Simvastatin regulates CXC chemokine formation in streptococcal M1 protein-induced neutrophil infiltration in the lung

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Simvastatin regulates CXC chemokine formation in streptococcal M1 protein-induced neutrophil infiltration in the lung. Am J Physiol Lung Cell Mol Physiol 300: L930–L939, 2011. First published March 25, 2011; doi:10.1152/ajplung.00422.2010.—Streptococcus pyogenes of the M1 serotype can cause streptococcal toxic shock syndrome and acute lung injury. Statins exert beneficial effects in septic patients although the mechanisms remain elusive. This study examined effects of simvastatin on M1 protein-provoked pulmonary inflammation and tissue injury. Male C57BL/6 mice were pretreated with simvastatin or a CXCR2 antagonist before M1 protein challenge. Bronchoalveolar fluid and lung tissue were harvested for determination of neutrophil infiltration, formation of edema, and CXC chemokines. Flow cytometry was used to determine Mac-1 expression on neutrophils. Gene expression of CXC chemokines was determined in alveolar macrophages by using quantitative RT-PCR. M1 protein challenge caused massive infiltration of neutrophils, edema formation, and production of CXC chemokines in the lung as well as upregulation of Mac-1 on circulating neutrophils. Simvastatin reduced M1 protein-induced infiltration of neutrophils and edema in the lung. In addition, M1 protein-induced Mac-1 expression on neutrophils was abolished by simvastatin. Furthermore, simvastatin markedly decreased pulmonary formation of CXC chemokines and gene expression of CXC chemokines in alveolar macrophages. Moreover, the CXCR2 antagonist reduced M1 protein-induced neutrophil expression of Mac-1 and accumulation of neutrophils as well as edema formation in the lung. These novel findings indicate that simvastatin is a powerful inhibitor of neutrophil infiltration in acute lung damage triggered by streptococcal M1 protein. The inhibitory effect of simvastatin on M1 protein-induced neutrophil recruitment appears related to reduced pulmonary generation of CXC chemokines. Thus, simvastatin may be a useful tool to ameliorate acute lung injury in streptococcal infections.

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Gram-negative bacteria dominated the microbial etiology of sepsis in the late 1970s and throughout the 1980s (10, 11, 31). However, the resurgence of Gram-positive bacterial infections has marked a dramatic change in the prevalence pattern of sepsis. Today, close to 50% of all cases of sepsis are caused by Gram-positive bacteria (5, 7, 31). One such example is septic shock caused by Streptococcus pyogenes, which is a severe condition associated with high mortality (9, 47). Although the pathophysiological mechanisms behind streptococcal toxic shock syndrome (STSS) remain elusive, convincing data have demonstrated that soluble streptococcal M protein of the M1 serotype is not only a potent activator of innate immunity, including activation of neutrophils (20) and monocytes (35), but also that the M1 serotype is predominantly linked to fatal STSS (18, 25). It is well recognized that the lung is the most sensitive and critical organ affected in STSS patients (51–53). In fact, acute lung injury continues to constitute a significant cause of morbidity and mortality in sepsis despite aggressive surgical interventions as well as antibiotic and immunomodulating therapies. In general, neutrophils are considered as the first line of defense against invading microorganisms. However, excessive accumulation of neutrophils is also considered to be a rate-limiting step in the pathophysiology of septic lung injury (37, 57). Bacterial antigens activate and upregulate cell adhesion molecules on neutrophils, such as Mac-1 (2). Tissue localization of neutrophils is coordinated by CXC chemokines, including macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC) (4, 27, 41, 43). CXCR2 (IL-8 receptor B) is the high-affinity receptor on murine neutrophils for MIP-2 and KC (21, 23, 24) and has been shown to regulate pulmonary accumulation of neutrophils in streptococcal infection (19). Thus, understanding the regulation M1 protein-induced inflammation and tissue damage in the lung may pave the way for more effective therapeutic strategies directed against STSS.

Besides antibiotics, current management of STSS is largely limited to supportive therapy. In this context, it is interesting to note that recent investigations have shown that statin intake may decrease hospital mortality and progression of bacterial infections in septic patients (55). In addition, recent data suggest that administration of statins increases survival in murine sepsis via improved cardiovascular functions (15, 33). Although statins are mainly used to regulate cholesterol synthesis in patients with increased risk of cardiovascular complications (8), the literature suggests that statins, such as simvastatin, may also exert anti-inflammatory effects, such as inhibition of cytokine formation, adhesion molecule expression, and reduction of nitric oxide production (17, 26, 49, 56), all of which could be of value in protecting against pathological inflammation in acute lung injury. A recent study reported that statins may also protect against lung infection by inhibiting bacterial invasion (40).

Based on the above considerations, the purpose of this study was to examine potential anti-inflammatory effects of simvastatin on M1 protein-induced neutrophil infiltration and tissue damage in the lung with special focus on adhesion molecule expression and CXC chemokines production.
MATERIALS AND METHODS

Animals. All experimental procedures were performed in accordance with the legislation on the protection of animals and were approved by the Regional Ethical Committee for Animal Experimentation at Lund University, Sweden. Male C57BL/6 mice weighing 20–25 g were housed on a 12:12-h light-dark cycle and fed a laboratory diet and water ad libitum. The mice were anesthetized with 75 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per kilogram body weight.

Experimental model of sepsis. M1 protein was purified from the isogenic mutant MC25 strain (derived from the AP1 S. pyogenes strain 40/58 from the WHO Collaborating Centre for references and Research on Streptococci, Institute of Hygiene and Epidemiology, Prague, Czech Republic) as described previously (20). Because the M1 protein samples were produced in a mutant S. pyogenes strain, this makes it unlikely to contain endotoxin. This was confirmed by the failure of the M1 protein samples to activate TLR4-transfected cells in separate experiments. Moreover, when the M1 protein samples were analyzed by gas chromatography, the content of endotoxin was below the detection limit (data not shown). Mice were intravenously injected with 15 μg of M1 protein in PBS. Sham mice received PBS intravenously only. Simvastatin (0.5 or 10 mg/kg; Sigma-Aldrich, Stockholm, Sweden) or the CXCR2 antagonist (SB-225002, 4 mg/kg; Calbiochem, Merck, Darmstadt, Germany) was administered intra-peritoneally 10 min before M1 protein challenge. Animals were reanesthetized 4 h after M1 protein challenge. The left lung was ligated and excised for edema measurement. The right lung was used for collecting bronchoalveolar lavage fluid (BALF) to quantify neutrophils. Next, the lung was excised, and one lobe was fixed in formaldehyde for histology, and the remaining lung tissue was snap-frozen in liquid nitrogen and stored at −80°C for later myeloperoxidase (MPO) assays and enzyme-linked immunosorbent assay (ELISA) as described subsequently.

Systemic leukocyte count. Blood was collected from the tail vein and mixed with Turks solution (0.2 mg gentian violet in 1 ml glacial acetic acid, 6.25% vol/vol) in a 1:20 dilution. Leukocytes were identified as monophonuclear (MLNs) and polymorphonuclear (PMNLs) cells in a Burker chamber.

Lung edema. The left lung was excised, washed in PBS, gently dried using a blotting paper, and weighed. The tissue was then dried at 60°C for 72 h and reweighed. The change in the ratio of the wet weight-to-dry weight was used as an indicator of lung edema formation.

MPO activity. Lung tissue was thawed and homogenized in 1 ml of 0.5% hexadecyltrimethylammonium bromide. Samples were freeze-thawed, after which the MPO activity of the supernatant was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H₂O₂ (450 nm, with a reference filter 540 nm, 25°C) as previously described (2). Values were expressed as MPO units per gram tissue.

ELISA. Levels of MIP-2 and KC in lung homogenates were analyzed by using double-antibody Quantikine ELISA kits (R & D Systems, Abingdon, Oxon, UK) using recombinant murine MIP-2 and KC as standards. The lower limit of the assay was 0.5 pg/ml.

Flow cytometry. For analysis of surface molecules expression on circulating neutrophils, blood was collected (1:10 acid citrate dextrose) 4 h after M1 protein challenge and incubated (10 min, RT) with an anti-CD16/CD32 antibody blocking FcγRIII/II receptors to reduce nonspecific labeling and then incubated with PE-conjugated anti-Gr-1 (clone RB6–8C5, rat IgG2b; eBioscience, San Diego, CA) and fluorescein isothiocyanate (FITC)-conjugated anti-Mac-1 (clone M1/70, integrin αM, China, rat IgG3b). The mean fluorescence intensity was determined by comparisons with appropriate isotype control (FITC-conjugated rat IgG2b). All antibodies were purchased from BD Biosciences Pharmingen (San Jose, CA) except where indicated. Cells were fixed, erythrocytes were lysed by BD lysis buffer, and then neutrophils were recovered following centrifugation. Flow-cytometric analysis was performed by first gating the neutrophil population of cells based on forward and side scatter characteristics, and then Mac-1 expression was determined on Gr-1− in these gated cells on a FACSCalibur flow cytometer (Becton-Dickinson, Mountain View, CA). A viable gate was used to exclude dead and fragmented cells.

Histology. Lung samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin-embedded. Sections (6 μm) were stained with hematoxylin and eosin. Lung injury was quantified in a blinded manner by adoption of a preexisting scoring system as described (6), including size of alveolar spaces, thickness of alveolar septa, alveolar fibrin deposition, and neutrophil infiltration graded on a zero (absent) to four (extensive) scale.

In vitro activation of neutrophils. Neutrophils were freshly isolated from healthy mice by aseptically flushing the bone marrow of femurs and tibias with complete culture medium RPMI 1640 and then subsequently isolated by using Ficoll-Paque Research Grade (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of the isolated neutrophils was higher than 70% as assessed in a hemacytometer. Isolated neutrophils were resuspended in culture medium until use. Blood was collected from healthy animals containing 1:10 acid citrate-dextrose. Whole blood and isolated neutrophils were incubated with M1 protein (1 μg/ml) and simvastatin (1 μM; Sigma-Aldrich) or CXCR2 antagonist (SB-225002, 500 nM; Calbiochem, Merck) at 37°C for 20 min. The simvastatin prodrug was activated as described previously (27). Cells were stained for flow cytometric analysis of Mac-1 expression on neutrophils (Gr-1−) as described above.

Isolation of alveolar macrophages. Alveolar macrophages were isolated from bronchoalveolar lavage as described in detail (59). Briefly, lungs were flushed three times with 1 ml of PBS supplemented with 0.5 mM EDTA. Alveolar fluids were then centrifuged at 400 g for 10 min at 4°C. The cells were then resuspended in RPMI 1640 complete culture medium and incubated at 37°C, 5% CO₂ in a 48-well plate. After 2 h, nonadherent cells were washed away by PBS. A total of ~2–3 × 10⁵ macrophages were obtained per mouse.

Quantitative RT-PCR. Total RNA was isolated from the alveolar macrophages using an RNeasy Mini Kit (Qiagen, West Sussex, UK) following the manufacturer’s protocol and treated with RNAse-free DNase (DNase I; Amersham Pharmacia Biotech, Solna, Sweden) to remove potential genomic DNA contaminants. RNA concentrations were determined by measuring the absorbance at 260 nm spectrophotometrically. Each cDNA was synthesized by reverse transcription from 10 μg of total RNA using the Stratascript First-Strand Synthesis System and random hexamer primers (Stratagene; AH diagnostics, Stockholm, Sweden). Real-time PCR was performed using a Brilliant SYBRgreen QPCR master mix and MX 3000P detection system (Stratagene). The primer sequences of MIP-2, KC, and β-actin were as follows: MIP-2 (forward) 5′-GCT TCC TCG GGC ACT CCA GAC-3′, MIP-2 (reverse) 5′-TTA GCC TTG CTT TGT AGT AT-3′, KC (forward) 5′-GCA AAT GAG CTG CGC TGT CAA TGC-3′, KC (reverse) 5′-CTT GGG GAC ACC TTT TAG CAT CTT-3′; and β-actin (forward) 5′-ATG TTT GAG ACC TTC AAC ACC-3′, β-actin (reverse) 5′-TCT CCA GGG AGG AAG AGG AT-3′. Standard PCR curves were generated for each PCR product to establish linearity of the RT-PCR reaction. PCR amplifications were performed in a total volume of 50 μl, containing 25 μl of SYBRgreen PCR 2× master mix, 2 μl of 0.15 μM each primer, 0.75 μl of reaction buffer, and one 1-μl cDNA as a template adjusted up to 50 μl with water. PCR reactions were started with 10 min denaturing temperature of 95°C, followed by a total of 40 cycles (95°C for 30 s and 55°C for 1 min), and 1 min of elongation at 72°C. The relative differences in expression between groups were expressed by using cycling time values. Cycling time values for the specific target genes were first normalized with that of β-actin in the same sample, and then relative differences between groups were expressed as a percentage of control.
RESULTS

Neutrophil recruitment and lung injury. Challenge with M1 protein increased pulmonary levels of MPO by $>12$-fold ($P < 0.05$ vs. sham, $n = 5$; Fig. 1A). Simvastatin treatment markedly decreased M1 protein-induced MPO activity in the lung ($P < 0.05$ vs. vehicle + M1 protein, $n = 5$; Fig. 1A). Microscopic analysis of BALF revealed that M1 protein enhanced the number of neutrophils in the alveolar compartment by more than sixfold ($P < 0.05$ vs. sham, $n = 5$; Fig. 1B). Administration of simvastatin significantly reduced BALF neutrophils ($P < 0.05$ vs. vehicle + M1 protein, $n = 5$; Fig. 1B). M1 protein challenge increased the lung wet-to-dry ratio, indicating induction of lung edema ($P < 0.05$ vs. sham, $n = 5$; Fig. 1C). Simvastatin also reduced the wet-to-dry ratio in M1 protein-treated mice ($P < 0.05$ vs. vehicle + M1 protein, $n = 5$; Fig. 1C). In addition, morphological examination revealed normal pulmonary microarchitecture in PBS-treated animals (Fig. 2A), whereas challenge with M1 protein caused severe destruction of the lung tissue characterized by extensive edema in the interstitial tissue, capillary congestion, necrosis, and massive infiltration of neutrophils (Fig. 2B). Treatment with simvastatin protected against M1 protein-induced destruction of tissue architecture, cellular damage, and neutrophil accumulation in the lung (Fig. 2, C and D). Quantification of the morphological lung damage revealed that M1 protein increased the lung injury score and that simvastatin significantly decreased the lung injury score in animals exposed to M1 protein (Fig. 2E). Challenge with M1 protein decreased both PMNL and MNL (Table 1). This M1 protein-induced neutropenia was significantly inhibited by administration of 10 mg/kg of simvastatin ($P < 0.05$ vs. vehicle + M1 protein, $n = 5$; Table 1).

Mac-1 expression. M1 protein challenge caused a distinct upregulation of Mac-1 on neutrophils compared with PBS-treated mice ($P < 0.05$ vs. sham, $n = 6$; Fig. 3, A and B). Simvastatin markedly antagonized M1 protein-induced expression of Mac-1 on the surface of neutrophils ($P < 0.05$ vs. vehicle + M1 protein, $n = 5$; Fig. 3B). We next asked whether this inhibitory effect of simvastatin was a direct effect on neutrophils. First, we stimulated isolated neutrophils with M1 protein in vitro and found no increase in Mac-1 expression (Fig. 3, C and D). Considering a previous study reporting that M1 protein binding to neutrophils may require the presence of fibrinogen (58), we repeated the experiments by use of whole blood. Indeed, M1 protein challenge increased Mac-1 expression on neutrophils in whole blood ($P < 0.05$ vs. sham, $n = 5$; Fig. 3, E and F). However, coincubation of simvastatin had no effect on M1 protein-provoked neutrophil expression of Mac-1 in whole blood, suggesting that the inhibitory effect of simvastatin on neutrophil recruitment to the lung is not due to direct inhibition of Mac-1 expression.

CXC chemokines in the lung. Baseline levels of CXC chemokines in the lungs of PBS-treated animals were low but detectable ($n = 5$; Fig. 4A). In contrast, challenge with M1 protein enhanced MIP-2 and KC levels in the lung by 56-fold and 30-fold, respectively ($P < 0.05$ vs. sham, $n = 5$; Fig. 4A). Administration of simvastatin (10 mg/kg) significantly decreased M1 protein-provoked production of MIP-2 and KC ($P < 0.05$ vs. vehicle + M1 protein, $n = 5$; Fig. 4A). We next isolated alveolar macrophages from the BALF in animals challenged with M1 protein and/or simvastatin. It was found that simvastatin markedly decreased mRNA levels of MIP-2.
and KC in the alveolar macrophages in M1 protein-treated animals ($P < 0.05$ vs. vehicle + M1 protein, $n = 5$; Fig. 4B).

**Role of CXCR2 in M1 protein-induced neutrophil infiltration and lung injury.** Although we established that CXC chemokines formation in the lung is a major target of simvastatin in M1 protein-provoked lung injury, it is not known whether CXC chemokines actually play a functional role in pulmonary recruitment of neutrophils triggered by M1 protein. To examine this, we used a CXCR2 antagonist (SB-225002) that blocks the function of both MIP-2 and KC. Administration of SB-225002 markedly decreased M1 protein-induced MPO activity and BALF neutrophils as well as edema formation in the lung ($P < 0.05$ vs. sham, $n = 5$; Fig. 5). Moreover, the CXCR2 antagonist markedly decreased M1 protein-induced expression of Mac-1 on the surface of neutrophils in septic animals ($P < 0.05$ vs. vehicle + M1 protein, $n = 5$; Fig. 6A). As shown above, stimulation of isolated neutrophils with M1 protein had no effect on Mac-1 expression ($n = 5$; Fig. 6B). In contrast, M1 protein challenge increased Mac-1 expression on neutrophils in whole blood ($P < 0.05$ vs. sham, $n = 5$; Fig. 6B). Coincubation with the CXCR2 antagonist did not influence M1 protein-induced Mac-1 expression on neutrophils in whole blood. Morphological examination with the lung injury score showed that administration of SB-225002 protected against M1 protein-induced destruction of tissue architecture, cellular damage, and neutrophil accumulation in the lung (Fig. 6D). Quantification of the histology samples revealed that SB-225002 significantly decreased the lung injury score in M1 protein-treated animals ($P < 0.05$ vs. vehicle + M1 protein, $n = 5$; Fig. 6E). In contrast, treatment with the CXCR2 antagonist had no effect.
of M1 protein-induced production of CXC chemokines in the lung (Fig. 6F).

**DISCUSSION**

Severe streptococcal infections, such as STSS, are frequently associated with *S. pyogenes* of the M1 serotype. Our present data show that administration of simvastatin can ameliorate acute lung injury induced by streptococcal M1 protein. The beneficial effect of simvastatin was due to reduced neutrophil accumulation in the lung. We found that simvastatin antagonized pulmonary infiltration of neutrophils via decreased formation of CXC chemokines in the lung. This reduction in CXC chemokines formation is a significant target of simvastatin considering our findings that inhibition of CXCR2 ameliorated M1 protein-induced neutrophil accumulation and tissue damage in the lung. Thus, these novel findings not only suggest that simvastatin may be useful in streptococcal infections but also reveal detailed anti-inflammatory mechanisms exerted by simvastatin in acute lung injury.

Severe streptococcal infections cause widespread activation of the host immune system. In streptococcal infections, M1 protein is shed from the surface of *S. pyogenes*, and convincing data have shown that M1 protein is a potent activator of innate and adaptive immunity, including neutrophils, monocytes, platelets, and T cells (20, 35, 36, 45). Acute lung injury is a critical component in STSS, and pulmonary infiltration of neutrophils is considered to be a rate-limiting step in septic lung injury (37, 46, 58). Recent data indicate that intravascular activation of neutrophils is a dominating feature in the onset of the M1 protein-provoked lung injury (16, 46, 58). In this context, it is interesting to note that an emerging body of literature suggests that statins

### Table 1. Systemic leukocyte differential counts

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<th>MNL</th>
<th>PMNL</th>
<th>Total</th>
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<tr>
<td>Sham</td>
<td>5.8 (5.2–6.1)</td>
<td>2.2 (2.2–2.7)</td>
<td>7.8 (7.5–8.5)</td>
</tr>
<tr>
<td>Vehicle + M1 protein</td>
<td>0.6 (0.6–0.8)*</td>
<td>0.4 (0.2–0.5)*</td>
<td>1.0 (1.0–1.3)*</td>
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<tr>
<td>Simvastatin (0.5 mg/kg) + M1 protein</td>
<td>2.8 (2.5–3.5)§</td>
<td>1.4 (1.2–1.7)§</td>
<td>4.2 (3.7–4.7)§</td>
</tr>
<tr>
<td>Simvastatin (10 mg/kg) + M1 protein</td>
<td>2.8 (2.5–3.1)§</td>
<td>1.2 (1.0–1.3)§</td>
<td>4.0 (3.5–4.8)§</td>
</tr>
<tr>
<td>SB-225002 + M1 protein</td>
<td>2.8 (2.6–2.9)§</td>
<td>0.8 (0.6–0.9)§</td>
<td>3.6 (3.2–3.8)§</td>
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Data are presented as medians (25–75 percentile), 10⁶ cells/ml and n = 5 experiments. Blood was collected from sham animals receiving PBS iv only as well as mice treated with simvastatin (0.5 or 10 mg/kg), SB-225002 (4 mg/kg), or vehicle before challenge with M1 protein for 4 h. Cells were identified as monomorphonuclear leukocytes (MNL) and polymorphonuclear leukocytes (PMNL). *P < 0.05 vs. sham and §P < 0.05 vs. vehicle + M1 protein.

Fig. 3. Simvastatin regulates M1 protein-induced Mac-1 expression on neutrophils. Mac-1 expression on neutrophils in vehicle- and simvastatin (0.5 or 10 mg/kg)-treated animals 4 h after M1 protein injection (A and B), isolated neutrophils incubated with M1 protein (1 μg/ml) and vehicle or simvastatin (1 μM) (C and D), and whole blood incubated with M1 protein (1 μg/ml) and vehicle or simvastatin (1 μM) (E and F). ctrl, Control; MFI, mean fluorescence intensity. Fluorescence intensity is shown on the x-axis and cell counts on the y-axis. Representative histogram from four samples. *P < 0.05 vs. sham and §P < 0.05 vs. vehicle + M1 protein.
exert powerful and pleiotropic anti-inflammatory effect besides their well-known potency to decrease levels of cholesterol (32). In the present study, we demonstrate that simvastatin attenuates pulmonary edema and tissue damage in response to M1 protein-induced acute lung damage. This finding is in line with recent investigations reporting that

Fig. 4. Simvastatin decreases CXC chemokines production in the lung and gene expression of CXC chemokines in alveolar macrophages. Animals were treated with simvastatin (0.5 or 10 mg/kg) or vehicle before M1 protein injection. Mice treated with PBS served as sham animals. A: enzyme-linked immunosorbent assay (ELISA) was used to quantify the levels of macrophage inflammatory protein-1 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC) in the lung of mice 4 h after M1 protein challenge. B: RT-PCR was used to determine the levels of mRNA expression of MIP-2 and KC in alveolar macrophages. Data are presented as medians (25–75 percentile). *P < 0.05 vs. sham and §P < 0.05 vs. vehicle + M1 protein, n = 5.

Fig. 5. Effect of CXCR2 antagonist in M1 protein-induced pulmonary infiltration of neutrophils. A–C: lung MPO levels (A), no. of BALF neutrophils (B), and edema formation (C) in the lung 4 h after M1 protein injection. Animals were treated with SB-225002 (4 mg/kg), which is a specific CXCR2 antagonist or vehicle, before M1 protein injection. Mice treated with PBS served as sham animals. Data are presented as medians (25–75 percentile). *P < 0.05 vs. sham and §P < 0.05 vs. vehicle + M1 protein, n = 5.
Simvastatin decreases vascular leakage in an endotoxin model of lung injury in mice (1, 22). In the present study, it was observed that simvastatin significantly reduced the lung activity of MPO, a marker of neutrophil recruitment, in M1 protein-treated animals. Simvastatin decreased the number of neutrophils in the bronchoalveolar space in mice challenged with M1 protein. Taken together, these findings suggest that simvastatin is a potent inhibitor of pulmonary infiltration of neutrophils in M1 protein-induced acute lung injury. In fact, this is the first study showing that simvastatin may interfere with neutrophil accumulation in the lung in streptococcal infections. Considering the intimate relationship between neutrophil infiltration and tissue injury, it may be suggested that part of the lung protective effect of simvastatin may be related to inhibition of pulmonary recruitment of neutrophils. It should be noted that this study...
was focused on the protective mechanisms of simvastatin and used a pretreatment design, which limits potential conclusions regarding the role of simvastatin in the treatment of sepsis.

It is widely held that neutrophil extravasation in the lung is a multistep process, including sequestration in the microcirculation and stationary adhesion on the vascular endothelium, followed by transendothelial and transmural migration (2, 39, 48). Leukocyte-endothelial interactions are mediated by specific adhesion molecules. For example, early interactions are supported by the selectin family of adhesion molecules and their ligands, including P-selectin glycoprotein ligand-1 (2, 30, 38, 50). Firm adhesion and transmigration are supported by $\beta_2$-integrins, such as Mac-1, in septic lung injury (3). Interestingly, a recent study reported that administration of the tetrapeptide Gly-Pro-Arg-Pro attenuates M1 protein-induced lung damage, suggesting that $\beta_2$-integrins may be useful targets in severe streptococcal infections (20). It was therefore of great interest to examine Mac-1 expression on neutrophils in the present study. Thus, we asked whether simvastatin might regulate neutrophil expression of Mac-1. We found that Mac-1 was upregulated on neutrophils in response to systemic challenge with M1 protein. Moreover, simvastatin treatment markedly reduced neutrophil expression of Mac-1 in mice exposed to M1 protein. Knowing that Mac-1 is an important adhesion molecule facilitating pulmonary accumulation of neutrophils in septic lung injury (3), it may be suggested that this downregulation of Mac-1 on the surface of neutrophils may help to explain the inhibitory effect of simvastatin on neutrophil recruitment and tissue injury in streptococcal M1 protein-induced lung damage. We next asked whether Mac-1 downregulation might be a direct effect of simvastatin on M1 protein-induced neutrophil activation. We found that isolated neutrophils did not increase expression of Mac-1 in response to M1 protein challenge. This lack of response in isolated neutrophils may be related to a previous study reporting that M1 protein binding to neutrophils appears to be dependent on the presence of fibrinogen (16). Indeed, this notion is supported by our data showing that M1 protein increased Mac-1 expression on neutrophils when using whole blood. However, simvastatin had no significant impact on M1 protein-induced neutrophil expression of Mac-1 in whole blood, suggesting that the inhibitory effect of simvastatin on neutrophil expression of Mac-1 is an indirect effect rather than a direct effect on neutrophils.

Activation and extravascular navigation of inflammatory cells at sites of inflammation is orchestrated by secreted chemokines (12, 28, 44). In particular, CXC chemokines, such as MIP-2 and KC, are known to activate and trigger chemotactic movement of neutrophils (13, 42). It has been reported that CXC chemokines production is increased in streptococcal infections (14, 54), and one recent study showed that M1 protein can increase MIP-2 and KC in the lung (58). We could confirm that M1 protein greatly increased formation of CXC chemokines formation in the lung in the present study. Notably, it was found that simvastatin markedly reduced M1 protein-provoked pulmonary production of CXC chemokines. Thus, this is the first study to show that simvastatin negatively regulates formation of CXC chemokines induced by M1 protein challenge. Interestingly, simvastatin markedly decreased gene expression of MIP-2 and KC in alveolar macrophages, suggesting that macrophage production of CXC chemokines may be an important target in the protective effect of simvastatin. Thus, inhibition of CXC chemokines production may be a direct target of simvastatin in reducing neutrophil activation (Mac-1 upregulation) and infiltration in the lung in response to M1 protein challenge. In this context, it should be mentioned that it is not known whether CXC chemokines play any functional role in M1 protein-induced inflammation and lung injury. Therefore, we explored if interference with CXCR2, which is the high-affinity receptor of MIP-2 and KC, can modulate proinflammatory changes induced by M1. We observed that CXCR2 inhibition markedly decreased M1 protein-provoked neutrophil activation (Mac-1 expression) and infiltration as well as edema formation and tissue damage in the lung in vivo, suggesting that CXC chemokines play an important role in lung inflammation and injury triggered by M1 protein. In contrast, it was found that the CXCR2 antagonist had no effect on M1 protein-induced Mac-1 expression on neutrophils in vitro. Considered together, CXC chemokines production in the lung is a functional and dominating target of simvastatin in acute lung injury caused by streptococcal M1 protein.

In conclusion, these novel findings demonstrate that simvastatin effectively protects against acute lung damage induced by M1 protein. The protective effect of simvastatin seems related to interference of neutrophil activation and recruitment to the lung. More specifically, our findings indicate that the anti-inflammatory mechanisms of simvastatin are due to attenuated formation of CXC chemokines in the lung. Thus, the effects and mechanisms described herein may not only help to understand the protective effects of statins in sepsis but also provide a new way to ameliorate acute lung injury in severe infections caused by S. pyogenes.

REFERENCES

SIMVASTATIN AND STREPTOCOCCAL INFECTION


