

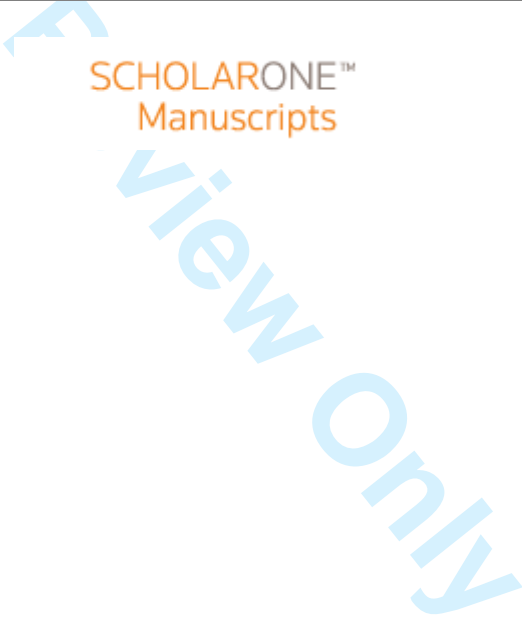


## **Statins and Pulmonary Fibrosis: The Potential Role of NLRP3 Inflammasome Activation**



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

## Activation

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M.N., Y.O., T.Y., J.C.R., R.S.J.E., A.A.D., B.E.H., C.E.C., F.J.M., M.K.H., D.A.L., J.D.C., E.K.S., I.O.R., and G.M.H.; statistical analysis and interpretation of the clinical data; G.R.W., H.H., F.J.M., M.K.H., D.A.L., J.D.C., E.K.S., I.O.R., and G.M.H.; administrative, technical, or material support; I.E.F., R.S.J.E., A.A.D., B.E.H., C.E.C., K.D., drafting the manuscript; J.F.X., G.R.W., K.N., S.W.R., A.M.K.C. and G.M.H.

### **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](http://www.atsjournals.org).

For Review Only

**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*<sup>-/-</sup> and *Casp1*<sup>-/-</sup> 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

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.

For Review Only



## 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-1 $\beta$  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

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*<sup>-/-</sup> and *Casp1*<sup>-/-</sup> 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

(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

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

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

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-1 $\beta$  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-1 $\beta$  expression (**Figure 2I**). Pravastatin alone had no impact on

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-1 $\beta$  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-1 $\beta$  and IL-18 secretion was dependent on caspase-1, as demonstrated by the loss of this effect on BMDMs from *Casp-1*<sup>-/-</sup> mice (**Figures 3D, E**). To determine which inflammasome pathway was involved in the promotion of caspase-1 activation by pravastatin, BMDM from *Nlrp3*<sup>-/-</sup> mice were subjected to LPS and ATP stimulation. The stimulatory effect of pravastatin pretreatment on secretion of IL-1 $\beta$  and IL-18 and caspase-1 activation was abolished in *Nlrp3*<sup>-/-</sup> BMDM (**Figures 3F-H**). Similarly, glyburide, a NLRP3 inflammasome inhibitor, prevented the enhancement of IL-1 $\beta$  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



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-1 $\beta$  (**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<sub>2</sub><sup>-</sup>) 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-1 $\beta$  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**

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

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-1 $\beta$  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)

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 COPD Gene 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)

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-1 $\beta$  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

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

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.

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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

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  $\mu$ m, 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  $\mu$ m, 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-1 $\beta$  and IL-18 in BALF at day 7 was measured by ELISA. (G, H) Concentration of IL-1 $\beta$  and IL-18 in lung homogenates was measured by

ELISA. (I) Lung homogenates were analyzed by immunoblotting for IL-1 $\beta$  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-1 $\beta$  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-1 $\beta$  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  $\mu$ 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 were analyzed by immunoblotting for caspase-1. (D, E) BMDM from caspase-1  $-/-$  mice were pretreated with pravastatin (10  $\mu$ M) for 24 h and then incubated with LPS (200 ng/ml) for 4 h,

followed by stimulation with ATP (5 mM) for 1 h. Secretion of IL-1 $\beta$  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  $\mu$ M) 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-1 $\beta$  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  $\mu$ 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 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  $\mu$ M) 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	
<b>Demographic Parameters</b>	n=1184 (87%)	n=172 (13%)	P value <sup>†</sup>
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
<b>Medications</b>			
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
Niacin	4 (<1%)	2 (1%)	0.17
Fish Oil	27 (2%)	6 (3%)	0.30
Statins			
All Statins	315 <sup>‡</sup> (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).



## REFERENCES:

1. Hebert PR, Gaziano JM, Chan KS, Hennekens CH. Cholesterol lowering with statin drugs, risk of stroke, and total mortality. An overview of randomized trials. *JAMA*. 1997;278(4):313-21.
2. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. *Lancet*. 2002;360(9326):7-22.
3. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet*. 1994;344(8934):1383-9.
4. Sacks FM, Pfeffer MA, Moye LA, Rouleau JL, Rutherford JD, Cole TG, Brown L, Warnica JW, Arnold JM, Wun CC, Davis BR, Braunwald E. The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators. *N Engl J Med*. 1996;335(14):1001-9.
5. Morimoto K, Janssen WJ, Fessler MB, McPhillips KA, Borges VM, Bowler RP, Xiao YQ, Kench JA, Henson PM, Vandivier RW. Lovastatin enhances clearance of apoptotic cells (efferocytosis) with implications for chronic obstructive pulmonary disease. *J Immunol*. 2006;176(12):7657-65.
6. Schonbeck U, Libby P. Inflammation, immunity, and HMG-CoA reductase inhibitors: statins as antiinflammatory agents? *Circulation*. 2004;109(21 Suppl 1):II18-26.

7. Hothersall E, McSharry C,Thomson NC. Potential therapeutic role for statins in respiratory disease. *Thorax*. 2006;61(8):729-34.
8. American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. *Am J Respir Crit Care Med*. 2002;165(2):277-304.
9. Tan A, Levrey H, Dahm C, Polunovsky VA, Rubins J,Bitterman PB. Lovastatin induces fibroblast apoptosis in vitro and in vivo. A possible therapy for fibroproliferative disorders. *Am J Respir Crit Care Med*. 1999;159(1):220-7.
10. Kim JW, Rhee CK, Kim TJ, Kim YH, Lee SH, Yoon HK, Kim SC, Lee SY, Kwon SS, Kim KH,Kim YK. Effect of pravastatin on bleomycin-induced acute lung injury and pulmonary fibrosis. *Clin Exp Pharmacol Physiol*.2010;37(11):1055-63.
11. Coward WR, Marei A, Yang A, Vasa-Nicotera MM,Chow SC. Statin-induced proinflammatory response in mitogen-activated peripheral blood mononuclear cells through the activation of caspase-1 and IL-18 secretion in monocytes. *J Immunol*. 2006;176(9):5284-92.
12. Gasse P, Riteau N, Charron S, Girre S, Fick L, Petrilli V, Tschopp J, Lagente V, Quesniaux VF, Ryffel B,Couillin I. Uric acid is a danger signal activating NALP3 inflammasome in lung injury inflammation and fibrosis. *Am J Respir Crit Care Med*. 2009;179(10):903-13.
13. Wilson MS, Madala SK, Ramalingam TR, Gochuico BR, Rosas IO, Cheever AW,Wynn TA. Bleomycin and IL-1beta-mediated pulmonary fibrosis is IL-17A dependent. *J Exp Med*. 2010;207(3):535-52.

14. Hoshino T, Okamoto M, Sakazaki Y, Kato S, Young HA, Aizawa H. Role of proinflammatory cytokines IL-18 and IL-1 $\beta$  in bleomycin-induced lung injury in humans and mice. *Am J Respir Cell Mol Biol*. 2009;41(6):661-70.
15. Fernandez AB, Karas RH, Alsheikh-Ali AA, Thompson PD. Statins and interstitial lung disease: a systematic review of the literature and of food and drug administration adverse event reports. *Chest*. 2008;134(4):824-30.
16. Washko GR, Hunninghake GM, Fernandez IE, Nishino M, Okajima Y, Yamashiro T, Ross JC, Estepar RS, Lynch DA, Brehm JM, Andriole KP, Diaz AA, Khorasani R, D'Aco K, Sciurba FC, Silverman EK, Hatabu H, Rosas IO. Lung volumes and emphysema in smokers with interstitial lung abnormalities. *N Engl J Med*. 2011;364(10):897-906.
17. Lederer DJ, Enright PL, Kawut SM, Hoffman EA, Hunninghake G, van Beek EJ, Austin JH, Jiang R, Lovasi GS, Barr RG. Cigarette smoking is associated with subclinical parenchymal lung disease: the Multi-Ethnic Study of Atherosclerosis (MESA)-lung study. *Am J Respir Crit Care Med*. 2009;180(5):407-14.
18. Washko GR, Lynch DA, Matsuoka S, Ross JC, Umeoka S, Diaz A, Sciurba FC, Hunninghake GM, San Jose Estepar R, Silverman EK, Rosas IO, Hatabu H. Identification of early interstitial lung disease in smokers from the COPD Gene Study. *Acad Radiol*. 2010;17(1):48-53.
19. Xu J, Nakahira K, Li H, Ryter S, Choi AM. 2011. Statins Promote NALP3 Inflammasome Activation Through Increased Mitochondrial ROS Generation In Macrophages. American Thoracic Society, 2011 Denver, Colorado.

20. Nakahira K, Haspel JA, Rathinam VA, Lee SJ, Dolinay T, Lam HC, Englert JA, Rabinovitch M, Cernadas M, Kim HP, Fitzgerald KA, Ryter SW, Choi AM. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat Immunol*. 2011;12(3):222-30.
21. Wang XM, Zhang Y, Kim HP, Zhou Z, Feghali-Bostwick CA, Liu F, Ifedigbo E, Xu X, Oury TD, Kaminski N, Choi AM. Caveolin-1: a critical regulator of lung fibrosis in idiopathic pulmonary fibrosis. *J Exp Med*. 2006;203(13):2895-906.
22. Zhou Z, Song R, Fattman CL, Greenhill S, Alber S, Oury TD, Choi AM, Morse D. Carbon monoxide suppresses bleomycin-induced lung fibrosis. *Am J Pathol*. 2005;166(1):27-37.
23. Zhou R, Yazdi AS, Menu P, Tschopp J. A role for mitochondria in NLRP3 inflammasome activation. *Nature*. 2011;475(7354):122.
24. Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA, Calverley P, Fukuchi Y, Jenkins C, Rodriguez-Roisin R, van Weel C, Zielinski J. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med*. 2007;176(6):532-55.
25. Kenchaiah S, Evans JC, Levy D, Wilson PW, Benjamin EJ, Larson MG, Kannel WB, Vasan RS. Obesity and the risk of heart failure. *N Engl J Med*. 2002;347(5):305-13.

26. R Development Core Team (2009) R: A Language and Environment for Statistical Computing. Vienna, Austria.
27. White CM. A review of the pharmacologic and pharmacokinetic aspects of rosuvastatin. *J Clin Pharmacol*. 2002;42(9):963-70.
28. Shitara Y, Sugiyama Y. Pharmacokinetic and pharmacodynamic alterations of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors: drug-drug interactions and interindividual differences in transporter and metabolic enzyme functions. *Pharmacol Ther*. 2006;112(1):71-105.
29. Ponnuswamy A, Manikandan R, Sabetpour A, Keeping IM, Finnerty JP. Association between ischaemic heart disease and interstitial lung disease: a case-control study. *Respir Med*. 2009;103(4):503-7.
30. Hubbard RB, Smith C, Le Jeune I, Gribbin J, Fogarty AW. The association between idiopathic pulmonary fibrosis and vascular disease: a population-based study. *Am J Respir Crit Care Med*. 2008;178(12):1257-61.
31. Gasse P, Mary C, Guenon I, Noulon N, Charron S, Schnyder-Candrian S, Schnyder B, Akira S, Quesniaux VF, Lagente V, Ryffel B, Couillin I. IL-1R1/MyD88 signaling and the inflammasome are essential in pulmonary inflammation and fibrosis in mice. *J Clin Invest*. 2007;117(12):3786-99.
32. Cassel SL, Eisenbarth SC, Iyer SS, Sadler JJ, Colegio OR, Tephly LA, Carter AB, Rothman PB, Flavell RA, Sutterwala FS. The Nalp3 inflammasome is essential for the development of silicosis. *Proc Natl Acad Sci U S A*. 2008;105(26):9035-40.

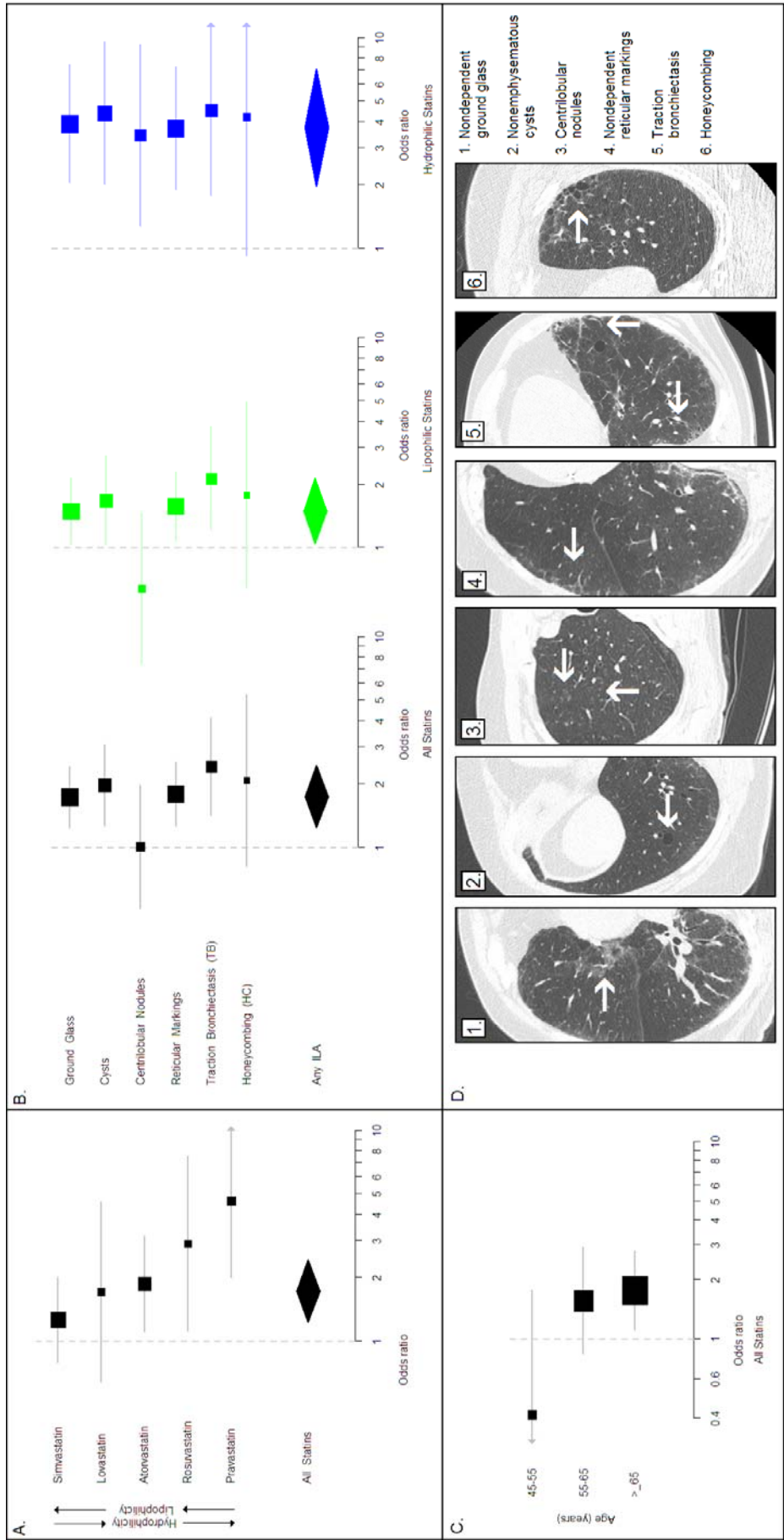
33. Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science*. 2008;320(5876):674-7.
34. Dirks AJ, Jones KM. Statin-induced apoptosis and skeletal myopathy. *Am J Physiol Cell Physiol*. 2006;291(6):C1208-12.
35. Ito M, Adachi T, Pimentel DR, Ido Y, Colucci WS. Statins inhibit beta-adrenergic receptor-stimulated apoptosis in adult rat ventricular myocytes via a Rac1-dependent mechanism. *Circulation*. 2004;110(4):412-8.
36. Bouitbir J, Charles AL, Echaniz-Laguna A, Kindo M, Daussin F, Auwerx J, Piquard F, Geny B, Zoll J. Opposite effects of statins on mitochondria of cardiac and skeletal muscles: a 'mitohormesis' mechanism involving reactive oxygen species and PGC-1. *Eur Heart J*. 2011;[Epub ahead of print].
37. Ou XM, Feng YL, Wen FQ, Huang XY, Xiao J, Wang K, Wang T. Simvastatin attenuates bleomycin-induced pulmonary fibrosis in mice. *Chin Med J (Engl)*. 2008;121(18):1821-9.
38. Pauwels NS, Bracke KR, Dupont LL, Van Pottelberge GR, Provoost S, Berghe TV, Vandenabeele P, Lambrecht BN, Joos GF, Brusselle GG. Role of IL-1{alpha} and the Nlrp3/caspase-1/IL-1{beta} axis in cigarette smoke-induced pulmonary inflammation and COPD. *Eur Respir J*. 2011;38(5):1019-28.
39. Doz E, Noulin N, Boichot E, Guenon I, Fick L, Le Bert M, Lagente V, Ryffel B, Schnyder B, Quesniaux VF, Couillin I. Cigarette smoke-induced pulmonary inflammation is TLR4/MyD88 and IL-1R1/MyD88 signaling dependent. *J Immunol*. 2008;180(2):1169-78.

40. Montero MT, Matilla J, Gomez-Mampaso E, Lasuncion MA.  
Geranylgeraniol regulates negatively caspase-1 autoprocessing: implication in the Th1 response against *Mycobacterium tuberculosis*. *J Immunol*. 2004;173(8):4936-44.
41. Montero MT, Hernandez O, Suarez Y, Matilla J, Ferruelo AJ, Martinez-Botas J, Gomez-Coronado D, Lasuncion MA. Hydroxymethylglutaryl-coenzyme A reductase inhibition stimulates caspase-1 activity and Th1-cytokine release in peripheral blood mononuclear cells. *Atherosclerosis*. 2000;153(2):303-13.
42. Kuijk LM, Mandey SH, Schellens I, Waterham HR, Rijkers GT, Coffier PJ, Frenkel J. Statin synergizes with LPS to induce IL-1 $\beta$  release by THP-1 cells through activation of caspase-1. *Mol Immunol*. 2008;45(8):2158-65.
43. Massonnet B, Normand S, Moschitz R, Delwail A, Favot L, Garcia M, Bourmeyster N, Cuisset L, Grateau G, Morel F, Silvain C, Lecron JC.  
Pharmacological inhibitors of the mevalonate pathway activate pro-IL-1 processing and IL-1 release by human monocytes. *Eur Cytokine Netw*. 2009;20(3):112-20.
44. Netea MG, Nold-Petry CA, Nold MF, Joosten LA, Opitz B, van der Meer JH, van de Veerdonk FL, Ferwerda G, Heinhuis B, Devesa I, Funk CJ, Mason RJ, Kullberg BJ, Rubartelli A, van der Meer JW, Dinarello CA. Differential requirement for the activation of the inflammasome for processing and release of IL-1 $\beta$  in monocytes and macrophages. *Blood*. 2009;113(10):2324-35.
45. Lommatzsch M, Cicko S, Muller T, Lucattelli M, Bratke K, Stoll P, Grimm M, Durk T, Zissel G, Ferrari D, Di Virgilio F, Sorichter S, Lungarella G, Virchow

- JC, Idzko M. Extracellular adenosine triphosphate and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2010;181(9):928-34.
46. Wagner BK, Kitami T, Gilbert TJ, Peck D, Ramanathan A, Schreiber SL, Golub TR, Mootha VK. Large-scale chemical dissection of mitochondrial function. *Nat Biotechnol*. 2008;26(3):343-51.
47. Rosas IO, Ren P, Avila NA, Chow CK, Franks TJ, Travis WD, McCoy JP, Jr., May RM, Wu HP, Nguyen DM, Arcos-Burgos M, MacDonald SD, Gochuico BR. Early interstitial lung disease in familial pulmonary fibrosis. *Am J Respir Crit Care Med*. 2007;176(7):698-705.
48. Steele MP, Speer MC, Loyd JE, Brown KK, Herron A, Slifer SH, Burch LH, Wahidi MM, Phillips JA, 3rd, Sporn TA, McAdams HP, Schwarz MI, Schwartz DA. Clinical and pathologic features of familial interstitial pneumonia. *Am J Respir Crit Care Med*. 2005;172(9):1146-52.
49. Gochuico BR, Avila NA, Chow CK, Novero LJ, Wu HP, Ren P, MacDonald SD, Travis WD, Stylianou MP, Rosas IO. Progressive preclinical interstitial lung disease in rheumatoid arthritis. *Arch Intern Med*. 2008;168(2):159-66.
50. Thompson PD, Clarkson P, Karas RH. Statin-associated myopathy. *JAMA*. 2003;289(13):1681-90.



Figure 1A-D.



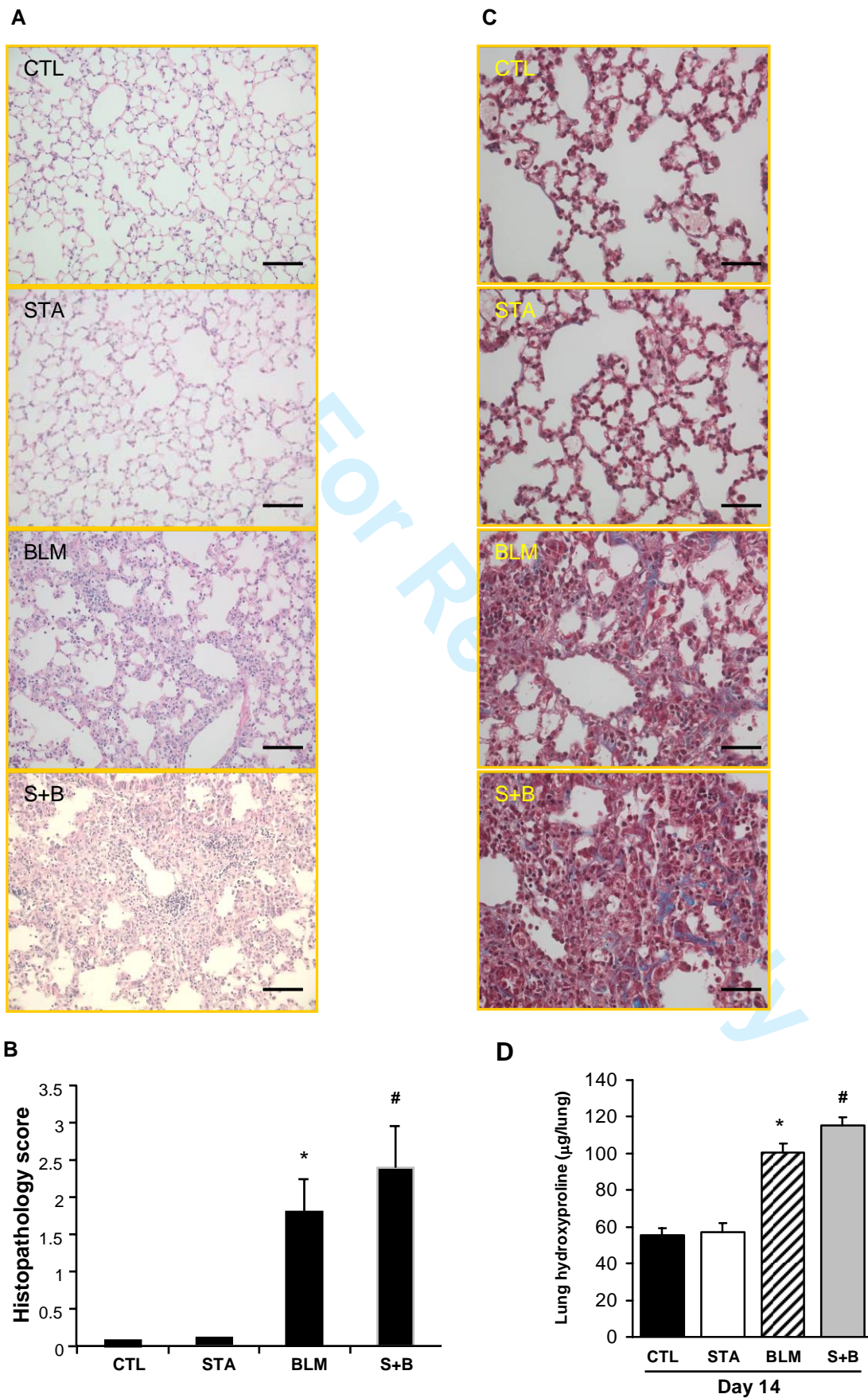


Figure 2A-D.

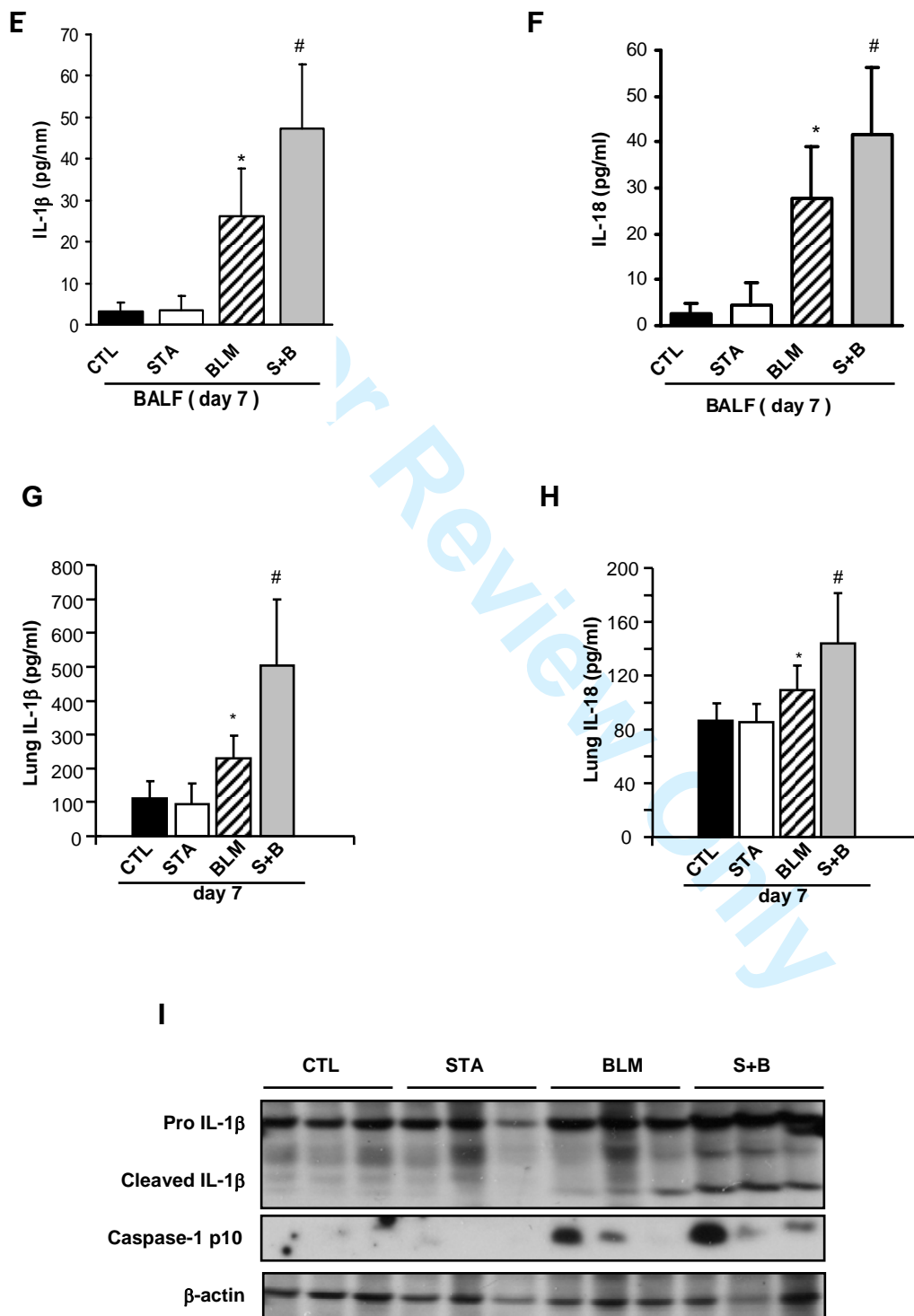


Figure 2E-I.

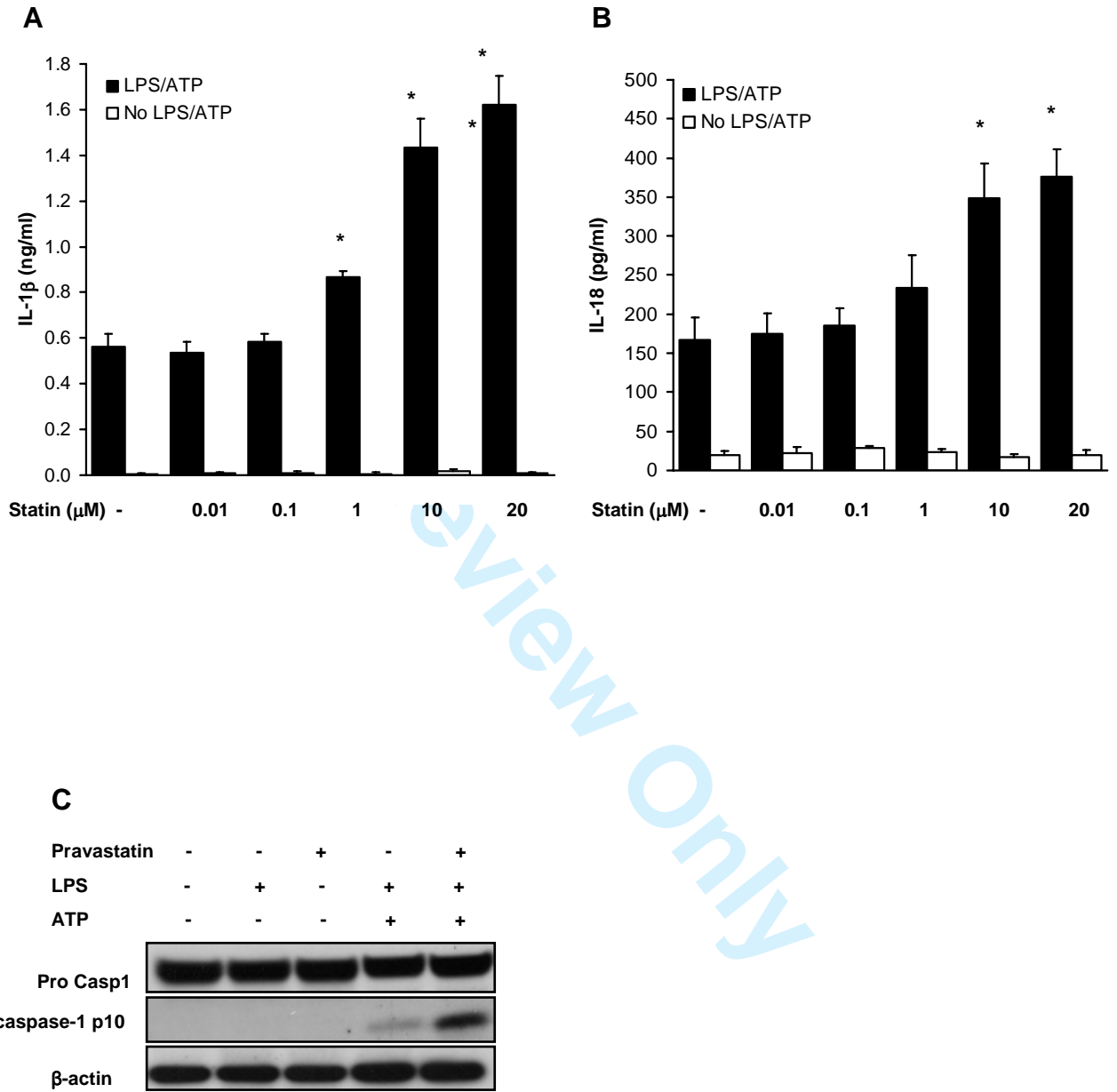


Figure 3A-C.

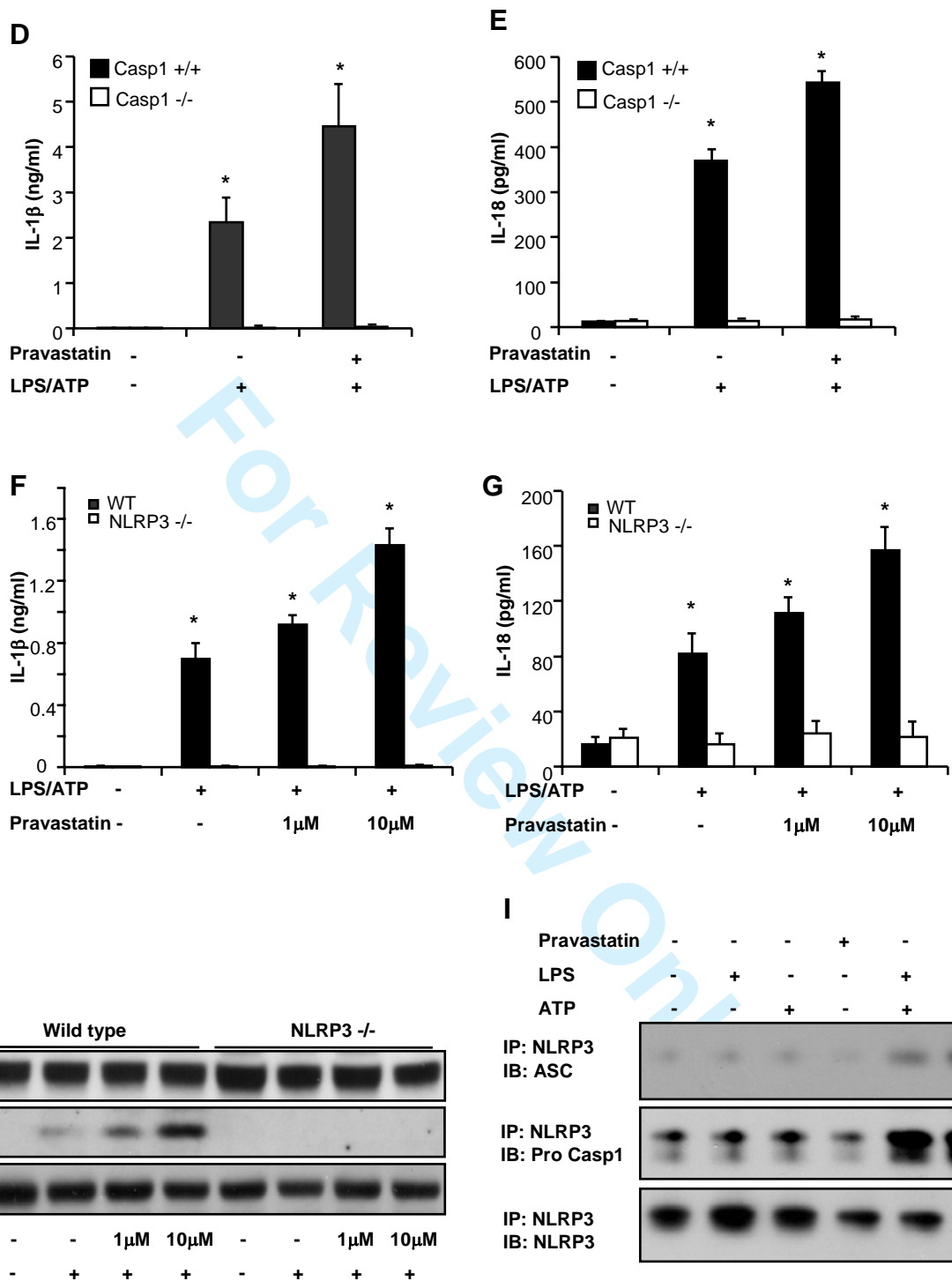


Figure 3D-I.

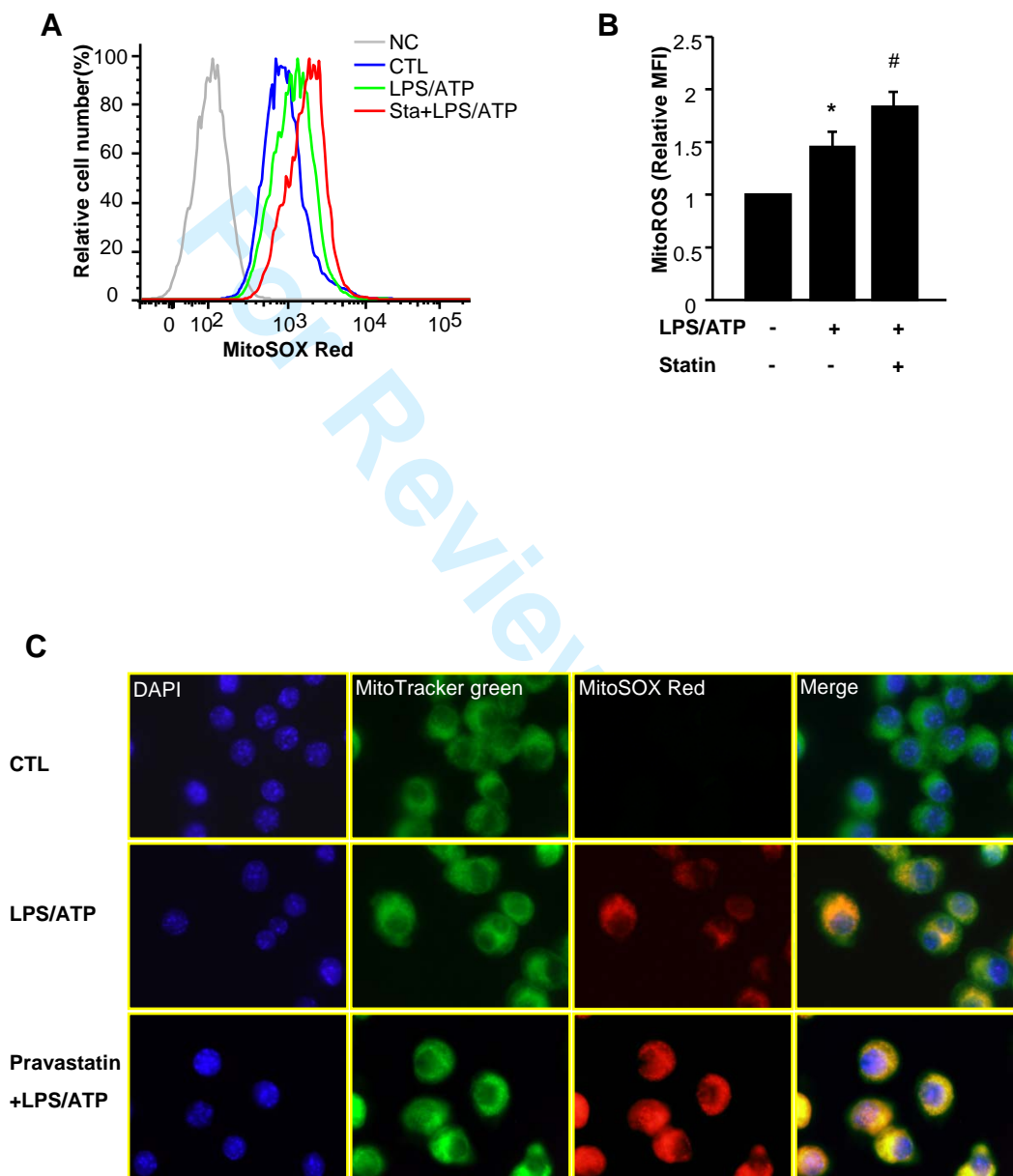


Figure 4A-C.