magnification, and classifying cell types as alveolar macrophage, neutrophil, eosinophil, lymphocyte, or "other" based upon standard morphological characteristics and staining profiles.

Lung Isolation and Fixation

Mice had their lungs fixed or immediately frozen and stored at -80 ^{o}C. Lungs were fixed for histological evaluation at 30 cm pressure with 1% paraformaldehyde for 1 hour, then immersed in fixative overnight (for at least 24 hours) at 4°C. After fixation the lungs were placed in 70% ethanol and prepared for paraffin embedding and then processed for light microscopy. Lung sections of 5 μm thickness were made with special attention to cutting through the larger lobar bronchi in parallel. Sections were then dried at 37°C in a standard fashion and prepared for staining.

Lung Histology

Lung sections were treated with xylene and alcohol to remove paraffin and rehydrate. Slides were then stained with hematoxylin & eosin (H&E) to qualitatively assess peribronchiolar inflammation (at 10x magnification). Two independent readers randomly examined the airways of slides in a blinded fashion.