Increased Expression of CD14 in Macrophages after Inhibition of the Cholesterol Biosynthetic Pathway by Lovastatin

Tiffany Frey[1] *and Antonio De Maio*[1,2]

[1]Department of Physiology, Johns Hopkins University, Baltimore, Maryland; and [2]Department of Surgery, University of California San Diego, La Jolla, California, USA

Sepsis, which is the product of a poorly controlled inflammatory response, is a major health problem. Adequate therapies for sepsis are unavailable, and patient care is mainly supportive. Statins, widely used for the treatment of hypercholesterolemia, have been found to be antiinflammatory, but the mechanisms responsible for this alteration in the inflammatory response are not well understood. We investigated the effect of statins on CD14 expression, the major binding site for bacterial lipopolysaccharide (LPS) on the macrophage surface. CD14 is found in both a membrane-bound form on the cell surface (mCD14) and in a soluble variant in circulation (sCD14). Treatment of RAW 264.7 macrophages with lovastatin resulted in elevated mCD14 levels and decreased sCD14 levels after LPS stimulation. The increase in mCD14 was dependent on depletion of geranylgeranyl pyrophosphate (GGPP) and subsequent inhibition of Rho GTPases, whereas the effect of lovastatin on sCD14 was independent of this pathway. The increase in mCD14 expression correlated with an enhanced response to LPS, at least at the level of tumor necrosis factor (TNF)-α secretion. These results suggest that statin treatment can modulate macrophage functon, which may have an impact on inflammation and the outcome from sepsis.

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INTRODUCTION

Sepsis, which is a systemic response to infection, is a major health problem with an incidence of approximately 750,000 cases per year in the United States and a mortality rate of 10–40% (1). Therapy for sepsis is mainly supportive, and the care of these patients is very expensive. Patients with sepsis may develop severe sepsis as well as septic shock (2), pathologies that may lead to diminished energy production, altered metabolic rate, and abnormal cellular processes, as well as an overwhelming inflammatory response. The combination of these conditions may lead to the development of multiple organ dysfunction syndrome and death (3). The inflammatory response is regulated by the balance between pro- and antiinflammatory mediators, including cytokines. High concentrations of either proinflammatory or antiinflammatory cytokines have been reported as predictors of a poor outcome after sepsis (4,5). A major inducer of the inflammatory response is bacterial lipopolysaccharide (LPS), or endotoxin. LPS is a component of the outer membrane of Gram-negative bacteria, which is shed during infection and has a dominant role in the septic process. LPS, in a complex with LPS-binding protein (LBP), interacts with CD14, toll-like receptor 4 (TLR4), and MD-2 on cells to initiate the release of cytokines and other effector molecules (6). CD14, which is the major LPS binding site on macrophages, is found in both a membrane-bound form on the cell surface (mCD14) and in a soluble variant in circulation (sCD14). Membrane-bound CD14 is anchored by a gylcosylphosphatidylinositol (GPI) tail, and is present in plasma membrane microdomains rich in cholesterol and sphingolipids that have been named detergent-resistant membranes (DRMs) or lipid rafts. The presence of CD14 within lipid rafts has been associated with cell activation, in particular with signal transduction (7).

Statins are widely prescribed for the treatment of hypercholesterolemia. These drugs are competitive inhibitors of 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase, catalyzing the rate-limiting step in cholesterol biosynthesis. Depletion of cellular cholesterol levels triggers the activation of sterol regulatory element binding proteins (SREBPs). SREBPs are endoplasmic reticulum membrane–bound transcription factors that are transported to the Golgi apparatus when cellular sterol levels are

Address correspondence and reprint requests to *Antonio De Maio, Department of Surgery, University of California San Diego, 9500 Gilman Drive, #0739, La Jolla, CA 92093-0739. Tel. 858-822-6502; Fax. 858-822-2981; E-mail: ademaio@ucsd.edu*
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low. In this organelle, SREBPs are cleaved, releasing the transcriptionally active N-terminal domain of the protein, which migrates to the nucleus and activates target genes by binding sterol response elements (SREs) in their promoters (8). The low-density lipoprotein (LDL) receptor is among the genes that are activated by SREBPs. Thus, a decrease in cellular cholesterol initiated by statins triggers the up-regulation of this receptor, increasing the uptake of lipoproteins and thereby reducing plasma LDL levels (9). Recent evidence suggests that statins may modulate the inflammatory process (10); statins have been shown to improve endothelial function, control thrombosis, enhance stability of atherosclerotic plaques, and decrease oxidative stress (11). These pleiotropic effects, while contributing to the beneficial effects on cardiovascular disease, also seem to play a role in other immune and inflammatory diseases. In human clinical studies, statins have been shown to reduce risks related to sepsis, such as mortality and disease progression and incidence (12–15). In murine models of sepsis, statin treatment has been shown to improve survival (16–19).

In the present study, we investigated the effect of statins on the capacity of macrophages to recognize proinflammatory mediators, such as LPS. We found that treatment of RAW 264.7 macrophages with lovastatin resulted in an increase in expression of mCD14, which was enhanced upon addition of LPS. In addition, lovastatin treatment resulted in a reduction of sCD14 in the supernatant after LPS stimulation. This effect of lovastatin on CD14 resulted in increased secretion of tumor necrosis factor (TNF)-α after stimulation with LPS.

MATERIALS AND METHODS

Materials

RAW 264.7 macrophages were obtained from American Type Culture Collection and maintained in RPMI 1640 containing 10% fetal bovine serum (FBS) and penicillin (50 IU/mL)/streptomycin (50 μg/mL). Mevalonic acid, GGPP, FPP, cholesterol-PEG 600, GGTI-298, FTI-277, MTT (thiazolyl blue tetrazolium bromide), SB203580, and *Escherichia coli* LPS (serotype 026:B6, lot # 023K4116) were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Lovastatin, toxin B, and U0126 were obtained from Calbiochem (San Diego, CA, USA). TNF-α was measured with a mouse ELISA kit from Invitrogen (Carlsbad, CA, USA). The CD14 antibodies (clone rmC5-3, nonconjugated and FITC-conjugated) and the corresponding FITC-conjugated isotype control were from Pharmingen (San Diego, CA, USA). The β-actin antibody was from Sigma-Aldrich. Complete mini-EDTA–free protease inhibitor cocktail pellets were obtained from Roche (Basel, Switzerland). RNA isolation was done with Trizol reagent from Invitrogen (Carlsbad, CA, USA).

Isolation of Peritoneal Macrophages

Mice were anesthetized with isofluorane and killed by cervical dislocation. Peritoneal macrophages were isolated from C57BL/6J and C57BL/6J CD14(-/-) mice by peritoneal lavage using 5 mL of serum-free RPMI 1640 containing penicillin/streptomycin (medium A). Cells were collected by centrifugation and plated at a density of 50,000 cells/well in a 96-well BD Falcon (BD Biosciences, San Jose, CA, USA) tissue culture plate. Cells were allowed to adhere in medium A in a 37°C, 5% CO_2 incubator for 1.5 hours and washed two times in PBS. The medium was replaced with RPMI 1640 containing 10% FBS and penicillin/streptomycin (medium B). Subsequently, cells were stimulated in medium B for 16 hours with 10 μM lovastatin followed by LPS stimulation for an additional five hours. Supernatants were then harvested and stored at –80°C for a TNF-α ELISA. An MTT cytotoxicity assay was then performed to assess viability and plating error. MTT (5 mg/mL) in PBS (filter sterilized) was diluted to 1.25 mg/mL in medium B, and 200 mL of this diluted solution was applied to the cells and incubated for one hour. The MTT-containing medium was then removed, and the formazan crystals were dissolved in 200 μL DMSO plus 25 mL Sorensen buffer (0.1 M glycine, 0.1 M NaCl, pH = 10.5)/well. The plate was then read in a microtiter plate reader at 560 nm. All animal procedures were part of protocol reviewed and approved by the Johns Hopkins Medical Institutions Animal Care and Use Committee.

Cellular Cholesterol Determination

RAW 264.7 macrophages were grown and treated in 60-mm cell culture plates (BD Falcon). After treatments, cells were washed once in PBS, followed by two extractions of cholesterol with hexane:isopropanol (3:2). Samples were dried under N_2 gas and resuspended in spectroanalyzed isopropanol. Cholesterol was measured using the Amplex Red Cholesterol Assay Kit from Molecular Probes/Invitrogen. Samples were normalized by protein remaining on the plate after extraction. The protein was solubilized in borate buffer (10 mM sodium borate, 1% SDS) and quantitated using the BCA protein assay kit from Pierce (Rockford, IL, USA).

Immunoblot Analysis

After treatment, RAW 264.7 macrophages were washed once in ice-cold PBS and scraped in ice-cold lysis buffer (50 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing the complete mini EDTA-free protease inhibitor cocktail from Roche. Protein concentration was determined using the BCA protein assay kit from Pierce. Cell lysates were mixed with 4X loading buffer, and 50 μg of total protein was separated using 7.5% SDS-PAGE. The separated proteins were transferred to PVDF membranes and blocked in 5% milk in TBS-0.1%T [100 mM Tris-HCL (pH 7.5), 150 mM NaCl, 0.1% Tween 20] for one hour at room temperature. Blots were probed with rat antimouse CD14 antibodies (1/500) or mouse β-actin antibodies (1/5000) in 5% milk TBS-0.1%T at 4°C overnight followed by three 10-min

washes in TBS-0.1% T at room temperature. Blots were then incubated with antirat or antimouse HRP antibodies (1/25,000) in 5% milk TBS-0.1%T for two hours at room temperature. After three 10-min washes in TBS-0.15% T, bands were detected by chemiluminescence using SuperSignal reagents from Pierce. After detection, blots were stripped by washing for 5 min in dH_20, 10 min in 0.2 M NaOH, and 5 min in dH_20.

RNA Isolation, Northern Blots, and Real-Time PCR

RNA was isolated from RAW 264.7 macrophages using Trizol reagent according to the manufacturer's instructions. For Northern blot analysis, 10 μg of total RNA was separated on a 1.2% agarose-formaldehyde gel in MOPS buffer and transferred to a positively charged nylon transfer membrane. Prior to probing, the transferred RNA was stained with methylene blue solution (0.03% methylene blue in 3 M sodium acetate) to visualize the 18 and 28 S rRNA. The CD14 probe was double labeled using [α-^{32}P] dCTP and [α-^{32}P] dATP (5 μCi each) with a random primer method as previously described (20). Blots were prehybridized at 42°C for 30 min in hybridization solution (50% formamide, 5X SSC, 5% SDS, 2.5X Denhardts, 0.2 mg/mL salmon sperm DNA, 1 mM EDTA, 20 mM $NaPO_4$, pH 6.5) and probed in hybridization solution at 42°C overnight. Blots were then washed three times in 6X SSC, 1% SDS at 42°C and developed using a Phosphorimager system (Molecular Dynamics, Amersham Pharmacia Biotech, Piscataway, NJ, USA).

For real-time PCR, RNA was reverse transcribed using the High-Capacity cDNA Archive Kit from Applied Biosystems (Foster City, CA, USA). The relative quantities of CD14, TLR4, and LDLR mRNA were quantified by real-time RT-PCR using primers and probes developed by Applied Biosystems (Taqman gene expression assays). The samples were each tested at least in duplicate and were normalized with GAPDH mRNA. Data were analyzed using the comparative C_T method and were confirmed by the standard curve method.

Immunostaining and Confocal Microscopy

RAW 264.7 macrophages were grown on sterile glass coverslips and treated for 16 hours with lovastatin (7.5 μM). Cells were then washed 1X in cold PBS and fixed for 20 min at –20°C in cold MeOH, followed by three washes in PBS. Nonspecific binding was blocked by incubating the coverslips in 10% goat serum for 30 min at room temperature, and CD14 was stained using an FITC-conjugated CD14 antibody or corresponding isotype control (Pharmingen) at a concentration of 1 μg/million cells for one hour at room temperature in 10% goat serum. Cells were washed three times in PBS, stained for 5 min at room temperature with 1 μg/mL DAPI, and again washed three times in PBS. Coverslips were mounted and cells were viewed using an Axiovert 200M microscope coupled to the Zeiss LSM 5 Pascal system. A Plan-Neofluar 40X/1.3 oil DIC objective was used with an optical section width of <1.0 μm.

Isolation of Detergent-Resistant Membranes

RAW 264.7 macrophages (8–10 million) were washed twice in cold PBS and lysed in 1 mL of ice-cold TNE buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA) containing 1% Triton X-100, 1 mM PMSF, and a mixture of protease inhibitors (Roche Molecular Biochemicals mini EDTA-free pellet) for 30 min on ice. Cell lysates were mixed with an equal volume of 85% sucrose in TNE and deposited in SW41Ti centrifuge tubes. The samples were overlaid with 4 mL of 35% sucrose in TNE, 1 mL of 5% sucrose in TNE, and 4.5 mL of TNE to fill the tube and then centrifuged for 18 hours at 39,000 rpm at 4°C. Then 3.5 mL of buffer was removed from the top, and 13 fractions (615 mL) were collected beginning at the top of the tube. Equal volumes of each fraction were mixed with 4X SDS-PAGE loading buffer and were analyzed by Western blot for CD14. To detect the GM1 raft marker, 10 μL aliquots of each fraction were spotted onto nitrocellulose and blocked for one hour at room temperature in 5% milk TBS-0.1% Tween 20. Blots were subsequently incubated at room temperature for two hours in 5% milk TBS-0.1% Tween 20 containing a 1/25,000 dilution of CTβ-HRP followed by extensive washing in TBS-0.15% Tween 20 and development with chemiluminescent reagents.

ELISA

The concentration of TNF-α in culture supernatants was measured using mouse TNF-α ELISA kits (Invitrogen, Carlsbad) according to the manufacturer's instructions. Culture supernatants were collected and frozen at –80°C until analysis.

RESULTS

Lovastatin Treatment Results in Increased CD14 Expression

RAW 264.7 macrophages were incubated with lovastatin (7.5 μM) for 16 hours at 37°C in media containing 10% FBS. Then, cells were lysed and CD14 levels were measured by Western blotting. As a result of lovastatin treatment, the protein levels of CD14 were increased about two-fold compared with untreated control cells (Figure 1A). To evaluate the specificity of lovastatin treatment, cells were coincubated with lovastatin and mevalonate (100 μM). Addition of mevalonate, which is the product of the reaction catalyzed by HMG-CoA reductase, to lovastatin-treated cells completely prevented the increase in CD14 levels. Treatment with mevalonate alone had no effect on CD14 expression (Figure 1A). This result indicated that the effect of lovastatin on CD14 expression is specific for inhibition of HMG-CoA reductase activity. The lovastatin-mediated increase of CD14 levels can also be abolished by the addition of farnesyl pyrophosphate (FPP, 10 μM), geranylgeranyl pyrophosphate (GGPP, 10 μM), or water-soluble cholesterol (cholesterol-PEG 600, 500 μM) to lovastatin-treated cells. Treatment of the cells with any of these intermediates in the absence of lovastatin had no affect on CD14 pro-

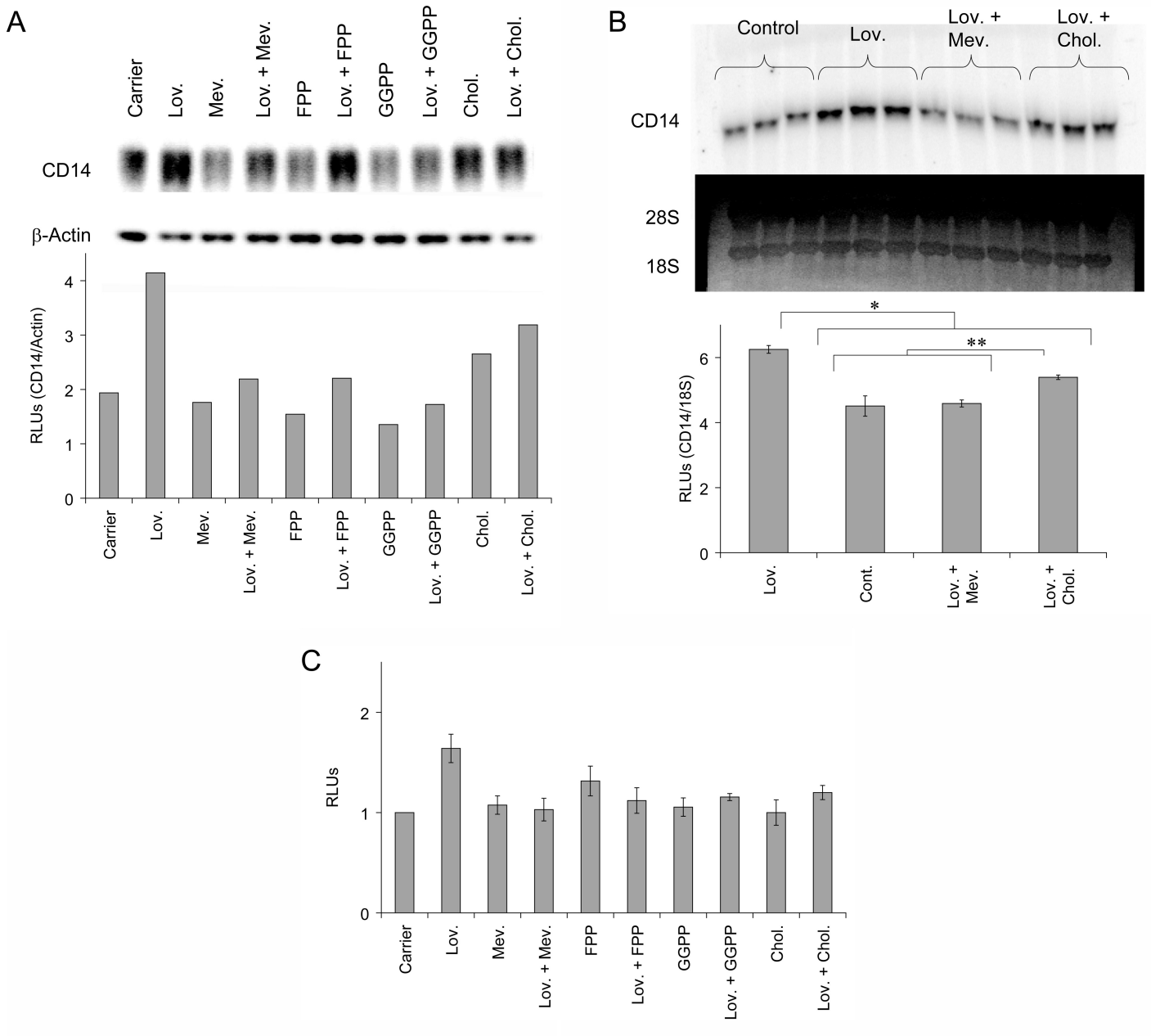

Figure 1. Lovastatin (Lov.) treatment results in increased CD14 expression. RAW 264.7 macrophages were treated with 7.5 μM lovastatin in the presence or absence of 100 μM mevalonate (Mev.), 10 μM FPP, 10 μM GGPP, or 500 μM cholesterol (Chol.) for 16 hours. Protein was isolated and CD14 levels were determined by Western blotting. After probing for CD14, the blot was stripped and reprobed for β-actin as a loading control. This blot is representative of two separate experiments (A). RAW 264.7 macrophages were treated with 7.5 μM lovastatin in the presence or absence of 100 μM mevalonate or 500 μM water-soluble cholesterol for 16 hours. RNA was isolated and CD14 levels were determined by Northern blot hybridization analysis. The membrane was stained with methylene blue and CD14 RNA levels were corrected by the 18 S ribosomal RNA band. Samples were run in triplicate for each treatment group, $^{*}P < 0.001$ (Lov. vs. control (Cont.), Lov. + Mev., Lov. + Chol.), $^{**}P < 0.01$ (Lov. + Chol. vs. Cont., Lov. + Mev.) by ANOVA/t-test (B). Macrophages were treated for 16 hours with 7.5 μM lovastatin in the presence or absence of 100 μM mevalonate, 10 μM FPP, 10 μM GGPP, or 500 μM cholesterol. RNA was isolated and reverse transcribed to cDNA. CD14 levels were quantified by real time PCR analysis using Taqman probes and primers developed by Applied Biosystems. Results were normalized to CD14 levels in carrier treated cells. CD14 levels were corrected by GAPDH levels. Results for each treatment group are the average of two separate samples (C).

tein levels, compared with untreated control cells (Figure 1A). In RAW 264.7 macrophages, treatment with various concentrations of pravastatin (0, 10, 50, 100 μM), a hydrophilic statin, unlike lovastatin, did not affect CD14 protein levels. To determine whether the effect of lovastatin on CD14 protein levels is due to an increase in gene expression, we measured CD14 mRNA levels. Macrophages were incubated with lovastatin, either alone or in combination with mevalonate or cholesterol. Total RNA was isolated from these treated cells. Northern blot/hybridization analysis (Figure 1B) revealed an increase in CD14 mRNA levels (1.4 fold) after lovastatin treatment. Coincubation with lovastatin and mevalonate (Figure 1B), however, did not increase CD14 mRNA levels. Coincubation with lovastatin and water-soluble cholesterol resulted in a reduction of CD14 mRNA compared with lovastatin-treated cells (Figure 1B). However, the addition of water-soluble cholesterol to lovastatin-treated cells did not decrease CD14 mRNA levels to those observed in control treated cells or cells coincubated with mevalonate and lovastatin (Figure 1B). These results were confirmed by real-time PCR analysis (Figure 1C). In addition, cells coincubated in lovastatin with GGPP or FPP did not show the increased CD14 mRNA levels observed in cells treated with lovastatin alone (Figure 1C).

CD14 is Present on the Cell Surface of Macrophages Treated with Lovastatin

We next examined the subcellular localization of CD14 in lovastatin-treated macrophages. RAW 264.7 macrophages were treated with lovastatin, and the presence of CD14 was visualized using a FITC-conjugated anti-CD14 antibody in permeabilized cells by confocal microscopy. CD14 was predominantly observed on the cell surface in both control and lovastatin-treated macrophages (Figure 2). Because CD14 has been found within lipid rafts, we investigated whether treatment with lovastatin affected the distribution of this glycoprotein within detergent-resistant membranes (DRMs). Macrophages were treated for 20 hours with lovastatin followed by isolation of DRMs by discontinuous sucrose density ultracentrifugation. The presence of DRMs was monitored by the presence of GM1 ganglioside. These membrane microdomains were not disrupted by lovastatin treatment. The localization of CD14 within the DRM fractions was not altered by lovastatin treatment, although higher levels of this glycoprotein were detected in DRM fractions (Figure 3).

Lovastatin Treatment Results in Increased Cellular CD14 and Decreased Soluble CD14 Protein Levels after LPS Stimulation

The soluble form of CD14, sCD14, plays a crucial role in the LPS response of cells lacking mCD14 (21–23). We investigated whether lovastatin treatment affected the expression of both mCD14 and sCD14 after stimulation with LPS. RAW 264.7 macrophages were treated with lovastatin, followed by incubation with LPS (100 ng/mL) for one to eight hours. LPS induced an increase in the expression of CD14 in untreated control cells (Figure 4). This LPS-induced increase in CD14 was enhanced in the presence of lovastatin (Figure 4). sCD14 levels were also elevated after incubation with LPS in untreated control cells. However, the release of sCD14 after LPS stimulation was reduced in the presence of lovastatin (Figure 4).

The effect of lovastatin and LPS on the expression of CD14 was also evaluated at the mRNA level by real-time PCR. Addition of LPS increased CD14 levels 3.3 fold with respect to nonstimulated control cells. Treatment with lovastatin alone also increased CD14 levels (1.5 fold). The combination of LPS and lovastatin did not show an additive effect of both reagents (Figure 5). Thus, CD14 mRNA levels after the combination of lovastatin and LPS were similar to LPS alone, indicating that the increase in CD14 protein levels following LPS stimulation in lovastatin-treated cells is likely regulated on the protein

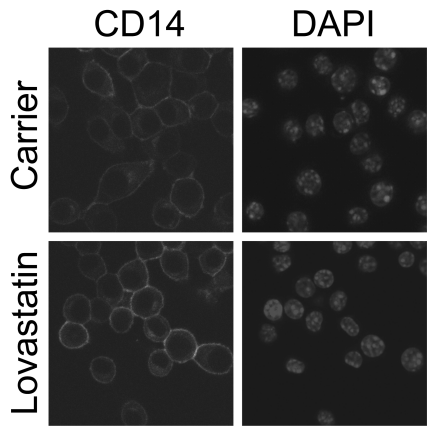

Figure 2. CD14 is located on the cell surface of lovastatin-treated macrophages. RAW 264.7 macrophages were treated with 7.5 μM lovastatin for 16 hours. Cells were fixed in methanol and stained with FITC-conjugated anti-CD14 antibodies and DAPI. An FITC-conjugated isotype control was used to assess nonspecific binding and did not result in any staining. Staining was visualized by confocal microscopy.

level. In addition, we investigated the effect of lovastatin and LPS on TLR4 mRNA levels by real-time PCR. TLR4 mRNA levels decreased slightly upon stimulation with LPS. An increase (1.9-fold) was observed after incubation with lovastatin. The combination of both lovastatin and LPS resulted in a decrease of TLR4 mRNA levels compared with lovastatin alone. TLR4 levels in cells treated with both lovastatin and LPS were higher than in cells treated with LPS alone, but not different from nonstimulated control cells (Figure 5).

Lovastatin Treatment of Macrophages Results in Elevated LPS-induced TNF-α Levels

We further investigated whether the increase in CD14 levels after lovastatin treatment resulted in an enhanced response of macrophages to LPS. Raw 264.7 macrophages were incubated with lovastatin (7.5 μM) for 16 hours prior to the addition of LPS (100 ng/mL) for an additional five hours. The extracellular medium was then collected and as-

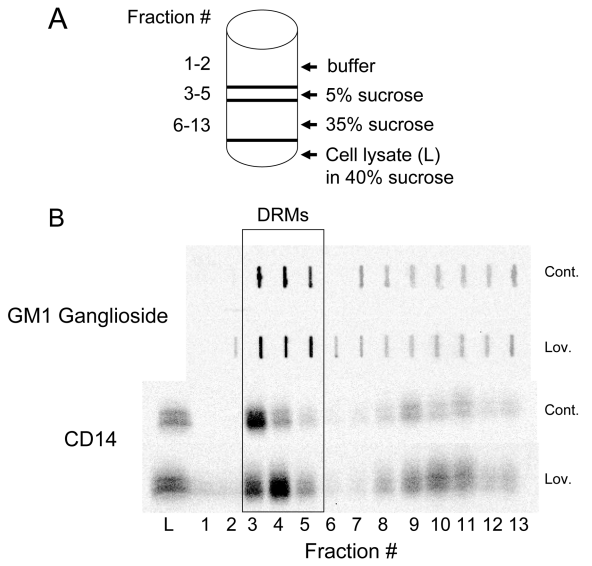

Figure 3. Localization of CD14 in detergent-resistant membranes (DRMs) is not disrupted by lovastatin treatment. RAW 264.7 macrophages were treated with 7.5 μM lovastatin for 20 hours followed by cell lysis at 4˚C in a buffer containing 1% Triton X-100. After one hour on ice, equal amounts of protein from each sample were subjected to discontinuous sucrose density ultracentrifugation to isolate DRMs (A). Samples were fractionated ,and equal amounts of each fraction were spotted onto nitrocellulose to detect GM1-ganglioside using HRP-conjugated cholera toxin β subunit (CTX-β-HRP). To detect CD14, equal amounts of each fraction were analyzed by Western blotting (B).

sayed for the presence of TNF-α. A 3.4 fold increase in TNF-α levels was observed in cells treated with lovastatin compared with untreated control cells (Figure 6). Addition of mevalonate to lovastatin-treated cells completely prevented the increased levels of LPS-induced TNF-α observed in the presence of lovastatin. Treatment with mevalonate alone had no effect on LPS-induced TNF-α levels compared with untreated control cells. In addition, co-

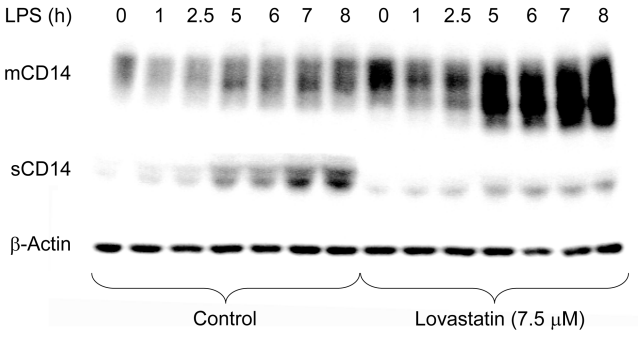

Figure 4. Lovastatin treatment increased mCD14 and decreased sCD14 after LPS stimulation. RAW 264.7 macrophages were treated with 7.5 μM lovastatin for 16 hours, then 100 ng/mL LPS was added for varying amounts of time (one to eight hours). At the appropriate time, supernatants and cellular protein were harvested and both mCD14 and sCD14 protein levels were analyzed by Western blotting. After probing for CD14, the cellular protein blot was stripped and reprobed for β-actin as a loading control.

incubation with water-soluble cholesterol (cholesterol-PEG 600, 500 μM) and lovastatin resulted in a reduction of LPS-induced TNF-α levels compared with lovastatin-treated cells (Figure 6). However, the addition of water-soluble cholesterol to lovastatin-treated cells did not reduce LPS-induced TNF-α to the levels observed in control treated cells, cells treated with mevalonate alone, or cells coincubated with mevalonate and lovastatin. Treatment with water-soluble cholesterol alone had no effect on LPS-induced TNF-α levels compared with untreated control cells. Coincubation of lovastatin-treated cells with FPP (10 μM) or GGPP (10 μM) also prevented the lovastatin-dependent increase in LPS-induced TNF-α levels (data not shown). The increase in LPS-induced TNF-α levels after lovastatin addition was reduced by incubation with interleukin-10 (IL-10), indicating that the antiinflammatory response of macrophages is not affected by the statin treatment (data not shown).

A Geranylgeranyl Transferase Inhibitor, GGTI-298, also Increases Expression of CD14

The isoprenoid intermediates FPP and GGPP are involved in the post-translational modification of more than 100 proteins, including members of the Ras, Rab, and Rho families (24). Modification of these proteins is critical for their biological function and is catalyzed by protein prenyltransferases. To further evaluate the role of the isoprenoid intermediates FPP and GGPP in the lovastatin-induced increase in CD14 expression, RAW 264.7 macrophages were treated with inhibitors of farnesyl transferase (FTI-277) and geranylgeranyl transferase (GGTI-298), and CD14 expression was evaluated by real-time PCR. Treatment with GGTI 298, but not FTI 277, led to an increase in CD14 mRNA levels comparable to the increase observed in lovastatin-treated cells (Figure 7A). We also evaluated the effect of these inhibitors on CD14 protein levels after stimulation with LPS by Western

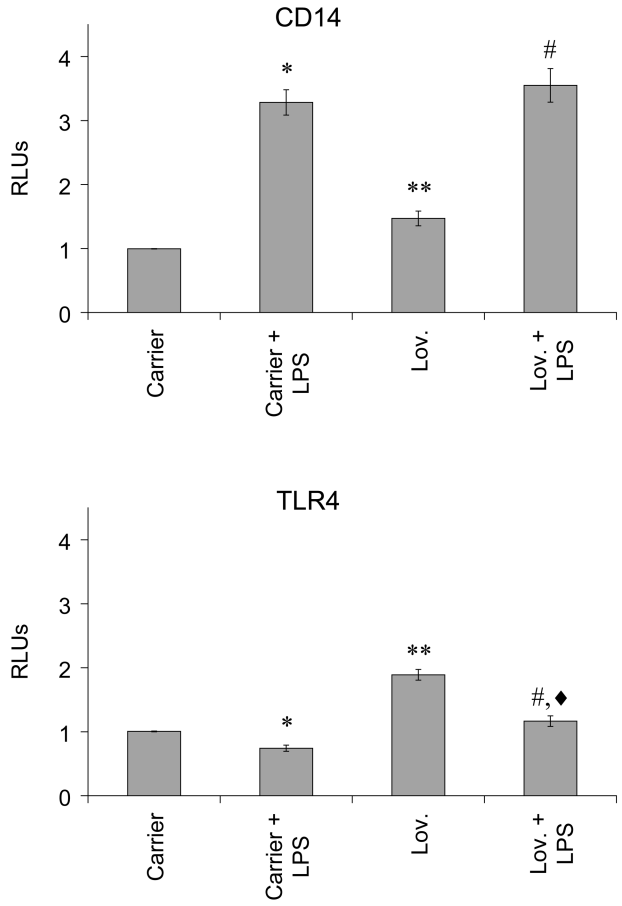

Figure 5. Lovastatin treatment did not additionally increase CD14 mRNA after LPS stimulation. RAW 264.7 macrophages were treated with 7.5 μM lovastatin for 16 hours followed by stimulation with 100 ng/mL LPS for an additional five hours. RNA was harvested and reverse transcribed to cDNA. CD14 and TLR4 levels were quantified by real-time PCR analysis using Taqman probes and primers developed by Applied Biosystems. Results were normalized to CD14 or TLR4 levels in carrier-treated cells. CD14 and TLR4 levels were corrected by GAPDH levels. Results for each treatment group are the average of five separate samples. For CD14, $*P < 0.001$ (Carrier compared with Carrier + LPS), $**P = .003$ (Carrier compared with Lov.), $\#P < 0.001$ (Lov. compared with Lov. + LPS). For TLR4, $*P < 0.001$ (Carrier compared with Carrier + LPS), $**P < 0.001$ (Carrier compared with Lov.), $\#P < 0.001$ (Lov. compared with Lov. + LPS), $\blacklozenge P < 0.01$ (Carrier + LPS compared with Lov. + LPS). Statistics were calculated by ANOVA/t-test.

blotting. Macrophages were treated with GGTI-298 (10 μM), followed by incubation with LPS (100 ng/mL) for 1 to 7.5 hours. Under these conditions, LPS also induced an increase in the expression of CD14 in untreated control cells (Figure 7B). This LPS-induced increase in CD14 was enhanced in the presence of GGTI-298 (Figure 7B). However, unlike lovastatin, GGTI-298 treatment did not inhibit the release of sCD14 following LPS stimulation compared with control treated cells (Figure 7B). Moreover, an inhibitor of Rho family GTPases, toxin B from *Clostridium difficile*, resulted in increased CD14 protein levels (both before and after LPS stimulation) without inhibition of sCD14 release (Figure 7C). Similar to the effect observed on the RNA level, treatment of macrophages with FTI-277 did not result in increased CD14 protein expression compared with control. Because GGTI 298 treatment resulted in increased CD14–expression, we evaluated the effect of this drug on LPS-induced TNF-α levels. Macrophages were treated with either GGTI 298 (10 μM) or FTI 277 (10 μM) for 16 hours followed by stimulation with LPS (100 ng/mL) for five hours. Supernatants were then harvested and TNF-α levels measured by ELISA. Treatment with GGTI 298, but not FTI 277 increased LPS-induced TNF-α levels (Figure 7D).

Lovastatin-Treated Peritoneal Macrophages Isolated from C57BL/6J CD14(–/–) Mice Do Not Exhibit Increased TNF-α Levels after LPS Stimulation

To investigate whether the increase in LPS-induced TNF-α levels after treatment with lovastatin depends on the presence of CD14, peritoneal macrophages derived from CD14(–/–) mice were used. Peritoneal macrophages were isolated from both C57BL/6J and C57BL/6J CD14(–/–) mice, and these cells were treated with lovastatin (10 μM) for 16 hours followed by stimulation with LPS (1 or 100 ng/mL) for an additional five hours, then TNF-α levels were measured in the extracellular medium. Peritoneal macrophages isolated from CD14(–/–) mice did not respond to a low LPS concentration (1 ng/mL), indicating that under these conditions the LPS response is dependent on the presence of CD14 (data not shown). Wild-type C57BL/6J peritoneal macrophages incubated with either 1 ng/mL or 100 ng/mL of LPS displayed the same pattern of increased TNF-α levels following lovastatin treatment observed in Raw 264.7 macrophages. In contrast, at a higher concentration of LPS (100 ng/mL), lovastatin-treated peritoneal macrophages isolated from CD14(–/–) mice did not exhibit the increased TNF-α levels observed in cells derived from wild-type mice. In fact, the levels of TNF-α from these cells were significantly decreased when incubated with lovastatin (Figure 8).

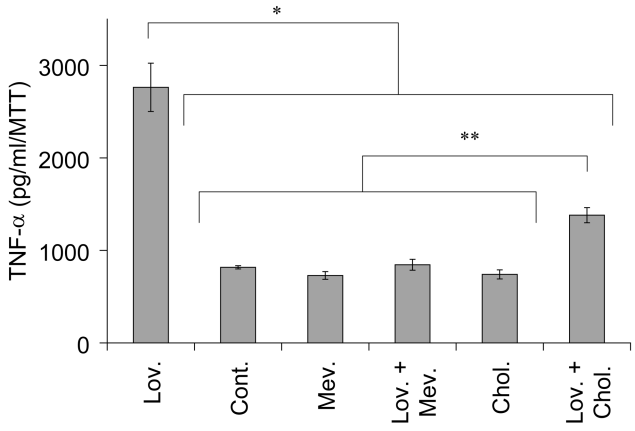

Figure 6. Lovastatin treatment results in increased levels of LPS-induced TNF-α. RAW 264.7 macrophages were treated with 7.5 μM lovastatin in the presence or absence of 100 μM mevalonate or 500 μM cholesterol for 16 hours, then 100 ng/mL LPS was added for an additional five hours. Supernatants were harvested and assayed for TNF-α by an ELISA. Cell viability and plating error were determined by the MTT cytotoxicity assay. Results are representative of three separate experiments, *$P < 0.01$ (Lov. vs. Cont., Mev., Lov. + Mev., Chol., Lov. + Chol.) and **$P < 0.01$ (Lov. + Chol. vs. Cont., Mev., Lov. + Mev., Chol.) by ANOVA/t-test.

Total Cellular Cholesterol Levels Are Not Significantly Affected by Incubation with Lovastatin

RAW 264.7 macrophages were treated with lovastatin (7.5 μM) for 16 hours and subsequently cholesterol was extracted from cells and quantitated. Lovastatin treatment did not result in a significant alteration of total cellular cholesterol levels as compared with untreated control cells (Table. 1).

CD14 Expression Does Not Appear to be Regulated by SREBPs

To determine if CD14 expression is under SREBP control, we treated macrophages under sterol depleting conditions (25,26). RAW 264.7 macrophages were incubated in 5% lipoprotein deficient serum (LPDS) containing 50 μM lovastatin and 50 μM mevalonate for 16 hours. After treatment, protein was isolated and CD14 expression was analyzed by Western blotting. As controls, cells were treated with lovastatin (7.5 μM) in medium containing 10% FBS. Under the sterol depleting conditions, CD14 protein levels were not elevated after treatment with lovastatin, which was in contrast to cells incubated with lovastatin in presence of FBS (Figure 9A). To test whether our treatment was indeed activating SREBPs, we evaluated the expression of LDL-receptor (LDLR) mRNA by real time PCR. Cells that were incubated in medium containing 5% LPDS for 16 hours had a small increase in LDLR mRNA levels as compared with cells incubated in medium containing 10% FBS. However, the sterol depleting conditions (5% LPDS, 50 μM lovastatin, and 50 μM mevalonate) resulted in an increase of LDLR mRNA levels, which were reduced upon coincubation with 25-hydroxycholesterol, an inhibitor of SREBP activation (27). CD14 mRNA levels did not increase significantly under the sterol depleting conditions, and the addition of 25-hydroxycholesterol did not alter mRNA levels (Figure 9B).

Increased CD14 Expression in Lovastatin-Treated Cells is Mediated by ERK

Because both extracellular signal-regulated kinase (ERK) and p38 have been previously shown to play a role in statin-mediated alterations in RAW 264.7 macrophages (28-31), we examined how inhibitors of these proteins affected the lovastatin-mediated increase in CD14 protein levels. RAW 264.7 macrophages were preincubated for 30 min with the MEK (activator of ERK) inhibitor U0126 (1 μM or 10 μM) or the p38 MAPK inhibitor SB203580 (3 μM or 10 μM) followed by addition of lovastatin (7.5 μM) for 16 hours, then LPS (100 ng/mL) was added and the mixture was incubated for five additional hours. At the lower concentration, the MEK inhibitor slightly reduced the increase in CD14 protein levels observed in the presence of lovastatin alone while at the higher concentration the lovastatin-induced increase was completely inhibited, indicating a role for ERK in the lovastatin-induced increase in CD14 expression. On the other hand, the presence of the p38 MAPK inhibitor did not alter the lovastatin-induced increase in CD14 protein levels (Figure 10).

DISCUSSION

Statins, which are widely used to decrease plasma cholesterol levels, also exhibit pleiotropic effects, including modulation of the inflammatory response (10). In particular, these drugs have been shown to reduce risks related to sepsis in both human clinical studies and murine experimental models (12-19). However, the relationship between statin treatment and the inflammatory response has not been completely elucidated. In this study, we investigated the effect of statins on the expression of CD14, the major binding site for LPS on macrophages. CD14 is present in two different forms: membrane bound (mCD14) and extracellular (sCD14). Our results indicate that treatment of macrophages with lovastatin increases mCD14 expression, which is the result of lovastatin-induced depletion of GGPP and subsequent inhibition of Rho GTPases. In contrast, the reduction in LPS-induced sCD14 levels following lovastatin treatment does not appear to be dependent on inactivation of Rho GTPases. This conclusion is supported by the use of the geranylgeranyl trans-

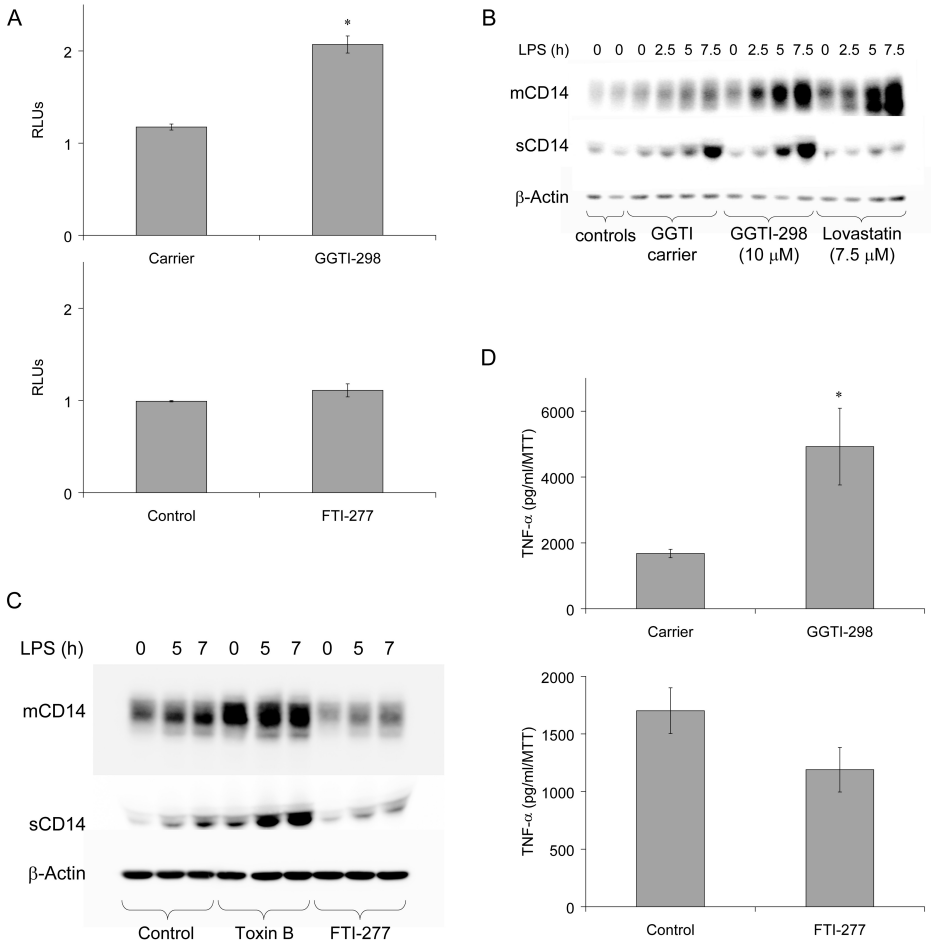

Figure 7. A geranylgeranyl transferase inhibitor, GGTI-298, results in increased CD14 expression and increased LPS-induced TNF-α levels. RAW 264.7 macrophages were treated with 10 μM FTI-277 or 10 μM GGTI-298 for 16 hours. RNA was isolated and reverse transcribed to cDNA. CD14 levels were quantified by real-time PCR analysis using Taqman probes and primers developed by Applied Biosystems. Results were normalized to CD14 levels in untreated control cells. CD14 levels were corrected by GAPDH mRNA levels. Results for each treatment group are the average of four separate samples, **P* < 0.001 (Carrier vs. GGTI-298) by t-test (A). RAW 264.7 macrophages were treated with either 10 μM GGTI-298 or 7.5 μM lovastatin for 16 hours, then 100 ng/mL LPS was added for varying amounts of time (2.5-7.5 hours). At the appropriate time, supernatants and cellular protein were harvested and both mCD14 and sCD14 protein levels were analyzed by Western blotting. After probing for CD14, the cellular protein blot was stripped and reprobed for β-actin as a loading control (B). RAW 264.7 macrophages were treated with 400 pM Toxin B or 10 μM FTI-277 for 16 hours, then 100 ng/mL LPS was added for varying amounts of time. At the appropriate time, supernantants and cellular protein were harvested and both mCD14 and sCD14 protein levels were analyzed by Western blotting. After probing for CD14, the blot was stripped and re-probed for β-actin as a loading control (C). RAW 264.7 macrophages were treated with 10 μM FTI-277 or 10 μM GGTI-298 for 16 hours, then 100 ng/mL LPS was added for an additional five hours. Supernatants were harvested and assayed for TNF-α by an ELISA. Cell viability and plating error were determined by the MTT cytotoxicity assay. Results are the average of three separate experiments, * *P* = .05 (Carrier vs. GGTI-298) by t-test (D).

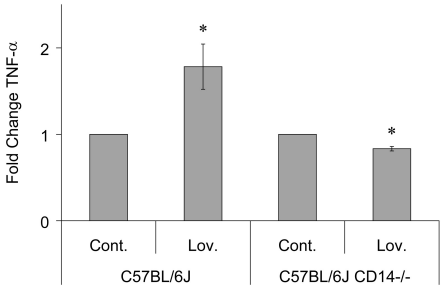

Figure 8. The lovastatin-induced increase in TNF-α after LPS stimulation was dependent on the presence of CD14. Peritoneal macrophages were isolated from C57BL/6J. and C57BL/6J. CD14(–/–) mice and treated with only 10 μM lovastatin for 16 hours, then either 1 ng/mL or 100 ng/mL LPS was added for an additional five hours. Supernatants were harvested and assayed for TNF-α by an ELISA. Cell viability and plating error were determined by the MTT cytotoxicity assay. Results for C57BL/6J. are the average of five separate experiments, **P* = .02 by t-test. Results of C57BL/6J. CD14(–/–) are the average of three separate experiments, **P* = .003 by t-test.

Table 1. Lovastatin Treatment Does Not Deplete Total Cellular Cholesterol.[a]

	Total cellular cholesterol (μg/mg protein)
Control	22.64 ± 2.99
Carrier	22.48 ± 1.20
Lovastatin (7.5 μm, 16 h)	21.84 ± 2.73

[a]RAW 264.7 macrophages were treated with 7.5 μM lovastatin for 16 hours followed by two 30-min extractions of cholesterol with hexane:isopropanol (3:2). Samples were dried under N_2 gas and resuspended in isopropanol. Cholesterol levels were measured using the Amplex Red Cholesterol Assay kit from Molecular Probes/Invitrogen. Protein remaining on the plate after extraction was solubilized, measured, and used to correct total cholesterol levels. Six samples were measured in each treatment group.

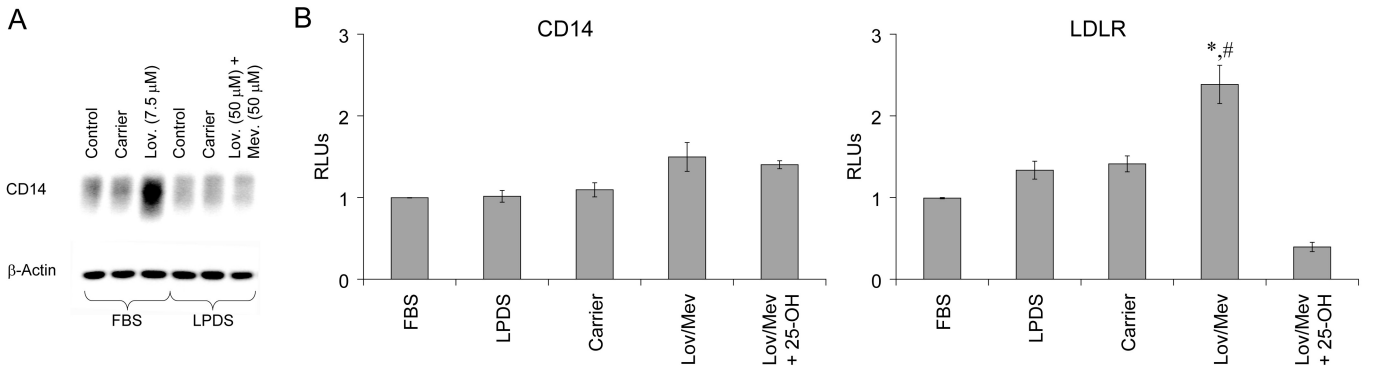

Figure 9. CD14 does not appear to be a SREBP target. RAW 264.7 macrophages were treated with either 7.5 μM lovastatin in medium containing 10 % FBS for 16 hours or 50 μM lovastatin and 50 μM mevalonate in medium containing 5% lipoprotein-deficient serum (LPDS) for 16 hours. Protein was isolated and CD14 levels were determined by Western blotting. After probing for CD14, the blot was stripped and re-probed for β-actin as a loading control (A). Macrophages were treated with 50 μM lovastatin and 50 μM mevalonate in 5% LPDS in the presence or absence of 1 μg/mL 25-hydroxycholesterol (25-OH-chol.) for 16 hours. RNA was isolated and reverse transcribed to cDNA. CD14 and LDLR levels were quantified by real time PCR analysis using Taqman probes and primers developed by Applied Biosystems. Results were normalized to CD14 or LDLR mRNA levels from cells grown in medium containing 10% FBS. CD14 and LDLR mRNA levels were corrected by GAPDH mRNA levels. Results for each treatment group are the average of four separate samples. For LDLR, $*P = .009$ (Carrier vs. Lov./Mev.) and $\#P < 0.001$ (Lov./Mev. vs. Lov./Mev. + 25-OH Chol.) by ANOVA/t-test (B).

ferase inhibitor GGTI-298 and the Rho GTPase inhibitor toxin B.

GGTI-298 treatment resulted in increased CD14 mRNA levels similar to those observed with lovastatin. Because geranylgeranylation is a common post-translational modification of many signal transduction molecules, including the Rho-family of small GTP binding proteins, it is possible that inhibition of lipid attachment to the signal transduction molecule may enhance CD14 expression. Moreover, the increase in mCD14 levels following lovastatin treatment was dependent on ERK MAPK, because an inhibitor of the upstream activator of ERK, MEK, inhibited the increase in CD14 protein levels observed with statin treatment alone. This observation is consistent with prior reports demonstrating that statin-induced alterations in RAW 264.7 macrophages were dependent on inhibition of small GTP binding proteins and subsequent activation of ERK MAPK and p38 MAPK (28–31). In our studies, however, an inhibitor of p38 MAPK did not alter the lovastatin-induced increase in CD14 protein levels, a result indicating that the increase in mCD14 levels in lovastatin-treated cells is mediated by Rho GTPases and ERK MAPK.

We observed that the lovastatin-induced increase in mCD14 protein levels after LPS stimulation parallels a decrease in sCD14. The mechanism for this decrease is unclear, but this observation suggests a potential defect in the processing of CD14. Although the mechanism of sCD14 release from cells is not completely understood, it has been reported to involve endocytosis of mCD14 (32). Thus, a decrease in endocytosis of mCD14 may reduce the release of sCD14. Depletion of cholesterol from the plasma membrane has been shown to have an effect on endocytosis, protein sorting, and signal transduction (33). For example, cholesterol perturbation by filipin led to redistribution of GPI-GFP from the cell surface to the Golgi complex (34), and depletion of cholesterol from the ER membrane resulted in impaired transport of secretory membrane proteins from the ER to the Golgi (35). In addition, lovastatin was found to enhance cell-surface expression and decrease endocytosis of another GPI-anchored protein, Ecto-5′-nucleotidase. This effect was reported to be dependent on decreased activity of Rho-GTPases, but independent of cholesterol (36). The decrease in sCD14 after LPS stimulation in lovastatin-treated cells that we report is not likely due to inhibition of Rho GTPase activity, because addition of GGTI-298 or toxin B, inhibitors of this activity, did not block sCD14 release.

The decreased release of SCD14 in lovastatin treated cells following LPS stimulation is an important finding in our study. High doses of sCD14 have been shown to play a role in neutralization of LPS (37), but low sCD14 concentrations (10–1000 ng/mL) are potentially harmful owing to delivery of LPS to endothelial cells (21). Increased sCD14 and decreased mCD14 in septic patients compared with healthy volunteers was recently reported (38), and another study revealed increased sCD14 released from peripheral blood mononuclear cells (PBMCs) from septic patients compared with healthy controls. These septic patients displayed potentially harmful sCD14 values of 10-20 ng/mL (39). Additionally, increased circulating levels of sCD14 have been associated with high mortality in Gram-negative septic shock (40). Therefore it is

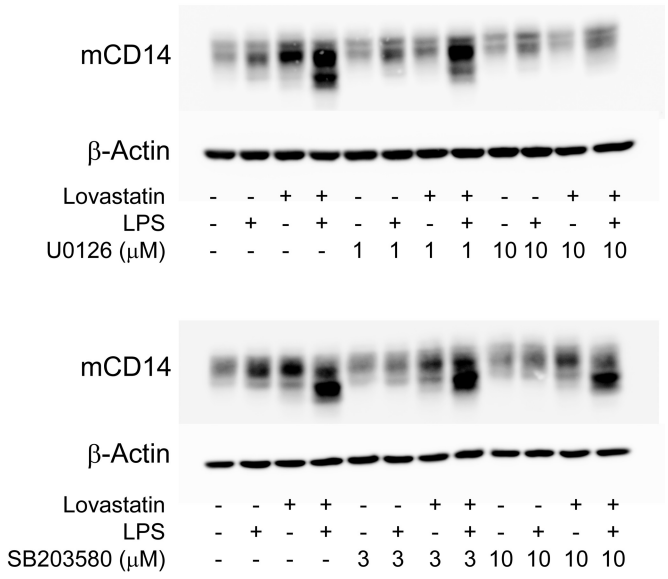

Figure 10. The increased expression of CD14 in lovastatin-treated cells is mediated by ERK, but not p38 MAPK. RAW 264.7 macrophages were pretreated for 30 min with either U0126 (1 μM or 10 μM), or SB203580 (3 μM or 10 μM), then lovastatin (7.5 μM) was added for an additional 16 hours. Cells were then treated with 100 ng/mL LPS for five hours and protein was harvested. mCD14 protein levels were analyzed by Western blotting. After probing for CD14, the cellular protein blot was stripped and reprobed for β-actin as a loading control.

possible that decreased levels of sCD14 contribute to the beneficial role of statins in septic patients.

Increased mCD14 expression observed after lovastatin incubation was strongly correlated with higher LPS-induced TNF-α levels after this treatment, a result confirmed by the response of peritoneal macrophages derived from CD14(–/–) mice. These cells did not display the stimulatory effect of lovastatin on LPS-induced TNF-α levels, indicating that the presence of CD14 is necessary for the lovastatin-induced increase in TNF-α levels after LPS stimulation.

The increase in LPS-induced TNF-α was prevented by coincubation of lovastatin-treated cells with mevalonate, indicating that the effect of lovastatin on TNF-α levels is at the level of HMG-CoA reductase inhibition. Coincubation of lovastatin-treated cells with FPP, GGPP, or water-soluble cholesterol also prevented the increase in LPS-induced TNF-α levels, suggesting that this effect may be regulated at multiple levels. A similar increase in LPS-induced TNF-α levels after lovastatin treatment in RAW 264.7 macrophages was previously reported (30). In addition, a number of other studies have indicated that statin treatment results in alterations in cytokine production. Both atorvastatin and simvastatin were shown to mediate the proinflammatory response in activated human PBMCs by activation of caspase-1 leading to IL-18 secretion in monocytes (41). Lipophilic statins have been reported to stimulate the production of MCP-1, IL-8, TNF-α, and IL-1β by human monocytes and to sensitize these cells to subsequent challenge with inflammatory agents (42). Mevastatin was shown to up-regulate TNF-α in microglia in cultured rat hippocampal slices (43). Simvastatin has been reported to enhance LPS-induced IL-12p40 production by RAW 264.7 macrophages by activation of the IL-12p40 promoter (44). Increased IL-1β and decreased IL-2 were detected in PBMCs treated with lovastatin and simvastatin, whereas decreased IL-1ra and IFNγ were detected in PBMCs treated with atorvastatin, lovastatin, and simvastatin (45). In contrast, other studies have shown the opposite effect. Lovastatin has been demonstrated to inhibit LPS-induced expression of TNF-α, IL-1β, and IL-6 in rat primary astrocytes, microglia, and macrophages (46). Lovastatin has also been shown to inhibit TNF-α release after LPS stimulation of THP-1 cells, a human monocytic line (47). Atorvastatin and simvastatin suppressed LPS-induced transcription of TNF-α, IL-6, and IL-12 and decreased expression of TLR4 in CD14+ monocytes from normocholesterolemic volunteers (48). Thioglycollate-elicited peritoneal macrophages from C57BL6 mice pretreated with simvastatin were shown to have decreased IL-6 mRNA after LPS stimulation, and this effect was dependent on peroxisome proliferator-activated receptor-α (49). These rather contradictory reports may be attributable to the diverse statins and cell systems that were used in these studies. For example, we have observed remarkable differences in the response of macrophages to hydrophobic (lovastatin) and hydrophilic (pravastatin) statins, observations that reflect the inability of hydrophilic statins to cross the plasma membrane in the absence of an active transport system (50).

The role of cholesterol depletion in altered CD14 expression after lovastatin treatment is unclear. We did not detect a change in total cellular cholesterol levels between control and lovastatin treated macrophages. Small changes in the cellular cholesterol pool, below the detection limit of our assay, may have contributed to the lovastatin-induced increase in mCD14 expression and/or the decreased release of sCD14. In fact, this increase in mCD14 expression was inhibited by coincubation of lovastatin-treated cells with water-soluble cholesterol. The expression of genes involved in cholesterol metabolism is regulated by the activation of SREBPs (8), but we were unable to show that CD14 is a SREBP target gene. Moreover, analysis of the CD14 upstream promoter region does not reveal any major

elements that could be regulated by lipid metabolism.

In summary, we observed a correlation between elevated expression of mCD14 and increased LPS-induced TNF-α levels after treatment with lovastatin. In addition, a parallel decrease in sCD14 after LPS stimulation was observed in lovastatin-treated cells. Because statins are widely used in our population and have been associated with antiinflammatory effects, the impact of these drugs on sepsis and related conditions of exaggerated inflammatory response is significant (51–55). The increase in LPS-induced TNF-α levels after lovastatin treatment that we observed is not necessarily detrimental. TNF-α is an important mediator of the innate immune response necessary for recovery after injury. It is the lack of TNF-α down-regulation as well as other inflammatory mediators that results in conditions such as sepsis. Our results do not argue against the concept that statin treatment is antiinflammatory, because the capacity of IL-10 to down-regulate TNF-α is not affected by the addition of lovastatin. Moreover, the decrease in sCD14 observed after lovastatin treatment may also be a part of the antiinflammatory effect.

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