Successful Treatment of Acute Lung Injury with Pitavastatin in Septic Mice: Potential Role of Glucocorticoid Receptor Expression in Alveolar Macrophages

Ken-ichi Takano, Seiji Yamamoto, Kengo Tomita, Michinori Takashina, Hiroki Yokoo, Naoyuki Matsuda, Yasuo Takano, and Yuichi Hattori

Departments of Molecular and Medical Pharmacology (K.Ta., S.Y., K.To., M.T., H.Y., N.M., Y.H.) and Diagnostic Pathology (Y.T.), Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan

ABSTRACT
There is growing evidence that the HMG-CoA reductase inhibitors (statins) provide some of the beneficial effects that are independent of their lipid-lowering effects. Recent animal experiments and clinical trials suggest that statin use may limit the development of sepsis and associated systemic inflammation. The aim of this study was to explore the potential role of statins in the prevention treatment of sepsis-induced acute lung injury (ALI). Mice were rendered septic by cecal ligation and puncture (CLP). An intraperitoneal injection of 3 mg/kg per day of pitavastatin was initiated 4 days before surgery and was maintained for life support afterward, which significantly improved the survival of CLP mice. Treatment with pitavastatin prevented the ALI development in CLP mice, as indicated by the findings that severe hypoxemia, increased pulmonary vascular permeability, and histological lung damage, including inflammatory cell infiltrate, were greatly remedied. This was associated with down-regulation of increased activity of nuclear factor-κB (NF-κB) in septic lungs. Although plasma cortisol showed a sharp rise, glucocorticoid receptor (GCR) expression in the lungs was strikingly reduced after the onset of CLP-induced sepsis. It is noteworthy that pitavastatin increased GCR expression with an increase in alveolar macrophages in which GCRs are localized, without modifying the sepsis-associated rise in plasma cortisol. These results confirm significant protection by pitavastatin on septic ALI and demonstrate that down-regulated NF-κB activation associated with the GCR expression increase consequent to the increased number of alveolar macrophages may explain, in part, the mechanisms responsible for favorable effects of statins on the ALI management.

Introduction
Sepsis can be defined as a systemic inflammatory response syndrome in which there is an identifiable focus of infection (Robertson and Coopersmith, 2006). Sepsis can be presented with a spectrum of severity. Severe sepsis is diagnosed once sepsis becomes complicated by factors, including acute organ dysfunction, tissue/organ hypoperfusion, hypotension, and coagulopathy (Tsioiotou et al., 2005). Septic shock represents the most severe form of host response to infection and has a poor prognosis (Das, 2000). It is associated with hypotension despite adequate fluid resuscitation. Severe sepsis and septic shock are among the most common causes of death in intensive-care units (Vincent et al., 2006). Under those conditions, the respiratory system is the most frequently affected organ system, and lung dysfunction is the first step in the development of multiple organ failure. Acute lung injury (ALI) and its most severe manifestation, the acute respiratory distress syndrome (ARDS), are a major cause of acute respiratory failure in critically ill patients and are characterized by acute severe hypoxemia, bilateral pulmonary infiltrates consistent with edema, and normal cardiac filling pressure (Ferguson et al., 2005). Despite significant advances in surgical technique and therapy, ARDS is a clinical entity with high morbidity and mortality. Thus, a major contributing factor to the poor outcome of this life-threatening syndrome is the lack of effective treatment.

ABBREVIATIONS: ALI, acute lung injury; CLP, cecal ligation and puncture; NF-κB, nuclear factor-κB; GCR, glucocorticoid receptor; ARDS, acute respiratory distress syndrome; LDL, low-density lipoprotein; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; IL, interleukin; CRP, C-reactive protein; IL-1ra, IL-1 receptor antagonist; TREM-1, triggering receptor expressed on myeloid cell 1; LBP, LPS-binding protein; TUNEL, terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling; BALF, bronchoalveolar lavage fluid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PI3K, phosphatidylinositol 3-kinase.
Statins are established drugs for the treatment of lipid disorders (Corsini et al., 1999). They inhibit the synthesis of cholesterol in the liver by blocking the conversion of HMG-CoA to L-mevalonate, the rate-limiting step in the cholesterol biosynthetic pathway, which leads to the up-regulation of the low-density lipoprotein (LDL) receptor and lowers plasma LDL cholesterol levels (Goldstein and Brown, 2009). A growing body of evidence suggests that statins exhibit many pleiotropic effects beyond their LDL cholesterol-lowering potencies. Indeed, statins have emerged as potentially powerful inhibitors of the inflammatory process (Greenwood and Mason, 2007). Some of these anti-inflammatory properties of statins are related to the inhibition of HMG-CoA reductase activity (Kwak et al., 2000; Romano et al., 2000), whereas others are independent of blocking HMG-CoA reductase activity (Weitz-Schmidt et al., 2001). It is noteworthy that the prospective observational cohort study has shown that statin therapy before the onset of acute bacterial infection is associated with a reduced rate of severe sepsis and intensive care unit administration (Almg et al., 2004). Moreover, simvastatin pretreatment has been reported to improve survival in a murine model of sepsis (Merx et al., 2004). We have also observed that fluvastatin therapy can significantly protect the rabbits against lipopolysaccharide (LPS)-induced septic death (Matuda et al., 2006). Taken together, statins might be considered an effective tool with therapeutic potential in sepsis.

In the present study, we assessed whether treatment with pitavastatin, a new synthetic statin with highly potent and efficacious HMG-CoA reductase inhibition (Kajinami et al., 2003), can reduce ALI in mice with cecal ligation and puncture (CLP)-induced polymicrobial sepsis. CLP-induced sepsis is an animal model that has high clinical relevance to humans, because it reproduces many hallmarks of sepsis that occur in patients (Hubbard et al., 2005). We found that treatment of CLP mice with pitavastatin provided a benefit in survival with mitigating ALI. Further studies were then undertaken to gain insight into the possible mechanism(s) underlying the beneficial effects of statins on severe inflammation such as septic ALI.

Materials and Methods

Experimental Model. All animal works were performed in accordance with the guidelines and the protocol was approved by the Animal Care and Use Committee of the University of Toyama. Male BALB/c mice (8–12-weeks-old) were quarantined in quiet, humidified, light-cycled rooms for at least 1 week before use. Mice were allowed ad libitum to food and water throughout quarantine. The surgical procedure to generate CLP-induced sepsis was carried out according to the method described in our previous studies with minor modification (Matuda et al., 2005, 2007). In brief, mice were anesthetized with 3 to 4% sevoflurane, and a middle abdominal incision was made. The cecum was mobilized, ligated at 5 mm from its top, and then perforated in two locations with a 21-gauge needle, allowing exposure of feces. The bowel was repositioned, and the abdomen was closed. Sham-operated control animals were subjected to the same surgical laparotomy, but the cecum was neither ligated nor perforated. Both were allowed ad libitum to food and water throughout the operation. The animals were killed, the lungs were perfused with heparinized saline (1 U/ml) via the pulmonary artery, and then the two portions of lung parenchyma were harvested. Both were weighted, and then one was desiccated at 60°C overnight before weighing the dry tissue. The other was immersed in formamide (4 ml/g wet weight) at 60°C for 18 h for extraction of the Evans blue dye. The absorbance of Evans blue dye extracted in formamide then was measured by spectrophotometry at a wavelength of 620 nm (ND-1000 Spectrophotometer, Nano Drop Technologies, Rockland, DE). The concentration of the Evans blue dye was then calculated from a standard curve and expressed as micrograms of Evans blue dye per gram dry weight of tissue.

Electrophoretic Mobility-Shift Assay. Nuclear protein extracts from freshly isolated lungs were obtained with a commercially available nuclear extraction kit (Sigma-Aldrich, St. Louis, MO) as described in the manufacturer’s manual. Electrophoretic mobility-shift assays were carried out with Odyssey Infrared electrophoretic mobility shift assay kit (LI-COR, Lincoln, NE) according to the manufacturer’s instruction. Double-stranded IRDye 700 infrared dye-labeled oligonucleotides with consensus sequences of nuclear factor-κB (NF-κB) (5'-AGT TGA GGG GAC TTT CCC AGG C-3' and 3'-TCA ACT CCC CTG AAA GGG TCC G-5') were used.

Western Blot Analysis. Immunoblotting was performed according to the method described previously (Matuda et al., 2007). Samples of homogenate (40 µg of protein) were run on 12.5% SDS polyacrylamide gel and transferred to a polyvinylidine difluoride filter membrane. Membranes were probed with anti-IκBα (Cell Signaling, Danvers, MA), anti-glucocorticoid receptor (GCR) (Abcam, Cambridge, MA), anti-assay kit (Cayman Chemical, Ann Arbor, MI) was used for quantification of cortisol in blood. This method showed little cross-reactivity with other steroid hormones such as progesterone (<0.01%). When IL-10 levels were assessed, mouse blood was centrifuged to obtain serum. The plate was read on a microplate reader (Nippon-InterMed, Tokyo, Japan). Assays were performed in duplicate.

Pulmonary Microvascular Leakage. Lung vascular leak was assessed by Evans blue dye extravasation. Evans blue dye (20 mg/kg) was injected to mice through the tail vein 30 min before termination of the experiment. The animals were killed, the lungs were perfused with heparinized saline (1 U/ml) via the pulmonary artery, and then one was desiccated at 60°C overnight before weighing the dry tissue. The other was immersed in formamide (4 ml/g wet weight) at 60°C for 18 h for extraction of the Evans blue dye. The absorbance of Evans blue dye extracted in formamide then was measured by spectrophotometry at a wavelength of 620 nm (ND-1000 Spectrophotometer, Nano Drop Technologies, Rockland, DE). The concentration of the Evans blue dye was then calculated from a standard curve and expressed as micrograms of Evans blue dye per gram dry weight of tissue.

Electrophoretic Mobility-Shift Assay. Nuclear protein extracts from freshly isolated lungs were obtained with a commercially available nuclear extraction kit (Sigma-Aldrich, St. Louis, MO) as described in the manufacturer’s manual. Electrophoretic mobility-shift assays were carried out with Odyssey Infrared electrophoretic mobility shift assay kit (LI-COR, Lincoln, NE) according to the manufacturer’s instruction. Double-stranded IRDye 700 infrared dye-labeled oligonucleotides with consensus sequences of nuclear factor-κB (NF-κB) (5'-AGT TGA GGG GAC TTT CCC AGG C-3' and 3'-TCA ACT CCC CTG AAA GGG TCC G-5') were used.

Western Blot Analysis. Immunoblotting was performed according to the method described previously (Matuda et al., 2007). Samples of homogenate (40 µg of protein) were run on 12.5% SDS polyacrylamide gel and transferred to a polyvinylidine difluoride filter membrane. Membranes were probed with anti-IκBα (Cell Signaling, Danvers, MA), anti-glucocorticoid receptor (GCR) (Abcam, Cambridge, MA), or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Santa Cruz, CA).

Immunofluorescence and Confocal Analysis. As described previously (Kamiyama et al., 2008), the trachea was cannulated, and lungs were gently inflation-fixed all together with 4% buffered formalin solution. Inflation-fixed lungs were harvested, postfixed, and paraffin- or frozen-sectioned. For immunohistochemical determination of target molecules, the tissue sections were exposed to the fluorescent antibody after overnight incubation with the primary antibody. The following commercially available antibodies were used: anti-Gr-1 (AbD Serotec, Oxford, UK), anti-NF-κB p65 antibody (Cell Signaling), anti-CI1c antibody (BD Biosciences, San Diego, CA), anti-GCR antibody (EMD Chemicals, Gibbstown, NJ), anti-C3d1 antibody (Millipore, Billerica, MA), and anti-cleaved caspase-3 antibody (Cell Signaling). The nucleus was counterstained with Hoechst 33258 dye (Nacalai Tesque, Kyoto, Japan). Immunofluorescent images were observed under a Leica TCS-SP5 confocal system.

Histologic Examination. Routine histology, sections from inflation-fixed lungs were prepared as described above. After depar
affinization, slides were stained with hematoxylin and eosin using standard methods.

**Apoptotic Cell Detection.** Terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) was performed using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore) according to the manufacturer’s instructions. Additional details are described elsewhere (Kamiyama et al., 2008).

**Statistical Analysis.** The data are presented as mean ± S.E. Data were analyzed using Prism software (ver. 4; GraphPad Software, Inc., San Diego, CA). Statistical analysis was performed using one- or two-way analysis of variance followed by Turkey’s multiple comparison test. A P value less than 0.05 was considered significant.

## Results

**Animal Survival, Hypotension, and Septic Biomarkers after CLP.** We initially examined whether the survival advantage after CLP can be conferred by pitavastatin treatment. As shown in Fig. 1A, survival of CLP mice declined sharply in the first 48 h. CLP mice administered pitavastatin at a daily dose of 1 mg/kg showed no significant survival benefit. However, the CLP animals given pitavastatin at 3 mg/kg daily survived significantly longer than the untreated CLP group. Therefore, all subsequent experiments were performed using 3 mg/kg per day as a therapeutic dose of pitavastatin.

The animals subjected to CLP showed a progressive fall in blood pressure. At 24 h after surgery, mean arterial blood pressure was significantly lower in the CLP group (53 ± 2 mmHg, n = 5) than in the sham group (76 ± 5 mmHg, n = 4). Administration of pitavastatin was without effect on CLP-induced hypotension (Fig. 1B).

We also investigated the changes in plasma concentrations of a number of diagnostic biomarkers for sepsis that have been used in clinical practice. CRP is a plasma protein produced by the liver and rises after an injury, infection, or inflammation. A time-dependent increase in CRP in the plasma was observed after CLP (Fig. 2A). An increase in the calcitonin precursor procalcitonin, a useful biomarker for the diagnosis of clinically relevant bacterial infections and sepsis, after CLP was also time-dependent (Fig. 2B). The member of the IL-1 cytokine family IL-1ra in plasma showed a great but transient increase at 12 h after CLP (Fig. 2C). TREM-1, which is an activating receptor expressed on neutrophils and monocyte macrophages and emerges as a regulator of innate immunity in sepsis syndrome, showed a time-dependent up-regulation after CLP (Fig. 2D). CD14 is a pattern-recognition receptor that has a central immunomodulatory role in proinflammatory signaling in response to a variety of ligand, including endotoxin. The plasma concentration of soluble CD14 reached a maximal elevation at 12 h after CLP (Fig. 2E). LBP, a soluble acute-phase plasma protein known to facilitate the diffusion of bacterial endotoxin, was significantly elevated from control at 6 h after CLP, with peak concentrations at 12 h (Fig. 2F). The rises in IL-1ra were suppressed significantly by pitavastatin treatment. However, pitavastatin was without effect on the elevations of procalcitonin, TREM-1, CD14, and LBP and rather enhanced the CRP response.

**Septic ALI.** To determine whether pitavastatin treatment can modify impaired pulmonary blood gas exchange during sepsis, blood gases were measured in arterial blood samples from mice 24 h after the onset of CLP-induced sepsis. As shown in Fig. 3A, septic mice displayed a severe hypoxemic condition, as indicated by markedly reduced PO<sub>2</sub> in comparison with sham control mice. This hypoxemia associated with sepsis was mitigated significantly by pitavastatin treatment. When pulmonary microvascular leakage was assessed by Evans blue dye extravasation, an approximate 3-fold increase in lung vascular permeability occurred 24 h after the onset of CLP-induced sepsis (Fig. 3B). Administration of pitavastatin resulted in a reduction in lung vascular permeability to the level observed in the sham-operated group.

According to histologic samples, CLP-induced septic mice displayed marked congestion of capillary vessels and inflammatory cell infiltration in septa in addition to erythrocytes originating from ruptured capillary vessels in the

**Fig. 1.** Effects of pitavastatin treatment on sepsis-associated mortality and hypotension. A, cumulative survival after CLP in animals given saline or pitavastatin at two different doses (1 and 3 mg/kg per day). Ten to 15 animals were used for each group. *, P < 0.01 (log rank test). B, changes in blood pressure at 24 h after CLP or sham operation in mice given saline or pitavastatin (3 mg/kg per day). Systolic (top), diastolic (bottom), and mean arterial blood pressure (middle line) are shown. All data are presented as mean ± S.E. (n = 3–5). ***, P < 0.001 compared with sham-operated control.**
lung tissues (Fig. 4A). When septic mice received treatment with pitavastatin, these histopathologic changes were minimized, as characterized by less distortion of alveolar architecture, scattered interstitial infiltrates, and rare areas of focal hemorrhage.

Further evidence for inflammatory cell infiltration in septic lungs was provided by the immunofluorescent image showing the presence of Gr-1-positive cells (Fig. 4B). Thus, a marked increase in the number of peripheral neutrophils, which were specifically immunostained with anti-Gr-1 antibody, was observed in septic lung sections. However, the sepsis-induced increase in immunofluorescent staining for peripheral neutrophils was blunted by pitavastatin treatment.

Cytokine Production and NF-κB Activity in Sepsis.
We examined the effect of pitavastatin treatment on blood levels of several cytokines. As shown in Fig. 5, A to C, the animals before CLP surgery had extremely low levels of the cytokines examined here. After induction of sepsis by CLP, the proinflammatory cytokines TNF-α and IL-6 as well as the anti-inflammatory cytokine IL-10 showed a progressive increase over a 24-h period. Pitavastatin treatment did not substantially alter the initial rise in blood levels of these cytokines but strongly inhibited their increased levels at 24 h after CLP-induced sepsis.

We then investigated whether pitavastatin treatment can modify sepsis-induced NF-κB activation in mouse lungs. NF-κB activation was assessed by NF-κB-DNA-binding activity and degradation of its inhibitory protein IκBα. As presented in Fig. 6A, the DNA binding activity of NF-κB was greatly increased in lung nuclear extracts from mice at 24 h after the onset of CLP-induced sepsis compared with those from sham-operated control mice. This increase was strongly eliminated by pitavastatin treatment. In addition, as shown in Fig. 6B, nuclear staining for NF-κB p65 was detected in the lung section from the animal with CLP-induced sepsis. No nuclear staining for p65 was found when pitavastatin was treated. Furthermore, treatment of septic mice with pitavastatin was associated with less degradation of IκBα (Fig. 6C).

Emergence of Alveolar Macrophages.
There was an interesting finding in lung sections from pitavastatin-treated mice. Thus, nearly spherical cells were detected in the alveoli at an unusually high level (Fig. 7A). To define what cell types they represent, histochemical staining experiments were performed.

---

**Fig. 2.** Effects of pitavastatin treatment on changes in plasma concentrations of sepsis markers after CLP. Time-dependent changes in plasma levels of CRP (A), procalcitonin (B), IL-1ra (C), TREM-1 (D), CD14 (E), and LBP (F) in mice that were subjected to CLP with or without pitavastatin treatment (3 mg/kg per day) are shown. All data are presented as means ± S.E. (n = 4–6). *, P < 0.05. n.s. = not significant.

**Fig. 3.** Effects of pitavastatin treatment on sepsis-associated hypoxemia and lung vascular hyperpermeability. A, for the measurement of arterial PO2, blood gas analysis was performed in mice 24 h after sham operation or CLP. Pitavastatin (3 mg/kg per day) was administered as described under Materials and Methods. The blood samples were collected by cardiac puncture under terminal anesthesia. B, lung vascular permeability was assessed by Evans blue dye extravasation at 24 h after CLP or sham operation in mice given saline or pitavastatin (3 mg/kg per day). All data are presented as mean ± S.E. (n = 4–9). *, P < 0.05 compared with sham-operated control. #, P < 0.01 compared with CLP alone.
formed. Although *Griffonia simplicifolia* isolectin B4 is reportedly useful as a marker for endothelial cells, this reagent has been used successfully for the histochemical demonstration of microglia/macrophages (Streit, 1990). As depicted in Fig. 7B, the cells in the alveoli, which were increased when mice were given pitavastatin, were labeled with isolectin B4. Likewise, anti-CD11c stained these cells. CD11c clearly recognizes dendritic cells, and it also labels certain macrophage subsets, including alveolar macrophages (Geissmann et al., 2003; Probst et al., 2005; Senft et al., 2007). Merging of isolectin B4 and CD11c showed colocalization of CD11c with isolectin B4. However, these cells were negative for the inflammatory macrophage marker CD11b (data not shown). Together, these data suggest that mature alveolar macrophages were increased remarkably in the lungs when mice were treated with pitavastatin.

**Pulmonary GCR Expression and Plasma Cortisol.** Confocal immunofluorescence microscopy was conducted to visualize GCRs, the receptors to which cortisol binds and other glucocorticoids, in lung sections. Double-labeling immunofluorescence showed that GCRs were present in alveolar macrophages identified by staining with anti-CD11c (Fig. 8). Both alveolar macrophages and GCRs were reduced strikingly after sepsis induction by CLP. It is noteworthy that alveolar macrophages, which do not express GCRs, were found in septic lungs. The reduction in GCR expression was prevented demonstrably by pitavastatin treatment with an increase in alveolar macrophages.

**Fig. 4.** Effects of pitavastatin treatment on histopathologic changes and neutrophilic infiltrate in lungs from septic mice. Mice underwent sham procedure or CLP with or without pitavastatin treatment (3 mg/kg per day). Tissues were harvested 24 h after surgery. A, lung tissue sections stained with hematoxylin and eosin. Magnified view is shown in each inset (scale bar, 20 μm). B, immunofluorescent images showing localization of neutrophils in lung tissues. Neutrophils were identified by rat anti-mouse Gr-1 monoclonal antibody (red). Nuclei were counterstained with Hoechst 33258 dye (blue). In the bottom trace, the summary of quantification of Gr-1-positive cell counts is presented. The average of Gr-1-positive cell number in three fields per sample was calculated. Means ± S.E. of data from five animals/group are shown. *, P < 0.001 compared with sham-operated control.

**Fig. 5.** Effects of pitavastatin treatment on sepsis-associated elevations in cytokines in blood. Time-dependent changes in blood levels of TNF-α (A), IL-6 (B), and IL-10 (C) in mice that were subjected to CLP with or without pitavastatin treatment (3 mg/kg per day) are shown. All data are presented as means ± S.E. (n = 4). *, P < 0.05.
When GCR expression in alveolar macrophages in BALF was assessed using Western blot assays, CLP-induced sepsis was found to result in weak immunoreactivity. Treatment with pitavastatin evidently up-regulated this reduced expression (Fig. 9A).

At 6 h after CLP, the plasma cortisol level was elevated...
from 195 ± 93 (n = 4) to 9367 ± 340 pg/ml (n = 4). Thus, plasma cortisol increased approximately 48-fold from baseline at 6 h after the onset of CLP-induced sepsis. The plasma level of cortisol slightly declined thereafter, but it remained unduly higher than the basal level. Treatment with pitavastatin did not substantially affect the sharp rise in plasma cortisol caused by sepsis induction (Fig. 9B).

From 195 ± 93 (n = 4) to 9367 ± 340 pg/ml (n = 4). Thus, plasma cortisol increased approximately 48-fold from baseline at 6 h after the onset of CLP-induced sepsis. The plasma level of cortisol slightly declined thereafter, but it remained unduly higher than the basal level. Treatment with pitavastatin did not substantially affect the sharp rise in plasma cortisol caused by sepsis induction (Fig. 9B).

**Cell Apoptosis in Septic Lungs.** To assess whether pitavastatin treatment can affect sepsis-induced apoptotic cell death in lungs, the tissue sections were labeled with an in situ TUNEL assay (Fig. 10, A and B). Induction of sepsis by CLP resulted in a marked appearance of TUNEL-positive cells. Apoptotic cells were identical morphologically to endothelial cells of capillary vessels in the alveolar septa and to epithelial type II cells (Matsuda et al., 2009a). In agreement with this finding, immunofluorescent staining for the cleaved form of caspase-3, a member of the caspase superfamily that initiates cell apoptotic events, showed that its protein expression was detectable also in cell types other than endothelial cells in lungs (Fig. 10C). In lungs from CLP mice given pitavastatin, TUNEL-positive cells were decreased sharply, providing a protective effect of pitavastatin treatment on pulmonary cell apoptosis mediated by sepsis (Fig. 10, A and B).

**Discussion**

In the present study, we employed the CLP model, because it closely resembles the pathophysiology of human sepsis and represents an indirect insult similar to the pathogenesis of ARDS (Hubbard et al., 2005). Indeed, in line with our previous report (Matsuda et al., 2005), we clearly demonstrated that our CLP mice exhibited marked hypoxemia, increased lung vascular permeability, and histological damage in lungs, including inflammatory infiltrate and hemorrhage. We found that these pathophysiologic consequences of ALI were greatly prevented by treatment with pitavastatin. The data are in good agreement with those obtained with other statins in LPS-challenged mice. Thus, simvastatin and pravastatin have been shown to significantly decrease intratracheal LPS-induced murine lung vascular leak and inflammation (Jacobson et al., 2005; Yao et al., 2006). Taken together, these results would implicate the potential for the class of drugs known as statins to serve as a novel therapeutic tool in ALI.

ALI represents a cytokine excess state that involves the microvascular dysfunction of multiple organs. The proinflammatory cytokines, such as TNF-α and IL-6, have been implicated in the pathogenesis of inflammatory lung injury, particularly under conditions of severe lung infection and sepsis (Bhatia and Mochhala, 2004). It has been shown that the elevated level of TNF-α in murine lung tissues after intratracheal LPS administration can be decreased by pravasta-
TNF-α induced sepsis causes a dramatic rise in circulating TNF-α levels. A recent study using NF-κB activation in vivo demonstrated this dramatic effect (Matsuda et al., 2009b). It is believed that NF-κB plays a key role in expression of many harmful genes responsible for the pathophysiology of sepsis-induced ALI (Liu and Malik, 2006). The present study is the first to demonstrate that pitavastatin strongly inhibited NF-κB activation in lungs of CLP-induced septic mice. We have previously revealed that in vivo transfection of NF-κB decoy oligonucleotides can suppress the transactivation of many NF-κB-associated genes and prevent ALI in CLP-induced septic mice (Matsuda et al., 2005). Therefore, it would be logical to conclude that NF-κB inhibition is involved in the beneficial effect of pitavastatin in the prevention of the septic ALI development.

It is noteworthy that we found that pitavastatin treatment increased the number of alveolar macrophages. Alveolar macrophages are regarded as the most abundant antigen-presenting cells in the airway and alveolar spaces, where they are critically involved in host defense, both as effector cells that bind and engulf pathogens and as sentinel cells that secrete proinflammatory cytokines and chemokines to recruit and activate inflammatory cells (Lambrecht, 2006). However, it has been indicated that alveolar macrophages may serve to limit deleterious inflammatory responses within the lung (Thepen et al., 1989). Furthermore, several studies using models of lung injury that vary from instillation of LPS or live bacteria to ischemia-reperfusion have shown that macrophage depletion can be associated with enhanced lung injury (Kooguchi et al., 1998; Knapp et al., 2003; Elder et al., 2005; Nakamura et al., 2005). They have concluded that these observations are due to the elimination of the suppressive effects of alveolar macrophages. Alveolar macrophages also exhibit an unusual phenotype compared with typical tissue macrophages. Thus, it has been found that alveolar macrophages express high levels of CD11c, a molecule that is not expressed by their counterparts in other body sites and is generally expressed by dendritic cells (Gonzalez-Juarrero et al., 2003). However, contrary to dendritic cells, alveolar macrophages are very unlikely to emigrate from the tissue and seem to have distinct roles in the initiation and maintenance of immune responses (Jakubzick et al., 2006). We noted that the CD11c marker was consistently expressed on alveolar macrophages that were increased by pitavastatin treatment.

Our immunofluorescent labeling study revealed that GCRs were specifically localized on alveolar macrophages. We have observed that GCR expression is significantly decreased in lungs as well as kidney and spleen from LPS-induced septic guinea pigs (Kamiyama et al., 2008). Consistent with this prior study is the present finding that CLP-induced sepsis resulted in a marked down-regulation of GCR expression in lungs. Treatment of septic mice with pitavastatin led to an offset effect on down-regulated pulmonary expression of GCRs. This was due primarily to the increase in alveolar macrophages. GCRs serve as a major modulator of the immune system as a result of their proficient anti-inflammatory and immunosuppressive activities (Baschant and Tuckermann, 2010). GCR trans-regulation, which is thought to be important for most of the anti-inflammatory functions, is based on the interaction with other transcription factors, including NF-κB, thereby repressing or potentiating their activity. Plasma cortisol levels were tremendously elevated during sepsis. Because of increased availability of the hormone binding sites after pitavastatin treatment, the increase in cortisol levels could efficiently operate in regulating inflammation. Therefore, the up-regulation of pulmonary ex-
pression of GCRs, which was accompanied by the increased number of alveolar macrophages may explain, at least in part, the preventive effect of pitavastatin on the development of septic ALI through NF-κB inhibition.

Recent accumulating evidence suggests that cell apoptosis plays an important role in the development of organ failure and mortality associated with sepsis, and it may be potentially detrimental in septic ALI. The Fas/Fas ligand system has been found to be up-regulated in pulmonary edema fluid and injured lung tissues from patients with ALI or ARDS (Albertine et al., 2002). Furthermore, it has been shown that silencing of Fas in lung epithelial cells can protect against lung inflammation in a mouse model of hemorrhagic shock followed by CLP (Perl et al., 2005). In a recent work, we have also reported that specific knockdown of Fas-associated death domain, an adaptor molecule to recruit procaspase-8 into the death-inducing signal complex, with small interfering RNA prevents the ALI development and improves the survival of CLP mice (Matsuda et al., 2009a). In the present study, pitavastatin administration reduced apoptosis in lungs as determined by TUNEL assay. The beneficial effect of pitavastatin is probably through its action on the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which is impaired under the septic condition (Matsuda et al., 2006, 2007). In this regard, our previous work has demonstrated that fluvastatin can restore the impairment of the PI3K/Akt pathway in the rabbit LPS-induced septic model (Matsuda et al., 2006). Akt is a key regulator of cell survival and reduces activation of caspase-8 and caspase-3 as a result of impaired recruitment of procaspase-8 to the death-inducing signaling complex (Jones et al., 2002). It is noteworthy to describe that treatment with the PI3K inhibitor wortmannin (1 mg/kg twice daily) hampered the ability of pitavastatin to reduce lung apoptosis and to improve survival in CLP mice (K. Takano and Y. Hattori, unpublished observation). Thus, it is most likely that the antiapoptotic action of pitavastatin is one of the principal mechanisms by which this statin reduced septic ALI. As seen in most studies for statin therapy in sepsis, extremely high-dose pitavastatin therapy was required for significant improvement of septic ALI. Although statins have proven to offer a relatively favorable safety profile, two common adverse effects, liver dysfunction and myositis, would be relevant in sepsis when high-dose statins are used. In this regard, because pitavastatin is processed only minimally in the liver (Kajinami et al., 2003), it may sidestep these side effects, which preclude the use of other statins. The cytochrome P450 system is of special interest in the context of the treatment of sepsis with statins because pitavastatin is metabolized only marginally by CYP2C9 and does not have any interaction with CYP3A4, a predominant isoform expressed in human liver, which serves as the major pathway for metabolism of simvastatin, atorvastatin, and lovastatin. However, our study using pitavastatin has some limitations. Despite the fact that the current drugs in the "statin" class are only available as an oral preparation, the intraperitoneal application route was used in this study. Appropriate routes of statin application for septic patients remains the subject of ongoing studies. Another concern is that our experiments were originally designed on the assumption that septic patients have always been treated with statins because of the widespread application of statins. To be available for wide use of statins as adjuvant therapy, the investigation of treatment after sepsis induction may well deserve further study. Nonetheless, we believe that our present results showing the potential effect of pitavastatin to mitigate typical ALI in a clinically relevant model of sepsis hold the promise of profound clinical significance of statin therapy in septic ALI.

In summary, we suggest that pitavastatin, a new synthetic statin with highly potent and efficacious HMG-CoA reductase inhibition, may serve as a potential therapeutic tool for septic ALI. This seemed to be attributed to down-regulated NF-κB activation in association with the up-regulation of GCR expression, which stemmed from the increased number of alveolar macrophages. In addition, the antiapoptotic action of pitavastatin is likely involved in the benefit of this statin to reduce the development of septic ALI. Additional work will be required to delineate the specific mechanism underlying increased alveolar macrophage accumulation as well as the phenotype/functional characterization of these cells.

Acknowledgments
We thank Hideki Origasa for expert advice on the statistical analysis; Dr. Hiromi Yamazaki for assistance with electrophoretic mobility-shift assay techniques; and Satoshi Ide, Kenji Miyazaki, and Yoshifumi Shimada for skillful technical assistance.

Authorship Contributions
Participated in research design: K. Takano, Matsuda, and Hattori. Conducted experiments: K. Takano, Yamamoto, Tomita, Takashina, Yokoo, and Y. Takano. Performed data analysis: K. Takano, Tomita, and Yokoo. Wrote or contributed to the writing of the manuscript: K. Takano and Hattori.

References
Jones RG, Elford AR, Parsons MJ, Wu L, Krawczyk CM, Yeh WC, Hakem R, Rottapel R, Woodgett JR, and Ohashi PS (2002) CD28-dependent activation of protein...


Address correspondence to: Dr. Yuichi Hattori, Department of Molecular and Medical Pharmacology, Graduate School of Medicine and Pharmaceutical Sciences, Univ. of Toyama, Toyama 930-0194, Japan. E-mail: yhattori@med.u-toyama.ac.jp.