

Lovastatin Enhances Clearance of Apoptotic Cells (Efferocytosis) with Implications for Chronic Obstructive Pulmonary Disease¹

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Statins are potent, cholesterol-lowering agents with newly appreciated, broad anti-inflammatory properties, largely based upon their ability to block the prenylation of Rho GTPases, including RhoA. Because phagocytosis of apoptotic cells (efferocytosis) is a pivotal regulator of inflammation, which is inhibited by RhoA, we sought to determine whether statins enhanced efferocytosis. The effect of lovastatin on efferocytosis was investigated in primary human macrophages, in the murine lung, and in human alveolar macrophages taken from patients with chronic obstructive pulmonary disease. In this study, we show that lovastatin increased efferocytosis in vitro in an 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase-dependent manner. Lovastatin acted by inhibiting both geranylgeranylation and farnesylation, and not by altering expression of key uptake receptors or by increasing binding of apoptotic cells to phagocytes. Lovastatin appeared to exert its positive effect on efferocytosis by inhibiting RhoA, because it 1) decreased membrane localization of RhoA, to a greater extent than Rac-1, and 2) prevented impaired efferocytosis by lysophosphatidic acid, a potent inducer of RhoA. Finally, lovastatin increased efferocytosis in the naive murine lung and ex vivo in chronic obstructive pulmonary disease alveolar macrophages in an HMG-CoA reductase-dependent manner. These findings indicate that statins enhance efferocytosis in vitro and in vivo, and suggest that they may play an important therapeutic role in diseases where efferocytosis is impaired and inflammation is dysregulated. *The Journal of Immunology*, 2006, 176: 7657–7665.

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- κ B 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 α (11), PPAR γ (4), and TGF β 1 (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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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; PI, 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 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 5.0×10^5 alveolar macrophages/well.

Murine thymocytes were isolated from the thymi of 3- to 4-wk-old, female ICR mice, by passing thymi through a 40- μ m 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 2×10^6 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 4×10^6 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 μ l of 100% ethanol, heated to 50°C, alkalized by adding 250 μ l 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 μ l 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 μ M 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 and for the indicated times: mevalonate (Sigma-Aldrich), GGPP (Sigma-Aldrich), and FPP (Sigma-Aldrich). Inhibitors of farnesyltransferase (FTI-276; Calbiochem-Novabiochem) and geranylgeranyltransferase I (GGTI-2133; Calbiochem-Novabiochem) were added to HMDMs 8 h before experimentation. FTI-276 inhibits farnesyltransferase with an IC₅₀ = 0.5 nM, and geranylgeranyltransferase I at a much higher IC₅₀ = 50 nM. GGTI-2133 inhibits geranylgeranyltransferase I with an IC₅₀ = 38 nM, and farnesyltransferase at a much higher IC₅₀ = 5.4 μ M.

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, 0.9% sodium chloride, 0.4% polysorbate 80, 0.9% benzyl alcohol in deionized water) by gavage and PBS by i.p. injection; 2) lovastatin group, treated with activated 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 μ l 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 expressed a PI. A minimum of 400 alveolar macrophages were counted blindly.

Recovered apoptotic thymocytes were determined as follows. Total lavage cells (including erythrocytes) were counted using a hemacytometer. The percentage of free thymocytes in the lavage was determined by FACS analysis (Fig. 5B). 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 Fc γ R staining, incubated with 5 μ g 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 FACScan cytometer using CellQuest Pro (BD Biosciences) and FloJo (Tree Star) software.

As used in FACS analysis were as follows: BD Biosciences/BD Pharmingen Abs include mouse monoclonal anti-human CD36 IgM, anti-human CD44 IgG1, anti-human integrin β_3 IgG1, anti-human CD32 (Fc γ RIIa) IgG2b, and mouse monoclonal IgG1, IgG2a, IgG2b, IgM, κ isotype controls (Chemicon International) Abs include mouse monoclonal anti-human integrin $\alpha_v\beta_3$ 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

HMDM membrane fractions were prepared and separated on SDS-PAGE as described previously (51, 52). Briefly, HMDMs 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 m. Supernatants were collected and spun-down at $10,000 \times g$ for 45 m. The pellet was solubilized in lysate buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM DTT, 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 $\mu\text{g}/\text{ml}$; Upstate Biotechnology) and Rac-1 (clone 23A8, 1 $\mu\text{g}/\text{ml}$; 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 \pm SEM.

Results

Lovastatin increases uptake of apoptotic cells (efferocytosis)

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

The effect of lovastatin on efferocytosis 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. 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 efferocytosis, confirming HMG-CoA reductase dependency. In contrast, lovastatin suppressed phagocytosis of IgG-opsonized erythrocytes through the Fc γ R, 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 efferocytosis receptors

We considered that lovastatin may exert its positive effect on efferocytosis by increasing expression of key uptake receptors, especially because statins were recently reported to increase the expression of CD36, a well-known efferocytosis receptor (55). Contrary to this report, we found that lovastatin had no effect on

expression of HMDM efferocytosis receptors (i.e., $\alpha_v\beta_5$, β_3 , CD91, calreticulin, CD36, CD44, CD14) or the Fc γ RIIIa (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 efferocytosis 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 efferocytosis, albeit with different potencies (Fig. 3, A and B). Even though these experiments suggested a role for both farnesylation and geranylgeranylation, they did not rule out the possibility that the effect of FPP was due to its conversion to GGPP and augmentation of geranylgeranylation. This is an important issue because the known Rho GTPase regulators of efferocytosis are only geranylgeranylated. 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 geranylgeranylation.

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 efferocytosis. Both inhibitors enhanced efferocytosis, 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 geranylgeranylated 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 geranylgeranylated proteins. Consistent with this possibility, combinations of GGTI-2133 and FTI-276 did not enhance efferocytosis more than lovastatin or GGTI-2133 alone (data not shown). Therefore, efferocytosis appears to be regulated by intracellular signaling molecules that are geranylgeranylated, 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 efferocytosis. Yet, in HMDMs, lovastatin consistently enhanced efferocytosis, implying that lovastatin may exert a prolonged, disproportionate effect on the prenylation and membrane localization of RhoA. To address this hypothesis, HMDMs 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 than Rac-1 (Fig. 4, A and B), suggesting a mechanism for the positive effect of lovastatin on efferocytosis.

Lovastatin reverses impaired efferocytosis by lysophosphatidic acid (LPA), a potent RhoA activator

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

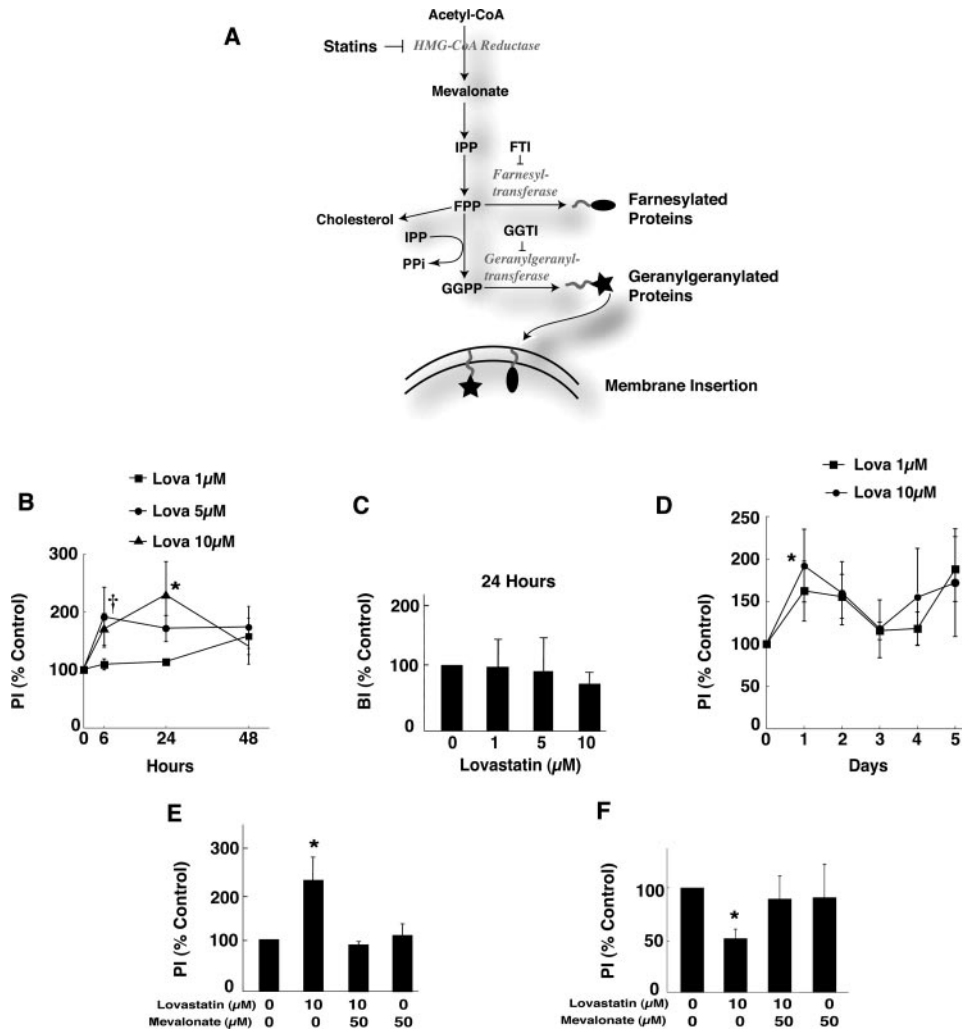


FIGURE 1. A, The cholesterol/prenylation pathway. Statins suppress the production of mevalonate, IPP, FPP, GGPP, and cholesterol by inhibiting HMG-CoA reductase. Farnesyltransferase and geranylgeranyltransferase prenylate Rho GTPases by catalyzing the attachment of FPP or GGPP, respectively. Statins broadly inhibit prenylation by decreasing levels of FPP and GGPP, while farnesyltransferase inhibitors (FTI) and geranylgeranyltransferase inhibitors (GGTI) more specifically affect the addition of their respective substrates. Prenylation ultimately regulates the activity of Rho GTPases and other proteins by influencing membrane insertion, localization, and protein:protein interaction. B–F, Lovastatin enhances efferocytosis, but suppresses Fc γ R phagocytosis, in an HMG-CoA reductase-dependent manner. HMDMs were treated with lovastatin for 6 h to 5 days, then cocultured with 2.5×10^6 apoptotic human neutrophils for an additional 40 min. Uningested neutrophils were then washed off, and a PI or binding index (BI) was determined by blinded visual inspection. The PI (or BI) is calculated by dividing the number of ingested (or bound) apoptotic cells by the total number of macrophages counted, multiplied by 100. B, Lovastatin (5 μ M) increased efferocytosis of apoptotic human neutrophils at 6 h. At 24 h, lovastatin increased efferocytosis in a dose-dependent fashion, exerting a maximum effect at 10 μ M. The mean PI as a percentage of control \pm SEM is shown for four to five replicates per group. Control mean PI: 13.7 ± 6.3 . *, Significantly different from control ($p < 0.05$); †, different from control ($p = 0.08$). C, Lovastatin had no effect on binding of apoptotic human neutrophils to HMDMs at 24 h. The mean BI as a percentage of control \pm SEM is shown for five replicates per group. Control mean BI: 7.9 ± 3.8 . D, Lovastatin increased efferocytosis of apoptotic human neutrophils at long at 5 days posttreatment. HMDMs were treated with 1 or 10 μ M lovastatin for 1–5 days and assessed for neutrophil efferocytosis. A total of 10 μ M lovastatin increased efferocytosis at day 1, whereas 1 μ M lovastatin increased efferocytosis at day 5. The mean PI as a percentage of control \pm SEM is shown for four to six replicates per group. Control mean PI: 8.5 ± 4.4 . *, Significantly different from control ($p < 0.05$; Student's *t* test). E, Mevalonate reversed the effect of lovastatin on uptake of apoptotic human neutrophils by HMDMs. The mean PI as percentage of control \pm SEM is shown for four replicates per group. Control mean PI: 6.1 ± 1.9 . *, Significantly different from control ($p < 0.05$). F, Lovastatin inhibited phagocytosis of Ig-G-opsonized erythrocytes through the Fc γ R, in an HMG-CoA reductase-dependent manner. Control mean PI: 81.5 ± 10.1 . *, Significantly different from control ($p < 0.05$).

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

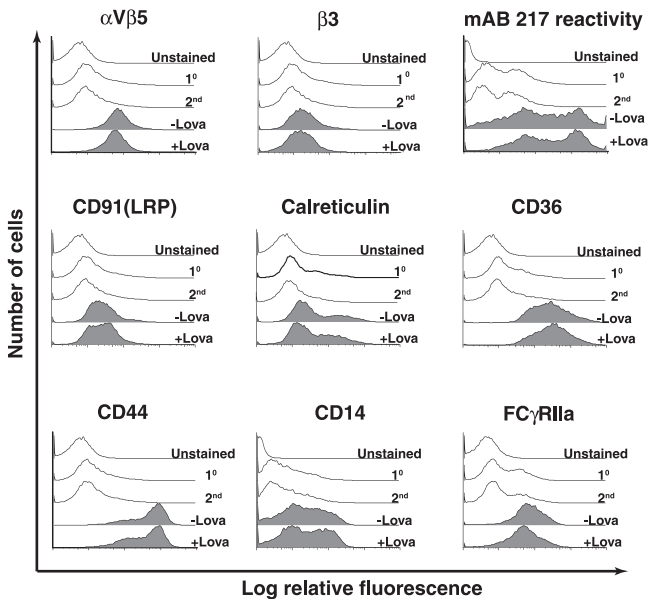


FIGURE 2. Lovastatin had no effect on expression of HMDM efferocytosis receptors. HMDMs were treated with and without lovastatin for 24 h and examined by flow cytometry for surface expression of a variety of receptors associated with efferocytosis and phagocytosis. Histograms for each receptor are representative of three replicates per group.

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 inflamma-

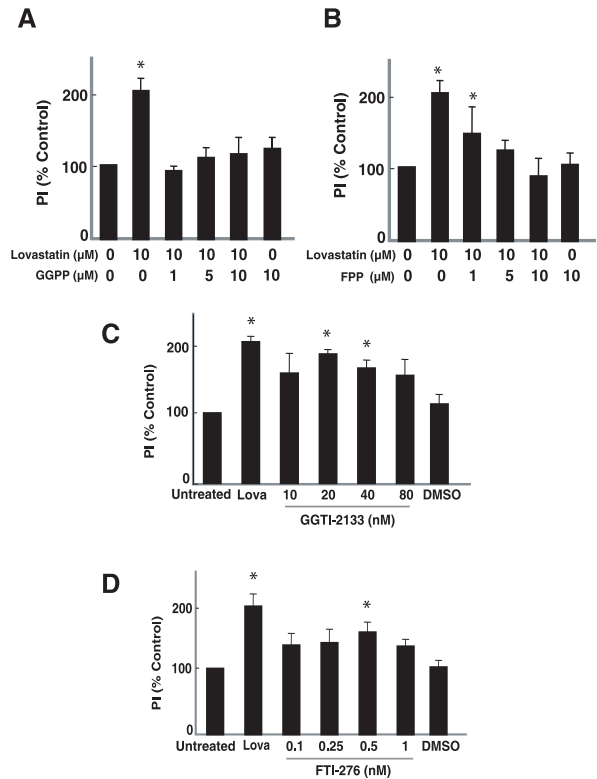


FIGURE 3. Lovastatin-enhanced efferocytosis is dependent on geranylgeranylation and farnesylation. *A*, GGPP (1, 5, and 10 μ M) and (*B*) FPP (5 and 10 μ M) prevented lovastatin from increasing efferocytosis of apoptotic human neutrophils by HMDMs, but 10 μ M GGPP or FPP alone had no effect on efferocytosis. The mean PI as a percentage of control \pm 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 μ M lovastatin for 24 h. DMSO vehicle had no effect on efferocytosis. The mean PI as a percentage of control \pm 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 ($p < 0.05$).

tion.” 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. Geranylgeranyltransferase I prenylates both RhoA and Rac-1, yet lovastatin suppressed membrane localization of RhoA to a greater extent than Rac-1. The reason(s) for this disproportionate effect is unclear, but it suggests that enzyme kinetics favor prenylation of Rac-1 over RhoA. Because lovastatin increased efferocytosis after 5 days of treatment, the effect appears to be sustained. Possible explanations for this

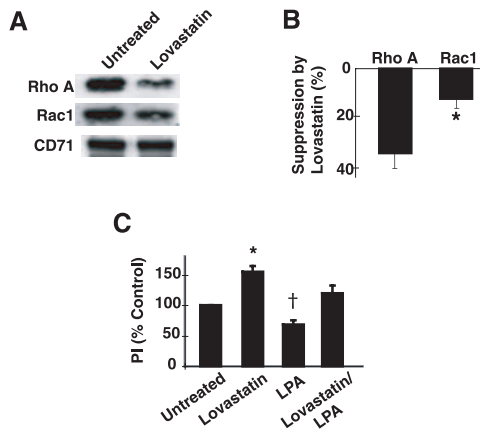


FIGURE 4. Lovastatin may enhance efferocytosis by suppressing membrane-bound RhoA. *A* and *B*, HMDMs were treated with and without lovastatin for 24 h and assessed for membrane-bound RhoA and Rac-1 by Western blot and densitometry. *A*, Representative Western blots from five separate experiments are shown. CD71 was used as a membrane marker and control for equal loading. *B*, Lovastatin significantly decreased membrane-bound RhoA greater than Rac-1 ($p < 0.05$). *C*, LPA (10 μ M), a potent RhoA activator, decreased ingestion of apoptotic Jurkat T cells by HMDMs in the absence, but not the presence, of lovastatin. Jurkat T cells were used in these experiments instead of neutrophils because they are ingested more avidly by HMDMs. The mean PI as a percentage of control \pm SEM is shown for six replicates per group. Control mean PI: 42.2 ± 10.3 . *, Significantly different from control ($p < 0.05$). †, Different from control ($p = 0.051$).

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 have 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

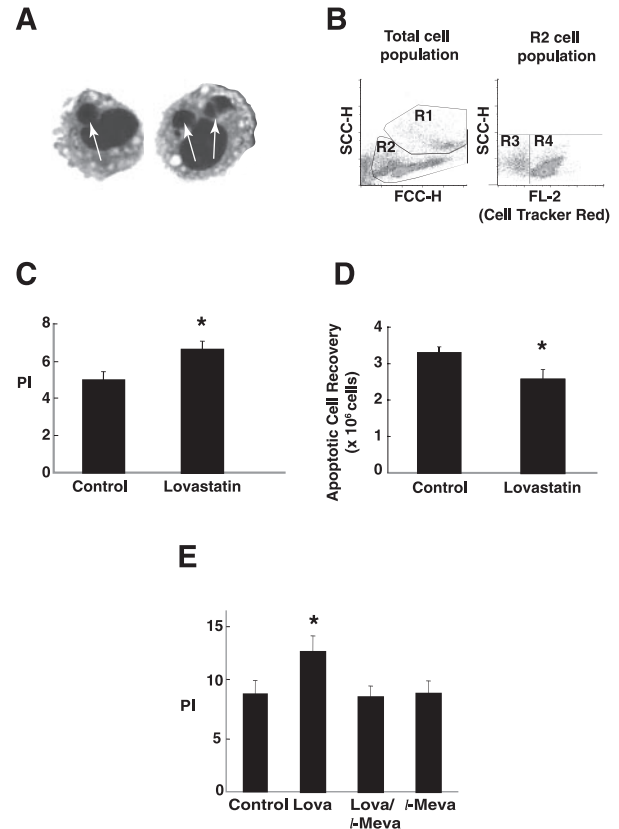


FIGURE 5. Lovastatin increases efferocytosis in vivo. Ten million, Cell Tracker Red-stained, apoptotic murine thymocytes were instilled intratracheally into anesthetized mice. Bronchoalveolar lavage was performed 40 min later, and was assessed for (*A*) the alveolar macrophage PI and (*B*) recovery of apoptotic thymocytes. *A*, Representative photomicrographs of Wright-Giemsa-stained murine alveolar macrophages (magnification, $\times 100$) with ingested apoptotic thymocytes (arrows). *B*, Plot on the left represents the forward-side scatter characteristics of bronchoalveolar lavage, where R1 = alveolar macrophages and R2 = recovered thymocytes + erythrocytes. Plot on the right represents side scatter-FL-2 (Cell Tracker Red) staining characteristics of R2, where FL-2-positive cells are recovered thymocytes (R4) and FL-2-negative cells are erythrocytes (R3). *C*, Lovastatin increased the alveolar macrophage PI (*, significantly different from control; $p = 0.035$) and (*D*) decreased the recovery of apoptotic thymocytes (*, different from control; $p = 0.054$) in vivo. Eight to 10 animals were used per group. *E*, Lovastatin increased the alveolar macrophage PI in the absence, but not the presence, of mevalonate, indicating that the effect of lovastatin was HMG-CoA reductase dependent in vivo. Six to 7 animals were used per group. *, Significantly different from control ($p < 0.05$).

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 PPAR γ , 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 μ M) 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 Fc γ R-mediated phagocytosis in an HMG-CoA reductase-dependent manner, confirming recent reports (53, 54). Interestingly, these authors all concluded that statins suppress Fc γ R-mediated phagocytosis by inhibiting cholesterol

Table I. Subject demographics^a

Characteristics	Values
<i>n</i>	3
Age (years)	57 ± 2.1
FEV ₁ (% predicted)	63.3 ± 8.2
FVC (% predicted)	80.4 ± 9.9
FEV ₁ /FVC (%)	60.2 ± 0.3
Smoking history (pack-years)	47 ± 15.9

^a FEV, Forced expiratory volume in 1 s; FVC, forced vital capacity.

biosynthesis, and not by inhibiting prenylation (53, 54). Like efferocytosis, prenylated proteins like Rac-1, Cdc42, and Rab11 are required for FcγR-mediated phagocytosis (80, 81), and a role for RhoA has been suggested (82). Therefore, we propose that under certain conditions statins might also influence FcγR phagocytosis through modulation of Rho GTPases. The effect of statins on FcγR-mediated phagocytosis is concerning because of its potential to impair host defense, but ultimately, its importance remains to be demonstrated in vivo. In a *Klebsiella pneumoniae* mouse model of pneumonia, lovastatin delayed bacterial clearance and enhanced dissemination (2). In contrast, statins have consistently improved survival in animal and human bacterial sepsis (16, 22, 83), implying that the beneficial effects of statins may outweigh their potential deleterious effects.

The dose and concentration of lovastatin used in the in vitro and in vivo experiments is certainly higher than is used clinically, suggesting that lovastatin may not augment efferocytosis when used in humans. In mice, however, lovastatin induces a 6- to 10-fold increase in hepatic microsomal HMG-CoA reductase after only 24 h, implying that higher doses may be required to produce clinical effects in mice compared with humans (48). In humans, prolonged administration of lovastatin at 80 mg/kg/day results in steady state concentrations ranging from 0.15 to 0.3 μM (84), which is marginally less than the lowest effective concentration of lovastatin used in our study (1 μM). The effectiveness of lovastatin in vivo, though, may depend on the clinical setting and remains to be determined in COPD. For example, it is possible that prolonged lovastatin treatment at clinically relevant doses may augment efferocytosis in humans with COPD, especially if RhoA activity is increased.

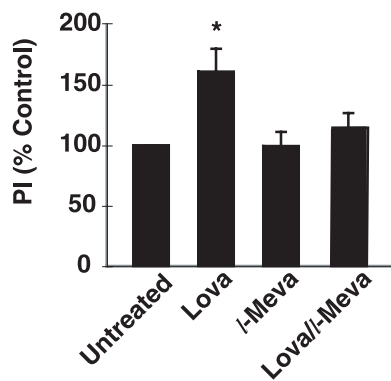


FIGURE 6. Lovastatin reverses impaired efferocytosis in COPD alveolar macrophages ex vivo. Human alveolar macrophages were isolated from patients with GOLD stage 2 COPD, then incubated with or without lovastatin or mevalonate for 24 h before coculture with apoptotic human neutrophils. Lovastatin (Lova; 10 μM) treatment enhanced efferocytosis by GOLD stage 2 alveolar macrophages (*n* = 3), but not in the presence of mevalonate (l-Meva; 50 μM). Control mean PI: 5.5 ± 1.9. *, Significantly different from control (*p* < 0.05).

The ability of statins to enhance efferocytosis suggests a new mechanism by which statins may modulate acute and chronic inflammatory diseases, and may help direct statin clinical trials to specific 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 apoptotic 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).

Disclosures

The authors have no financial conflict of interest.

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