

Simvastatin inhibits goblet cell hyperplasia and lung arginase in a mouse model of allergic asthma: a novel treatment for airway remodeling?

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Airway remodeling in asthma contributes to airway hyperreactivity, loss of lung function, and persistent symptoms. Current therapies do not adequately treat the structural airway changes associated with asthma. The statins are cholesterol-lowering drugs that inhibit the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase, which is the rate-limiting step of cholesterol biosynthesis in the mevalonate (MA) pathway. These drugs have been associated with improved respiratory health, and ongoing clinical trials are testing their therapeutic potential in asthma. We hypothesized that simvastatin treatment of ovalbumin (OVA)-exposed mice would attenuate early features of airway remodeling by a mevalonate-dependent mechanism. BALB/c mice initially were sensitized to OVA and then exposed to 1% OVA aerosol for 2 weeks after sensitization for 6 exposures. Simvastatin (40 mg/kg) or simvastatin plus MA (20 mg/kg) were injected intraperitoneally before each OVA exposure. Treatment with simvastatin attenuated goblet cell hyperplasia, arginase-1 protein expression, and total arginase enzyme activity, but it did not alter airway hydroxyproline content or transforming growth factor- β 1. Inhibition of goblet cell hyperplasia by simvastatin was mevalonate-dependent. No appreciable changes to airway smooth muscle cells were observed in any control or treatment groups. In conclusion, in an acute mouse model of allergic asthma, simvastatin inhibited early hallmarks of airway remodeling, which are indicators that can lead to airway thickening and fibrosis. Statins are potentially novel treatments for airway remodeling in asthma. Additional studies using subchronic or chronic allergen exposure models are needed to extend these initial findings. (Translational Research 2010;156:335–349)

Abbreviations: AHR = airway hyperreactivity; ANOVA = analysis of variance; ARG1 = arginase-1; BCA = bicinchoninic acid; BM = basement membrane; BSA = bovine serum albumin; Cdyn = Dynamic lung compliance; DistEpi = distal airway epithelium; EDTA = ethylenediaminetetraacetic acid; EtOH = ethanol; FA = filtered air; FPP = farnesylpyrophosphate; GGPP = geranylgeranylpyrophosphate; HEPA = high efficiency particulate air; HMG-CoA = 3-hydroxy-3-methyl-glutaryl-CoA; HRP = horseradish peroxidase; IL = interleukin; i.p. = intraperitoneal; MA = mevalonate; MCh = methacholine; NS = not significant; OVA = ovalbumin; PAS = periodic acid-Schiff; PBS = phosphate buffer saline; ppb = parts per billion; ProxEpi = proximal airway epithelium; RLU = relative light units; Rrs = respiratory system resistance; SIM = simvastatin; SMC = smooth muscle cell; TGF β 1 = transforming growth factor- β 1

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AT A GLANCE COMMENTARY

Zeki A, et al.

Background

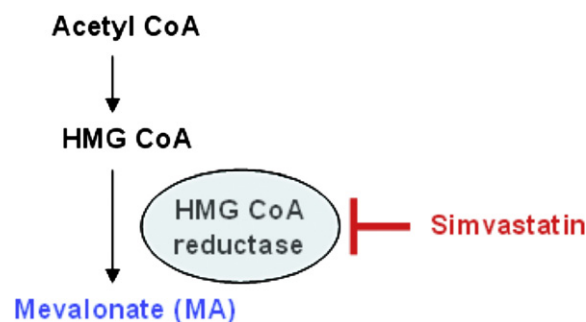
Current antiinflammatory agents do not treat airway remodeling in asthma. The statin drugs are cholesterol-lowering agents that have pleiotropic antiinflammatory and antiproliferative properties. Multiple observational clinical studies indicate that statins may have a novel therapeutic application to asthma and chronic obstructive pulmonary disease.

Translational Significance

The statins inhibit allergic airway inflammation and airway hyperreactivity in mouse models of asthma. Our current study suggests that the statins also may attenuate or prevent airway remodeling by modulating goblet cell hyperplasia and profibrogenic mediators. Although additional studies in a chronic model are warranted, the statins may be a novel treatment for airway remodeling in human asthma.

Asthma is a chronic inflammatory disease that can progress to irreversible airflow obstruction. The constellation of structural airway changes that occur in persistent asthma, termed “airway remodeling,” contributes to a loss of lung function and occur in multiple different lung compartments, manifesting as goblet cell hyperplasia, smooth muscle cell (SMC) proliferation and hypertrophy, subepithelial fibrosis/collagen deposition, loss of airway epithelial barrier, angiogenesis, and fibroblast-to-myofibroblast changes.^{1,2} Airway remodeling has not been eradicated or prevented despite a wide use of antiinflammatory agents.³ Asthmatic patients who have established structural changes to their airways are poorly responsive to corticosteroid therapy,⁴ and no other current asthma therapy is effective in treating this remodeling.

The statin drugs are cholesterol-lowering agents that inhibit the enzyme 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, the rate-limiting step of cholesterol biosynthesis in the mevalonate (MA) pathway (Scheme 1). These drugs also have pleiotropic antiinflammatory, antiproliferative, and immunomodulatory effects beyond their lipid-lowering capacity.^{5,6} Statin use in patients with asthma and chronic obstructive pulmonary disease has been associated with improved lung health in several observational clinical studies.^{7,8} Statins also have been proposed as potential new



Scheme 1. The MA pathway and statin mechanism of action. Sim inhibits the rate-limiting enzyme HMG-CoA reductase to prevent the conversion of HMG-CoA to MA. Reversal of observed statin effects with MA cotreatment indicates that HMG-CoA reductase is the likely target enzyme of the statin.

treatments for lung disease.^{9,10} Two short-term (4 to 8 weeks) clinical trials in asthma did not show a clinical benefit to statin treatment despite improvements in sputum markers of inflammation.^{11,12} However, the effects of statins on preestablished asthmatic airway remodeling may not be apparent after such a brief treatment duration in which lung function already has declined significantly in response to chronic inflammation.

In smoke-exposed rats, simvastatin treatment can attenuate small airway thickening¹³ and inhibit the development of emphysema.¹⁴ However, to date, the effects of statins on the development of asthmatic airway remodeling remain unknown in both animal models and in humans. We previously showed that simvastatin inhibits airway hyperreactivity (AHR) in a mouse model of acute allergic inflammation.¹⁵ This statin-mediated improvement in lung function was *independent* of inflammatory cell influx in which simvastatin likely had additional effects on airway resident cells. Because statins also have cellular antiproliferative effects,⁵ this observation led us to investigate whether statins can attenuate the development of airway remodeling in the ovalbumin (OVA) mouse model.

One of the earliest changes in allergen-induced asthma is goblet cell hyperplasia,¹⁶ which is an important component of long-term remodeling. A host of mechanisms may be responsible for “early remodeling,” such as goblet cell hyperplasia, but recent interest has focused on arginase and transforming growth factor- β 1 (TGF β 1).¹⁷⁻²⁰ Arginase enzymes convert the substrate L-arginine to ornithine, which can be metabolized further to proline and hydroxyproline, important components of collagen. The Th2 cytokines interleukin (IL)-4 and IL-13 induce arginase expression in macrophages and lung tissue leading to subepithelial airway fibrosis.^{21,22} Similarly, TGF β 1 is a critical link

between inflammation and fibrosis, a complex mediator that has both pro- and antiinflammatory properties, as well as being profibrogenic.

Given the clinical implications for asthma treatment, we examined whether systemic treatment with simvastatin, in an acute (2 week) OVA-exposure mouse model, attenuates airway remodeling such as goblet cell hyperplasia and profibrogenic changes. We *hypothesized* that simvastatin treatment inhibits airway goblet cell hyperplasia as well as lung arginase-1 (Arg1) and TGF β 1 protein expression by a MA-dependent mechanism. We used simvastatin to inhibit HMG-CoA reductase and provided MA simultaneously to reverse the statin effect to test the hypothesis that simvastatin was acting specifically on the target enzyme HMG-CoA reductase (ie, via the MA pathway [Scheme 1]).

We found that simvastatin attenuated airway goblet cell hyperplasia and inhibited lung Arg1 protein expression and enzyme activity. The simvastatin effect on goblet cell hyperplasia was reversed with MA cotreatment, suggesting a MA-dependent mechanism for this early hallmark of remodeling. The inhibition of Arg1 by simvastatin is a novel finding in allergic asthma. The statin drugs may be an important modulator of airway remodeling with implications for longer term asthma treatment.

METHODS

Animals. All procedures were performed as per an Institutional Animal Care and Use Committee approved protocol, following institutional standards and regulations for animal care and use. All mice were maintained in a high efficiency particulate air (HEPA)-filtered laminar flow cage rack with a 12-h light/dark cycle and allowed free access to food and water. Animals were housed and cared for by the veterinary staff of Animal Resource Services at the University of California, Davis in Assessment and Accreditation of Laboratory Animal Care accredited facilities in plastic cages over autoclaved bedding in HEPA-filtered cage racks. Animals were screened routinely for health status by serology and histology by our veterinary animal resources facility. Eight-week-old BALB/c mice were purchased from Charles River and allowed to acclimatize for 1 week prior to the initiation of OVA sensitization protocol. All experiments conform to the relevant ethical guidelines for animal research.

Exposure of mice to OVA aerosol. All mice were sensitized by intraperitoneal (i.p.) injection of chicken egg albumin (OVA, grade V, $\geq 98\%$ pure; Sigma, St. Louis, Mo, $2 \times 10 \mu\text{g}/0.1 \text{ mL}$, 2 weeks apart) with alum as an adjuvant²³ starting on day 0. After a 4-week period of sensitization, mice then were exposed to aerosolized

OVA (for 30 min) 3 times a week for 2 weeks (for 6 total exposures or “6OVA”). Mice were divided into the following treatment groups: 6OVA + ethanol (EtOH) (where EtOH solution is the drug vehicle), 6OVA + Simvastatin (Sim), 6OVA + Sim + MA, filtered air (FA) + EtOH, FA + Sim, and FA + phosphate buffer saline (PBS). Sim was purchased from Sigma-Aldrich and prepared as described in Zeki et al 2009.¹⁵ OVA-exposed groups received i.p. injections of 0.3 mL of 10% EtOH (Sim 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). FA-exposed mice also received their corresponding i.p. injections of 0.3 mL of PBS (1 $\times$), 0.3 mL of 10% EtOH, or 0.3 mL of Sim (40 mg/kg). Drug injections were administered 30 min before exposure to FA or 1% OVA aerosol in PBS (10 mL of a 10-mg/mL OVA solution).

To determine whether HMG-CoA reductase inhibition by Sim alters measured endpoints, we exposed mice to inhaled OVA or FA for 2 weeks and treated them with Sim (40 mg/kg, with and without MA [20 mg/kg] cotreatment) or drug vehicle (EtOH) 30 min before all exposures. We previously reported no statistically significant differences in inflammatory cell influx between the 6OVA + EtOH and 6OVA + MA groups or their respective air controls.¹⁵ To decrease the unnecessary use of mice, the experiments in the present study do not include these MA control groups (6OVA + MA and FA + MA).

Exposures to OVA aerosols were performed with chambers and generators as described previously.²⁴ Aerosol delivery was conducted via a side stream nebulizer (Invacare Corp., Elyria, Ohio) and air compressor (Invacare Corp., Sanford, Fla). Mass concentration and aerodynamic size distributions were determined with a Mercer-type cascade impactor.²⁴ Histological evaluations of OVA-exposed and FA-exposed control mice lungs demonstrated that we could induce airway inflammation, epithelial cell sloughing, and goblet cell hyperplasia in the OVA-exposed mice.

After completion of the final exposure, all mice underwent lung physiology studies with a full-body plethysmograph for restrained animals. Immediately thereafter, mice were killed with intraperitoneally administered Beuthanasia-D (0.3 mL at 1:6 dilution) followed by a collection of tissue for analysis (blood draw, lung lavage, and lung isolation).

Airway inflammation. Lungs were lavaged with 2 1-mL aliquots of PBS, at pH 7.4. Each aliquot was passaged twice through the lungs. The lavage fluid was centrifuged (at 2500 rpm for 10 min) to pellet the cells, and the supernatant was removed for inflammatory cytokine profiling. The remaining cell pellet was

suspended in an AKC lysis buffer (0.15 M NH_4Cl , 1 mmol/L KHCO_3 , 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 7.3) to lyse red blood cells and finally was resuspended in 0.5-mL PBS.

Total lavage live cell numbers were determined using Trypan blue exclusion, and the total live cells per mL lavage was calculated using a hemacytometer. Aliquots of 100 μL of the remaining cell suspension were processed onto slides using a cytocentrifuge at 1650 rpm for 15 min. Slides were air dried and stained with a Hema3 stain set as described in the manufacturer's instructions (Fisher Scientific, Kalamazoo, Mich) and sealed using Cytoseal (Richard Allen Scientific, Kalamazoo, Mich). Cell percent differentials for alveolar macrophages were determined by counting 10 fields under a 40 $\times$ objective based on morphological characteristics and staining profiles.

Histological preparation. Half of the animals had their lungs fixed for histological evaluation at 30-cm pressure using 1% paraformaldehyde in PBS (pH 7.5). After 24 h of fixation, the left lung lobes were placed in 70% EtOH and embedded in paraffin. Lung sections of 5- μm thickness were made with special attention to cutting through the larger lobar bronchi in parallel then slides were dried at 37°C overnight. Lung sections were deparaffinized and processed for Arg1- or TGF β 1-specific immunohistochemistry or Alcian Blue-Periodic acid-Schiff (PAS) staining to quantify the number of mucus-containing goblet cells.

Whole lung lavage cytokine assays. The concentrations of helper T-cell type 2 (Th2) cytokines (IL-4, IL-13) and TGF β 1 in lung lavage supernatant were measured with commercially available multiplex assays (Millipore, St. Charles, Mo). The assay for TGF β 1 measured the active dimerized form of the protein. For cytokine measurements that were below the lower detection limit of the assay, results were assigned a value equal to the minimal detection limit for the specific assay to facilitate statistical analysis.

Lung compliance and resistance measurements. Mice were anesthetized and sedated with medetomidine 0.5 mg/kg (Dormitor; Orion Pharma, Espoo, Finland) as well as tiletamine/zolpidem 50 mg/kg (Telazol; Fort Dodge Laboratories, Fort Dodge, Iowa) and ventilated at 7 to 8 cm^3/kg with a mouse ventilator (MiniVent, Harvard Apparatus, Cambridge, Mass) at a frequency of 150 breaths per minute for the duration of the procedure. Dynamic lung compliance (C_{dyn}, as mL/cm of H_2O) and respiratory system resistance (R_{rs}, as cm of $\text{H}_2\text{O}^*\text{s/mL}$) measurements were made at baseline after serial 3-min nebulizations of saline and methacholine (MCh) at doses of 0, 0.5, 1.0, and 2.0 mg/mL. Dynamic compli-

ance and R_{rs} were measured using a plethysmograph for restrained animals (Buxco Inc., Troy, NY) as described previously.²⁵ The *percent change* (% decrease in C_{dyn} or % increase in R_{rs}) is calculated as the *difference* between the physiologic parameter (C_{dyn} or R_{rs}) measured at baseline and after the third MCh bronchial challenge, *divided by* the baseline value (for % decrease in C_{dyn}) or by the value measured after the third MCh bronchial challenge (for % increase in R_{rs}).

Exhaled nitric oxide (FeNO) measurements. Prior to the MCh dose-response challenge, a 5-min sample of exhaled gases was collected from the cannulated mice (immediately after the insertion of a mouse into the plethysmograph) via the ventilator exhalation port during the PBS exposure and recovery periods (ie, saline time-point). Samples were collected in specially constructed Mylar bags, and the fraction of exhaled nitric oxide (FeNO) was measured in parts per billion by a chemiluminescence assay using the Sievers Nitric Oxide analyzer (Sievers Inst., Boulder, Col). Placement of the Mylar bag did not affect pressure measurements.²⁶

Measurement of nitrate/nitrite in whole lung homogenate. Nitrate and nitrite in an isolated whole lung tissue homogenate were measured after reduction with acidified vanadium III using the Sievers NO Analyzer (Sievers, Boulder, Col) as an indicator of NO production. For specimens, 20 μg of total protein was deproteinized using 10% trichloroacetic acid. Samples were centrifuged at 14,000 rpm on a tabletop centrifuge, and the supernatant was used to measure nitrate and nitrite content.

Alcian blue-PAS staining. Left lung sections prepared as described were immersed in a 1% alcian blue and 3% glacial acetic acid solution (pH 2.5) for 30 min and then immediately rinsed in tap water. Slides then were immersed in a 1% periodic acid solution for 7 min. Slides were rinsed 3 times in distilled water and immersed in Schiff reagent solution consisting of 0.45% basic fuchsin, 10% HCl, and 0.45% sodium bisulfite for 15 min. Slides were immersed repeatedly in running tap water until clear and then counterstained by flash immersion in Harris' hematoxylin²⁷ and immediately rinsed repeatedly in running tap water. Slides then were dehydrated and mounted in cytooseal.

Each animal was represented by a single section of lung; 5 randomly selected regions were evaluated (2 segments of the primary conducting airway, 2 segments from separate secondary conducting airways, and 1 segment from a tertiary conducting airway). A minimum of 100 sequential airway epithelial cells were counted from each region, and the total number of PAS-positive cells per total epithelial cells was determined

for each region. These regional values then were averaged to give a final PAS score per animal termed "mean goblet cell index."

Western blot analysis of tissue. Western blot methods are described in Bratt et al with changes in methods described subsequently.²⁸ Antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, Calif) unless otherwise stated. Right lung lobes were sonicated in a homogenization buffer and centrifuged on ice at 10,000 rpm for 20 min. The supernatant was stored at -80°C until it was used again. The total protein concentration was measured for each sample using the Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, Ill). Samples (75 μg total protein) were electroporated under reducing conditions and transferred to a polyvinylidene difluoride membrane. Membranes were probed using 0.8 $\mu\text{g}/\text{mL}$ rabbit, antimouse NOS2; 0.6 $\mu\text{g}/\text{mL}$ rabbit, antimouse NOS3; 0.4 $\mu\text{g}/\text{mL}$ of goat, antimouse Arg1; 0.4 $\mu\text{g}/\text{mL}$ rabbit, antimouse TGF β 1; or 0.4 $\mu\text{g}/\text{mL}$ rabbit, antimouse α -Actinin IgG in 5% dry milk in PBS and 40 ng/mL horseradish peroxidase (HRP)-conjugated goat antirabbit IgG (Pierce Biotechnology, Rockford, Ill) or 40 ng/mL HRP-conjugated donkey antigoat IgG (Pierce Biotechnology, Ill) secondary antibodies in 5% milk in PBS. Bands were visualized using Western Lightning Plus-ECL substrate kit (PerkinElmer, Shelton, Conn) and band intensity was measured using the Fujifilm Image Reader LAS-3000 V2.1 (Fuji Photo Film Co, Cypress, Calif).

Measurement of total arginase activity. The arginase activity assay of tissue homogenates was adapted from Xu et al²⁹ In brief, right lung lobe homogenates from OVA-exposed mice were aliquoted into volumes containing 10- μg total protein, and FA-exposed animals were aliquoted to volumes containing 30- μg total protein. Tissue homogenate volumes were brought to a volume of 50 μL using homogenization buffer as described in the section on Western blot analysis.

The homogenate mixture was incubated with 100 μL 0.1% Triton X-100 for 30 min at room temperature on a shaker. After incubation, 100 μL of 25 mmol/L Tris-HCl (pH 7.5) and 20 μL of 10 mmol/L MnCl_2 was added to each sample and incubated at 56°C for 10 min. Samples then were incubated with 100 μL 0.5 M L-arginine at 37°C for 1 h, and the reaction was stopped with 900 μL of a solution containing 8.7% H_2SO_4 , 23.2% H_3PO_4 , and 68% H_2O (1:3:7 of 96% H_2SO_4 : 85% H_3PO_4 : water). The sample was mixed with 40 μL of 9% isonitrosopropiophenone dissolved in EtOH and heated to 95°C for 30 min. The relative urea concentration was calculated using a spectrophotometer at 540

nm, using a linear standard curve of arginase activity derived from a liver homogenate dilution series. The unit of measure for the total arginase activity was represented by absorbance at 540 nm.

Arg1 and TGF β 1 immunohistochemistry. Left lung sections were prepared as described. Slides were incubated in 1 mmol/L EDTA, pH 7.5 at 100°C for 20 min to decloak the antigen. After rinsing in deionized water, sections were processed using the R&D Systems Cell and tissue staining kit HRP-DAB System (Minneapolis, Minn) for rabbit antibodies. Sections were incubated overnight at 4°C in 1 $\mu\text{g}/\text{mL}$ goat antimouse Arg1 IgG diluted in 5% goat serum in 1% bovine serum albumin (BSA), 1.33 $\mu\text{g}/\text{mL}$ rabbit antimouse TGF β 1 IgG, 1 $\mu\text{g}/\text{mL}$ goat IgG (isotype control), or serum + BSA only (negative control). All other reagents were diluted as per the manufacturer's instructions. Incubation times were as follows: 1 h in biotinylated goat antirabbit or biotinylated donkey antigoat secondary antibody, 30 min in high sensitivity streptavidin-conjugated HRP, and 15 min in diaminobenzidine.

Results were scored independently by 2 blinded observers for TGF β 1 immunohistochemical staining, with the lung tissue divided into the following compartments: proximal airway epithelium (ProxEpi), distal airway epithelium (DistEpi), airway and vascular SMCs, parenchyma, macrophages, and basement membrane (BM). For the Arg1 assessment, the lung tissue compartments were divided into airway epithelial and subepithelial compartments for the qualitative assessment of immunohistochemical staining. Arg1 staining was defined as present or absent, and if present, it also was defined as weak, moderate, or strong. For TGF β 1, a grading system of 0 to 5 was established prior to the grading based on prestained slide standards. All slides were scored under $\times 200$ power using a linear intensity grading scale: (0 being no stain as compared with a primary antibody negative control to 5 being dramatically increased staining).

Hydroxyproline measurement in isolated airways. The colorimetric assay method was based on methods derived from Woessner³⁰ with the modifications described subsequently. In brief, fresh right lung lobes were transferred to EtOH and microdissected, producing a preparation of airways separate from the associated parenchyma. These preparations consisted of the right mainstem bronchus to the terminal bronchioles of the four right lobes. Airways then were homogenized in 500- μL milliQH $_2\text{O}$ with a 50- μL aliquot removed for bicinchoninic acid (BCA) assay of total protein content and the remaining sample centrifuged at 3000 rpm for 10 min. The supernatant

was removed and the pellet resuspended in 100 μ L of 6 M HCl. The suspension was transferred to a glass tube, sealed, and incubated overnight at 110°C. Tubes then were cooled to room temperature and opened. A volume of 30 μ L 0.1% phenol red was added to each sample. Samples were neutralized with 80 μ L 6 M NaOH and 100 μ L of solution was transferred to glass test tubes for analysis. Samples were normalized to the total protein content and treatment groups were compared using statistical tests as described in the Results section.

Statistical analysis. Results are presented as mean values $\pm$ standard error of the mean. Means were compared by the unpaired Student *t* test or analysis of variance (ANOVA, 1-way or 2-way), with Tukey's or Bonferroni correction for multiple comparisons applied where appropriate, using the Prism 5 software package (Graphpad, Inc., San Diego, Calif). Nonparametric analyses were done using the Kruskal–Wallis test with Dunn's posttest correction or the Mann–Whitney test. Standard linear regression analysis was done for all 2-dimensional correlations. Linear regression analysis of data was derived from individual animal measurements to determine correlations. The D'Agostino and Pearson omnibus test was used to evaluate data for normality (an $\alpha < 0.05$ was used to indicate normality). $P < 0.05$ was taken to indicate statistical significance. Values that differed by more than 2 standard deviations from the mean were excluded from the statistical analysis where appropriate.

RESULTS

Fraction of exhaled FeNO. Sim reduced *in vivo* FeNO by 61.7% ($P = 0.0019$) to air control levels, suggesting that NO production is altered by Sim treatment (Fig 1, A). Although a trend was noted of increased FeNO production after MA cotreatment, this small reversal of Sim's effect on FeNO did not reach statistical significance ($P =$ not significant [NS]), indicating a possible MA-independent mechanism of action.

Nitrate/nitrite in whole lung homogenate. Treatment with Sim did not alter whole lung nitrate/nitrite content, an indicator of NO production ($P =$ NS) (Fig 1, B). A trend was noted toward higher nitrate/nitrite levels in the air control groups compared with the OVA groups. This observation was confirmed when the groups were analyzed in aggregate (ie, all OVA animals compared with all FA animals) in which the FA animals on average had 22.2% higher nitrate/nitrite levels than the OVA animals ($P = 0.0164$ by Mann–Whitney test, data not shown).

Lung NOS2 and NOS3 protein expression. Statins can alter the expression of both NOS2 and NOS3 in various

tissues including lungs, airway epithelial cells, and endothelium.^{31–35} In our experimental model, Sim treatment did not alter NOS2 or NOS3 protein expression (data not shown) in whole lung homogenate as measured by Western blot ($P =$ NS by 1-way ANOVA). A trend was noted toward higher NOS2 expression in the OVA groups compared with their corresponding FA controls, but this value was not statistically significant. However, when all combined OVA groups are compared with all FA groups, the OVA mice had 38% higher NOS2 protein expression than the FA mice ($P = 0.031$ by *t* test, data not shown). NOS1 was not assessed in this experiment.

Airway goblet cell hyperplasia. Airway goblet cell hyperplasia is one of the earliest hallmarks of airway remodeling in the OVA mouse model. In OVA-exposed mice, Sim treatment reduced goblet cell hyperplasia (measured as mean goblet cell index) by 33% ($P < 0.005$), with MA cotreatment reversing this inhibitory statin effect ($P < 0.05$) (Fig 2, *i*). Alcian Blue-PAS staining of representative lung sections showed marked reductions in mucus staining with Sim treatment as well as a reversal of this statin effect with MA cotreatment (Fig 2, *ii*). As expected, the air controls showed no evidence of mucus-producing goblet cells staining.

Lung arginase expression, enzyme activity, and immunohistochemistry. To determine whether Sim treatment affects lung arginase, we evaluated Arg1 protein expression, arginase enzyme activity, and Arg1 tissue localization. Arg1 protein expression and total enzyme activity were evaluated using whole lung homogenate. Sim treatment reduced Arg1 protein expression, as measured by Western blot, by 59.4% ($P < 0.05$) (Fig 3, A), with MA cotreatment partially reversing this inhibitory statin effect; however, this result was NS ($P =$ NS). Total arginase enzyme activity (Arg1 and Arg2) followed the same pattern as the Arg1 protein expression, with Sim reducing arginase activity by 57% ($P < 0.005$) in OVA-exposed mice (Fig 3, B), with MA cotreatment reversing the statin effect ($P = 0.0275$ by *t* test). Arg1 protein expression was barely detectable in the air control (FA) groups (Fig 3, A) with concordant low total arginase enzymatic activity (Fig 3, B). The total arginase enzyme activity correlated positively with Arg1 protein expression ($r^2 = 0.70$, $P < 0.0001$) (Fig 3, C).

Immunohistochemical staining of lung sections for the localization of Arg1 protein expression showed no Arg1 staining in the FA control groups (Fig 4, D); however, intense staining occurred of inflammatory alveolar macrophages and components of the airway subepithelial compartment in the OVA groups (Fig 4, A–C). An apparent trend was noted with Sim reducing Arg1 subepithelial staining in the OVA group (Fig 4, B) and with

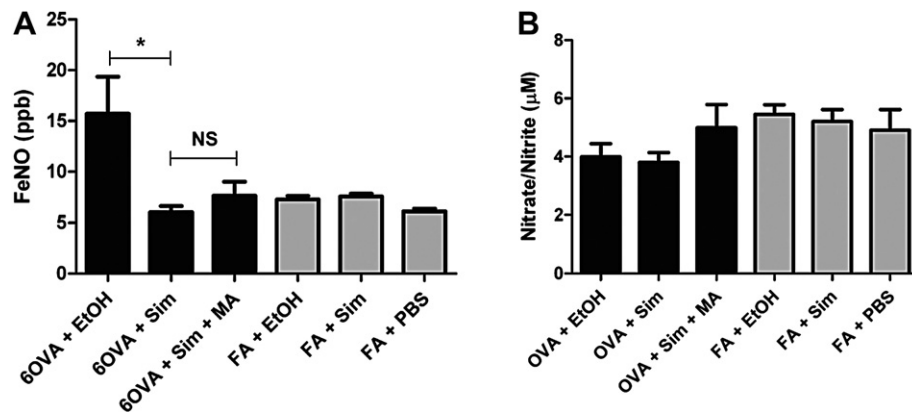


Fig 1. (A) FeNO, The effect of Sim on *in vivo* FeNO measurements as described in Methods. Sim (40 mg/kg) treatment decreased FeNO by 61.7% (* $P < 0.0019$ by Kruskal–Wallis test) down to air control levels. Sim and MA (20 mg/kg) cotreatment did not reverse the inhibitory statin effect ($P = \text{NS}$, Kruskal–Wallis test). Each treatment group had between 5 and 8 mice. (B) Whole lung homogenate nitrate/nitrite levels. Treatment with Sim did not alter the whole lung nitrate/nitrite content, an indicator of NO production ($P = \text{NS}$ by 1-way ANOVA). Each treatment group had between 6 and 9 mice.

MA cotreatment only partially abrogating this Sim effect (Fig 4, C); however, no difference was noted in the intensity of Arg1 staining in peribronchiolar and alveolar macrophages. Changes observed in the Arg1 staining of the subepithelial compartment were likely a result of the Sim-dependent reduction in peribronchiolar inflammation, including a 48.3% reduction in lung lavage macrophages ($P < 0.005$) that is MA-dependent as shown in Fig 7, B. Most eosinophils did not stain positively for Arg1 in the OVA groups. All treatment groups showed no evidence of Arg1 staining in airway SMCs. Nearly all OVA-exposed mice showed no appreciable Arg1 staining in the airway epithelium (Fig 4, A–C); however, we did not distinguish between columnar ciliated cells and goblet cells. Negative control experiments with an isotype control antibody showed no brown color staining (data not shown).

Correlations of arginase to inflammation and lung function. Because IL-13 and IL-4 are known to increase Arg1 expression, we evaluated whether Arg1 protein expression correlated with inflammation or lung lavage IL-4 and IL-13 concentrations. We previously published IL-4/IL-13 measurements;¹⁵ however, these current comparisons with Arg1 are new and not previously reported. Whole lung Arg1 positively correlated with lung lavage total cell count ($r^2 = 0.56$, $P < 0.0001$), eosinophil count ($r^2 = 0.56$, $P < 0.0001$), macrophage count ($r^2 = 0.42$, $P < 0.0001$), and lymphocyte count ($r^2 = 0.46$, $P < 0.0001$) (data not shown). Both IL-4 and IL-13 also positively correlated with Arg1 protein expression ($r^2 = 0.78$, $P < 0.0001$ for IL-4 and $r^2 = 0.56$, $P = 0.0003$ for IL-13; Fig 5).

Arg1 protein expression was also positively correlated with AHR as calculated by the percent increase in Rrs ($r^2 = 0.45$, $P = 0.0044$) (Fig 6, A) and the percent decrease in Cdyn ($r^2 = 0.78$, $P < 0.0001$) (Fig 6, B), in response to the MCh bronchial challenge. We previously published aspects of this lung physiology data (Cdyn and Rrs); however, the data presented as “percent increase” or “percent decrease” and the comparisons to Arg1 are new and not previously reported.

Airway hydroxyproline content. The measurement of the total hydroxyproline content from microdissected airways indicated no significant differences among all 6 treatment groups at the time point examined ($P = \text{NS}$ by 1-way ANOVA). In general, the OVA groups did not demonstrate a higher hydroxyproline content compared with air controls. In addition, no significant difference was found between the combined OVA versus the combined FA groups ($P = 0.128$ by t test, data not shown). Sim did not alter the hydroxyproline content in the OVA-exposed mice, nor was it different from the other OVA groups or air controls ($P = \text{NS}$ by 1-way ANOVA) (data not shown).

Lung TGFβ1: Lung lavage concentration, protein expression, and immunohistochemistry. TGFβ1 protein is expressed in several different forms in tissues where we focused on the precursor and on the active, dimerized forms of this protein. We evaluated the levels and locations of TGFβ1 expression in mouse lung and in different compartments after OVA and FA exposures with Sim and MA cotreatment.

In the lung lavage fluid, the 6OVA + EtOH group had a significantly higher TGFβ1 concentration than any of the FA controls ($P < 0.005$), but Sim treatment or MA

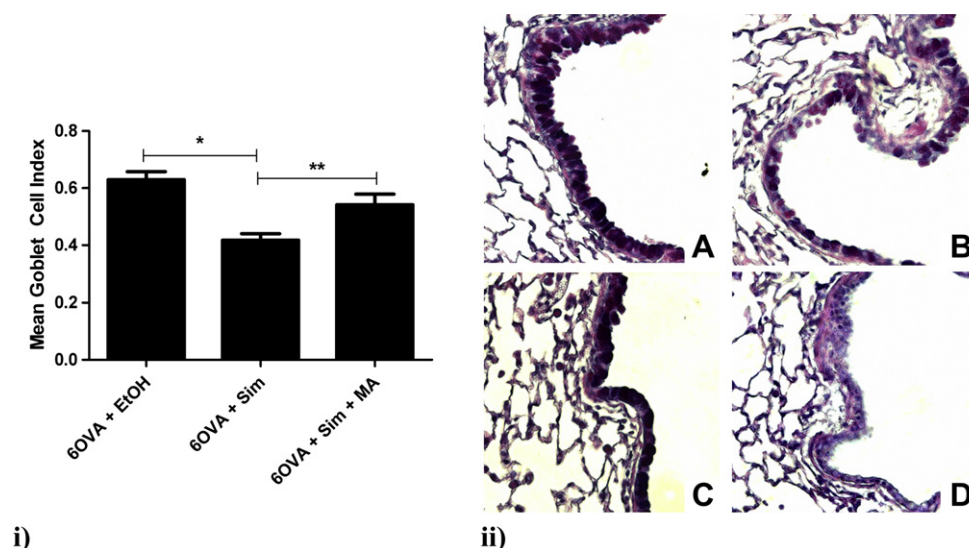


Fig 2. Airway goblet cell hyperplasia. (i) Treatment with Sim (40 mg/kg) reduced the mean goblet cell index (see Methods section) by 33% (* $P < 0.005$ by 1-way ANOVA). Cotreatment with MA (20 mg/kg) reversed this inhibitory statin effect (** $P < 0.05$ by 1-way ANOVA). The air controls are not shown because virtually no goblet cells were visualized (see Fig 2 (ii) image (D)). Each treatment group had between 4 and 6 mice. (ii) The following different treatment groups observed at an original magnification of $\times 400$ were stained with Alcian Blue-PAS to show goblet cells in the airway epithelium: (A) 6OVA + EtOH, (B) 6OVA + Sim, (C) 6OVA + Sim + MA, and (D) FA + PBS. The OVA group treated with Sim (6OVA + Sim) has significantly less goblet cell staining corresponding to the 33% decrease in mean goblet cell index as described in (i).

cotreatment did not significantly affect lavage TGF β 1 concentration (Fig 7, A).

No statistically significant differences were noted in the total TGF β 1 (precursor and dimer) protein expression between the OVA and FA control groups ($P = \text{NS}$ by Kruskal–Wallis test with Dunn posttest correction, data not shown). The “total” TGF β 1 protein was calculated as the sum of the precursor and dimer protein levels as measured by Western blot simultaneously (ie, relative light units [RLU] or band intensity). The combined OVA groups versus the FA groups showed a trend of higher total TGF β 1 expression in the OVA animals, but this trend did not reach statistical significance ($P = 0.061$ by Mann–Whitney test, data not shown). Separate evaluations of the TGF β 1 precursor and dimer protein expression also did not yield any statistically significant differences among the groups ($P = \text{NS}$ by 1-way ANOVA, data not shown). Sim (40 mg/kg) treatment and MA cotreatment both did not alter the total TGF β 1 protein expression in the OVA groups (by 1-way ANOVA, data not shown). In the OVA treatment groups, each group had 16 to 18 mice, and in the air controls, each group had 6 mice.

Sim treatment decreased the lung lavage absolute macrophage count by 48.3% ($P < 0.005$) in OVA-exposed animals, with MA cotreatment reversing the Sim inhibitory effect ($P = 0.0029$ by t test) (Fig 7, B). By qualitative immunohistochemical assessment,

macrophages seem to be the main inflammatory cells that stain intensely for TGF β 1, as compared with eosinophils or lymphocytes (Fig 7, C).

A semiquantitative assessment of TGF β 1 immunohistochemical staining in 6 different lung compartments was completed as described in the Methods section. Sim treatment did not significantly alter TGF β 1 staining in any of the 6 different lung compartments evaluated ($P = \text{NS}$) (Fig 8). In the ProxEpi, the air controls (FA + Sim and FA + PBS) had a greater TGF β 1 content compared with the OVA groups, especially 6OVA + EtOH ($P < 0.05$) (Figs 8, *i* and *ii*). In contrast, the lung parenchyma of all 3 air control groups had a much lower TGF β 1 content compared with the 6OVA + EtOH group ($P < 0.005$) but with the 6OVA + Sim + MA group showing a reduced TGF β 1 content compared with the 6OVA + EtOH group ($P < 0.005$) (Figs 8, *iii* and *iv*).

In the BM, the air controls had significantly less TGF β 1 content than the OVA groups, specifically 6OVA + EtOH ($P < 0.05$) and 6OVA + Sim ($P < 0.05$) (Fig 8, *vi*). The DistEpi (Fig 8, *v*) and the airway and vascular SMCs showed no differences among the 6 treatment groups ($P = \text{NS}$, by 1-way ANOVA) (data not shown).

Macrophages had a 2-fold higher TGF β 1 content in the combined OVA groups compared with the air controls ($P = 0.0003$ by t test, data not shown), but this finding did not demonstrate an individual significance

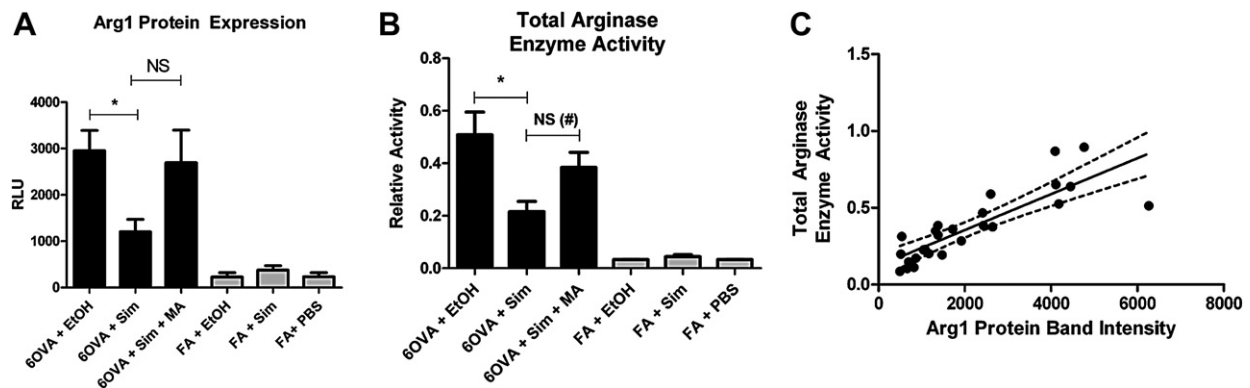


Fig 3. (A) Arg1 protein expression in whole lung homogenate by Western blot. Sim (40 mg/kg) reduced Arg1 protein expression by 59.4% ($*P < 0.05$ by 1-way ANOVA). Cotreatment with MA (20 mg/kg) reversed the inhibitory statin effect on Arg1 expression, but this was not statistically significant ($P = NS$ by 1-way ANOVA, $P = 0.057$ by t test). Each treatment group had between 8 and 12 mice. (B) Total arginase enzyme activity in whole lung homogenate. Sim (40 mg/kg) reduced total arginase enzyme activity by 57% in the OVA group ($*P < 0.005$ by 1-way ANOVA). Cotreatment with MA (20 mg/kg) reversed the statin effect (not significant by 1-way ANOVA but significant by t test [$*P = 0.0275$]). Each treatment group had between 6 and 9 mice. (C) Correlation between total arginase enzyme activity and Arg1 protein expression. Arg1 protein expression is represented by protein band intensity (measured by Western blots of whole lung homogenates). Total arginase enzyme activity correlated positively with Arg1 protein expression ($r^2 = 0.70$, $P < 0.0001$). The dotted lines above and below the best-fit line represent the 95% confidence interval. Each dot represents one mouse.

across the different treatment groups ($P = NS$, by 1-way ANOVA) (data not shown). These lung macrophages seem to be the predominant inflammatory cells responsible for TGF β 1 production during OVA-induced allergic inflammation, with TGF β 1 immunohistochemically staining strongest in lung macrophages and little or no staining occurring in eosinophils or lymphocytes. Negative control experiments with isotype control antibody showed no brown color staining (data not shown).

DISCUSSION

In our previous study, we established that treatment with Sim decreases the total inflammatory cell load and eosinophilic airway inflammation via a MA-dependent mechanism and that the MA-independent inhibition of AHR by Sim was likely a result of additional effects on airway resident cells.¹⁵ Using this same acute mouse model of allergic airway inflammation, we now have studied the effects of Sim on the development of *early* hallmarks of airway remodeling. We investigated whether Sim reduces the expression of remodeling markers such as goblet cell hyperplasia as well as profibrogenic mediators in the lung such as Arg1, TGF β 1, and hydroxyproline. Given the “pleiotropic effects” of statins, we also performed experiments with simultaneous Sim and MA cotreatment to test whether Sim’s mechanism of action was via an inhibition of the target enzyme HMG-CoA reductase (Scheme 1).

The key findings from this study are that systemically administered Sim attenuated goblet cell hyperplasia as

well as inhibited lung Arg1 protein expression and total arginase enzyme activity. Arg1 protein expression correlated positively with measurements of AHR (increased lung resistance and decreased lung compliance) and with increased lung lavage IL-4 and IL-13 concentration. Despite a reduction of exhaled NO by Sim, we did not see changes in NOS2 or NOS3 protein content in the lung or in the lung tissue intracellular nitrite/nitrate content. Histologic examination of lung sections did not reveal significant airway SMC hypertrophy or hyperplasia in any of the 2-week OVA-exposed mice.

For all measured endpoints, the Sim effect was MA-independent, except for goblet cell hyperplasia in which the inhibitory statin effect was MA-dependent, indicating a statin inhibition of HMG-CoA reductase (Fig 2, Scheme 1). The MA pathway leads to cholesterol and other crucial lipid intermediates (the isoprenoids FPP and GGPP) that are necessary for small guanosine triphosphatases (eg, Ras and RhoA, respectively) cell-membrane-anchoring, and subsequent intracellular signal transduction. Cellular inflammation, transmigration, and proliferation depend on the availability of these isoprenoids downstream of MA. This finding suggests that FPP and/or GGPP may be playing a role with respect to goblet cell hyperplasia.

Kim et al also found a reduction in the percentage of goblet cells (approximately a 69% reduction) in BALB/c mice treated with Sim; however, the authors did not suggest a potential mechanism for this inhibition other than a decrease in IL-5.³⁶ The reduction in goblet cell

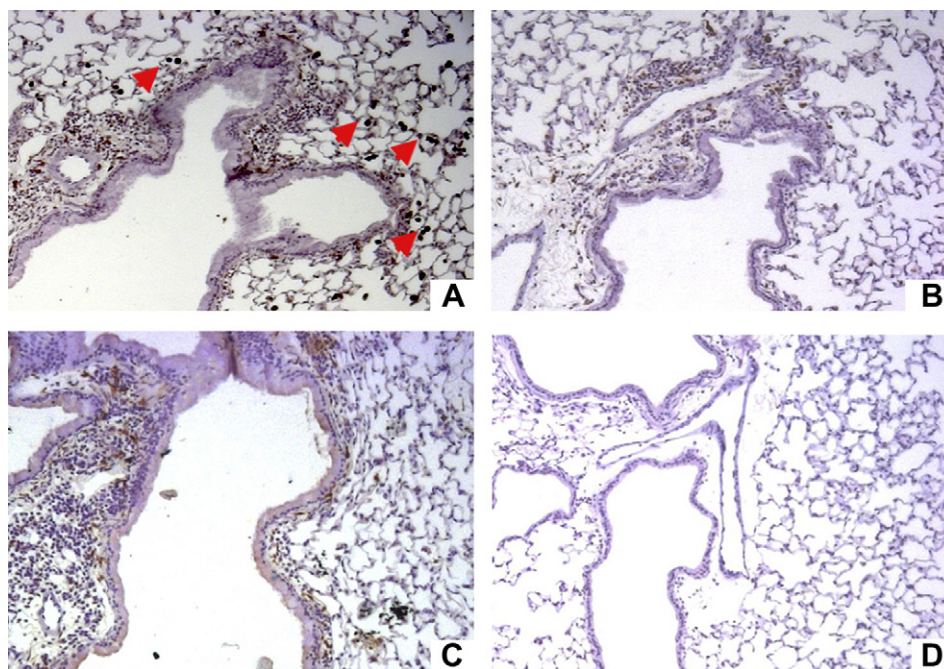


Fig 4. Immunohistochemical staining of Arg1. The 4 different treatment groups are observed at an original magnification of $\times 100$, where Arg1 stains brown. We evaluated the epithelial and subepithelial compartments, of which the latter contains the airway SMCs, inflammatory cells, and extracellular space. No Arg1 staining of airway SMCs occurred in any of the treatment groups. (A) 6OVA + EtOH. Arg1 stains dark brown and localizes to the subepithelial space and in macrophages (red arrow heads). Little-to-no Arg1 staining was visualized in airway epithelial cells. (B) 6OVA + Sim. Sim treatment (40 mg/kg) of OVA-exposed mice attenuated Arg1 staining in the subepithelial space. No differences were observed compared with 6OVA + EtOH in macrophage Arg1 staining by this qualitative assessment. (C) 6OVA + Sim + MA. Cotreatment with MA (20 mg/kg) partially abrogated the Sim effect on Arg1 staining in the subepithelial compartment. No significant differences were observed with respect to macrophage Arg1 staining. (D) FA + PBS. No Arg1 staining was noted in this air control group.

hyperplasia found by Kim et al is more than twice that in our study (69% vs 33%), perhaps because of methodological differences. In a rat model of lipopolysaccharide-induced airway mucus hypersecretion, Ou et al suggest that the inhibition of goblet cell hyperplasia by Sim is dose-dependent and is mediated via an inhibition of Muc5AC expression and RhoA/p38 pathways.³⁷ Alternatively, the statin effect on goblet cells may be driven by potent IL-13 inhibition,¹⁵ which is a key cytokine that drives Muc5AC expression, mucin synthesis, and ciliated cell to goblet cell phenotypic change.³⁸ More work is needed to elucidate the underlying mechanism(s) of this observation because goblet cell hyperplasia and mucus production are key elements of airway remodeling and fatal asthma.³⁹

Arginase plays an important role in asthma and has been correlated with allergic lung inflammation, AHR, and airway remodeling in both humans and in animal models.^{40,41} Treatment with Sim significantly reduced lung Arg1 protein expression and overall arginase enzyme activity, which is a novel finding in asthma

(Figs 3, A and B), and potentially may be mediated by the MA pathway. The arginase activity assay measures total arginase (which includes both isoforms Arg1 and Arg2); however, linear regression analysis of arginase activity versus Arg1 protein expression shows a strong correlation between these 2 parameters (Fig 3, C), indicating that the Arg1 isoform contributes to a significant portion of the total arginase enzyme activity in the inflamed lung.

Immunohistochemical staining showed Arg1 throughout the lung in which positive, strong staining was observed in the subepithelial peribronchiolar space, particularly among infiltrating inflammatory cells (predominantly macrophages, Fig 4, A), consistent with previous reports in mouse models.¹⁸⁻²⁰ Although no changes were observed in the intensity of Arg1 staining in macrophages, Sim reduced Arg1 staining in the subepithelial peribronchiolar space (Fig 4, B) by reducing the total macrophages as depicted in lung lavage macrophage counts (Fig 7, B).

Most OVA-exposed mice showed no appreciable Arg1 staining in the airway epithelium (Figs 4, A–C).

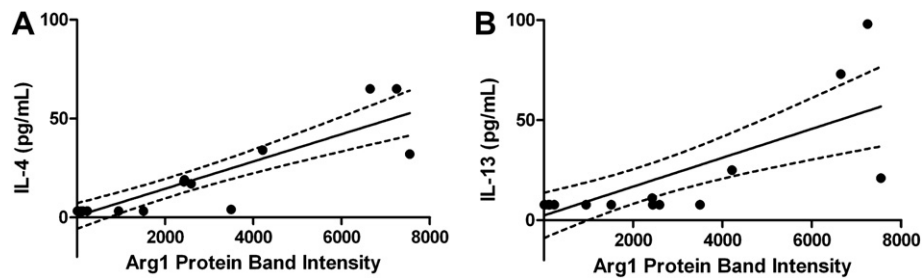


Fig 5. Correlations between Th2 cytokines and Arg1 protein expression. Arg1 protein expression is represented by protein band intensity (equivalent to RLU) as measured by Western blots of whole lung homogenates. The dotted lines above and below the best-fit line represent the 95% confidence interval. Each dot represents 1 mouse. (A) The whole lung lavage IL-4 levels correlated positively with increased Arg1 protein expression in whole lung ($r^2 = 0.78$, $P < 0.0001$). (B) The whole lung lavage IL-13 levels correlated positively with increased Arg1 protein expression in whole lung ($r^2 = 0.56$, $P = 0.0003$).

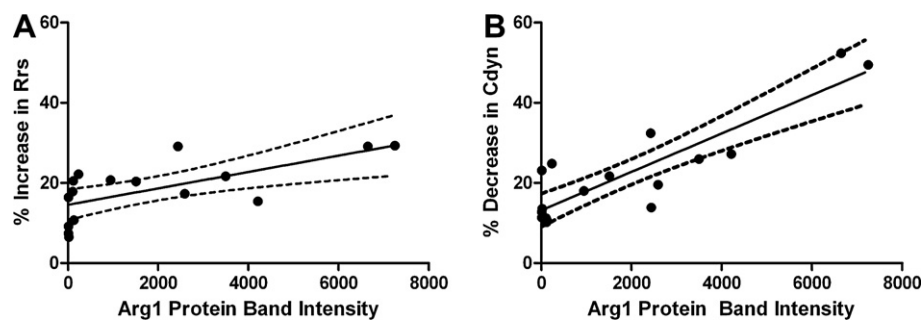


Fig 6. Correlations between lung physiology and Arg1 protein expression. Arg1 protein expression is represented by protein band intensity (equivalent to RLU) as measured by Western blots of whole lung homogenates. The dotted lines above and below the best-fit line represent the 95% confidence interval. Each dot represents 1 mouse. (A) The percent increase in Rrs correlated positively with increased Arg1 protein expression in whole lung ($r^2 = 0.45$, $p = 0.0044$). (B) The percent decrease in Cdyn correlated positively with increased Arg1 protein expression in whole lung ($r^2 = 0.78$, $P < 0.0001$).

When evaluating specimens obtained from human asthmatics and mouse models of allergic asthma, North et al found significant Arg1 staining in the airway epithelium of both acute and chronic OVA-exposed mice, perhaps more intensely in the chronically (12-week) exposed mice.¹⁹ Their acute exposure was for 3 weeks, whereas ours was for 2 weeks, but it is not clear whether the time course alone can explain the difference between our results and that of North et al. Evidence to support this possibility comes from Takemoto et al, where *Dermaphagoides farinae* (Df)-exposed extract mice at day 12 also showed no evidence of Arg1 staining in airway epithelial cells.²⁰ Similarly, in an OVA exposure model for 12 days, no Arg1 staining in the airway epithelium was noted.¹⁸ Zimmermann et al noted a mostly subepithelial Arg1 mRNA signal (by *in situ* hybridization) in OVA and *Aspergillus fumigatus* extract mouse models of asthma; however, patchy areas of Arg1 mRNA positive airway epithelial cells were noted in an endobronchial specimen from a human asthmatic.⁴² One potential explanation for the lack of Arg1 expression in airway

epithelia in our model is the high density of goblet cells observed at 2 weeks of OVA exposure, which we previously showed to be as high as 74.3% of all epithelial cells at this time point.¹⁶ The goblet cell percentage decreases to 9.2% after 8 weeks of OVA exposure; thus, a chronic exposure model is more likely to reveal the presence (or absence) of Arg1 staining. It is plausible that, in more chronic allergen exposure models (or different mouse strains) and established human asthma, the airway epithelium expresses Arg1 more consistently than that observed in more acute models such as ours.⁴³

The mechanism of Sim's inhibition of Arg1 protein expression and enzyme activity is not clear from our experiments. One possible explanation is a reduction in IL-13, which is known to be a strong inducer of Arg1 in asthma.⁴² In addition to IL-13, the Th2 cytokine IL-4 also is known to induce arginase expression.⁴⁴ This finding is supported by positive linear correlations between these 2 cytokines and Arg1 protein expression (Fig 5). We and others previously have shown a strong inhibition of lung lavage IL-4 and IL-13 concentration

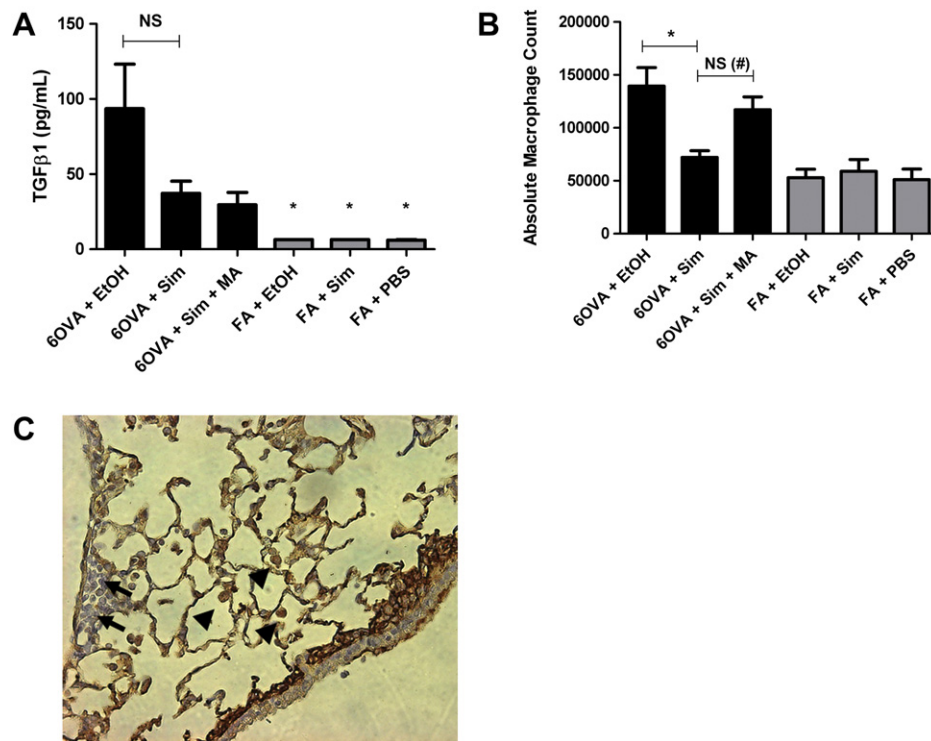


Fig 7. (A) Concentration of TGFβ1 in whole lung lavage. The group 6OVA + EtOH had significantly higher TGFβ1 concentration than any of the FA controls ($*P < 0.005$ by 1-way ANOVA). Sim (40 mg/kg) reduced the TGFβ1 concentration in lung lavage by 60.4% in the OVA group ($P = \text{NS}$ by 1-way ANOVA or t test). Cotreatment with MA (20 mg/kg) did not reverse this inhibitory statin effect on TGFβ1. Each treatment group had between 5 and 6 mice. (B) Whole lung lavage absolute macrophage counts. Sim (40 mg/kg) treatment decreased lung lavage absolute macrophage count by 48.3% ($*P < 0.005$ by 1-way ANOVA) in OVA exposed animals. Cotreatment with MA (20 mg/kg) reversed the Sim inhibitory effect ($P = \text{NS}$ by 1-way ANOVA, but $\#P = 0.0029$ by t test). Each treatment group had between 10 and 17 mice. (C) TGFβ1 immunohistochemical staining of lung and inflammatory cells. By qualitative immunohistochemical assessment, macrophages seem to be the main inflammatory cells responsible for TGFβ1 production under OVA-induced allergic inflammation (image shows the 6OVA + EtOH treatment group: representative slide where TGFβ1 stains brown seen at $\times 400$ magnification). The arrows represent inflammatory cells with little-to-no staining for TGFβ1 (likely eosinophils or lymphocytes). The arrow heads represent macrophages that stain densely brown for TGFβ1.

by Sim.^{15,45} However, it is likely that more complex mechanisms are involved in which the MA pathway also may play a role. In endothelial cells, the RhoA/ROCK pathway induces arginase activity.^{46,47} Signaling via the RhoA/ROCK pathway is a critical step for Arg1 and Arg2 mRNA and protein expression in gut endothelial cells.⁴⁸ In a breast cancer cell line (MCF-7), both Sim and fluvastatin inhibited Arg2 expression, which was reversed with MA cotreatment.⁴⁹ Other mechanisms could involve the inhibition of asymmetric dimethylarginine^{50,51} or the modulation of transcription factors such as CCAAT/enhancer-binding protein.⁵²⁻⁵⁷

The cytokine TGFβ1 is a complex cytokine that plays a key role in asthma and serves as an important link between inflammation and fibrosis. In addition to inducing Arg1, TGFβ1 also can stimulate varied cytokines

important in airway remodeling⁵⁸ as well as induce goblet cell hyperplasia, fibroblast proliferation, and myofibroblast differentiation.⁵⁹ Our data suggest that TGFβ1 is unlikely to be the prime mediator of Sim's inhibition of goblet cell hyperplasia or Arg1 expression/function at the 2-week time point (Figs 3, 7, and 8). We conclude that other mechanisms are likely at play and that a more chronic model of OVA exposure (ie, 4 to 6 weeks) may yield different results with respect to statins and TGFβ1.

Several important limitations pertain in this study. First, although our model manifests some early hallmarks of airway remodeling, it is not a chronic OVA exposure model, and therefore, not all aspects of airway remodeling and potential statin effect(s) can be investigated thoroughly. This limitation is of special concern for testing whether Sim inhibits collagen synthesis

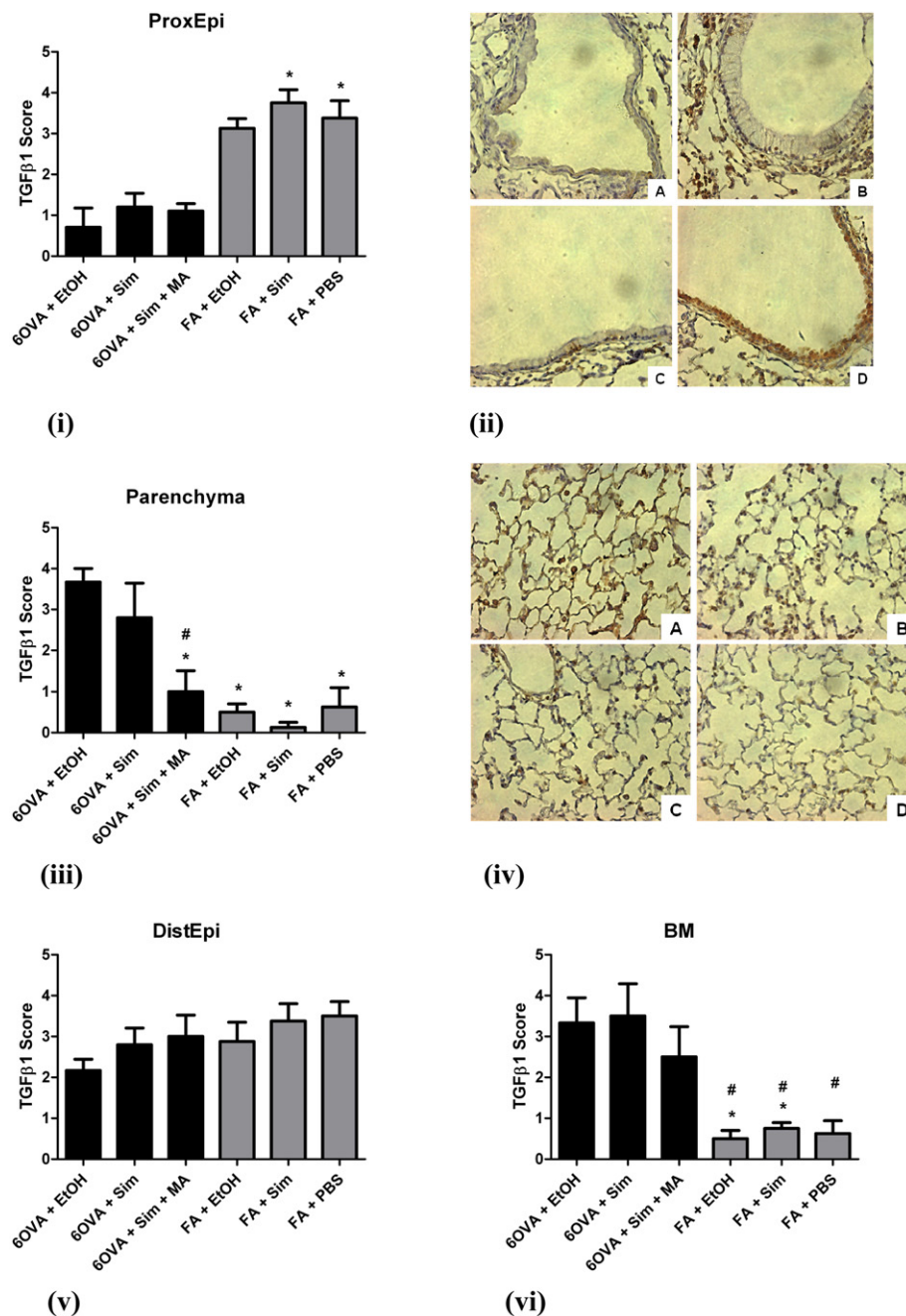


Fig 8. TGFβ1 immunohistochemical staining and scoring in different lung compartments. The following groups illustrated in the histology images are shown at an original magnification of $\times 400$, where TGFβ1 stains brown: (ii) and (iv) (A) 6OVA + EtOH, (B) 6OVA + Sim, (C) 6OVA + Sim + MA, and (D) FA + PBS. Sim (40 mg/kg) had no statistically significant effect on the total TGFβ1 content in the different lung compartments evaluated. Each treatment group had between 4 and 6 mice. All analyses were done by 1-way ANOVA. (i) In the ProxEpi, the air controls (FA + Sim and FA + PBS) had a greater TGFβ1 content compared with the OVA groups, specifically 6OVA + EtOH ($*P < 0.05$). (ii) Histology corresponding to (i) where TGFβ1 stains brown most intensely in the epithelium of the FA + PBS group. (iii) In the lung parenchyma, all 3 air control groups had much lower TGFβ1 content compared with the 6OVA + EtOH group ($*P < 0.005$). The treatment group 6OVA + Sim + MA showed less TGFβ1 content than the 6OVA + EtOH group ($^{\#}P < 0.005$). (iv) Histology corresponding to (iii) shows the highest intensity TGFβ1 staining in group (A) 6OVA + EtOH, greater than the other OVA groups and significantly greater than all air controls. (v) In the DistEpi, no statistically significant differences were noted among all 6 groups ($P = NS$). (vi) In the BM, the air controls had significantly less TGFβ1 content than the OVA groups, specifically 6OVA + EtOH ($*P < 0.05$) and 6OVA + Sim ($^{\#}P < 0.05$).

and airway fibrosis in asthma by analogy with previously reported effects in other animal models of lung fibrosis.^{13,60-62} Second, although, for some features of remodeling, the Sim effect seems to be MA-dependent (ie, goblet cell hyperplasia), the detailed mechanism remains unknown. For the MA-independent observations, we did not confirm whether MA actually was present or absent in the tissue of interest after i.p. injection; the presumption is that MA was distributed ubiquitously. Third, we assessed *whole lung* tissue for most endpoints, restricting some of our observations to the whole lung rather than to the specific lung compartment or cell types. Fourth, extrapolation of these results to human disease would be premature, as more animal studies are needed to understand the mechanisms and to show consistent efficacy.

Sim likely has many cellular targets and future studies must use a chronic exposure model with a special focus on SMC hyperplasia/hypertrophy, fibroblast-to-myofibroblast differentiation, and airway fibrosis while exploring the underlying mechanism(s) of action. Given the potential for innovative therapy in asthma and airway remodeling, such investigations are needed urgently.

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