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Lovastatin Enhances Clearance of Apoptotic Cells (Efferocytosis) with Implications for Chronic Obstructive Pulmonary Disease

Konosuke Morimoto,* William J. Janssen,† Michael B. Fessler,† Kathleen A. McPhillips,‡ Valeria M. Borges,§ Russell P. Bowler,‖ Yi-Qun Xiao,‡ Jennifer A. Kench,‡ Peter M. Henson,‡ and R. William Vandivier2*

Statins are potent, cholesterol-lowering agents with newly recognized, broad anti-inflammatory properties (1). For example, statins suppress the innate immune response in vitro by inhibiting neutrophil migration (2), oxidative stress (3), NF-κB activation (4), proinflammatory mediator release (5, 6), expression of matrix metalloproteinases (7–9), and by increasing expression of constitutive NO synthase (10), peroxisome proliferator-activated receptor (PPAR)3 protein (11), PPARγ (4), and TGFβ1 (12). Statins also suppress the adaptive immune response by inhibiting IFN-γ-inducible MHC class II expression (13), decreasing expression of CD40/CD40L (14), and by direct blockade of LFA-1 (15). These pleiotropic, anti-inflammatory effects have important therapeutic implications, because 1) statins effectively treat animal models of sepsis, rheumatoid arthritis, acute lung injury, asthma, and emphysema (2, 16–19), and 2) clinically, statins have a promising therapeutic role in the acute coronary syndrome, stabilization of carotid artery plaques, sepsis syndrome, lung allograft rejection, and rheumatoid arthritis (7, 20–24). Therefore, statins appear to be emerging as a new class of immunomodulators, surpassing their originally envisaged role as cholesterol lowering drugs.

The immunomodulatory effects of statins are largely cholesterol independent; instead, they appear to depend upon the ability of statins to posttranslationally modify an extensive array of intracellular signaling molecules, including the Rho family of GTPases (1). Rho GTPases (e.g., RhoA, Rac-1, and Cdc42) are molecular switches, which for function, depend upon the covalent attachment of lipid adducts (prenylation) that direct membrane insertion, localization, and protein:protein interaction (25, 26). Statins regulate prenylation of Rho GTPases by blocking 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which decreases production of mevalonate, and downstream prenylation substrates, including farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (see Fig. 1). These statin properties suggest a cholesterol-independent mechanism through which they may have far-reaching, regulatory effects.

Phagocytosis of apoptotic cells (efferocytosis) is integrally involved with the regulation of the inflammatory response and maintenance of lung homeostasis by 1) removing dead cells before the onset of necrosis (27), by 2) inducing release of anti-inflammatory mediators (27) and antiproteases (28), and by 3) increasing production of growth factors (29, 30). Perhaps not surprisingly, impaired efferocytosis appears to be involved in the pathogenesis of a variety of chronic lung and systemic inflammatory diseases.

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1 COPD Center, Division of Pulmonary Sciences and Critical Care Medicine, University of Colorado Health Sciences Center, Denver, CO 80262; and Department of Medicine and Department of Immunology, National Jewish Medical and Research Center, Denver, CO 80206

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2 Address correspondence and reprint requests to Dr. R. William Vandivier, COPD Center, Division of Pulmonary Sciences and Critical Care Medicine, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, C272, Denver, CO 80220. E-mail address: Bill.Vandivier@uchsc.edu

3 Abbreviations used in this paper: PPAR, peroxisome proliferator-activated receptor; HMG-CoA, 3-hydroxyl-3-methylglutaryl coenzyme A; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; COPD, chronic obstructive pulmonary disease; HMDM, human monocyte-derived macrophage; PL, phagocytic index; IPP, isopentenyl pyrophosphate; LPA, lysophosphatidic acid.
because it is defective in systemic lupus erythematosus, rheumatoid arthritis, cystic fibrosis, bronchiectasis, asthma, and chronic obstructive pulmonary disease (COPD) (31–37). The regulation of efferocytosis is tightly controlled by the Rho family GTPases, in that RhoA inhibits (38–40) and Rac-1/Cdc42/RhoG promotes (41–43) the process. Because statins robustly inhibit RhoA, a negative regulator of efferocytosis, we hypothesized that statins would enhance efferocytosis, and sought to determine whether statins might have therapeutic potential in COPD, a disease of impaired efferocytosis (31, 32).

Indeed, our data demonstrate that statins are potent inducers of efferocytosis in vitro and in vivo in an HMG CoA-reductase dependent manner. Statins appear to exert their effect, not by suppressing cholesterol, but by disproportionately suppressing the prenylation and membrane localization of RhoA. Finally, these data may have therapeutic implications for the treatment of chronic inflammatory diseases of the lung, because statins augment efferocytosis in the naive murine lung and in alveolar macrophages obtained from patients with COPD.

Materials and Methods

Human subjects

The study was approved by, and performed in accordance with, the ethical standards of the institutional review board on human experimentation at National Jewish Medical and Research Center. Written informed consent was obtained from each subject.

Experimental animals

Mice were housed and studied under institutional animal care and use committee-approved protocols in the animal facility of National Jewish Medical and Research Center. Experiments were performed on 8- to 12-wk-old, age-matched, female ICR mice (Harlan Sprague Dawley).

Primary cell isolation and culture

Human monocyte-derived macrophages (HMDMs) and neutrophils were isolated and prepared from normal blood, as previously described (44). HMDMs were cultured in X-vivo medium (Cambrex Bioscience) with 10% pooled human serum at 37°C in 10% CO2 for 7 days before use. Human alveolar macrophages were isolated by bronchoalveolar lavage from patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage 2 COPD as described (31, 45). The right middle lobe was lavaged with 100 ml of 0.9% saline solution at room temperature. Human alveolar macrophages were resuspended in X-vivo medium (Cambrex BioScience) and were plated on baked glass coverslips in 24-well tissue culture plates (BD Biosciences) at 5.0 × 105 alveolar macrophages/well. Murine thymocytes were isolated from the thymi of 3- to 4-wk-old, female ICR mice, by passing thymy through a 40-μm strainer (Fisher Scientific) to separate individual cells.

Cell lines and culture

The human Jurkat leukemia T cell line was obtained from the American Type Culture Collection and cultured in RPMI 1640 with 10% FBS, supplemented with penicillin-streptomycin-glutamine, and incubated at 37°C in 10% CO2 (31, 46).

Induction of apoptosis

Apoptosis was induced in human neutrophils, Jurkat T cells, and murine thymocytes by exposure to UV irradiation at 312 nm (Fotodyne) for 10 min, as previously described (31). Human neutrophils were cultured in RPMI 1640 with 0.5% low-endotoxin BSA (Sigma-Aldrich) at 37°C in 10% CO2 for 2.5 h before use. Jurkat T cells were cultured in RPMI 1640 with 10% FCS at 37°C in 5% CO2 for 3.5 h before use. Neutrophils and Jurkat T cells treated in this way were ~80% apoptotic by nuclear condensation. Thymocytes were cultured in RPMI 1640 with 10% FCS at 2 × 106 cells/ml at 37°C in 5% CO2 for 3 h. In Fig. 5E, thymocytes were cultured in RPMI 1640 with 0.5% low-endotoxin BSA at 4 × 106 cells/ml overnight without exposure to UV irradiation. Thymocytes exposed to UV irradiation were ~90% annexin V positive and 30% propidium iodide positive. Thymocytes cultured overnight were ~80% annexin V positive and 60% propidium iodide positive.

Lovastatin activation

Lovastatin (Sigma-Aldrich) was converted to its active form by dissolving 25 mg of the lactone form in 500 μl of 100% ethanol, heated to 50°C, alkalinized by adding 250 μl of 0.6 M NaOH, and incubated at 50°C for 2 h. After incubation, the solution was neutralized with 0.4 M HCl at pH 7.5. Aliquots of stock solution were stored frozen at −20°C until used (47, 48).

In vitro phagocytosis assays

Phagocytic assays were performed on day 7 HMDMs, as previously described (31). Briefly, apoptotic human neutrophils were added to HMDMs at a 5:1 ratio (apoptotic cell to HMDM) and incubated at 37°C in 10% CO2 for 40 min in 500 μl of X-Vivo medium. HMDMs were washed gently with cold PBS to remove uningested cells, fixed, and stained with a modified Wright-Giemsa (Fisher Scientific). Phagocytosis was determined by visual inspection of samples and was expressed as the phagocytic index (PI), as described (49). Each condition was tested in duplicate and a minimum of 400 HMDMs were counted per condition. In all cases, during analysis, the reader was blinded to the sample identification.

HMDMs were pretreated with 0–10 μM lovastatin for 4–120 h before experimentation. Phagocytosis assays were then performed in the presence and absence of the following reagents at the indicated concentrations: for the indicated times: mevalonate (Sigma-Aldrich), GPPG (Sigma-Aldrich), and FPP (Sigma-Aldrich). Inhibitors of farnesyltransferase (FTI-276; Calbiochem-Novabiochem) and geranylgeranyltransferase 1 (GGTI-2133; Calbiochem-Novabiochem) were added to HMDMs 8 h before experimentation. FTI-276 inhibits farnesyltransferase with an IC50 of 0.5 nM, geranylgeranyltransferase 1 at a much higher IC50 = 50 nM. GGTI-2133 inhibits geranylgeranyltransferase 1 with an IC50 = 38 nM, and farnesyltransferase at a much higher IC50 = 5.4 μM.

In vivo phagocytosis assays

To test the effect of lovastatin and mevalonate on uptake of apoptotic cells, mice were divided into four groups and treated as follows: 1) control group, treated with vehicle (0.5% carboxymethylcellulose sodium, 0.09% sodium chloride, 0.4% polysorbate 80, 0.09% benzyl alcohol in deionized water) by gavage and PBS by i.p. injection; 2) lovastatin group, treated with lovastatin (10 mg/kg) in vehicle by gavage and PBS by i.p. injection; 3) mevalonate group, treated with vehicle by gavage and 10 mg/kg l-mevalonate by i.p. injection; 4) lovastatin/mevalonate group, treated with lovastatin (10 mg/kg) in vehicle by gavage and mevalonate (10 mg/kg) by i.p. injection. Mice were treated three times, spaced within 30 h, before the time of experimentation.

Apoptotic thymocytes were instilled intratracheally as previously described (45). Briefly, mice were anesthetized with Avertin, following which 10 × 10^6 Cell Tracker Red-stained (Molecular Probes) apoptotic thymocytes, suspended in 50 μl of PBS, were instilled intratracheally using a modified animal feeding needle (Fisher Scientific). Forty minutes later, whole lung bronchoalveolar lavage was performed with a total of 5 ml of ice-cold PBS. Lavage cells were fixed and stained with modified Wright’s Giemsa (Fisher Scientific). Phagocytosis was determined by visual inspection of samples (see Fig. 5A), as previously described (45, 50), and was presented as a PI. A minimum of 400 alveolar macrophages were counted blindly.

Recovered apoptotic thymocytes were determined as follows. Total lung cells (including erythrocytes) were counted using a hemacytometer. The percentage of free thymocytes in the lavage was determined by FACS analysis (Fig. 5E). Macrophages were excluded, based upon macrophage forward-side scatter characteristics, F4/80 staining, and autofluorescence. Total recovered thymocytes were calculated by multiplying total lavage cells by the percentage of Cell Tracker Red-positive cells.

FACS analysis

FACS analysis was done as previously described (31). Briefly, HMDMs were suspended in HBSS containing 2% FCS (Gemini Bio-Products), blocked with human serum, except cells for FcγR staining, incubated with 5 μg of the primary Ab for 30 min on ice, washed twice, then incubated with the secondary Ab (1/50 dilution) on ice for 30 min. Washed macrophages were analyzed on a FACSScan cytometer using CellQuest Pro (BD Biosciences) and FloJo (Tree Star) software.

Abs used in FACS analysis were as follows: BD Biosciences/BD Pharmingen Abs include mouse monoclonal anti-human CD36 IgM, anti-human integrin β2 IgG1, anti-human integrin αM IgG1, and anti-human integrin αβ2 IgG1 (BD Pharmingen). IgG2a, IgG2b, and mouse monoclonal IgG1, IgG2a, IgM, κ isotype controls (Chemicon International) Abs include mouse monoclonal anti-human integrin αβ1, IgG1 (Affinity BioReagent). Abs include chicken
polyclonal anti-human calreticulin IgY (American Diagnostica) Abs include mouse monoclonal anti-human α-chain CD91 IgG1. Mouse monoclonal anti-human PS recognition structure IgM (217) was prepared in this laboratory as previously described (27). Jackson ImmunoResearch Laboratories Abs include chicken IgY isotype control, Cy-3 goat IgG anti-mouse IgM, and Cy-3 goat IgG anti-mouse IgG, and Cy-3 goat anti-chicken IgY.

Western blotting
HMMD membrane fractions were prepared and separated on SDS-PAGE as described previously (51, 52). Briefly, HMMDs plated on 10-cm tissue-culture dish were harvested and resuspended in PBS. Pelleted cells were lysed by Reporter Lysis Buffer (Promega) and by repeated freeze-thaw cycles. Cell lysates were spun down at 3,000 rpm for 10 min. Supernatants were collected and spun-down at 10,000 × g for 45 min. The pellet was solubilized in lystate buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM DTTL, 1% Triton X-100). Samples were run on a 7.5% SDS-PAGE gel, transferred to nitrocellulose, blocked with 3% milk, and sequentially incubated with primary and secondary Abs. Total protein was measured by the BCA Protein Assay kit (Pierce) and equivalent amounts were loaded into gels. Immunoblotting was performed using mAbs against RhoA, RhoB, and RhoC (clone 55, 3 μg/ml; Upstate Biotechnology) and Rac-1 (clone 23A8, 1 μg/ml; Upstate Biotechnology), and immunodetection was accomplished using a mouse anti-mouse HRP-conjugated secondary Ab (1/10,000). Membranes were developed using Amersham ECL system (Amersham Biosciences).

Statistics
The means were analyzed using ANOVA for multiple comparisons; when ANOVA indicated significance, the Dunnett's method was used to compare groups with an internal control. For all other experiments in which two conditions were being compared, a Student's t test assuming equal variance was used. All data were analyzed using JMP (version 3) Statistical Software for Macintosh (SAS Institute) and are presented ± SEM.

Results
Lovastatin increases uptake of apoptotic cells (effерocytosis)
We tested the effect of statins on effеrcytosis by incubating HMMDs with lovastatin for 6–48 h. Lovastatin (5 μM) increased effеrcytosis as early as 6 h (Fig. 1B). However, at 24 h, lovastatin increased effеrcytosis in a dose-dependent fashion, exerting a maximum effect at 10 μM. In contrast, lovastatin had no effect on binding of apoptotic cells to HMMDs (Fig. 1C). Lovastatin (1 μM) also increased effеrcytosis in HMMDs after 5 days of treatment compared with untreated controls, suggesting that the effect was prolonged (Fig. 1D).

The effect of lovastatin on effеrcytosis is dependent on HMG-CoA reductase
Most, but not all, statin effects are related to their ability to competitively block HMG-CoA reductase, and thereby decrease production of multiple intermediates in the cholesterol biosynthetic pathway. Mevalonate is the initial product of HMG-CoA reductase (Fig. 1A) and mevalonate levels are decreased by statin therapy. Most, but not all, statin effects are related to their ability to competitively block HMG-CoA reductase, and thereby decrease production of multiple intermediates in the cholesterol biosynthetic pathway. Mevalonate is the initial product of HMG-CoA reductase (Fig. 1A) and mevalonate levels are decreased by statin therapy. We performed “rescue” experiments with mevalonate to determine whether lovastatin was acting through an HMG-CoA reductase-dependent pathway (Fig. 1E). Mevalonate reversed the ability of lovastatin to potentiate effеrcytosis, confirming HMG-CoA reductase dependency. In contrast, lovastatin suppressed phagocytozyis of IgG-opsonized erythrocytes through the FcγR, as has recently been shown (53, 54), and this also appeared to be dependent on HMG-CoA reductase (Fig. 1F).

Lovastatin does not affect expression of effеrcytosis receptors
We considered that lovastatin may exert its positive effect on effеrcytosis by increasing expression of key uptake receptors, especially because statins were recently reported to increase the expression of CD36, a well-known effеrcytosis receptor (55). Contrary to this report, we found that lovastatin had no effect on expression of HMMD effеrcytosis receptors (i.e., α5β3, CD91, calreticulin, CD36, CD44, CD14) or the FcγRIIa (Fig. 2). Lovastatin also had no effect on staining by mAb 217. The protein target of mAb 217 was originally thought to be the phosphatidylserine receptor, but is now not known.

Lovastatin-enhanced effеrcytosis is dependent on prenylation
Statins regulate the posttranslational modification of hundreds of proteins by controlling the production of key substrates of protein prenylation, such as FPP and GGPP. Because mevalonate is an upstream precursor of both FPP and GGPP, statins ultimately decrease their levels as well. We performed dose-response rescue experiments with GGPP and FPP, and found that both reversed lovastatin-enhanced effеrcytosis, albeit with different potencies (Fig. 3, A and B). Even though these experiments suggested a role for both farnesylation and geranylation, they did not rule out the possibility that the effect of FPP was due to its conversion to GGPP and augmentation of geranylation. This is an important issue because the known Rho GTPase regulators of effеrcytosis are only geranylated. Incomplete inhibition of HMG-CoA reductase could potentially allow low level synthesis of the 5-carbon isopentenyl pyrophosphate (IPP), which normally condenses with 15-carbon FPP to produce 20-carbon GGPP (Fig. 1A), thereby creating a pathway for FPP repletion to influence geranylation.

Specific inhibitors of geranylgeranyltransferase I (GGTI-2133; IC50 = 38 nM) and farnesyltransferase (FTI-276; IC50 = 0.5 nM) were used to further examine the role of these separate prenylation pathways on effеrcytosis. Both inhibitors enhanced effеrcytosis, but the effect of FTI-276 appeared to be more modest than GGTI-2133 (Fig. 3, C and D). It is important to note that farnesyltransferase inhibitors may increase the production of geranylated proteins, presumably by shunting excess FPP to GGPP (56). This suggests that farnesyltransferase inhibitors may not solely act by decreasing farnesylated proteins, but instead they may also change the balance in favor of geranylated proteins. Consistent with this possibility, combinations of GGTI-2133 and FTI-276 did not enhance effеrcytosis more thanLovastatin or GGTI-2133 alone (data not shown). Therefore, effеrcytosis appears to be regulated by intracellular signaling molecules that are geranylated, and possibly by those that are farnesylated.

Lovastatin suppresses membrane-bound RhoA greater than Rac-1
Statins inhibit prenylation and membrane localization of a variety of Rho GTPases, including positive (Rac-1) and negative (RhoA) regulators of effеrcytosis. Yet, in HMMDs, lovastatin consistently enhanced effеrcytosis, implying that lovastatin may exert a prolonged, disproportionate effect on the prenylation and membrane localization of RhoA. To address this hypothesis, HMMDs were treated with and without lovastatin for 24 h, and membrane fractions were assessed for RhoA and Rac-1 staining by Western blot. Lovastatin decreased membrane-bound RhoA greater then Rac-1 (Fig. 4, A and B), suggesting a mechanism for the positive effect of lovastatin on effеrcytosis.

Lovastatin reverses impaired effеrcytosis by lysophosphatidic acid (LPA), a potent RhoA activator
We next used the potent RhoA activator, LPA to address whether lovastatin could reverse impaired effеrcytosis in vitro (57). LPA-suppressed effеrcytosis by HMMDs, and this suppression was prevented by lovastatin (Fig. 4C), suggesting that lovastatin, or for that matter other Rho pathway inhibitors, could play a therapeutic

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role in diseases where suppression of efferocytosis contributes to disease pathogenesis.

**Lovastatin increases efferocytosis in vivo**

We tested whether statins could enhance efferocytosis by treating mice with lovastatin (10 mg/kg) three times over 30 h. Ten million, Cell Tracker Red-labeled apoptotic murine thymocytes were then instilled intratracheally and clearance was assessed. This model has previously been used to evaluate efferocytosis by macrophages and epithelial cells in vivo (45, 50). Defective efferocytosis is suggested by either decreased uptake into alveolar macrophages (i.e., decreased PI; Fig. 5A), or by increased recovery of apoptotic cells in the bronchoalveolar lavage (Fig. 5B). Lovastatin modestly increased efferocytosis in the naive murine lung as measured by an increase in the alveolar macrophage PI (Fig. 5C) and by a decrease in the recovery of apoptotic thymocytes (Fig. 5D).

To examine whether the action of lovastatin on efferocytosis in vivo was HMG-CoA reductase dependent, mice were treated with...
Lovastatin (10 mg/kg), three times over 30 h, in the presence or absence of rescue mevalonate, and clearance of apoptotic thymocytes by alveolar macrophages was assessed. Lovastatin again increased efferocytosis by alveolar macrophages in vivo, and this effect was prevented by mevalonate (Fig. 5E). Together, these results indicate that lovastatin enhances efferocytosis in the naive murine lung in an HMG-CoA reductase-dependent manner, thus confirming in vitro results.

**Lovastatin enhances efferocytosis by human alveolar macrophages taken from patients with COPD**

Accumulating evidence suggests that efferocytosis is dysregulated in chronic inflammatory lung diseases, such as COPD, and may contribute to disease pathogenesis. For example, several animal models of COPD are associated with increased accumulation (58–61) and impaired removal (45) of apoptotic cells. Likewise, apoptotic cells are increased in COPD lungs (31, 62–65) and efferocytosis is defective in COPD alveolar macrophages ex vivo (32). Therefore, we tested the effect of lovastatin on efferocytosis by alveolar macrophages isolated from GOLD stage 2 (66) COPD patients (Table I). Lovastatin enhanced efferocytosis in these alveolar macrophages in an HMG-CoA reductase-dependent fashion (Fig. 6). Taken together, these data suggest that statins may have therapeutic potential in diseases, such as COPD, where efferocytosis is suppressed and inflammation is dysregulated.

**Discussion**

Apoptotic cell clearance defects are increasingly recognized in diseases of chronic inflammation (31, 32) and autoimmunity (33, 34, 67, 68), suggesting that effective efferocytosis may be necessary for the maintenance of homeostasis. This concept is based on the role efferocytosis plays in suppressing both the innate (69, 70) and adaptive (68) immune response and in removing autoantigens (71). Interestingly, this concept is not new. Over 100 years ago, Metchnikoff (72) recognized this removal process and its importance to homeostasis, which he termed “physiologic inflammation.” Therefore, therapies that enhance efferocytosis may offer a unique therapeutic benefit, especially if impaired efferocytosis is a determinant of disease pathogenesis. Our findings indicate that lovastatin enhances efferocytosis in vitro and in vivo. Lovastatin’s effect on efferocytosis depends on its ability to inhibit HMG-CoA reductase, decrease prenylation substrates, and to alter the balance of Rho GTPases. Lovastatin may have therapeutic potential in chronic inflammatory diseases with impaired efferocytosis because it reverses impaired efferocytosis in vitro and in vivo, and enhances efferocytosis by alveolar macrophages from patients with COPD.

Up to 2% of expressed cellular proteins are prenylated and over 150 prenylated proteins have been identified (73, 74), suggesting that the effect of statins on efferocytosis is likely to be complex. Our findings indicate that lovastatin enhanced efferocytosis in vitro, in part, by altering the membrane balance of RhoA and Rac-1, two key regulators of efferocytosis. Geranylgeranylation and farnesylation. Lovastatin-enhanced efferocytosis is dependent on geranylgeranylation and farnesylation. A, GGPP (1, 5, and 10 μM) and (B) FPP (5 and 10 μM) prevented lovastatin from increasing efferocytosis of apoptotic human neutrophils by HMDMs, but 10 μM GGPP or FPP alone had no effect on efferocytosis. The mean PI as a percentage of control ± SEM is shown for five replicates per group. Control mean PI: 3.8 ± 0.8. * Significantly different from control (p < 0.05). C, GGTI-2133 (20 and 40 nM) and (D) FTI-276 (0.5 nM) enhanced efferocytosis of apoptotic human neutrophils by HMDMs. As a positive control, HMDMs were treated with 10 μM lovastatin for 24 h. DMSO vehicle had no effect on efferocytosis. The mean PI as a percentage of control ± SEM is shown for 3–10 replicates/group. Control mean PI for C: 3.9 ± 0.3, and D: 4.9 ± 1.0. * Significantly different from control (p < 0.05).
prolonged effect include: 1) geranylgeranyltransferase I may prenylate Rac-1 more efficiently than RhoA, or 2) prenylation of Rac-1 may be less substrate dependent. Even during lovastatin treatment, prenylation substrates (GGPP or FPP) would be expected to be present in small quantities due to incomplete blockade of HMG-CoA reductase, or due to salvage pathway activity (75). Alternatively, 3) the half-life of prenylated Rac-1 may be longer than prenylated RhoA. This possibility is less likely, because lovastatin enhanced efferocytosis as long as 5 days after treatment. We also noted that the ability of lovastatin to increase efferocytosis by HMDMs waned during days 3 and 4 of treatment, but increased again at day 5. We do not have a clear explanation for this observation, but it was consistent across both concentrations tested.

Our data suggest that farnesylated proteins might negatively regulate efferocytosis, because inhibition of farnesyltransferase modestly increased efferocytosis, and FPP repletion reversed lovastatin-enhanced efferocytosis. RhoB is an attractive candidate for this effect, because it is both farnesylated and geranylgeranylated (76) and it plays a known role in the phagocytosis of Pneumocystis (77). Whether farnesyltransferase inhibitors also shift the balance toward production of other geranylgeranylated proteins that could enhance efferocytosis, like Rac-1, Cdc42, or RhoG (43, 78), remains to be determined.

Growing evidence from animal models indicates that statins may play a role in the treatment of inflammatory lung diseases, including acute lung injury (2), asthma (18), and emphysema (19). Our data supports this notion, because lovastatin increased efferocytosis in the lungs of naive mice. In vivo, lovastatin may enhance efferocytosis by altering the balance of membrane-bound RhoA and Rac-1, as was demonstrated in vitro. Alternatively, in vivo and especially in an inflammatory environment, lovastatin may enhance efferocytosis by suppressing oxidative stress, because oxidative stress inhibits efferocytosis by activating RhoA (K. A. McPhillips, manuscript in preparation). Lovastatin also suppresses matrix metalloproteinase-9 (8), which inhibits efferocytosis in vitro (R. W. Vandivier, unpublished data), and is an important component of lung inflammation. Finally, statins increase PPARγ, which has also been shown to increase efferocytosis (79).

Lovastatin did not enhance efferocytosis by altering apoptotic cell binding or by increasing expression of efferocytosis receptors. In contrast, Ruiz-Velasco et al. (55) found that lovastatin treatment (10 μM) increased CD36 surface expression and mRNA in human monocytes at 24 h and THP-1 cells at 48 h. The disparity between our findings may relate to intrinsic differences between human monocytes and HMDMs.

Lovastatin suppressed FcγR-mediated phagocytosis in an HMG-CoA reductase-dependent manner, confirming recent reports (53, 54). Interestingly, these authors all concluded that statins suppress FcγR-mediated phagocytosis by inhibiting cholesterol
biosynthesis, and not by inhibiting prenylation (53, 54). Like effec-
terocytosis, prenylated proteins like Rac-1, Cdc42, and Rab11 are
rated to impair host defense, but ultimately, its importance remains
to be demonstrated in vivo. In a Klebsiella pneumoniae mouse model of
n the presence of malonate (1-Meva; 50 μM). Control mean PI: 5.5 ± 1.9. *, Signifi-
cantly different from control (p < 0.05).
FIGURE 6. Lovastatin reverses impaired effec-

The ability of statins to enhance efferocytosis suggests a new
mechanism by which statins may modulate acute and chronic in-
flammatory diseases, and may help direct statin clinical trials to
specify diseases. For example, cystic fibrosis, bronchiectasis, and
COPD are attractive targets for statin therapy because they are all
associated with accumulation, and defective clearance, of apo-
tic cells (31, 32). Indeed, our data provides “proof of principle” for
this approach because lovastatin increased efferocytosis by human
alveolar macrophages taken from patients with COPD. Lee et al. (19)
have also shown that statin treatment inhibits the development of
cigarette smoke-induced emphysema in rats. However, we
would not suggest that potential therapeutic targets be limited to
these chronic inflammatory lung diseases, because accumulation of
apoptotic cells and failed efferocytosis has also been implicated in
systemic inflammatory diseases, like glomerulonephritis (34),
rheumatoid arthritis (36), and systemic lupus erythematosus (35),

Table I. Subject demographicsa

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<td>Smoking history (pack-years)</td>
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a FEV₁, Forced expiratory volume in 1 s; FVC, forced vital capacity.


