Statins and Pulmonary Fibrosis: The Potential Role of NLRP3 Inflammasome Activation

Complete List of Authors:	Xu, Jin-Fu; Brigham and Women's Hospital, Pulmonary and Critical Care Medicine
	Washko, George; Brigham and Women's Hospital, Pulmonary and Critical Care Medicine
	Nakahira, Kiichi; Brigham and Women's Hospital, Pulmonary and Critical Care Medicine
	Hatabu, Hiroto; Brigham and Women's Hospital, Department of Radiology
	Patel, Avignat; Brigham and Women's Hospital, Pulmonary and Critical Care Medicine
	Fernandez, Isis; Brigham and Women's Hospital, Pulmonary and Critical Care Medicine
	Nishino, Mizuki; Brigham and Women's Hospital, Department of Radiology
	Okajima, Yuka; Brigham and Women's Hospital, Department of Radiology
	Yamashiro, Tsuneo; Brigham and Women's Hospital, Department of Radiology
	Ross, James; Brigham and Women's Hospital, Surgical Planning Laboratory, Department of Radiology
	Estepar, Raul; Brigham and Women's Hospital, Surgical Planning Laboratory, Department of Radiology
	Diaz, Alejandro; Brigham and Women's Hospital, Pulmonary and Critical Care Medicine
	Li, Hui-Ping; Tongji University School of Medicine, Department of Pulmonary Medicine
	Qu, Jie-Ming; Huadong Hospital, Fudan University, Department of Pulmonary Medicine
	Himes, Blanca; Brigham and Women's Hospital, Channing Laboratory
	Come, Carolyn; Brigham and Women's Hospital, Department of Pulmonary Medicine
	D'Aco, Katherine; Brigham and Women's Hospital, Department of Pulmonary Medicine
	Martinez, Fernando; University of Michigan, Department of Internal

	Medicine
	Han, MeiLan; University of Michigan, Department of Internal Medicine Lynch, David; National Jewish Medical and Research Center, Department of Radiology Crapo, James; National Jewish Medical and Research Center, Department of Medicine Morse, Danielle; Brigham and Women's Hospital, Department of Pulmonary Medicine Ryter, Stephan; Brigham and Women's Hospital, Department of Pulmonary Medicine Silverman, Edwin; Brigham and Women's Hospital, Channing Laboratory Rosas, Ivan; Brigham and Women's Hospital, Department of Pulmonary Medicine Choi, Augustine; Brigham and Women's Hospital, Department of Pulmonary Medicine Hunninghake, Gary; Brigham and Women's Hospital, Pulmonary and Critical Care Medicine
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Statins and Pulmonary Fibrosis: The Potential Role of NLRP3 Inflammasome

Activation

Jin-Fu Xu,[1,2][*] George R. Washko,[1][*] Kiichi Nakahira,[1] Hiroto Hatabu,[3, 4] Avignat S. Patel,[1] Isis E. Fernandez,[1] Mizuki Nishino,[3, 4] Yuka Okajima,[4] Tsuneo Yamashiro,[3, 4] James C. Ross,[5,6] Raúl San José Estépar,[4,5] Alejandro A. Diaz,[1, 7] Hui-Ping Li,[2] Jie-Ming Qu,[8] Blanca E. Himes,[6,9] Carolyn E. Come,[1] Katherine D'Aco,[1,6] Fernando J. Martinez,[10] MeiLan K. Han,[10] David A. Lynch,[11] James D. Crapo,[12] Danielle Morse[1], Stefan W. Ryter[1], Edwin K. Silverman,[1, 6] Ivan O. Rosas,[1] Augustine M.K. Choi,[1][*] and Gary M. Hunninghake[1, 6][*] and the COPDGene Investigators

[1]Pulmonary and Critical Care Division, Brigham and Women's Hospital, Harvard Medical School, Boston MA; [2]Department of Pulmonary Medicine, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, China, 200433. [3]Center for Pulmonary Functional Imaging, Brigham and Women's Hospital, Boston MA; [4]Department of Radiology, Brigham and Women's Hospital, Boston MA; [5]Surgical Planning Laboratory, Department of Radiology, Brigham and Women's Hospital, Boston MA; [6]Channing Laboratory, Brigham and Women's Hospital, Boston MA; [7]Department of Pulmonary Diseases. Pontificia Universidad Católica de Chile, Santiago, Chile; [8]Department of Pulmonary Medicine, Huadong Hospital, Fudan University, Shanghai, China, [9]Center for Genomic Medicine, Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts; [10]Department of Internal Medicine, University of Michigan, Ann Arbor MI; [11]Department of Radiology, National Jewish Medical and Research Center, Denver CO and; [12]Department of Medicine, National Jewish Medical and Research Center, Denver CO.

*These authors contributed equally to this manuscript.

Corresponding Authors: Augustine M. K. Choi, MD
Gary M. Hunninghake M.D., M.P.H.
Division Pulmonary and Critical Care Division
Department of Medicine
Brigham and Women's Hospital
75 Francis Street
Boston, MA 02115

Email: amchoi@rics.bwh.harvard.edu, ghunninghake@partners.org
Phone: 617-732-7599, 617-525-9687
Fax: 617-732-7421, 617-264-5133

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At a Glance Commentary:

Scientific Knowledge on the Subject – While HMG-CoA reductase inhibitors (statins) have immunomodulatory and anti-inflammatory properties that in theory could be beneficial in the treatment of respiratory disease, they have also been implicated in the development of interstitial lung disease (ILD).

What This Study Adds to the Field – Our findings demonstrate that statin use is associated with interstitial lung abnormalities (ILA) among current and former smokers in the COPDGene study. In addition, we found that statin pretreatment enhanced bleomycin-induced lung inflammation and fibrosis *in vivo,* augmented mtROS generation, and enhanced NLRP3 inflammasome activation.

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This article has an online supplement, which is accessible from this issue's table

of contents at www.atsjournals.org.

Abstract: (n=247)

Rationale: The role of HMG-CoA reductase inhibitors (statins) in the development and/or progression of interstitial lung disease (ILD) is controversial.

Objectives: To evaluate the association between statin use and ILD.

Methods: We used regression analyses to evaluate the association between statin use and interstitial lung abnormalities (ILA) in a large cohort of smokers from COPDGene. Next, we evaluated the effect of statin pretreatment on bleomycin-induced fibrosis in mice and explored the mechanism behind these observations *in vitro.*

Results: In COPDGene, 38% of subjects with ILA were taking statins compared to 27% of subjects without ILA. Statin use was positively associated in ILA (odds ratio [OR] 1.60, 95% confidence interval [CI] 1.03-2.50, P=0.04) after adjustment for covariates including a history of high cholesterol or coronary artery disease. This association was modified by the hydrophilicity of statin and the age of the subject. Next, we demonstrate that statin administration aggravates lung injury and fibrosis in bleomycin-treated mice. Statin pretreatment enhances caspase-1-mediated immune responses *in vivo* and *in vitro*; the latter responses were abolished in bone marrow-derived macrophages (BMDMs) isolated from *Nlrp3^{-/-}* and *Casp1^{-/-}* mice. Finally, we provide further insights by demonstrating that statins enhance NLRP3-inflammasome activation by increasing mitochondrial reactive oxygen species generation in macrophages.

Conclusions: Statin use is associated with ILA among smokers in the COPDGene study and enhances bleomycin-induced lung inflammation and

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fibrosis in the mouse through a mechanism involving enhanced NLRP3-

inflammasome activation. Our findings suggest that statins may influence the

susceptibility to, or progression, of ILD.

Introduction

Statins (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors) are commonly prescribed medications whose major indications include the treatment of hypercholesterolemia in the primary(1, 2) and secondary(3, 4) prevention of cardiovascular disease(CVD)-related morbidity and mortality. In addition to reducing cholesterol levels, statins may have immuno-modulatory(5) and anti-inflammatory(6) properties that in theory could be beneficial in the treatment of some respiratory diseases.(7)

The role of statins in the development of interstitial lung disease (ILD), a group of respiratory diseases characterized by varying degrees of pulmonary interstitial fibrosis and inflammation,(8) is controversial. While some studies evaluating both human lung fibroblasts,(9) and mice(10) suggest that statins could be beneficial in the treatment of fibrotic lung disease, contrasting observations suggest that statins enhance monocyte secretion of inflammasome-regulated cytokines (e.g. IL-1β and IL-18)(11-13) that may play important roles in the progression of pulmonary fibrosis,(14) and numerous case-reports suggest that statins could cause various types of ILD.(15)

Recently, we characterized a group of current and former smokers from the COPDGene study who, although previously undiagnosed with ILD, demonstrated chest CT patterns of increased lung density (which we have previously defined as interstitial lung abnormalities [ILA]).(16) We demonstrated that the subjects

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with ILA had reductions in total lung capacity (TLC), and increases in respiratory

symptoms.(16) Based on both case reports of statin-associated ILD(15) and

prior data suggesting that smoking is associated with ILA,(16-18) we

hypothesized that statins would increase the risk for ILA in populations of

smokers. To test this hypothesis we first evaluated the association between

statin use and ILA in a large cohort of current and former smokers from the

COPDGene study. Next, to provide experimental evidence that statins could

contribute fibrotic lung disease we demonstrated that statin administration

aggravates lung injury and fibrosis in bleomycin-treated mice. To explore the

mechanism behind these observations we demonstrate that statin pretreatment

enhanced caspase-1-mediated immune responses *in vivo* and *in vitro*; the latter

responses were abolished in bone marrow-derived macrophages (BMDMs)

isolated from *Nlrp3*^{-/-} and *Casp1*^{-/-} mice. Finally, we provide further insights by

demonstrating that statins enhance *Nlrp3*-inflammasome activation through

mitochondrial reactive oxygen species (mtROS) generation in macrophages.

Some of this work was previously presented in abstract form.(19)

Methods (n=498)

For additional details, *see* the **supplementary text**.

Clinical Data

Study Design

The protocols for subject recruitment in COPDGene have been previously

described.(16) In brief, volumetric chest CTs were evaluated by three readers

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(including two chest radiologists and one pulmonologist) using a sequential

reading method.(16) ILA were defined as nondependent changes affecting >5%

of any lung zone including, nondependent ground-glass or reticular

abnormalities, diffuse centrilobular nodularity, nonemphysematous cysts,

honeycombing, or traction bronchiectasis. Qualitative CT assessment was

performed by readers blind to any additional information including current use of

medications. Disease-related demographic parameters and information about

use of current medications were designated by self-report. The COPDGene

study was approved by the institutional review boards of all participating centers.

Laboratory Data

Cell culture

J774A.1 macrophages and bone marrow-derived macrophages (BMDMs) were

prepared and maintained as described previously.(20) Cells were pretreated with

statins 24 h prior to incubation with LPS (500 ng/ml) for 4 h, followed by

stimulation with ATP. Glyburide was added to the medium 15 minutes before

ATP treatment.(20) MitoTEMPO was added to the medium 1 before LPS priming

as described before.(20)

Mice

Male C57B/L6 mice (8 weeks old, 18-22 g) were used for *in vivo* experiments.

Bleomycin (Hospira Inc., Adelaide, Australia) at a dose of 0.1U/mice in 50 μl

normal saline was instilled into lung intratracheally to induce lung inflammation

and fibrosis, as previously described.(21, 22) Mice were treated with Pravastatin

(40 mg/kg per day) or PBS intraperitoneally daily starting from three days before bleomycin instillation. All experiments were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal care and use for all experiments was approved by the Harvard Medical Area Standing Committee on Animals of Harvard Medical School.

Fluorescence Staining

After treatment, cells were fixed with 4% paraformaldehyde, and followed with the immunofluorescence staining protocol as described previously.(23) MitoTracker green, MitoSOX red and DAPI were used to label total mitochondria, mtROS and the nuclei respectively. Stained samples were fixed onto the slides and viewed with Olympus Fluoview-FV10i Confocal and Olympus FSX100 fluorescence microscopy. Fluorescence picture was simultaneously captured by standard confocal imaging techniques.

Statistical Analysis

In COPDGene, Bivariate analyses were conducted with Fisher's exact tests (for categorical variables), and two-tailed t tests or Wilcoxon rank-sum tests (for continuous variables) where appropriate. Logistic regression models were used in multivariate analyses to study the relation between ILA and statin use. All of the adjusted models included age, sex, race, pack-years of smoking, current smoking status, COPD (defined as $\geq$ GOLD stage 2),(24) self-report of either high cholesterol or coronary artery disease and additional covariates where

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indicated. We estimated the risk of ILA attributable to statin use in smokers from COPDGene.(25) All analyses were performed using Statistical Analysis Software version 9.1 (SAS Institute, Cary, NC). Forest Plots were generated utilizing the rmeta package as implemented in R version 2.9.(26) For the laboratory data, means$\pm$SD are reported. Student's t-test was used for statistical analysis. P values <0.05 were considered statistically significant.

Results

Of the 2,508 subjects from COPDGene, 2207 (88%) provided information about current use (and type of) statin prescribed. Of these 2207 subjects, 2115 (96%) had a CT available and were included in these analyses. Cardiovascular disease characteristics and medications of subjects stratified by ILA status are presented in **Table 1** (additional baseline characteristics have been published previously,(16) and baseline characteristics stratified by statin use are included in **Table E1**). In addition to increases in statin use, in univariate analyses subjects with ILA were more likely to have coronary artery disease, diabetes, high blood pressure, and to be taking beta-blockers (see **Table 1**).

Statins and Interstitial Lung Abnormalities (ILA)

In COPDGene, 38% of subjects with ILA were taking statins compared to 27% of subjects without ILA (see **Table 1, Figure 1A**). Compared to those not taking statins, statin users had a 60% increase in their odds to have ILA after adjustment for relevant covariates including a history of high cholesterol or

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coronary artery disease (see **Table 2**). There was no significant decrement in

the association between statins and ILA in models adjusting for additional

cardiovascular medications and diseases (see **Table 2**). With the exception of

statin use, in multivariate models no additional cardiovascular medication or

disorder was positively associated with ILA (in contrast, current use of

angiotensin converting enzyme inhibitors was inversely associated with ILA [OR

0.56, 95% CI 0.32-0.98, P=0.04]). In COPDGene, the risk of ILA attributable to

statin use was 14% (95% CI, 1% to 22%).

Hydrophilic vs. Lipophilic Statins

The association between statin use and ILA varied by statin type (see **Table 2**

and **Figure 1A**). The prevalence of ILA varied from 8% in subjects on

simvastatin to 23% for subjects on pravastatin (see **Table E2**). There was

evidence that statins with increased hydrophilicity (as measured by decreasing

log$D$,(27, 28) see **Figure 1A, B**) were associated with increases in the odds for

ILA (P<0.001 for trend). Pravastatin (a hydrophilic statin) was the statin drug

most strongly associated with ILA (OR 4.61, 95% CI 1.99-10.70, P<0.001).

There was no evidence for increased coronary artery disease among subjects

taking hydrophilic statins (OR 0.90, 95% CI 0.49-1.66, P=0.73).

Statins, Specific Radiologic Features, and Age

Most CT radiologic features of ILA were associated with statin use (**Figure 1B,**

C, and Table E3). In addition to radiologic features that can be identified in

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inflammatory lung diseases (e.g. ground-glass), statin use was also associated with radiologic features more typical of pulmonary fibrosis (e.g., statin users had a 125% increase in their odds of having traction bronchiectasis, OR 2.25, 95% CI, 1.33-3.82, P=0.003). While there was no evidence for an interaction between statin use and many relevant covariates (including both current use and pack-year history of tobacco smoke exposure), there was significant evidence that age modified the association between statin use and ILA (P = 0.04 for the interaction, see **Figure 1D**, and **supplemental text**).

Statins Exacerbate Bleomycin Induced Fibrosis in the Mouse

To investigate the effect of statins on lung injury and fibrogenesis in an experimental model, mice were pretreated with pravastatin (based on our clinical findings) prior to intratracheal bleomycin administration. Lungs from mice treated with both pravastatin and bleomycin showed increased lung fibrosis (**Figures 2A**, **B**), HT15 Trichrome staining (**Figure 2C**), and collagen deposition (**Figure 2D**) at day 14 compared to mice exposed to bleomycin alone (similar findings were noted in the mice at day 7, **Supplemental Figures 1A, B)**. Comparably, pretreatment with pravastatin enhanced weight loss and inflammatory cell recruitment in mice (**Supplemental Figures 1C,D**). The pravastatin and bleomycin experimental group also had significant increases in IL-1β and IL-18 in the BALF and in the lung homogenate (**Figures 2E-H**) compared to mice treated with bleomycin alone; this correlated with increased caspase-1 activation and cleaved IL-1β expression (**Figure 2I**). Pravastatin alone had no impact on

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histological changes and collagen deposition in the absence of bleomycin instillation (**Figures 2A-D**).

Statins Enhance Activation of the NLRP3 Inflammasome

To further explore the mechanism behind these observations, J774A.1 macrophages were pretreated with pravastatin for 24 hours followed by stimulation with both LPS and ATP.[20] Pretreatment with pravastatin increased IL-1β and IL-18 secretion in a dose-dependent manner (**Figures 3A, B**; similar findings were noted with atorvastatin, **Supplemental Figures 2A, B**) and resulted in accumulation of the cleaved form of caspase-1 (p10) in stimulated cells (**Figure 3C**, **Supplemental Figure 2C**). No effect of statin was noted in the absence of LPS and ATP stimulation (**Figures 3A-C, Supplemental Figures 2A, B**). The effect of pravastatin on IL-1β and IL-18 secretion was dependent on caspase-1, as demonstrated by the loss of this effect on BMDMs from *Casp-1*^{-/-} mice (**Figures 3D, E**). To determine which inflammasome pathway was involved in the promotion of caspase-1 activation by pravastatin, BMDM from *Nlrp3*^{-/-} mice were subjected to LPS and ATP stimulation. The stimulatory effect of pravastatin pretreatment on secretion of IL-1β and IL-18 and caspase-1 activation was abolished in *Nlrp3*^{-/-} BMDM (**Figures 3F-H**). Similarly, glyburide, a NLRP3 inflammasome inhibitor, prevented the enhancement of IL-1β and IL-18 secretion by pravastatin (**Supplemental Figures 2D, E**). Pravastatin pretreatment also enhanced the molecular interactions between NLRP3 and ASC (an NLRP3 inflammasome co-factor necessary to activate caspase-1), and between NLRP3

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and caspase-1, further supporting a role for NLRP3 in pravastatin-mediated inflammasome activation (**Figure 3I**)**.** Pravastatin had no effect on the protein expression of P2X7 receptor, ASC and LPS-induced pro-IL-1β (**Supplemental Figure 2F**).

Activation of NLRP3 by Statins is Dependent on Upregulation of mtROS

MtROS is a critical factor for NLRP3 inflammasome activation.(20, 23) To determine the effect of pravastatin on mtROS, MitoSOX Red (a membrane permeable fluorogenic dye for the selective detection of mitochondrial O_2^-) was used to detect mtROS. Pravastatin enhanced mtROS generation in stimulated cells (**Figures 4A, B**). Confocal fluorescence microscopy (using MitoTracker green to label total mitochondria) confirmed that mtROS colocalized to mitochondria in stimulated cells (**Figure 4C**). Scavenging of mtROS by MitoTEMPO (a derivative of the antioxidant TEMPO that concentrates in the mitochondrial matrix) resulted in reduced fluorescence intensity of MitoSOX (**Figure 4D**, **Supplemental Figures 3A,B**) and inhibited IL-1β and IL-18 secretion in a dose-dependent manner (**Figures 4E,F**), implying a direct role for mtROS in inflammasome activation by pravastatin. In contrast, mitoTEMPO did not affect TNF secretion, a cytokine not influenced by caspase-1 activation (**Supplemental Figure 3C**).

Discussion

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Our findings in a large cohort of smokers demonstrate that statin use may increase the risk of developing radiographic evidence of ILD including findings characteristic of pulmonary fibrosis. This risk may be modified by the hydrophilicity of statin and the age of the subject. In support of our clinical findings, we demonstrate that statin use exacerbates bleomycin-induced lung fibrosis in mice. Further, our study demonstrates that statin pretreatment increases mtROS in stimulated cells, resulting in increased NLPR3 inflammasome-mediated immune responses.

Our data provides support to evidence from numerous case-reports[15] suggesting that statins may cause ILD. While prior case-control studies limited to the association between statin use and idiopathic pulmonary fibrosis (IPF) alone[29, 30] have not demonstrated significant associations, these studies are limited by small sample size,[29] the potential for selection bias in controls,[29] and include cases selected by diagnostic codes alone.[30] In contrast, our study includes the CT characterization of a large cohort (>2100 subjects), and presents associations between statins and ILA scored by multiple readers blind to information about current medication use.

While our data, and those of others,[30] support an association between cardiovascular disease the development of fibrotic lung disease, several lines of evidence suggest that cardiovascular disease alone is unlikely to entirely explain our findings: (1) the association between statin use and ILA is independent of

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both presence of cardiovascular disease and additional medications commonly prescribed for cardiovascular disease, (2) while hydrophilic statin users in COPDGene were at greater risk for ILA this was not coupled with an increased report of coronary artery disease (in fact, hydrophilic statin users were slightly less likely to report coronary artery disease compared to lipophilic statin users), and (3) we demonstrate experimentally that statin use can exacerbate lung fibrosis in mice.

Comparable to our clinical findings, our results in mice indicate that statin administration enhances bleomycin-induced caspase-1-mediated immune response in the lung. Moreover, we show that enhanced activation of caspase-1 correlates with an increase in fibrotic change in the lung treated with bleomycin and pravastatin. The effect of statin administration on cytokine secretion was exerted on upstream steps of NLRP3 based on our following observations: (1) NLRP3 deficiency completely impaired the effect of statin pretreatment on IL-1β and IL-18 secretion,(2) formation of NLRP3 inflammasome induced by LPS and ATP was increased by statin pretreatment, and (3) the effect of statin on the cytokine secretion was inhibited by glyburide which suppresses the activation pathway upstream of the NLRP3 inflammasome but downstream of P2X7 receptor. These results suggest that statins target the activation pathway upstream of NLRP3 inflammasome, and further implicate activation of the NLRP3 inflammasome in fibrotic lung disease.(31-33)

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While mtROS are important for various mitochondrial functions including biosynthesis of many molecules and catabolic pathways, it has been shown that excess mtROS generation hyper-activates immune responses.(20, 23) Our data show that statin administration increases immune responses in our inflammasome-activating models however, it is still unclear how statins enhance mtROS in the stimulated macrophages. Of note, in blocking the synthesis of cholesterol, statins block the synthesis of ubiquinone which is essential in mitochondrial electron transport.(34) While not all studies demonstrate that statins increase mtROS(35) some of these discrepancies may be explained by different stimuli and differences in tissue specificity.(35, 36)

Our findings contrast with two previous reports which suggest that statins could ameliorate bleomycin-induced lung injury,(10, 37) these studies differ in the type of statin and the dose of bleomycin used (Ou et al., employed simvastatin and instilled bleomycin 15-20-fold higher [0.3 U/10 g] than standard dosing for such experiments),(37) and in the timing of statin administration (Kim *et al.* administered pravastatin coincident with bleomycin instillation).(10) Importantly, it should be noted that pravastatin alone did not enhance NLRP3 inflammasome activation *in vitro* or aggravate the lung injury *in vivo*, in the absence of pro-inflammatory challenge. Moreover, it may be relevant that all subjects from COPDGene were current or former smokers, as cigarette smoke alone may result in pulmonary inflammation through NLRP3 inflammasome mediated pathways in humans(38) and in mouse models.(39)

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One limitation of study is the lack of correlative data allowing us to relate in human samples the mechanisms described in the mouse. Many prior studies have demonstrated that statins contribute to the release of inflammasome related cytokines including IL-1β and IL-18 in human peripheral blood monocytes through a caspase-1 dependent mechanism. Upregulation of the inflammasome in response to statins is blocked by the reintroduction of downstream products of the cholesterol synthesis pathway including mevalonate and geranylgeraniol.(40-43) In these studies human peripheral blood monocytes frequently require a stimulus such as TLR ligands (e.g. lipopolysaccharide) to activate the inflammasome**.** However, in contrast to peripheral blood monocytes (or monocytic cell lines), recent evidence suggests that human alveolar macrophages may require adenosine triphosphate (ATP) as an additional stimulus to activate the inflammasome.(44) This may have relevance to our findings as studies have demonstrated that, while smoking upregulates extracellular ATP,(45) the regulation of intracellular ATP by statins is drug dependent (e.g. simvastatin, lovastatin, and fluvastatin result in decreases in cellular ATP levels, while atorvastatin, rosuvastatin, and pravastatin do not).(46) Our study has several additional limitations. First, while biopsies were not obtained in this cohort, it is important to note that biopsies on similarly ascertained cohorts of patients with ILA(47-49) have demonstrated histopathologic evidence of ILD (idiopathic interstitial pneumonias in particular). Second, although we did not find evidence that the association of statin use and ILA was modified by either current use of tobacco, or the intensity of tobacco

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smoke exposure, it is worth noting that all subjects in COPDGene have a history

of at least 10 pack-years of smoking. Our group(16, 18) and others(17) have

previously demonstrated that smoking is associated with ILA. Therefore it is

possible that the association between statin use and ILA is limited to current and

former smokers. Third, we do not have information on the duration of therapy or

drug dosage in a majority of the patients on statins. However, it is unlikely that

the variability in effective drug dosage alone explains the increased odds for ILA

we demonstrate among subjects taking hydrophilic statins (at commonly

prescribed doses there is a large difference in the expected cholesterol reduction

between pravastatin and rosuvastatin).(27) Fourth, while we provide

experimental evidence in support of our clinical findings, further experimental

work in mice exposed to tobacco smoke would be helpful to more precisely

define the combined effect of smoking and statin use.   Finally, although prior

studies have implicated a role for the NLRP3 inflammasome in fibrotic lung

disease,(31-33) further studies in people will be required to determine the extent

to which NLRP3 inflammasome activation plays a role in statin-induced ILD, and

ILD in general.

We urge caution in extrapolating our findings to the care of patients. While

increases in the risk of ILA, and radiologic features of pulmonary fibrosis, are

causes for concern, these risks do not likely outweigh the substantial benefits of

statin therapy in patients with cardiovascular disease. In addition, our findings do

not rule out the possibility that statin use could benefit some patients with

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respiratory disease. Instead, we believe that clinicians should be aware that radiographic evidence of interstitial lung disease, much like myopathy,(50) can occur in some patients on statins.

CONCLUSIONS

In summary, our study demonstrates that statin use is associated with ILA among current and former smokers in the COPDGene study. We found that statin pretreatment enhanced bleomycin-induced lung inflammation and fibrosis *in vivo,* augmented mtROS generation, and enhanced NLRP3-inflammasome activation. While our study raises concerns about the potential role for statins in the development and/or progression of ILD in settings of enhanced NLRP3 inflammasome activation, these risks likely do not outweigh the substantial benefits of statin therapy in patients with CVD. Instead, our findings suggest that the use of statins in patients with ILD should be re-evaluated.

Acknowledgements

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FIGURE LEGENDS:

Figure 1: Statin use is associated with interstitial lung abnormalities (ILA).

(**A**) Odds ratios (OR) for the association between individual statins (arranged in

order of increasing hydrophilicity as measured by decreasing log$D$) and ILA. ORs

and 95% confidence intervals (CIs) are represented by boxes (with their size

proportional to the sample size) and bars respectively. The overall association

between statin use and ILA is represented by a diamond. The upper limit of the

95% CI for the association between pravastatin and ILA is > 10 (95% CI 1.99-

10.70). (**B**) Odds ratios (OR) for the association between statins and specific

radiologic features. Black boxes (with their size proportional to the sample size)

and bars represent ORs and 95% CIs for the association between statins overall

and specific radiologic features. Green boxes and bars represent ORs and 95%

CIs for the association between lipophilic statins and specific radiologic features.

Blue boxes and bars represent ORs and 95% CIs for the association between

hydrophilic statins and specific radiologic features. The association between

statin use (including all statins, lipophilic, and hydrophilic statins) and ILA in

general are represented by diamonds. The upper limits of the 95% CIs for the

association between hydrophilic statins and bronchiectasis, and honeycombing

are > 10 (95% CI 1.78-11.40, and 95% CI 0.89-19.70, respectively). (**C**) Axial

volumetric chest computed tomographic (CT) images of the hemithorax

representing specific radiologic findings of ILA present in COPDGene subjects on

statin medications. B1: Nondependent ground glass present in a subject taking

simvastatin. B2: Nonemphysematous cysts present in a subject taking

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lovastatin. B3: Centrilobular Nodules present in a subject taking rosuvastatin.

B4: Nondependent reticular markings present in a subject taking atorvastatin, B5:

Traction Bronchiectasis present in a subject taking rosuvastatin. B6:

Honeycombing present in a subject taking pravastatin. (**D**) Odds ratios (OR) for

the association between statins and ILA stratified by age (including subjects aged

45-55 years [n=415], subjects aged 55-65 years [n=451], and in subjects > 65

years old [n=490]). Black boxes (with their size proportional to the sample size)

and bars represent ORs and 95% CIs for the association between statins overall

and ILA stratified by age.

Figure 2: Statin increases bleomycin-induced lung inflammatory response

and fibrotic changes in mice.

(**A**) Sections of paraffin-embedded lung tissue from mice with different treatments

were stained with H.E. Images were shown at a 200x magnification. Bar, 20 µm,

day 14; CTL- Control, STA - Pravastatin, BLM - Bleomycin, S+B – Pravastatin +

Bleomycin. (**B)** Semiquantitative histopathology score was shown. (**C**) Sections

of paraffin-embedded lung tissue from mice treated with different treatments

were stained with Masson Trichrome. Images were shown at a 400x

magnification. Bar, 40 µm, day 14; CTL- Control, STA - Pravastatin, BLM -

Bleomycin, S+B – Pravastatin + Bleomycin. (**D**) Pulmonary collagen deposition

was quantified and expressed as micrograms of hydroxyproline per left lung. (**E,**

F) Concentration of IL-1β and IL-18 in BALF at day 7 was measured by ELISA.

(**G, H**) Concentration of IL-1β and IL-18 in lung homogenates was measured by

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ELISA. (**I**) Lung homogenates were analyzed by immunoblotting for IL-1β and

caspase-1. CTL, mice received with intraperitoneal injection of PBS and

intratracheal instillation of PBS; STA, mice received with intraperitoneal injection

of pravastatin and intratracheal instillation of PBS; BLM, mice received with

intraperitoneal injection of PBS and intratracheal instillation of bleomycin; S+B,

mice received with intraperitoneal injection of pravastatin and intratracheal

instillation of bleomycin. Day 7: CTL, n=5; STA, n=5; BLM, n=8; S+B, n=9. Day

14: CTL, n=5; STA, n=5; BLM, n=11; S+B, n=11. * $P < 0.05$ when compared with

CTL group. # $P < 0.05$ when compared with BLM group.

Figure 3: Statin enhances NLRP3 inflammasome activation in

macrophages.

(**A, B**) Pravastatin pretreatment enhances the secretion of IL-1β and IL-18 in

macrophages stimulated with LPS and ATP. J774A.1 macrophages were

pretreated with pravastatin or vehicle (PBS) for 24 h and then incubated with LPS

(500 ng/ml) for 4 h, followed by stimulation with ATP (5 mM) for 1 h. Secretion of

IL-1β and IL-18 into the media was measured by ELISA. *$P < 0.01$, versus cells

treated with LPS and ATP. (**C**) Pravastatin pretreatment increases caspase-1

activation. J774A.1 macrophages were pretreated with pravastatin (10 μM) for 24

h and then incubated with LPS (500 ng/ml) for 4 h, followed by stimulation with

ATP (5 mM) for 15 min. Cell lysates were analyzed by immunoblotting for

caspase-1. (**D, E**) BMDM from caspase-1 -/- mice were pretreated with

pravastatin (10 μM) for 24 h and then incubated with LPS (200 ng/ml) for 4 h,

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followed by stimulation with ATP (5 mM) for 1 h. Secretion of IL-1β and IL-18 was

analyzed by ELISA. *P < 0.01, versus caspase-1 -/- cells treated with LPS and

ATP. (**F-H**) NLRP3 inflammasome is involved in the role of pravastatin on

Caspase-1 activation. BMDM from *NLRP3* -/- mice were pretreated with

pravastatin (10 μM) for 24 h and then incubated with LPS (200 ng/ml) for 4 h,

followed by stimulation with ATP (5 mM) for 15 min (**H**) or 1 h (**F,G**). Secretion of

IL-1β and IL-18 was analyzed by ELISA. *P < 0.01, versus NLRP3 -/- cells

treated with LPS and ATP. Cell lysates were analyzed by immunoblotting for

caspase-1. (**I**) Pravastatin increases interaction of NLRP3 inflammasome-

associated molecules. J774A.1 macrophages were pretreated with pravastatin

(10 μM) for 24 h and then incubated with LPS (500 ng/ml) for 4 h, followed by

stimulation with ATP (5 mM) for 15 min. Cell lysates were analyzed for interaction

of NLRP3 and ASC or NLRP3 and pro caspase-1 by immunoprecipitation.

Figure 4: **Statin pretreatment increases mitochondrial ROS generation.**

(**A**) LPS-primed J774A.1 macrophages were stained with MitoSOX for 15 min

before stimulation with ATP in the absence or presence of pravastatin, and then

analyzed by flow cytometric analyses. Representative histograms are shown.

(**B**) Relative mean fluorescence intensity (MFI) of MitoSOX was represented. *P

< 0.01, versus untreated cells. #P < 0.05, versus cells stimulated with LPS and

ATP. (**C**) MitoTracker was used to show mitochondria, MitoSOX Red was used

to show ROS, DAPI was used to show the nuclei of the cells. MitoSOX Red

labeled ROS was shown in mitochondria and was increased by pravastatin

pretreatment, compared with LPS/ATP treatment only. (**D**) J774A.1

macrophages were pretreated with MitoTEMPO (500 μM) 1h before LPS

treatment in the absence or presence of pravastatin. Level of mtROS in cells was

analyzed by MitoSOX labeling. (**E, F**) Macrophages were pretreated with

MitoTEMPO 1h before LPS treatment in the absence or presence of statin,

followed by ATP stimulation for 1 h. Cytokine secretion was analyzed by ELISA. *

P < 0.01, versus cells treated with LPS and ATP. [#] *P* < 0.01, versus statin-

pretreated cells stimulated with LPS and ATP.

Table 1: Characteristics of Smokers from COPDGene Stratified by the

Presence of Radiographic Interstitial Lung Abnormalities (ILA)

Variable*	Number(%) or Median (Interquartile Range) where appropriate		
	No ILA	ILA	
Demographic Parameters	n=1184 (87%)	n=172 (13%)	P value[†]
High Cholesterol	504 (43%)	82 (48%)	0.22
Coronary Artery Disease	69 (6%)	19 (11%)	0.02
Diabetes	141 (12%)	30 (17%)	0.05
High Blood Pressure	481 (41%)	90 (52%)	0.005
Previous Myocardial Infarction	59 (5%)	12 (7%)	0.27
Previous Cerebrovascular Accident	29 (2%)	8 (5%)	0.12
Previous Thromboembolic Disease	42 (4%)	9 (5%)	0.28
Medications			
Aspirin	186 (16%)	37 (22%)	0.06
Beta Blockers	139 (12%)	30 (17%)	0.04
ACE inhibitors	155 (13%)	18 (10%)	0.39
Gemfibrozil	1 (<1%)	0 (0%)	1.0
Niacain	4 (<1%)	2 (1%)	0.17
Fish Oil	27 (2%)	6 (3%)	0.30
Statins			
All Statins	315[‡] (27%)	66 (38%)	0.002
Lipophilic Statins	280 (24%)	51 (32%)	0.03
Hydrophilic Statins	33 (4%)	15 (12%)	<0.001

* Data on disease related demographic, and medication variables were determined

by self-report. Data missing for high blood pressure, previous cerebrovascular

accident, previous thromboembolic disease (n=1).

† P values compare those with ILA to those without ILA using Fisher's exact tests.

‡ Three subjects reporting statin medication use did not provide specific drug

information and are included in this group.

Table 2: Univariate and Multivariate Analyses of Association Between Statins and ILA

	Odds Ratio (95% Confidence Interval) P value, where appropriate		
	Unadjusted	Adjusted Model 1*	Adjusted Model 2†
All Statins	1.72 (1.23-2.40) 0.002	1.60 (1.03-2.50) 0.04	1.62 (1.02-2.58) 0.04
Lipophilic Statins	1.49 (1.04-2.14) 0.03	1.36 (0.85-2.18) 0.20	1.37 (0.83-2.26) 0.22
Hydrophilic Statins	3.73 (1.96-7.09) <0.001	3.39 (1.64-7.02) 0.001	4.00 (1.86-8.59) <0.001

* Model 1: A multivariate model evaluating the association between statin use and

ILA including adjustment for age, sex, race, BMI, pack years of smoking, current

smoking status, COPD ($\geq$ GOLD Stage 2), and having either high cholesterol or

coronary artery disease.

† Model 2: A multivariate model evaluating the association between statin use and

ILA including adjustment with the same covariates as model 1 and additional

adjustments for high blood pressure, diabetes, histories of myocardial infarction,

cerebrovascular accident, venous thromboembolism, and additional cardiac

medications (including aspirin, beta-blockers, ACE inhibitors, gemfibrozil, niacin, and

fish oil).

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Figure 1A-D.
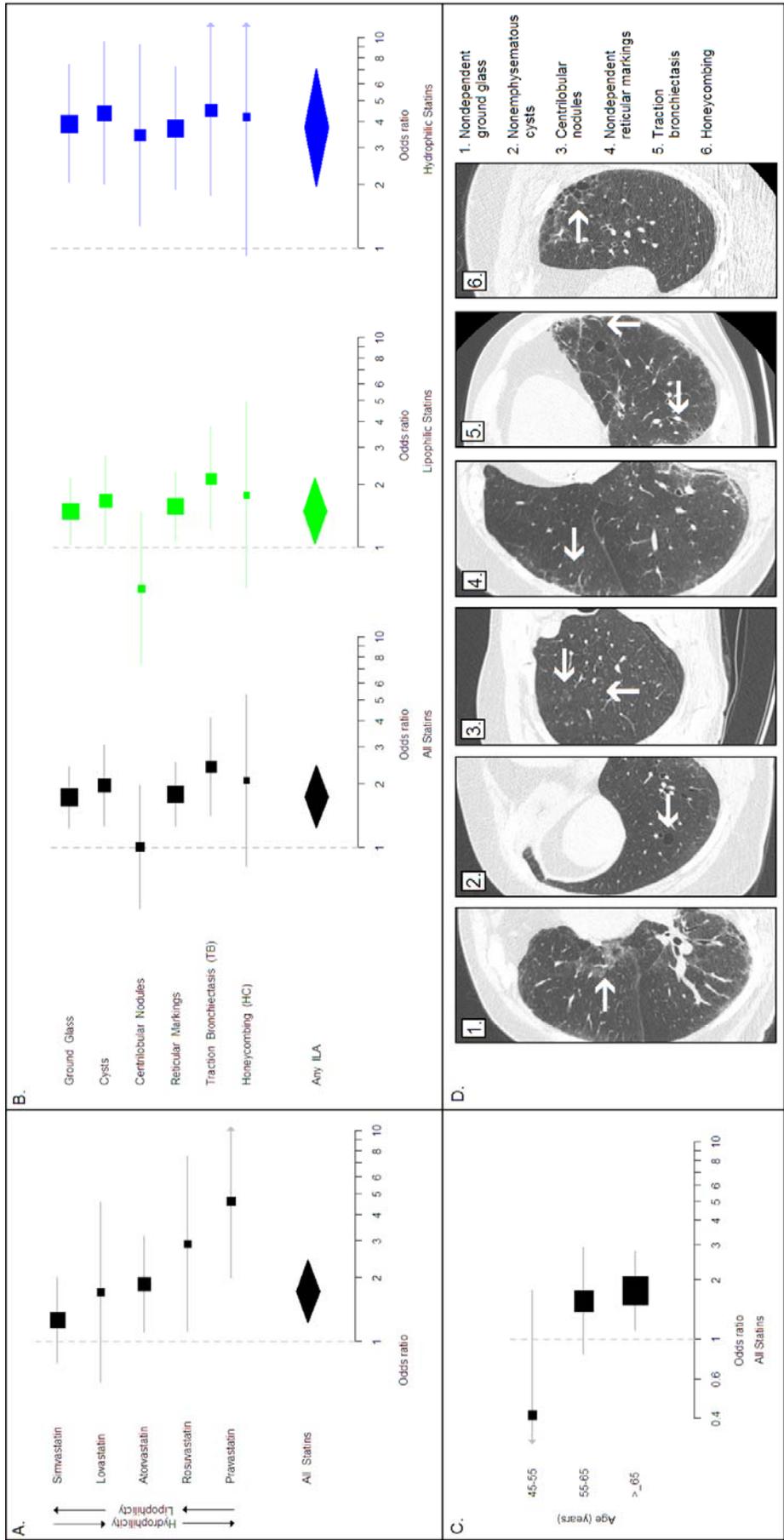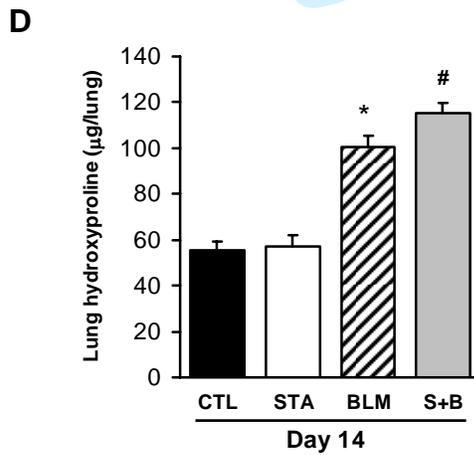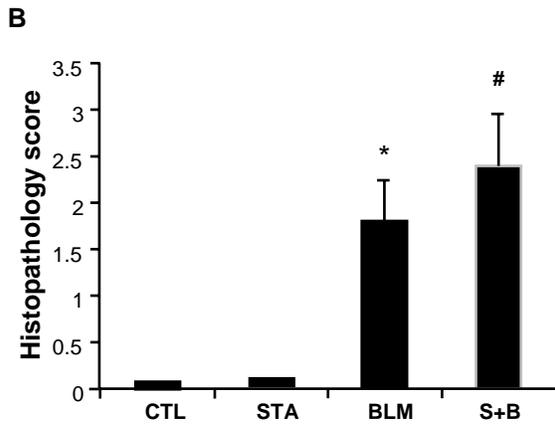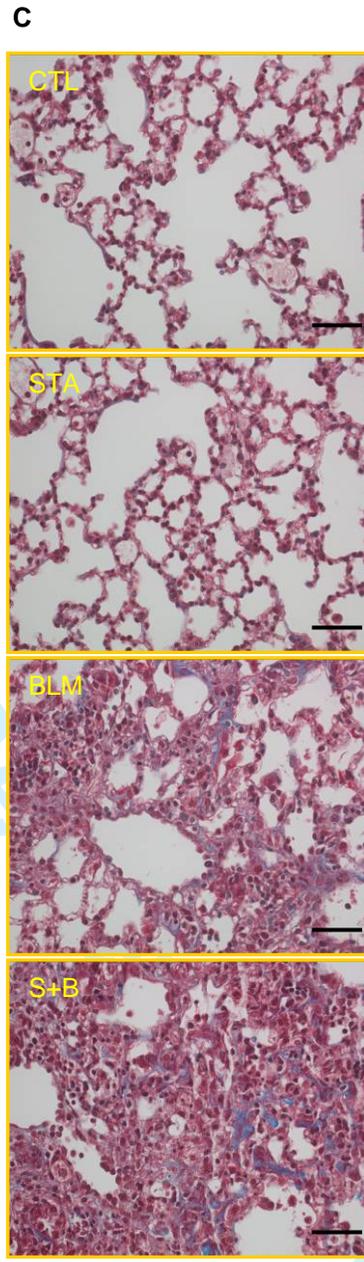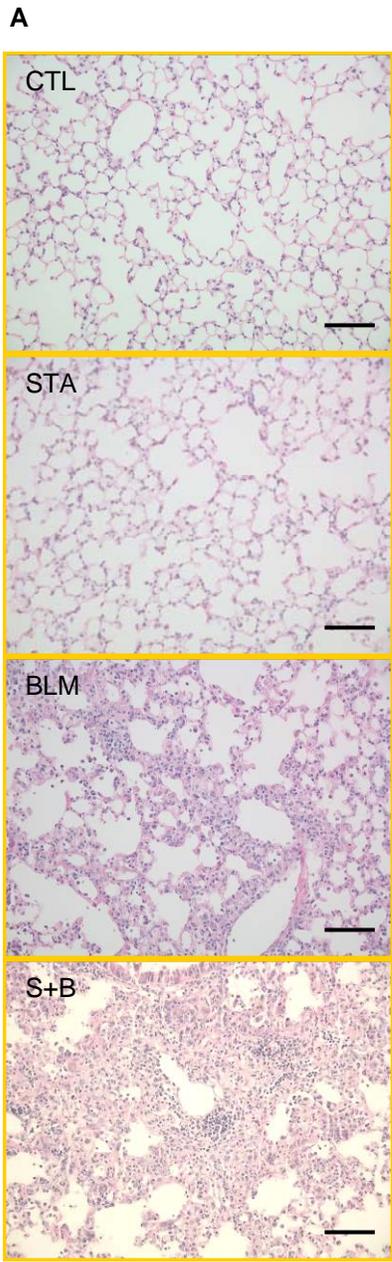
Figure 2A-D.

Figure 2E-I.

A

B

C
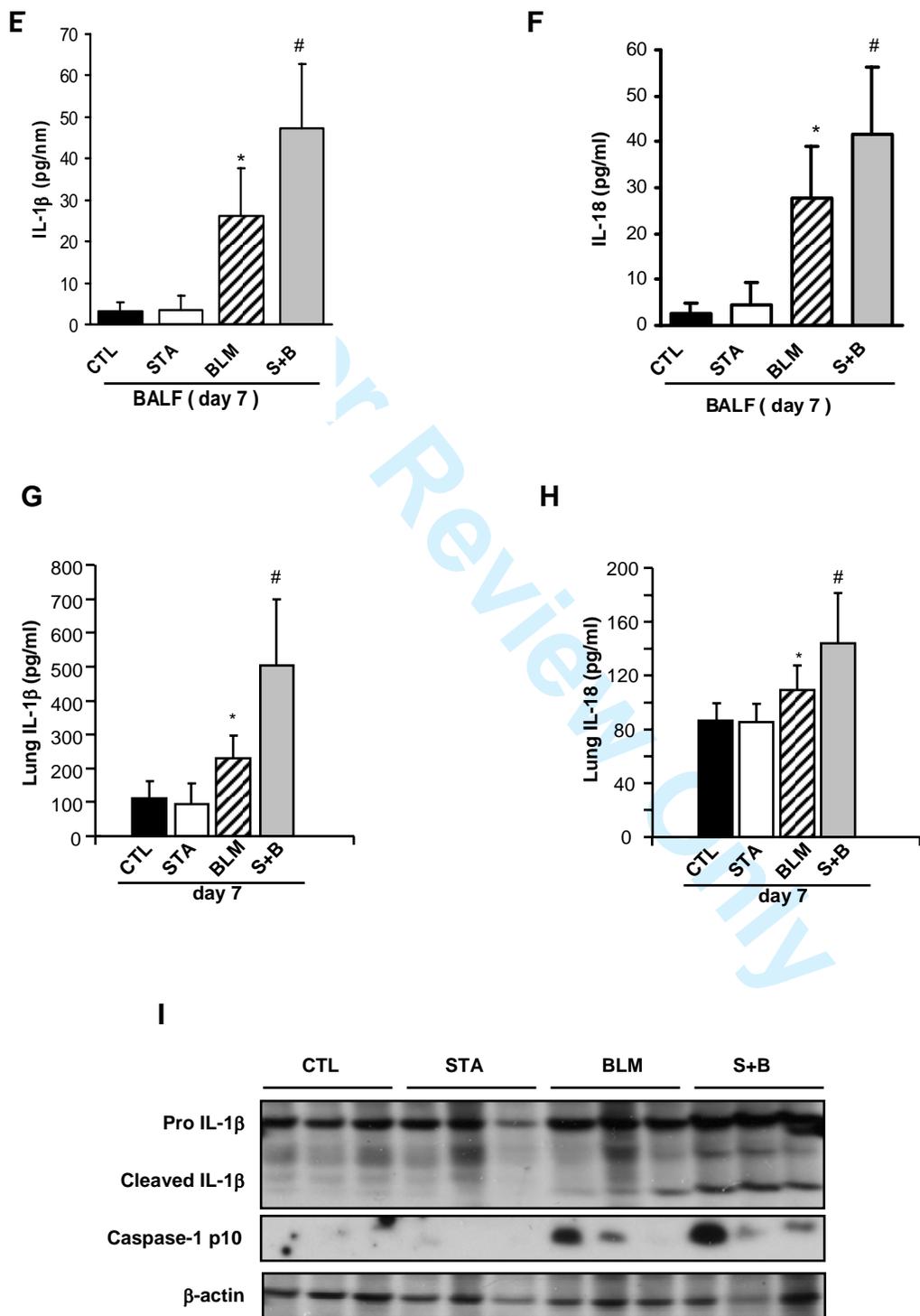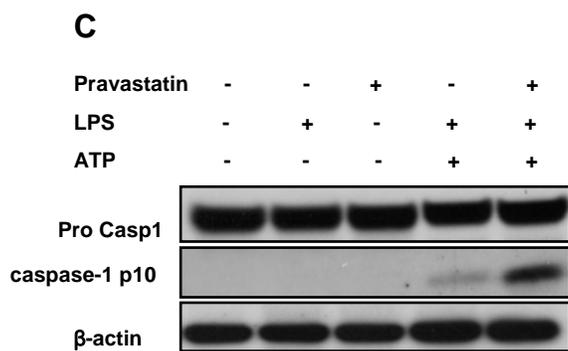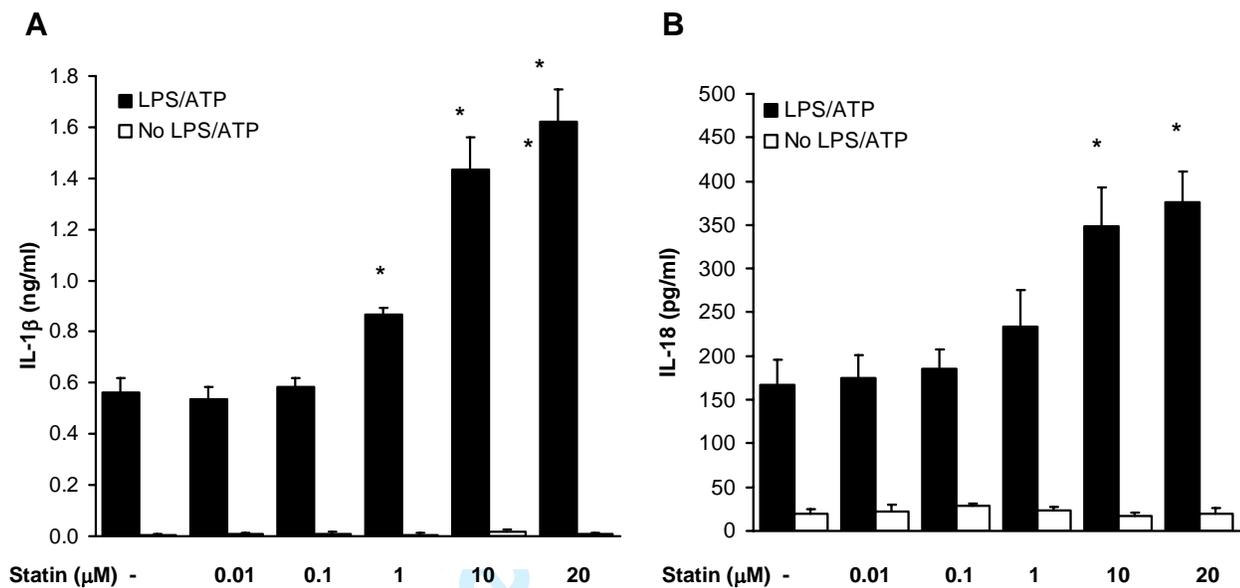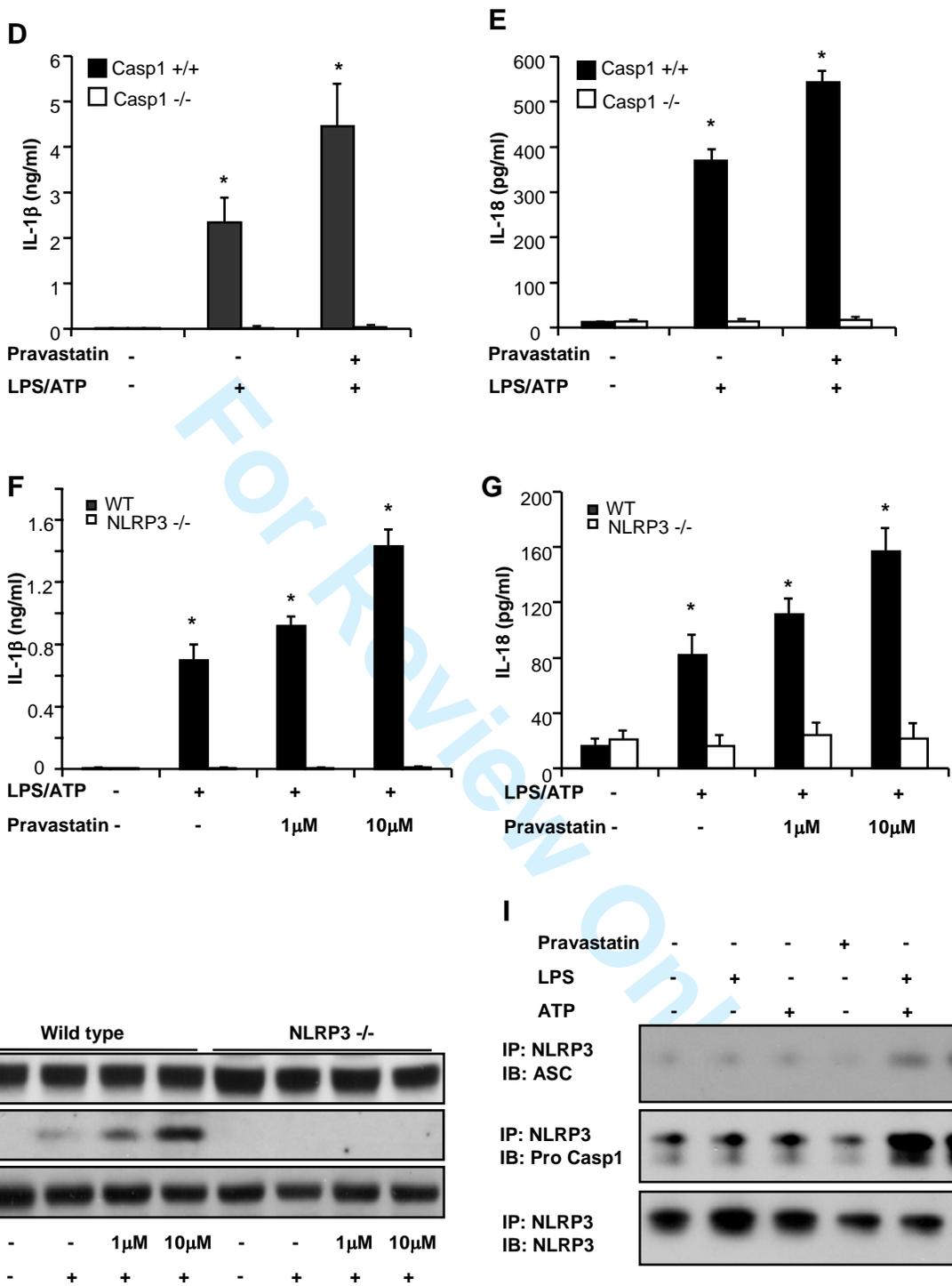

Figure 3A-C.

Figure 3D-I.

Figure 4A-C.

D

E

F
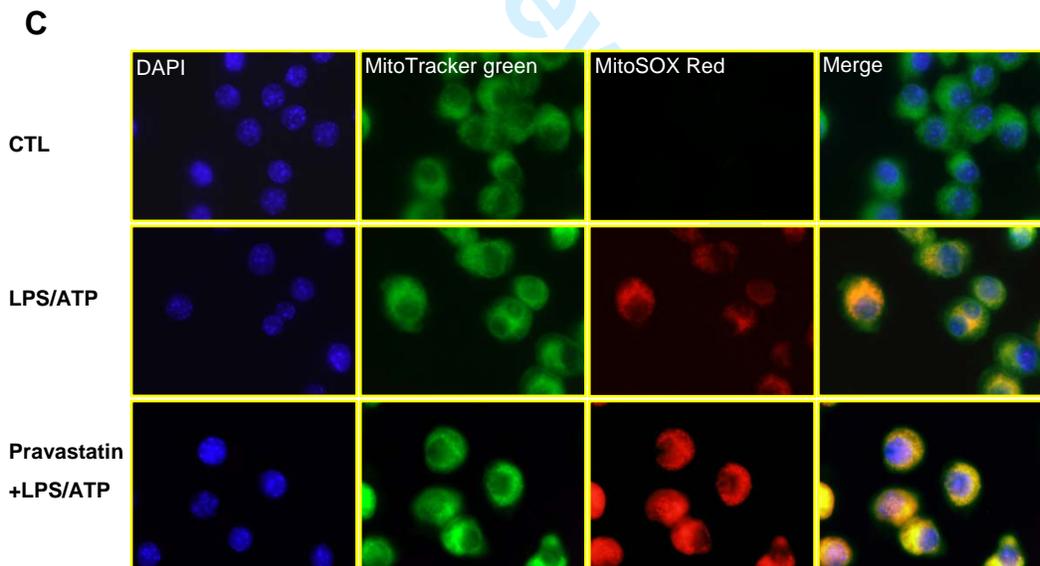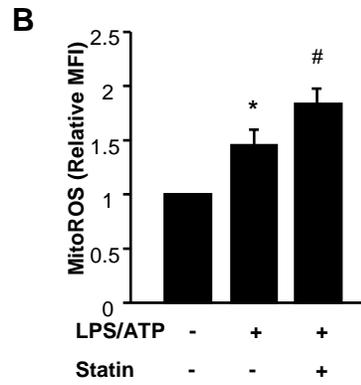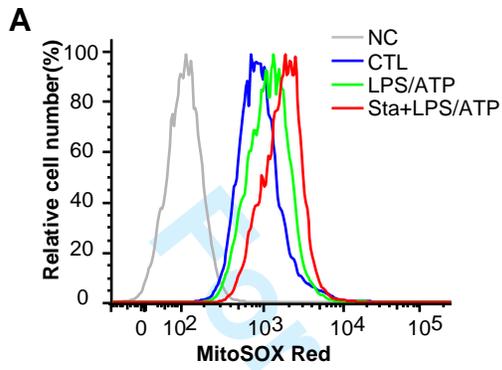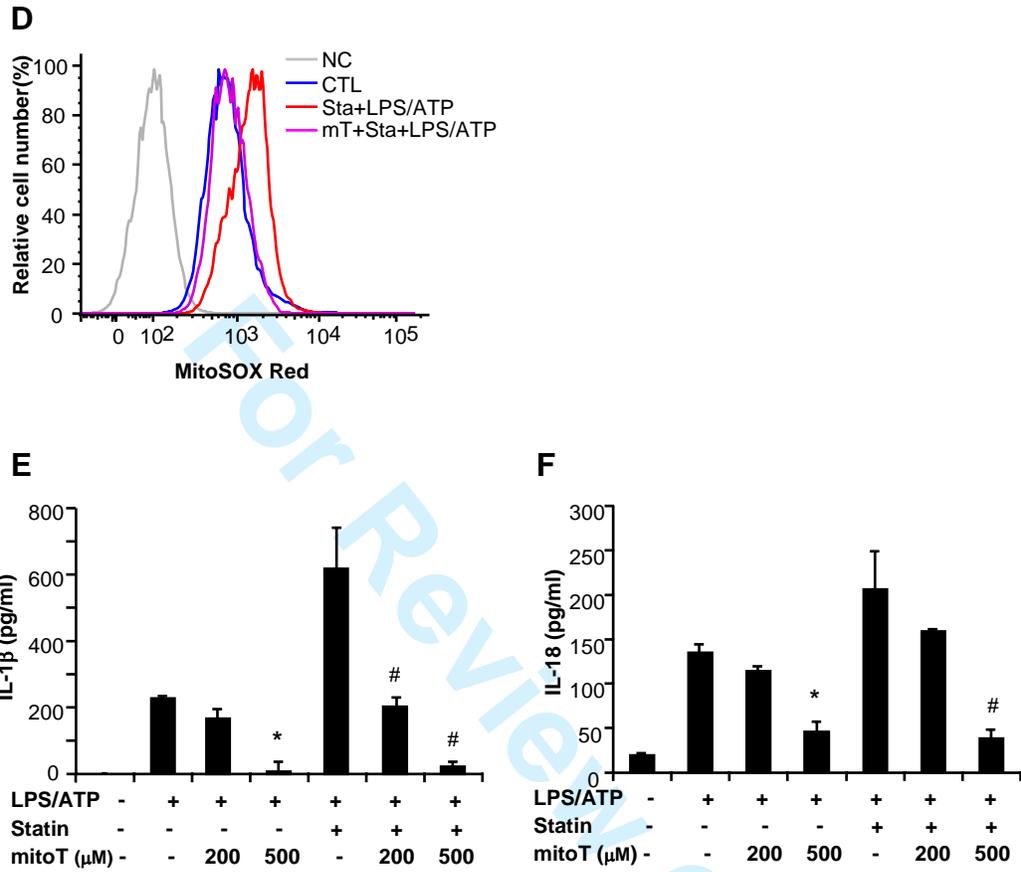
Figure 4D-F.

Statins Contribute to Pulmonary Fibrosis by Enhancing NLRP3

Inflammasome Activation

Supplementary Information

Jin-Fu Xu,[1,2]* George R. Washko,[1]* Kiichi Nakahira,[1] Hiroto Hatabu,[3, 4] Avignat S. Patel,[1] Isis E. Fernandez,[1] Mizuki Nishino,[3, 4] Yuka Okajima,[4] Tsuneo Yamashiro,[3, 4] James C. Ross,[5,6] Raúl San José Estépar,[4,5] Alejandro A. Diaz,[1, 7] Hui-Ping Li,[2] Jie-Ming Qu,[8] Blanca E. Himes,[6,9] Carolyn E. Come,[1] Katherine D'Aco,[1,6] Fernando J. Martinez,[10] MeiLan K. Han,[10] David A. Lynch,[11] James D. Crapo,[12] Danielle Morse[1], Stefan W. Ryter[1], Edwin K. Silverman,[1, 6] Ivan O. Rosas,[1] Augustine M.K. Choi,[1]* and Gary M. Hunninghake[1, 6]* and the COPDGene Investigators

[1]Pulmonary and Critical Care Division, Brigham and Women's Hospital, Harvard Medical School, Boston MA; [2]Department of Pulmonary Medicine, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, China, 200433. [3]Center for Pulmonary Functional Imaging, Brigham and Women's Hospital, Boston MA; [4]Department of Radiology, Brigham and Women's Hospital, Boston MA; [5]Surgical Planning Laboratory, Department of Radiology, Brigham and Women's Hospital, Boston MA; [6]Channing Laboratory, Brigham and Women's Hospital, Boston MA; [7]Department of Pulmonary Diseases. Pontificia Universidad Católica de Chile, Santiago, Chile; [8]Department of Pulmonary Medicine, Huadong Hospital, Fudan University, Shanghai, China, [9]Center for Genomic Medicine, Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts; [10]Department of Internal Medicine, University of Michigan, Ann Arbor MI; [11]Department of Radiology, National Jewish Medical and Research Center, Denver CO and; [12]Department of Medicine, National Jewish Medical and Research Center, Denver CO.

*These authors contributed equally to this manuscript.

Methods: COPDGene Clinical Data

From November of 2007 to April of 2010, 2500 non-Hispanic White (n=1,860, 74%), and African-American (n=640, 26%) smokers (with at least 10 pack years of smoking) between the ages of 45 and 80 were enrolled into COPDGene, an ongoing and previously described(1, 2) study designed to investigate the genetic and epidemiologic associations of COPD and other smoking related lung diseases. Prior to the release of the April 2010 version of the COPDGene dataset we identified 8 subjects, with chest computed tomographic (CT) evidence of extensive honeycomb changes and no history of interstitial lung disease (ILD), originally recruited to the COPDGene study. Although these 8 subjects ultimately did not meet criteria for inclusion in the April 2010 COPDGene dataset (subjects with history of lung disease other than asthma, emphysema, or COPD were excluded from COPDGene), they met our study inclusion criteria and are therefore included in this analysis. Further inclusion and exclusion criteria are available online (www.copdgene.org). The COPDGene study was approved by the institutional review boards of all participating centers.

Medication History

Medication use was designated by self-report.

Coronary Artery Disease, High Cholesterol, and 10-year Cardiovascular Disease Risk

Coronary artery disease, high cholesterol, diabetes, high blood pressure, and histories of myocardial infarction, cerebrovascular accident, or venous thromboembolic disease were designated by self-report.

Pulmonary Function Testing

Spirometry was performed at each of the COPDGene clinical centers with an NDD EasyOne[TM] Spirometer (Zurich, Switzerland) in accordance with American Thoracic Society/European Respiratory Society recommendations.(3) However, subjects whose spirometry did not meet reproducibility and acceptability criteria were allowed to remain in the study if they passed a central review. After baseline spirometry, two puffs (180 mcg) of Albuterol were administered, and spirometry was repeated twenty minutes later.

Volumetric CT Scanning Protocol

Volumetric CTs (CTs) were performed at full inflation and at relaxed exhalation using a 16 or 64 detector Siemens, GE, or Phillips CT scanner.

Quantitative CT Analysis

Quantitative measures of total lung volume and emphysema were performed using Airway Inspector (www.airwayinspector.org) for each inspiratory and expiratory CT scan as described previously.(4, 5) Briefly, the tracheobronchial tree is automatically extracted using a region growing approach starting from a seed point automatically placed in the tracheal lumen. Following segmentation of

the central airways, the lungs were automatically identified and segmented from the chest.(6) The left and right lung was also extracted. The volume of the lung minus the central airways for each CT scan was then calculated and reported at full inspiration (TLC) and at relaxed exhalation (LV_{re}). The percentage of emphysematous lung was defined as the volume of lung with a CT attenuation value of less than -950 Hounsfield units (HU)(4) divided by the total lung volume at full inflation, multiplied by 100. Quantitative measures of lobar segmented emphysema were obtained using VIDA software (VIDA Diagnostics, Iowa City, IA).

Visual CT Analysis

All inspiratory CT images were reviewed on Picture Archiving Communication Systems (PACS) workstations (Centricity, GE Healthcare) using axial images with a window level of -700 HU and a window width of 1500 HU. The CTs were evaluated by three readers (including one pulmonologist and two chest radiologists) using a sequential reading method as previously described.(2) CTs were scored as follows: no evidence of ILA, indeterminate, and ILA. In brief, for each block of 100 CT scans, reader 1 would review all of the 100 CT scans. Reader 2, who was blinded to the initial interpretation, would review all of the scans labeled as ILA, indeterminate, and 20% of the normal scans. Finally, reader 3, also blinded to the previous interpretations, provided majority opinion on those scans discordantly scored. Readers rotated positions after each block of 100 CT scans were evaluated. ILA were defined as changes affecting >5% of

any lung zone including, nondependent ground-glass or reticular abnormalities, diffuse centrilobular nodularity, nonemphysematous cysts, honeycombing, or traction bronchiectasis.(2, 7, 8) Indeterminate scans were defined as focal or unilateral ground-glass attenuation, focal or unilateral reticulation, and patchy ground-glass abnormality (<5% of the lung). Qualitative CT assessment was performed by readers blind to any additional information including current use of medications.

All subjects with ILA had 8 separate radiologic features characterized by consensus opinion of three readers who were blinded to the subject's clinical characteristics. These 8 features include; the presence of non-dependent ground glass, or reticular abnormalities, traction bronchiectasis, honeycombing, non-emphysematous cysts, centrilobular nodules, the predominant location of abnormalities (including upper lobe predominant, lower lobe predominant, diffuse, or multifocal), and the distribution of these abnormalities (including predominantly centrilobular, subpleural, or mixed centrilobular and subpleural abnormalities).

We divided the subjects with ILA into four major radiologic subtypes including 1) predominant centrilobular and/or peribronchial ground glass opacities sparing the peripheral lung parenchyma (Centrilobular), 2) reticular/nodular and/or ground glass opacities in a predominant subpleural distribution (Subpleural), 3) mixed centrilobular and subpleural abnormalities (Mixed) and, 4) extensive radiologic

changes consistent with firm evidence of ILD based on ATS guidelines(9)

(Radiologic ILD).(10)

Statistical Analysis

Bivariate analyses were conducted with Fisher's exact tests (for categorical

variables), and two-tailed t tests or Wilcoxon rank-sum tests (for continuous

variables) where appropriate. Logistic regression models were used in

multivariate analyses to study the relation between ILA status and statin use. In

all models, subjects for whom ILA could not be confidently included or excluded

(n=759, 36% [indeterminate]) were removed from analysis. All of the adjusted

models include age, sex, race, pack-years of smoking, current smoking status,

COPD (defined as $\geq$ GOLD stage 2)(11), and self-report of either high cholesterol

or coronary artery disease unless specifically indicated. We estimated the

population risk of ILA attributable to statin use in COPDGene using the following

formula.(12)

$$PAR = pd[(OR-1)/OR]x100,$$

where "pd" is the prevalence of statin use among subjects with ILA and "OR" is

the adjusted odds ratio(13) obtained from multivariate logistic regression.   All

analyses were performed using Statistical Analysis Software version 9.1 (SAS

Institute, Cary, NC). P values < .05 were considered statistically significant.

Laboratory Data

Reagents. The following antibodies were used: mouse monoclonal antibody to

NLRP3 (Alexis Biochemicals), mouse monoclonal antibody to ASC (Millipore), anti-rabbit P2X7 Receptor antibody (APR-004; Alomone labs, Jerusalem, Israel); Rabbit anti-mouse caspase-1 (sc-514; Santa Cruz Biotechnology, Santa Cruz, CA); and rabbit anti-mouse IL-1β (5129-100; Biovision). The Diff-Quik Stain Set was from Dade Behring Inc. (Newark, DE). Mito-TEMPO was from Alexis. LPS (*Escherichia coli*) was from Invivogen (San Diego, CA). MitoTracker green and MitoSOX red stain were from Invitrogen (Carlsbad, CA). Protein A/G plus–Agarose was from Santa Cruz Biotechnology. The mIL-1β and TNF ELISA kits were from R&D Systems (Minneapolis, MN). The mIL-18 ELISA kit was from MBL (Woburn, MA). Bleomycin was from Hospira Inc. (Adelaide, Australia). Pyrexplus tube was from Corning Incorporated (Corning, NY). Pravastatin, Atorvastatin, ATP, Accustain Trichrome stains (Masson, HT15), hydroxyproline standard, chloramine-T, 4-(Dimethl-amino) benzaldehyde, DAPI, and all other reagent grade chemicals were from Sigma (St. Louis, MO).

Mice. Pathogen free C57B/L6 male mice were housed in a pathogen-free facility. The *Nlrp3*^{-/-} and *Casp-1*^{-/-} mice were generated as previously described.(14) All experiments were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal care and use for all experiments was approved by the Harvard Medical Area Standing Committee on Animals of Harvard Medical School.

Cell culture. J774A.1 macrophages were maintained in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Bone marrow-derived macrophages (BMDMs), were prepared from wild-type, $Nlrp3^{-/-}$ and $Casp1^{-/-}$ mice as described previously.(14) Briefly, bone marrow collected from mouse femurs and tibias was plated on sterile petri dishes and then incubated for 7 days in DMEM containing 10% (*vol/vol*) heat-inactivated FCS, and 25% (*vol/vol*) conditioned medium from L929 mouse fibroblasts, penicillin and streptomycin.

For the induction of inflammasome activation, macrophages were cultured in DMEM containing 10% FBS and antibiotics. Cells were pretreated with statins for 24 h prior to treatment with LPS (500 ng/ml). MitoTEMPO was added to the medium 1 before LPS priming as described before.(14)

Co-immunoprecipitation and Western blot analysis. Proteins were isolated from cell cultures in immunoprecipitation assay buffer [1x PBS, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, 0.1 mg/ml phenylmethylsulfonyl fluoride, 30 µl/ml aprotinin, and 1 mM sodium orthovanadate]. Protein concentration was determined using the Coomassie plus protein assays (Pierce, Rockford, IL, USA) on Beckman Counter DU640 spectrophotometer and was equalized among all samples. For co-immunoprecipitation, samples were pre-cleared with Protein A/G plus–Agarose beads (Santa Cruz Biotechnology), then 1 µg of antibody was added to 500 µg of total protein in 500 µl, rotated overnight at 4°C, and then incubated with 25 µl of

beads for 2–4 h, spun down at 500 x g, and washed 5 times with immunoprecipitation buffer. Then, 50 µl of loading buffer (100 mM Tris-HCl, 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, and 20% glycerol) was added. For SDS-PAGE, samples containing equal amounts of protein were boiled in the loading buffer and separated on SDS-PAGE, followed by transfer to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk and stained with the primary antibodies for 2 to 12 h at 1:250 to 1:2000 dilutions. After 5 washes in PBS with 0.2% Tween 20, the horseradish peroxidase-conjugated secondary antibody was applied, and the blot was developed with enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ, USA). All secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

ELISA for cytokines. The mouse IL-1β ELISA kit was from R&D Systems. Mouse IL-18 ELISA kit was from MBL. Mouse cytokines in culture supernatants, serum, BALF or lung tissue lyses were measured with ELISA kits according to the manufacturer's protocol.

Flow cytometry. Mitochondrial ROS were measured in cells by MitoSOX (Invitrogen) staining (5 µM for 15 min at 37 °C), as previous reported.(14) Briefly, for measurement of mitochondrial ROS, cells were stained for 15 min at 37 °C with 5µM MitoSox red, followed by 20 min of ATP treatment. Cells were washed with PBS, treated with trypsin and resuspended in PBS containing 1% (vol/vol)

heat-inactivated FBS. Data were acquired with a FACSCanto II (BD Biosciences) and were analyzed with FlowJo analytical software (TreeStar).

Fluorescence staining. After treatment, cells were fixed with 4% paraformaldehyde, and followed with the immunofluorescence staining protocol as described previously.(15) MitoTracker green, MitoSOX red and DAPI were used to label total mitochondria, mtROS and the nuclei respectively. Stained samples were fixed onto the slides and viewed with Olympus Fluoview-FV10i Confocal and Olympus FSX100 fluorescence microscopy. Fluorescence picture was simultaneously captured by standard confocal imaging techniques.

In vivo experiments. Male mice (8 weeks old, 18-22 g) were used for *in vivo* experiments. Bleomycin (Hospira Inc., Adelaide, Australia) at a dose of 0.1U/mice in 50 µl normal saline was instilled into lung intratracheally to induce lung inflammation and fibrosis, as previously described.(16, 17) Pravastatin (40 mg/kg per day, intraperitoneally) was administered intraperitoneally to the mice from three days before bleomycin instillation and treated daily for 2 or 3 weeks (S+B group). Mice instilled with normal saline and injected with PBS daily were taken as control (CTL group). Mice instilled with bleomycin and injected with PBS daily were taken as the bleomycin group (BLM group). Mice injected with pravastatin daily following instillation with normal saline were taken as pravastatin group (STA group). At day 7 or day 14 after bleomycin instillation, mice were killed with an overdose of intraperitoneally administered Beuthanasia-

D (pentobarbital sodium and phenytoin sodium). After blood collection, lungs were lavaged three times with a total volume of 1 ml PBS (pH 7.4), immediately placed on ice, and then centrifuged at 2,500 rpm on a tabletop centrifuge for 10 minutes. Supernatants of BALF were stored for further assays. The cell pellets were used to count the total cell number after eradicating the red blood cells. We then performed cytospins (Shandon Cytospin 3, USA) with cell suspensions and stained cytospins in Diff-quick (Dade Behring Inc., Newark, DE) and differential cell counts were determined. BALF protein concentrations were detected using the Coomassie plus protein assays. Whole left lung were used for hydroxyproline assays, as previous reported.(16) Mice had their right lungs fixed or immediately frozen and stored at -80 ℃. Lungs were fixed for histological evaluation with 1% paraformaldehyde, and then immersed in fixative overnight. After fixation and paraffin embedding, the lungs were stained with hematoxylin and eosin to assess peribronchiolar inflammation and fibrosis scores as previously reported.(18, 19) Briefly, HE stained sections were observed at light microscopy and the lesions were defined as follows: Score 0, no lesions; Score 1, occasional small localized subpleural fibrotic foci; Score 2, thickening of intra-alveolar septa and subpleural fibrotic foci; and Score 3, thickened continuous subpleural fibrous foci and intra-alveolar septa. Masson Trichome staining was performed as previously reported.

Statistical analysis. Data represent the mean±SD. Student's t-test was used for statistical analysis. *P* values <0.05 were considered statistically significant.

RESULTS

While there was no difference in the prevalence of ILA among those who provided information about statin use compared to those who did not (13% vs. 11%, respectively) those who provided information about statin use tended to be older (median age 62 vs. 54, respectively) with an increased frequency of conditions associated with aging (e.g. COPD in 43% vs. 30%, respectively). The only statistically significant difference in baseline characteristics among those with ILA who provided information about statin use compared to those who did not was an increased report of high cholesterol (48% vs. 13%, respectively). Of these 2207 subjects, 2115 (96%) had a CT available and were included in these analyses. Information about the concordance between readers for the identification of ILA has been published previously.(10) Baseline characteristics of subjects stratified by statin use and ILA status are presented in **Supplemental Table E1**. Of the 2115 subjects, 29% (n=615) reported taking statin medications including simvastatin (n=305, 50%), atorvastatin (n=179, 29%), rosuvastatin (n=41, 7%), lovastatin (n=41, 7%), pravastatin (n=39, 6%), fluvastatin (n=6, 1%), and cervastatin (n=1, 0.2%). Three subjects reporting statin medication use did not provide specific drug information.

As expected, statin use was associated with increasing age, a history of high cholesterol levels, and numerous cardiovascular risk factors, outcomes, and associated medications. Consistent with prior studies, statin use was less

common among women and African-Americans.(20, 21) While statin users had

greater degrees of tobacco smoke exposure, they were 20% less likely to be

smoking at the time of study entry (see **Table E1**).   Although statin users had a

greater degree of emphysema on CT, there were no significant differences in

COPD (defined as $\geq$ GOLD stage 2)(11), or measurements of lung volumes.

Statins and Interstitial Lung Abnormalities (ILA)

In COPDGene, 22 subjects (<1%) were taking medications known to cause

interstitial lung disease (ILD) (including methadone (n=10), methotrexate (n=9),

nitrofurantoin (n=2), and carbemazepime (n=1)). We observed an association of

borderline statistical significance between use of medications known to cause

ILD and ILA (odds ratio [OR] 3.03, 95% confidence interval [CI] 0.86-10.69,

P=0.08).

Statin use remained significantly associated with ILA in models adjusting for

relevant covariates including a history of high cholesterol or coronary artery

disease, high blood pressure, diabetes, a history of myocardial infarction, a

history of a cerebrovascular accident, a history of venous thromboembolic

disease, and excluding the 22 subjects taking medications known to cause ILD

(OR 1.58, 95% CI 1.002-2.49, P=0.05).

The association between statin use and ILA varied by statin type (see

Supplemental Table E2). The prevalence of ILA varied from 8% in subjects on

simvastatin to 23% for subjects on pravastatin (see **Supplemental Table E2**).

Subjects on hydrophilic statins (rosuvastatin and pravastatin) had an ~3.4 fold

increase in their odds for having ILA compared to an ~1.4 fold increase in the

odds for ILA in subjects on lipophilic statins (cerivastatin, simvastatin, lovastatin,

fluvastatin, and atorvastatin) (P for difference between hydrophilic and lipophilic

statins in the prediction of ILA = 0.008).

Statins and Age

While there was no evidence for an interaction between statin use and many

relevant covariates (including both current use and pack-year history of tobacco

smoke exposure), there was significant evidence that age modified the

association between statin use and ILA (P = 0.04 for the interaction, see **Figure

1C**). For example, in subjects 45-55 years old (where the prevalence of ILA is

6%), statin users were less likely to have ILA after adjusting for relevant

covariates (although this result was not statistically significant, OR 0.36, 95% CI

0.07-1.78, P = 0.21), while in subjects > 65 years old (where the prevalence of

ILA is 11%), statin users were more likely to have ILA after adjusting for relevant

covariates (OR 1.96, 95% CI 1.06-3.61, P=0.03).

Race and ILA

Previous analyses by our group did not demonstrate a significant association

between race (white vs. African-American) and ILA.(10) However, in models

adjusting for relevant covariates including a history of high cholesterol or

coronary artery disease *and statin use* we note African-Americans had ~2.3 fold

increase in their odds for ILA compared to white subjects (OR 2.32, 95% CI 1.53-

3.51, P = <0.001). At least part of the differences between our previous

association between race and ILA(10) and our current association can be

explained by differences in statin use between white and African-American

subjects (see **Supplemental Table E1**).

Coronary Artery Disease and Subtypes of ILA

While a history of coronary artery disease was associated with ILA at baseline

(OR 2.06, 95% CI 1.22-3.46, P=0.007), after adjustment for relevant covariates

this association was of borderline significance (OR 1.67, 95% CI 0.94-2.98,

P=0.08), and was further attenuated by the inclusion of statins into the model

(OR 1.44, 95% CI 0.77-2.66, P=0.25).

There was no significant association between a history of coronary artery

disease and subtypes of ILA including centrilobular, subpleural, and mixed after

adjustment for relevant covariates. In contrast, a history of coronary artery

disease was associated with Radiologic ILD (OR 5.23, 95% CI 1.23-22.29,

P=0.03) after adjustment for relevant covariates.

Statins Exacerbate Bleomycin Induced Fibrosis in the Mouse

Comparable to our measurements of hydroxyproline, IL-1□, and IL-18, the lungs

of mice treated with both pravastatin and bleomycin as well as bleomycin alone

demonstrated increases in collagen-1 transcript level (p values <0.02) and TGF-

□ protein measurement (p values <0.001) relative to controls. Although similar

increases in the mean levels of collagen-1 transcript level and TGF-□protein

measurement were noted in mice treated with both pravastatin and bleomycin

compared to mice treated with bleomycin alone these findings were not

statistically significant.

Figure legends

Supplemental Figure 1: Pravastatin increases bleomycin-induced lung inflammatory response and fibrotic changes in mice. (**A**) Sections of paraffin-embedded lung tissue from mice treated with pravastastin and bleomycin were stained with H.E. Images were shown at a 200x magnification. Bar, 20 μm, day 7; CTL- Control, STA - Pravastatin, BLM - Bleomycin, S+B – Pravastatin + Bleomycin. (**B**) Sections of paraffin-embedded lung tissue from mice treated with pravastastin and bleomycin were stained with Masson Trichome. Images were shown at a 400x magnification. Bar, 40 μm, day 7; CTL- Control, STA - Pravastatin, BLM - Bleomycin, S+B – Pravastatin + Bleomycin. (**C**) The weight change was monitored at day 7 and 14 after bleomycin instillation. (**D**) The number of PMN and lymphocyte in BALF at day 7. (**E**) Protein concentration in BALF at day 7 and 14 was measured.

Supplemental Figure 2: Statin pretreatment increased caspase-1 activation in macrophages stimulated with LPS and ATP. J774A.1 macrophages were pretreated with atorvastatin or vehicle (DMSO) for 24 h at indicated concentration (μM) and then incubated with LPS (500 ng/ml) for 4 h, followed by stimulation with ATP (5 mM). Secretion of IL-1β (**A**) and IL-18 (**B**) into the media was measured by ELISA. *$P < 0.01$, versus cells treated with LPS and ATP. (**C**) J774A.1 macrophages were pretreated with pravastatin at indicated concentrations for 24 h and then incubated with LPS (500 ng/ml) for 4 h, followed by stimulation with ATP (5 mM) for 15 min. Cell lysates were analyzed by

immunoblotting for caspase-1. (**D,E**) LPS-primed macrophages were pretreated with glyburide (10-100 ☐M) or vehicle (DMSO) 15min before ATP treatment in the absence or presence of pravastatin. Secretion of IL-1β (**D**) and IL-18 (**E**) was analyzed by ELISA. *$P < 0.01$, versus pravastatin-pretreated cells primed with LPS and ATP. (**F**) J774A.1 macrophages were pretreated with pravastatin (10 ☐M) for 24 h and then incubated with LPS (500 ng/ml) for 4 h, followed by stimulation with ATP (5 mM) for 15 min. Cell lysates and supernatants were analyzed by immunoblotting for IL-1β, ASC, NLRP3 and P2X7 receptor.

Supplemental Figure 3: Statin pretreatment increased MtROS generation in LPS and ATP stimulated macrophages (**A**) J774A.1 Macrophages were pretreated with MitoTEMPO (500 ☐M) 1h before LPS treatment in the absence or presence of pravastatin. Level of mtROS in cells was analyzed by MitoSOX labeling. (**B**) Relative MFI of MitoSOX was represented. * $P < 0.01$, versus cells treated with LPS and ATP. [#] $P < 0.01$, versus statin-pretreated cells stimulated with LPS and ATP. (**C**) Macrophages were pretreated with MitoTEMPO 1h before LPS treatment in the absence or presence of statin, followed by ATP stimulation for 1 h. TNF secretion was analyzed by ELISA. * $P < 0.01$, versus cells treated with LPS and ATP. [#] $P < 0.01$, versus statin-pretreated cells stimulated with LPS and ATP.

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The members of the COPDGene® study group as of June 2010

Ann Arbor VA: Jeffrey Curtis, MD (PI), Ella Kazerooni, MD (RAD)

Baylor College of Medicine, Houston, TX: Nicola Hanania, MD, MS (PI), Philip Alapat, MD, Venkata Bandi, MD, Kalpalatha Guntupalli, MD, Elizabeth Guy, MD, Antara Mallampalli, MD, Charles Trinh, MD (RAD), Mustafa Atik, MD

Brigham and Women's Hospital, Boston, MA: Dawn DeMeo, MD, MPH (Co-PI), Craig Hersh, MD, MPH (Co-PI), George Washko, MD, Francine Jacobson, MD, MPH (RAD)

Columbia University, New York, NY: R. Graham Barr, MD, DrPH (PI), Byron Thomashow, MD, John Austin, MD (RAD)

Duke University Medical Center, Durham, NC: Neil MacIntyre, Jr., MD (PI), Lacey Washington, MD (RAD), H Page McAdams, MD (RAD)

Fallon Clinic, Worcester, MA: Richard Rosiello, MD (PI), Timothy Bresnahan, MD (RAD)

Health Partners Research Foundation, Minneapolis, MN: Charlene McEvoy, MD, MPH (PI), Joseph Tashjian, MD (RAD)

Johns Hopkins University, Baltimore, MD: Robert Wise, MD (PI), Nadia Hansel, MD, MPH, Robert Brown, MD (RAD), Gregory Diette, MD

Los Angeles Biomedical Research Institute at Harbor UCLA Medical Center, Los Angeles, CA: Richard Casaburi, MD (PI), Janos Porszasz, MD, PhD, Hans Fischer, MD, PhD (RAD), Matt Budoff, MD

Michael E. DeBakey VAMC, Houston, TX: Amir Sharafkhaneh, MD (PI), Charles Trinh, MD (RAD), Hirani Kamal, MD, Roham Darvishi, MD

Minneapolis VA: Dennis Niewoehner, MD (PI), Tadashi Allen, MD (RAD), Quentin Anderson, MD (RAD), Kathryn Rice, MD

Morehouse School of Medicine, Atlanta, GA: Marilyn Foreman, MD, MS (PI), Gloria Westney, MD, MS, Eugene Berkowitz, MD, PhD (RAD)

National Jewish Health, Denver, CO: Russell Bowler, MD, PhD (PI), Adam Friedlander, MD, David Lynch, MB (RAD), Joyce Schroeder, MD (RAD), John Newell, Jr., MD (RAD)

Temple University, Philadelphia, PA: Gerard Criner, MD (PI), Victor Kim, MD, Nathaniel Marchetti, DO, Aditi Satti, MD, A. James Mamary, MD, Robert Steiner, MD (RAD), Chandra Dass, MD (RAD)

University of Alabama, Birmingham, AL: William Bailey, MD (PI), Mark Dransfield, MD (Co-PI), Hrudaya Nath, MD (RAD)

University of California, San Diego, CA: Joe Ramsdell, MD (PI), Paul Friedman, MD (RAD)

University of Iowa, Iowa City, IA: Geoffrey McLennan, MD, PhD (PI), Edwin JR van Beek, MD, PhD (RAD), Brad Thompson, MD (RAD), Dwight Look, MD

University of Michigan, Ann Arbor, MI: Fernando Martinez, MD (PI), MeiLan Han, MD, Ella Kazerooni, MD (RAD)

University of Minnesota, Minneapolis, MN: Christine Wendt, MD (PI), Tadashi Allen, MD (RAD)

University of Pittsburgh, Pittsburgh, PA: Frank Sciurba, MD (PI), Joel Weissfeld, MD, MPH, Carl Fuhrman, MD (RAD), Jessica Bon, MD

University of Texas Health Science Center at San Antonio, San Antonio, TX: Antonio Anzueto, MD (PI), Sandra Adams, MD, Carlos Orozco, MD, Mario Ruiz, MD (RAD)

Administrative Core: James Crapo, MD (PI), Edwin Silverman, MD, PhD (PI), Barry Make, MD, Elizabeth Regan, MD, Sarah Moyle, MS, Douglas Stinson

Genetic Analysis Core: Terri Beaty, PhD, Barbara Klanderman, PhD, Nan Laird, PhD, Christoph Lange, PhD, Michael Cho, MD, Stephanie Santorico, PhD, John Hokanson, MPH, PhD, Dawn DeMeo, MD, MPH, Nadia Hansel, MD, MPH, Craig Hersh, MD, MPH, Jacqueline Hetmanski, MS, Tanda Murray

Imaging Core: David Lynch, MB, Joyce Schroeder, MD, John Newell, Jr., MD, John Reilly, MD, Harvey Coxson, PhD, Philip Judy, PhD, Eric Hoffman, PhD, George Washko, MD, Raul San Jose Estepar, PhD, James Ross, MSc, Rebecca Leek, Jordan Zach, Alex Kluiber, Jered Sieren, Heather Baumhauer, Verity McArthur, Dzimitry Kazlouski, Andrew Allen, Tanya Mann, Anastasia Rodionova

PFT QA Core, LDS Hospital, Salt Lake City, UT: Robert Jensen, PhD

Biological Repository, *Johns Hopkins University, Baltimore, MD*: Homayoon Farzadegan, PhD, Stacey Meyerer, Shivam Chandan, Samantha Bragan

Data Coordinating Center and Biostatistics, *National Jewish Health, Denver, CO*: James Murphy, PhD, Douglas Everett, PhD, Carla Wilson, MS, Ruthie Knowles, Amber Powell, Joe Piccoli, Maura Robinson, Margaret Forbes, Martina Wamboldt

Epidemiology Core, *University of Colorado School of Public Health, Denver, CO*: John Hokanson, MPH, PhD, Marci Sontag, PhD, Jennifer Black-Shinn, MPH, Gregory Kinney, MPH

Supplemental Table E1: Baseline characteristics of Smokers from

COPDGene Stratified by Statin Use.

Variable*	Number(%) or Median (Interquartile Range) where appropriate		
Demographic Parameters	Not Taking Statins n=1500 (71%)	On Statins n=615 (29%)	P value[†]
Age (years)	60 (52-67)	66 (60-72)	<0.001
Gender (female)	762 (51%)	282 (46%)	0.04
Race (African-American)	404 (27%)	67 (11%)	<0.001
Pack years of smoking	39 (28-54)	45 (33-65)	<0.001
Body Mass Index	27 (23-31)	29 (26-33)	<0.001
Current Smoker	711 (47%)	168 (27%)	<0.001
High Cholesterol	383 (26%)	533 (87%)	<0.001
Coronary Artery Disease	36 (2%)	113 (18%)	<0.001
Diabetes	121 (8%)	152 (25%)	<0.001
High Blood Pressure	554 (37%)	371 (60%)	<0.001
Previous Myocardial Infarction	37 (2%)	73 (12%)	<0.001
Previous Cerebrovascular Accident	27 (2%)	25 (4%)	0.003
Previous Thromboembolic Disease	53 (4%)	39 (6%)	0.007
Medications			
Aspirin	164 (11%)	202 (33%)	<0.001
Beta Blockers	130 (9%)	163 (27%)	<0.001
ACE inhibitors	129 (9%)	142 (23%)	<0.001
Gemfibrozil	6 (<1%)	1 (<1%)	0.68
Niacin	3 (<1%)	8 (1%)	0.003
Fish Oil	23 (2%)	27 (4%)	<0.001
Spirometric Parameters			
COPD ($\geq$ GOLD Stage 2)	631 (42%)	282 (46%)	0.12
FEV_1 (% of predicted)‡	79 (54-94)	76 (52-93)	0.52
FVC (% of predicted)‡	88 (75-99)	87 (74-98)	0.33
FEV1/FVC %‡	69 (51-78)	67 (51-76)	0.14
Chest CT Parameters			
Emphysema % (-950 HU)§‖	3.3 (1.1-11.0)	5.1 (1.6-13.0)	<0.001
Total Lung Capacity (TLC: Liters)‖	5.5 (4.6-6.6)	5.6 (4.7-6.5)	0.39
No ILA Indeterminate ILA	869 (58%) 525 (35%) 106 (7%)	315 (51%) 234 (38%) 66 (11%)	0.007**

* Data on disease related demographic, and medication variables were determined by self-report. Data missing for high blood pressure, previous cerebrovascular

accident, previous thromboembolic disease, COPD status and pulmonary function testing (n=1), emphysema % and TLC (n=18, <1%).

† P values compare those taking statins to those not taking statins using Fisher's exact tests (for binary variables), and paired t-tests or Wilcoxon rank-sum tests (for continuous variables where appropriate).

‡ Post-bronchodilator pulmonary function measurements presented. Predicted values for FEV_1 and FVC are derived from Crapo et al.(22)

§ HU: Hounsfield units.

‖ Quantitative metrics of emphysema and TLC were performed using Airway Inspector (www.airwayinspector.org) for each inspiratory CT.

** P value for ILA reflects the comparison in statin use between those with ILA and those with either no ILA or indeterminate status. After excluding subjects characterized as indeterminate for ILA n=759 (36%), 11% of subjects not taking statins had evidence of ILA and 17% of those taking statins had evidence of ILA (p value = 0.002).

Supplemental Table E2: Prevalence of Radiologic Interstitial Lung Abnormalities (ILA) by Statin Type*

	Number (%)					
	Not Taking Statins	Simvastatin	Lovastatin	Atorvastatin	Rosuvastatin	Pravastatin
No ILA	869 (58%)	163 (54%)	24 (59%)	88 (49%)	17 (41%)	16 (41%)
Indeterminate	525 (35%)	116 (38%)	12 (29%)	71 (40%)	18 (44%)	14 (36%)
ILA	106 (7%)	25 (8%)	5 (12%)	20 (11%)	6 (15%)	9 (23%)
ILA (excluding indeterminate)	106 (11%)	25 (13%)	5 (17%)	20 (19%)	6 (26%)	9 (36%)

* Information not presented for 3 subjects taking a statin of unknown type.

Supplemental Table E3: Chest CT radiologic features of Radiologic Interstitial Lung Abnormalities (ILA) Stratified by Statin Use.

Variable*	Number(%) or Median (Interquartile Range) where appropriate		
Specific Radiologic Features	Not Taking Statins n=1500 (71%)	On Statins n=615 (29%)	P value*
Non-Dependent Ground Glass	102 (7%)	64 (10%)	0.007
Non-Dependent Reticular Markings	89 (6%)	58 (9%)	0.006
Non Emphysematous Cysts	49 (3%)	36 (6%)	0.007
Centrilobular Nodules	32 (2%)	12 (2%)	0.87
Traction Bronchiectasis (TB)	30 (2%)	27 (4%)	0.003
Honeycombing (HC)	10 (1%)	8 (1%)	0.19
TB or HC	31 (2%)	27 (4%)	0.005
Specific Radiologic Features	Not Taking Statins n=1500 (74%)	On Lipophilic Statins n=532 (26%)	P value*
Non-Dependent Ground Glass	102 (7%)	49 (9%)	0.08
Non-Dependent Reticular Markings	89 (6%)	45 (8%)	0.05
Non Emphysematous Cysts	49 (3%)	27 (5%)	0.06
Centrilobular Nodules	32 (2%)	7 (1%)	0.27
Traction Bronchiectasis (TB)	30 (2%)	21 (4%)	0.02
Honeycombing (HC)	10 (1%)	6 (1%)	0.39
TB or HC	31 (2%)	21 (4%)	0.02
Specific Radiologic Features	Not Taking Statins n=1500 (95%)	On Hydrophilic Statins n=80 (5%)	P value*
Non-Dependent Ground Glass	102 (7%)	15 (19%)	<0.001
Non-Dependent Reticular Markings	89 (6%)	13 (16%)	0.001
Non Emphysematous Cysts	49 (3%)	9 (11%)	0.002
Centrilobular Nodules	32 (2%)	5 (6%)	0.04
Traction Bronchiectasis (TB)	30 (2%)	6 (8%)	0.008
Honeycombing (HC)	10 (1%)	2 (3%)	0.12
TB or HC	31 (2%)	6 (8%)	0.009

* P values compare the association between statin use and those with specific

ILA features to those without these specific features of ILA using Fisher's exact

tests.

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A

CTL

STA

BLM

S+B

B

CTL

STA

BLM

S+B

Supplemental Figure 1A-B.

C

Body weight (mg) — Day 7: CTL, STA, BLM*, S+B#; Day 14: CTL, STA, BLM*, S+B#

D

Cell count at day 14 ($\times 10^4$/ml) — Total cell, PMN, Lymphocyte; CTL, STA, BLM, S+B
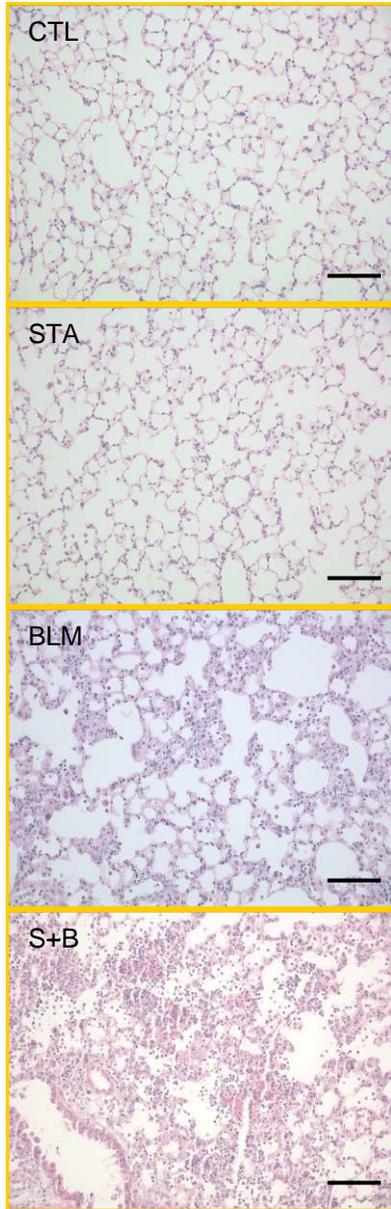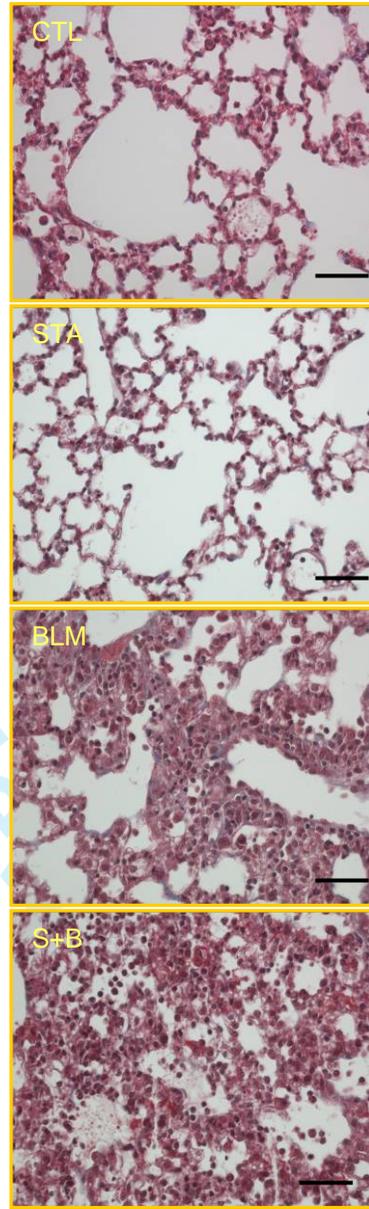
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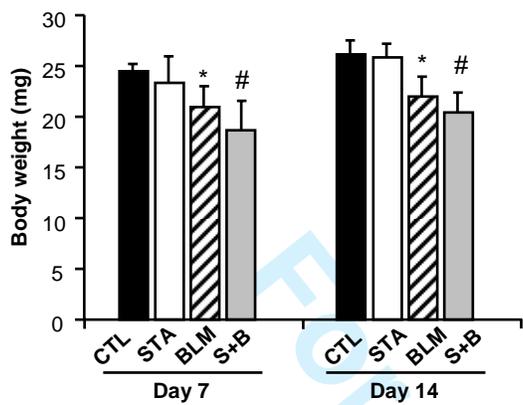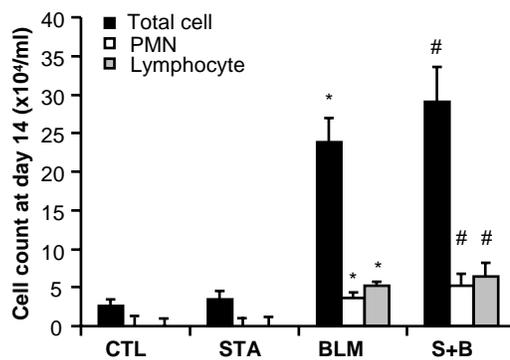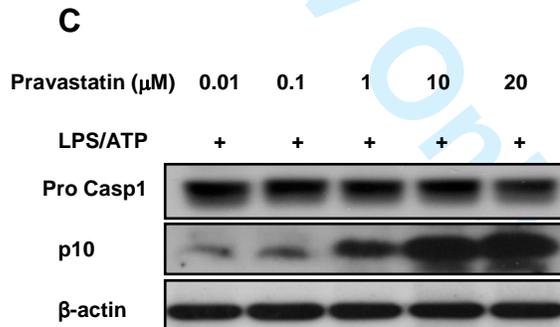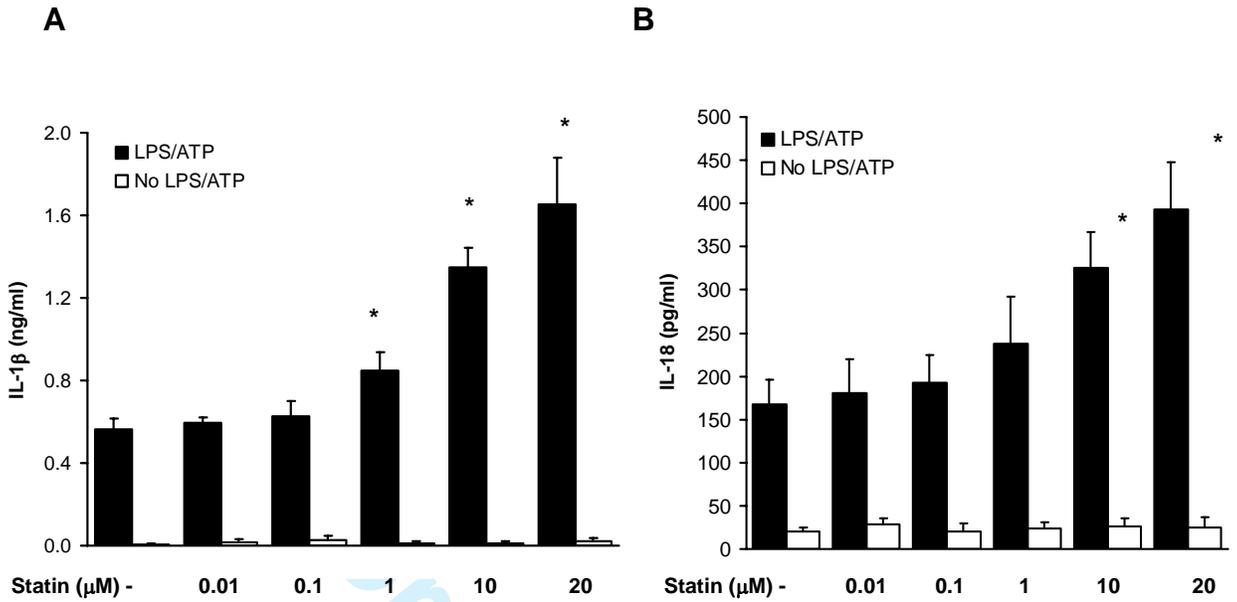
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D
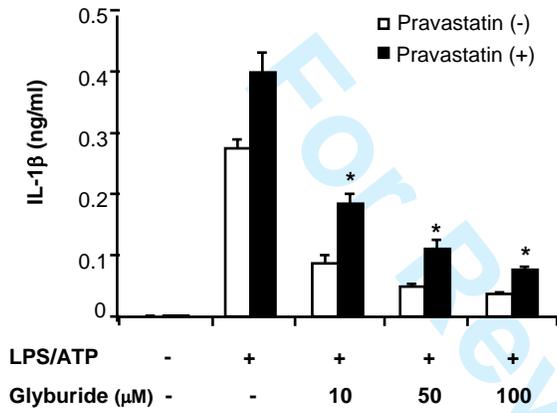
E

F
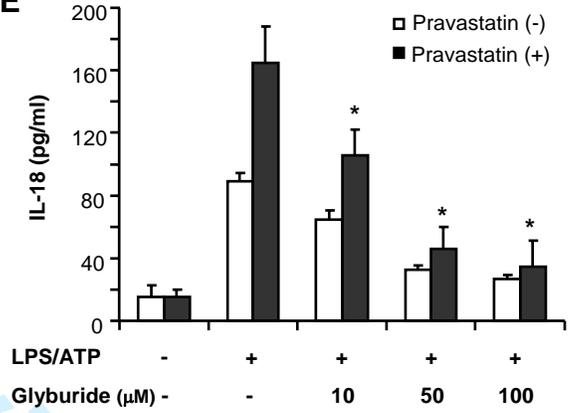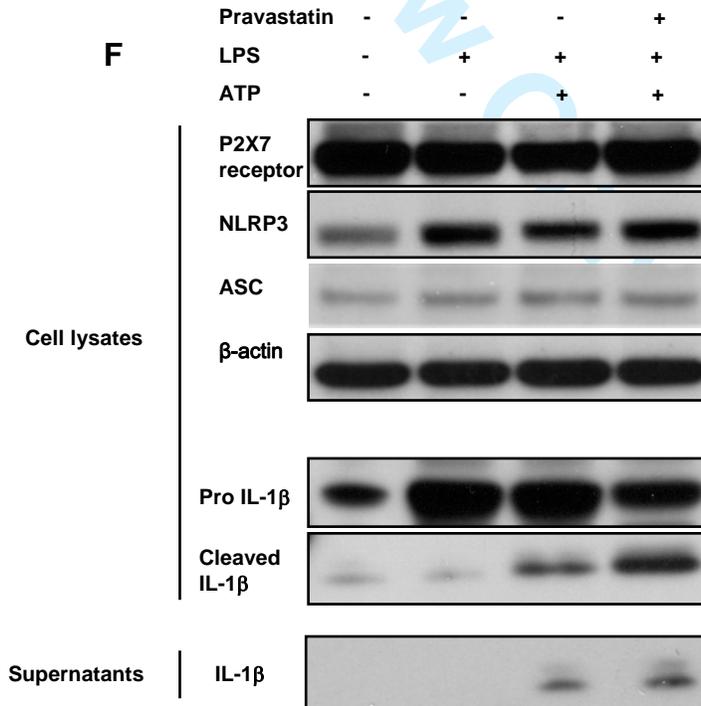
Supplemental Figure 2D-F.

Supplemental Figure 3A-C.