

FLAGELLIN FROM GRAM-NEGATIVE BACTERIA IS A POTENT MEDIATOR OF ACUTE PULMONARY INFLAMMATION IN SEPSIS

Lucas Liaudet,^{*†‡} Csaba Szabó,^{*†§} Oleg V. Evgenov,^{*||} Kanneganti G. Murthy,^{*}
Pál Pacher,^{*} László Virág,^{*} Jon G. Mabley,^{*} Anita Marton,^{*}
Francisco G. Soriano,^{*†} Mikhail Y. Kirov,^{||¶} Lars J. Bjertnaes,^{||} and
Andrew L. Salzman^{*}

^{*}Inotek Pharmaceuticals Corporation, Beverly, MA 01915; [†]Department of Surgery, New Jersey Medical School, UMDNJ, Newark, NJ 01703; [‡]Critical Care Division, Department of Internal Medicine, University Hospital, Lausanne, Switzerland; [§]Institute of Human Physiology and Clinical Experimental Research, Semmelweis University, Budapest, Hungary; ^{||}Department of Anesthesiology, Faculty of Medicine, University of Tromso, Tromso, Norway; and [¶]Northern State Medical University, Arkhangelsk, Russia

Received 5 Apr 2002; first review completed 29 Apr 2002; accepted in final form 3 Jul 2002

ABSTRACT—Flagellin is a recently identified bacterial product that elicits immune response via toll-like receptor 5. Here, we demonstrate that flagellin is an extraordinarily potent proinflammatory stimulus in the lung during sepsis. *In vitro*, flagellin triggers the production of interleukin (IL)-8 by human lung epithelial (A549) cells, with 50% of the maximal response obtained at a concentration of 2×10^{-14} M. Flagellin also induces the expression of ICAM-1 *in vitro*. Intravenous administration of flagellin to mice elicited a severe acute lung inflammation that was significantly more pronounced than following lipopolysaccharide (LPS) administration. Flagellin induced a local release of proinflammatory cytokines, the accumulation of inflammatory cells, and the development of pulmonary hyperpermeability. These effects were associated with the nuclear translocation of the transcription NF- κ B in the lung. Flagellin remained active in inducing pulmonary inflammation at doses as low as 10 ng/mouse. In the plasma of patients with sepsis, flagellin levels amounted to 7.1 ± 0.1 ng/mL. Plasma flagellin levels showed a significant positive correlation with the lung injury score, with the alveolar-arterial oxygen difference as well as with the duration of the sepsis. Flagellin emerges as a potent trigger of acute respiratory complications in gram-negative bacterial sepsis.

KEYWORDS—Endotoxin shock, chemokines, bacterial infection, inflammation, lung

INTRODUCTION

Acute lung inflammation culminating in acute respiratory distress syndrome (ARDS) is a major complication of gram-negative bacterial sepsis (1). The mechanisms linking a distant focus of infection to remote lung inflammation remain elusive. The commonly held view is that bacterial toxins released into the circulation activate interconnected inflammatory cascades in the lung, ultimately leading to diffuse alveolar damage (2). Bacterial lipopolysaccharide (LPS), a component of the outer cell wall of gram-negative bacteria, has been so far held responsible for triggering this chain of events (3, 4). Whether additional bacterial toxins play a role in the development of acute pulmonary inflammation during gram-negative sepsis remains an unresolved issue.

Recent investigations have indicated that flagellin, the principal component of bacterial flagella (5), can induce the expression of proinflammatory mediators by monocytes (6–8) and intestinal epithelial cells (9–11) *in vitro*, resulting from the activation of the transcription factor NF- κ B (9, 12). The signal transduction pathway mediating these effects has been recently identified by Hayashi and colleagues (12), who showed that flagellin is the unique ligand of the Toll-like receptor 5 (TLR5). TLR5 is a member of a family of innate immune

receptors that recognize specific pathogen-associated molecular patterns (PAMPs) (13). For instance, LPS, the prototype gram-negative PAMP, requires TLR4 to exert its biological activity after an initial binding to the CD 14 receptor (14, 15). Flagellin was found to be continuously released into the culture supernatant of gram-negative bacteria (9, 11, 16) and was reported to circulate as a free protein in the plasma of rats with gram-negative sepsis (9). These findings suggested that flagellin may contribute to localized as well as disseminated inflammatory responses during gram-negative bacterial infection. Therefore we investigated the potential role of flagellin as a mediator of lung inflammation using both *in vitro* and *in vivo* experimental models. In addition, we have measured circulating flagellin levels in human patients with sepsis, and correlated the plasma levels of flagellin with various severity indices of lung injury and shock.

MATERIALS AND METHODS

Preparation of recombinant *Salmonella muenchen* flagellin

Purified recombinant *S. muenchen* flagellin was prepared as previously described (9). To remove any possible contaminant LPS, the purified flagellin preparation was passed through a polymyxin B column, resulting in an LPS-free flagellin preparation. Previous studies confirmed the lack of LPS contamination in the flagellin preparations used in our studies (9).

Cell culture

Human alveolar epithelial cells (A549), an adenocarcinoma cell line with the alveolar type 2 phenotype (18), were grown in 96- or 6-well plates in RPMI 1650 medium containing 10% fetal calf serum (FCS). Confluent cells were stimulated

Address reprint requests to Andrew L. Salzman, MD, Inotek Pharmaceuticals Corporation, Suite 419 E, 100 Cummings Center, Beverly, MA 01915.
DOI: 10.1097/01.shk.0000046635.25163.19

with recombinant *S. muenchen* flagellin (concentration range: 10^{-17} to 10^{-8} g/mL, 2×10^{-19} to 2×10^{-10} M) for 24 h. The production of interleukin (IL)-8 in the medium was measured using a commercial ELISA (R&D Systems, Minneapolis, MN). Cells in 6-well plates were scraped and washed twice in ice-cold phosphate-buffered saline (PBS). Staining was performed on ice with fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-human ICAM-1 antibody and isotype-matched monoclonal antibody (both from Pharmingen, San Diego, CA) as a control for 1 h. Cells were then washed twice in ice cold PBS and fixed in 1% paraformaldehyde. Samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA), and ICAM-1 expression was determined as an increase in mean fluorescence intensity.

In vivo experiments

In vivo experiments were performed in accordance with National Institutes of Health guidelines. Male BALB/c mice received an intravenous injection of *S. muenchen* flagellin ($n = 8$) or *S. enteritidis* ($n = 7$) LPS (4 mg/kg, 100 μ g/mouse) via the tail vein. A group of $n = 5$ mice challenged with vehicle was used for control. After 4 h, the mice were anesthetized with ketamine/xylazine intraperitoneally, and were bled by transection of the inferior vena cava to reduce hemorrhage into the lungs. Bronchoalveolar lavage (BAL) was performed by the intratracheal instillation of 1 mL of PBS, reinfused three times into the lungs. The BALF (BALF) was centrifuged, and the cell-free supernatant was frozen at -70°C . The cells were resuspended in 0.5 mL of 0.4 mL of PBS and 0.1 mL of 0.4% Trypan blue and were counted with a hemocytometer. In a second series of experiments, mice were challenged with 400 μ g/kg (10 μ g/mouse) or 40 μ g/kg (1 μ g/mouse) of recombinant flagellin. BAL was performed after 1, 4, 12, and 24 h in 4–5 mice/group. In a third series of experiments, mice received 4 μ g/kg (100 ng/mouse) or 400 ng/kg (10 ng/mouse) of flagellin, and BALF was obtained after 4 h.

The BALFs were assayed for the concentrations of various cytokines and chemokines (tumor necrosis factor- α [TNF- α], IL-1 β , IL-6, macrophage inflammatory protein 1 α [MIP1 α], and MIP-2) using commercial ELISAs. The protein content was measured by the Bradford assay. Myeloperoxidase (MPO) activity, an indicator of neutrophil accumulation, was directly measured in the BALF by mixing an aliquot of BALF (20 μ L) with 1.6 mM tetra-methyl-benzidine and 1 mM hydrogen peroxide. Activity was measured as the change in absorbance at 650 nm, and was expressed as milliunits of MPO activity/mL BALF. The concentrations of nitrate and nitrite, stable end-products of nitric oxide (NO), were measured by the modified Griess reaction (9).

In an additional study, isolation of lung nuclear proteins and NF- κ B electromobility shift assay was performed as follows. Mice challenged with 10 μ g of flagellin ($n = 12$) were killed after 1, 4, 12, or 24 h (three mice per time-point). Mice injected with vehicle served as control. Lungs were harvested and frozen in liquid nitrogen and stored at -70°C . For the isolation of nuclear proteins, lungs (100 mg) were homogenized in ice-cold Tris-buffer saline, pH 7.5. After centrifugation, the cell pellet was resuspended in 0.8 mL of ice-cold hypotonic buffer (HEPES 10 mM, pH 7.9, EDTA 0.1 mM, EGTA 0.1 mM, KCl 10 mM, dithiothreitol [DTT] 1 mM, phenylmethyl sulfonide [PMSF] 0.5 mM, and 1 μ M each of aprotinin, leupeptin, and pepstatin). After the addition of 50 μ L of Nonidet-P40, the tubes were vortexed for 30 s and centrifuged. After a single wash in hypotonic buffer without Nonidet-P40, the pellet was resuspended in ice-cold hypertonic buffer (HEPES 20 mM, pH 7.9, EDTA 1 mM, EGTA 1 mM, NaCl 0.4 M, DTT 1 mM, PMSF 1 mM, and 1 μ M of each protease inhibitor). The tubes were then incubated for 30 min at 4°C with frequent vortexing. After centrifugation, the supernatants (containing the nuclear proteins) were collected and used for electromobility shift analysis. An NF- κ B oligonucleotide probe (5'-AGTTGAGGGGACTTTCCAGG-3'; Promega, Madison, WI) was labeled with γ - ^{32}P ATP using T4 polynucleotide kinase (Promega) and was purified on a Bio-Spin Chromatography column (Bio-Rad, Hercules, CA). Nuclear proteins (2.5 μ g) were preincubated with EMSA buffer (12.5 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1.25 mM MgCl_2 , 0.5 mM EDTA, 0.5 mM DTT, 50 ng/mL poly[d(I-C)], and 4% glycerol) for 10 min at room temperature, followed by the addition of the radiolabeled probe for 15 min. Samples were resolved on a non-denaturing polyacrylamide gel consisting of 6% acrylamide and were run in 0.5 \times TBE (1 mM EDTA, 45 mM boric acid, and 45 mM Tris-HCl) at constant voltage (250 V) for 1 h. Gels were transferred to Whatman 3M paper, dried under vacuum at 80°C for 1 h, and exposed to photographic film at -70°C with an intensifying screen. Because this assay was conducted in whole-lung homogenates, it is likely that vascular and nonvascular elements, as well as circulating blood cells, all contribute to the findings observed.

Human studies

The Ethics Committee of the Northern State Medical University (Arkhangelsk, Russia) approved the study. Written informed consent was obtained from the patient or from the next of kin of the patient. Sixteen patients (mean age 58.0 ± 4.7 years) admitted to the Intensive Care Unit of the City Hospital #1 of Arkhangelsk from 1998 to 1999 were enrolled. The patients were diagnosed with severe sepsis and septic shock according to the modified criteria of the American College of Chest

Physicians and the Society of Critical Care Medicine consensus conference (17, 18). Septic shock was defined as severe sepsis associated with a mean arterial pressure (MAP) of <70 mmHg for at least 30 min despite fluid resuscitation, or with requirement for infusion of dopamine >5 mg/kg/min and/or norepinephrine >0.05 mg/kg/min and/or epinephrine >0.05 mg/kg/min, for at least 30 min to maintain MAP between 70 and 90 mmHg (18). Patients were eligible to enter the study if they received mechanical ventilation, had the pulmonary artery catheters in place, and the duration of severe sepsis was <72 h, and of septic shock was <24 h.

Bacterial and fungal cultures (blood, respiratory secretion, urine, peritoneal fluid, and wound discharge) were routinely assayed. Bacterial infections were treated with selective antibiotics, with preference given to third-generation cephalosporins, quinolones, or carbapenems. The concomitant therapy included substitution of fluids, mechanical ventilation, anticoagulants, sedation, nutritional support, and hemodialysis in cases of acute renal failure. If necessary, all patients received surgery and advanced cardiac life support.

Hemodynamic and respiratory parameters, gas exchange, and organ function variables were assessed as previously described (18). The Simplified Acute Physiology Score (SAPS) II (19), the Sepsis-Related Organ Failure Assessment (SOFA) score (20), and the Lung Injury Score (LIS) (21) were determined. Duration of septic shock, mechanical ventilation, hospital and ICU stays, and survival rate at day 28 were recorded as well. Plasma levels of flagellin were measured using ELISA as previously described (9).

Statistical analysis

All the results are presented as means \pm SEM. Parameters were statistically compared by analysis of variance, followed by Bonferroni adjustment. Linear regression analysis was used to evaluate the relationship between plasma flagellin concentrations and organ dysfunction scores as well as hemodynamic, respiratory, and clinical parameters. Statistical significance was assigned to a P value <0.05 .

RESULTS

Flagellin induces the expression of IL-8 and ICAM-1 in human alveolar epithelial cells

The recruitment of inflammatory cells into the lung is a multistep process, involving adhesion to the endothelium, transendothelial migration, and trafficking into the lung interstitium (22). Airway epithelial cells, which can express adhesion molecules and release chemoattractant factors such as chemokines, play an important role in this process (23). The stimulation of human lung epithelial (A549) cells with flagellin resulted in a massive production of the neutrophil chemokine IL-8 (Fig. 1a), as well as the expression of the adhesion molecule ICAM-1 (Fig. 1b). The effect of flagellin on IL-8 synthesis was extraordinarily potent, as indicated by a half-maximal stimulation obtained at a concentration of 2×10^{-14} M (1 pg/mL), and a maximal effect at 2×10^{-10} M. In addition, the threshold concentration of flagellin inducing ICAM-1 expression was 2×10^{-13} M (10 pg/mL).

The systemic administration of flagellin induces severe acute pulmonary inflammation

We next determined whether flagellin administered *in vivo* is able to induce an inflammatory response in the lung, and how this response differs from that induced by LPS. In a first series of experiments, we selected relatively high doses of LPS and flagellin (4 mg/kg, 100 μ g/mouse) that correspond to the usual doses of LPS used in murine models (24, 25). In these conditions, flagellin induced a severe form of acute lung inflammation, indicated by a massive release of TNF- α (Fig. 2a), IL-1 β (Fig. 2b), and of the chemokines MIP-1 α (Fig. 2c) and MIP-2 (Fig. 2d). These effects were associated with the recruitment of leukocytes—most notably polymorphonuclear granulocytes—into the alveolar spaces, evidenced by increased myeloperoxidase activity (Fig. 3, a and b). Also, the development of significant lung hyperpermeability was observed, as indicated by an

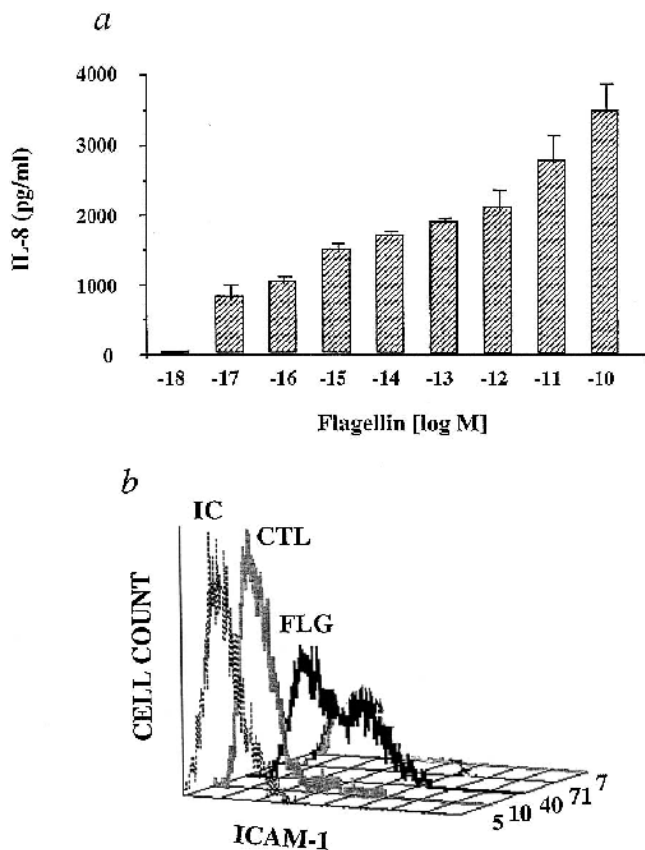


FIG. 1. Flagellin induces the production of IL-8 and the expression of ICAM-1 by human alveolar epithelial (A549) cells. The column data represent the means \pm SEM of IL-8 concentrations measured in the culture medium from 5 to 6 wells/condition (a). Half-maximal stimulation of IL-8 production was obtained at concentrations of flagellin of 2×10^{-14} M. Incubation of the cells in the presence of LPS did not induce IL-8 production (not shown). The histograms display fluorescence intensity values reflecting ICAM-1 expression (b). Cells were either left untreated (CTL) or stimulated with 1 ng/mL flagellin (FLG). ICAM-1 expression was determined by flow cytometry using phycoerythrin-labeled anti-human ICAM-1 antibody. A sample was also stained with an isotype-matched phycoerythrin-labeled indifferent antibody as negative control (IC). Data shown are representative of three independent experiments.

increased protein content in the BALF (Fig. 3c). Flagellin also upregulated the pulmonary production of NO (Fig. 3d). In agreement with previous studies (3, 4, 26), LPS also produced inflammatory changes in the lung, but the magnitude of its effects was significantly smaller than seen in response to flagellin. In marked contrast to the phenomena observed in the lung, the systemic release of proinflammatory mediators was markedly larger after LPS than after flagellin, as indicated by the appearance of TNF- α (Fig. 2, insert) and IL-6 (not shown) in the plasma of these animals.

In further experiments, the time course of the proinflammatory effects of flagellin was evaluated using smaller doses of flagellin (1 and 10 μ g/mouse). These doses still produced a marked increase in the pulmonary production of TNF- α , IL-6, MIP-1 α , and MIP-2 (Fig. 4 a-d), starting at 1 h, and reaching a peak after 4 h, followed by a progressive decrease over the next 20 h. Significant levels of MIP-1 α were still measurable after 24 h in mice challenged with 10 μ g of flagellin. Additional dose-response experiments indicated that flagellin retained its proinflammatory potential even when administered

at doses as low as 10 ng/mouse, as indicated by significant detectable levels of MIP-1 α and MIP-2 at 4 h (Fig. 5, a and b).

Flagellin activates NF- κ B nuclear translocation in vivo

The recognition of flagellin triggers the nuclear mobilization of NF- κ B (9, 12). Consistently, with this mechanism, we found an early activation of NF- κ B in the lung after the injection of flagellin (Fig. 6).

Flagellin circulates in patients with sepsis in vivo

In the patients with sepsis, mean \pm SEM values for LIS were 1.6 ± 0.2 points, SAPS II was 56.4 ± 4.5 points, SOFA score was 10.0 ± 0.8 points, PaO₂/FiO₂ was 196 ± 27 mmHg, MAP was 78.5 ± 3.9 mmHg, cardiac index was 4.6 ± 0.4 l/min/m², systemic vascular resistance index was 1335 ± 95 dyne/s/cm⁵, mean pulmonary arterial pressure was 20.4 ± 1.7 mmHg, pulmonary vascular resistance index was 216 ± 30 dyne/s/cm⁵, duration of mechanical ventilation was 78.6 ± 13.0 h, and mortality at day 28 was 50%.

In healthy human volunteers, plasma flagellin levels were below the detection limit of our ELISA (<0.1 ng/mL). In 16 patients with sepsis, mean plasma flagellin levels amounted to 7.06 ± 0.14 ng/mL. We observed a statistically significant correlation between plasma flagellin concentrations and lung injury score ($P < 0.05$), plasma flagellin concentrations, and alveolar-arterial oxygen difference (AaPO₂), as well as between plasma flagellin concentrations and the duration of septic shock (Fig. 7). There was also a tendency for a correlation ($P = 0.07$) between the plasma flagellin concentrations and the degree of leukopenia (Fig. 7). In contrast, no correlation was noted between plasma flagellin concentrations and SAPS II, SOFA score, mean arterial blood pressure, systemic vascular resistance index, or the degree of hypoxemia, as defined by the PaO₂/FiO₂ ratio (not shown). Clearly, the human component of the present study was conducted on a small sample size, and further studies are required to confirm the present preliminary findings.

DISCUSSION

It is generally accepted that LPS released from the bacterial cell wall is a key trigger of the inflammatory response to gram-negative pathogens (27). However, the many differences distinguishing bacterial and LPS-induced inflammation and shock (28), as well as the failure of anti-LPS antibodies to increase survival from sepsis in pivotal clinical trials (29) suggest that additional microbial stimuli distinct from LPS may contribute to inflammatory injury in gram-negative sepsis. In this respect, the recent identification of the flagellin/TLR5 signaling (12) pathway has established a novel, potentially important concept in the interactions of eukaryotic organisms with environmental pathogens.

In the present study, we identify flagellin as a potent trigger of acute inflammatory processes in the lung. *In vitro*, flagellin induced the production of IL-8 and the expression of ICAM-1, two mediators playing a critical role in the recruitment and activation of polymorphonuclear cells (30). The importance of IL-8 in the pathophysiology of ARDS has been emphasized in several clinical studies (31, 32), showing a direct correlation

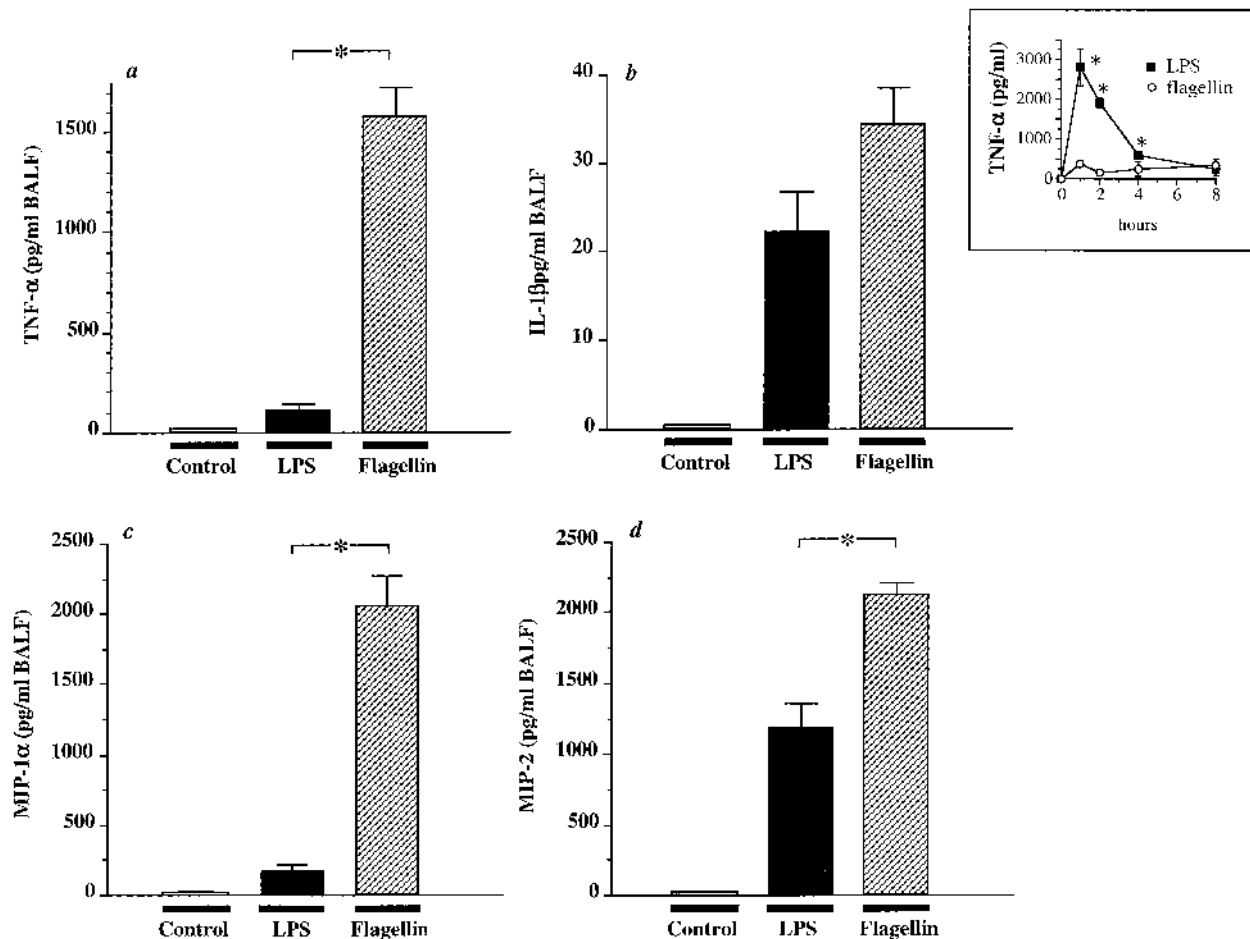


FIG. 2. **Flagellin is a more potent stimulus than LPS for the release of proinflammatory cytokines in the lung.** Mice were challenged with 100 μ g of *Salmonella muenchen* flagellin (n = 8) or LPS (n = 7) and the levels of TNF- α (a), IL-1 β (b), MIP-1 α (c), and MIP-2 (d) were determined in bronchoalveolar lavage fluid after 4 h. Similar measures were made in a group of normal mice (control, n = 5). In separate experiments, the appearance of TNF- α in plasma after LPS or flagellin (insert) was determined in 7 to 8 mice per group at the indicated time-points. Flagellin induced the formation of significantly larger amounts of cytokines than did LPS in the lung. In contrast, LPS was a stronger stimulus for the systemic release of TNF- α . * $P < 0.05$ indicates significant difference between mice receiving flagellin or LPS.

between the levels of IL-8 in BAL, the severity of ARDS, and mortality. A particularly striking observation was the extraordinary potency of flagellin—it was biologically active at concentrations in the femtomolar range. This finding supports the proposal that flagellin may represent a major eliciting bacterial stimulus for the recruitment of immune effector cells in the lung.

The *in vitro* observations are substantiated by our *in vivo* findings demonstrating that flagellin induced a severe form of lung inflammation characterized by the acute release of proinflammatory cytokines and the upregulation of NO production. These events were associated with alveolar-capillary damage, indicated by a marked degree of protein leak into the alveolar spaces. A particularly interesting finding was the massive production of the chemokines MIP-1 α and MIP-2, reminiscent of the *in vitro* effects of flagellin on IL-8. Furthermore, MIP-1 α and MIP-2 were still expressed when minute doses (10 ng) of flagellin were administered. Chemokines play a central role in the pathophysiology of ARDS by directing the migration of inflammatory cells through chemotactic gradients (22). In rodents, where no homolog of the human IL-8 exists, the CXC chemokines MIP-2 and KC, and the CC chemokine

MIP-1 α serve as powerful granulocyte-recruiting chemokines (33, 34). Indeed, we observed an early accumulation of leukocytes, notably neutrophils, after flagellin administration. Therefore, our findings indicate that the upregulation of mechanisms regulating leukocyte trafficking is a critical consequence of flagellin signaling.

In line with previous data obtained *in vitro* (12), we found that the relevant mechanism underlying the proinflammatory actions of flagellin was the early pulmonary activation of NF- κ B. It is important to note here that Northern blot analysis reveals that TLR5 is expressed predominantly in liver and lung, with low-level expression in most other tissues examined (35). The preferential expression of this receptor in the pulmonary tissue may be consistent with the hypothesis that signaling through TLR5 is highly compartmentalized. Such compartmentalization would make sense when one considers the considerable potency of flagellin, for such a mechanism would help the resolution of localized infection while avoiding overwhelming systemic inflammatory responses. This type of difference between local and systemic inflammatory pathologies induced by flagellin may mechanistically explain some of the local inflammatory pathologies associated with systemic

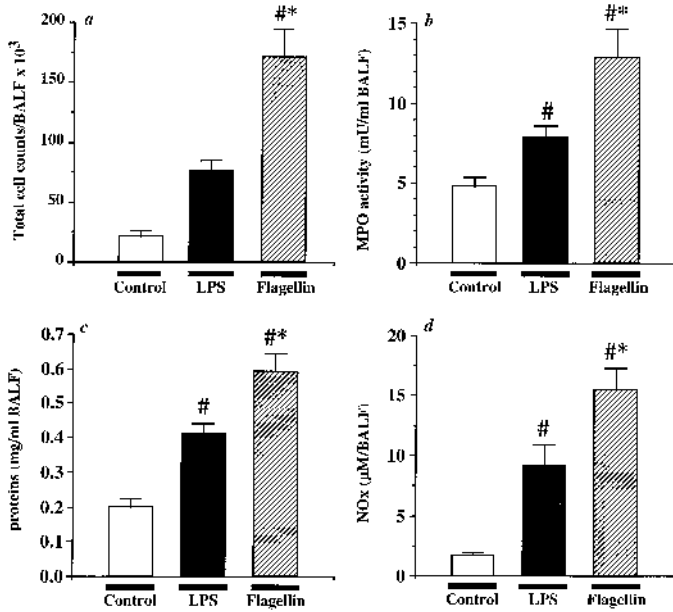


FIG. 3. Flagellin stimulates the accumulation of neutrophils, the development of high permeability edema, and the production of NO in the lung. Data are means \pm SEM of values measured in normal animals (control, n = 5), as well as at 4 h after the injection of 100 μ g of *Salmonella muenchen* flagellin (n = 8) or LPS (n = 7). Flagellin induced an important recruitment of leukocytes (a), notably polymorphonuclear cells (b, MPO activity) in the alveolar spaces. This was associated with a marked degree of protein leak (c) indicative of lung hyperpermeability, as well as with an increased pulmonary formation of NO. *P < 0.05 indicates significant differences compared with control. *P < 0.05 indicates significant difference between mice receiving flagellin or LPS.

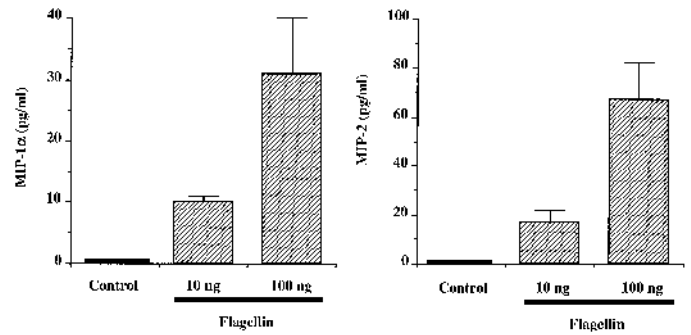


FIG. 5. Minute quantities of flagellin are sufficient to trigger the production of chemokines in the lung. Mice received *Salmonella muenchen* flagellin at a dose of 10 (n = 4) or 100 ng (n = 6). The chemokines MIP-1 α and MIP-2 were measured in the bronchoalveolar lavage fluid 4 h later. Normal animals (control, n = 5) did not express these chemokines. Flagellin at the doses indicated induced a small but significant release of MIP-1 α and MIP-2. *P < 0.05 indicates significant differences (P 0.05) vs. control.

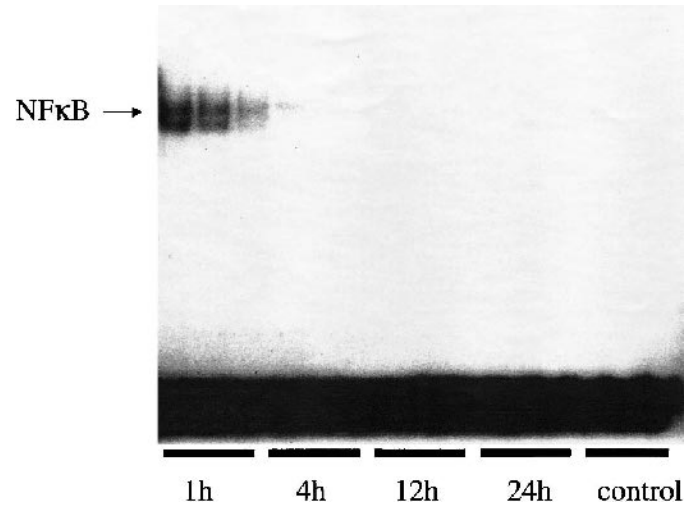


FIG. 6. Flagellin induces the nuclear translocation of NF- κ B in the lung. EMSA studies in lung nuclear extracts from mice demonstrate that flagellin elicited the nuclear mobilization of NF- κ B 1 h (but not 12 or 24 h) after its administration (n = 3 animals at each timepoint).

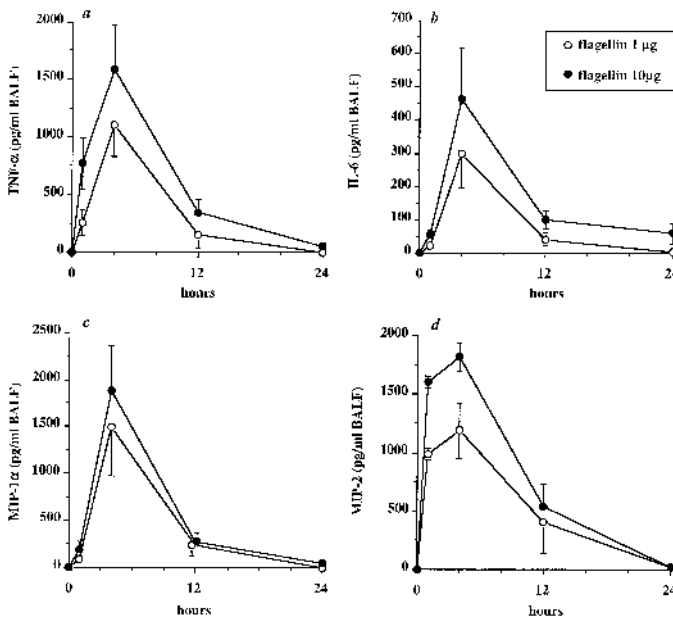


FIG. 4. The release of proinflammatory cytokines in the lung is an early consequence of flagellin administration. Each data point represents the mean \pm SEM of n = 4–5 mice challenged with *Salmonella muenchen* flagellin at a dose of 1 or 10 μ g. The appearance of TNF- α (a), IL-6 (b), MIP-1 α (c), and MIP-2 (d) in bronchoalveolar lavage followed a similar time course, starting within 1 h of flagellin administration, peaking after 4 h, and returning toward baseline over the next 20 h. A significant amount of IL-6 was still measurable after 24 h in mice receiving 10 μ g of flagellin.

inflammatory conditions. It is noteworthy that monocytes/macrophages also express the TLR5 receptor and respond to flagellin. Further studies are required to determine the relative contribution of parenchymal cells, nonprofessional immune cells (e.g., lung epithelium) versus immune cells to the flagellin-induced immune/inflammatory responses.

It is noteworthy that some of the flagellin-induced responses developed very rapidly (e.g., the tachypnoe seen within minutes after injection). Although the mechanism of this response remains to be determined, it is unlikely that these responses are related to the TLR5-NF- κ B-cytokine/chemokine axis. It is noteworthy that bacterial LPS (endotoxin) also exerts some rapid physiological responses, which are mediated through the rapid production of lipid mediators (e.g., thromboxane or platelet-activating factor) and/or NO (36–39). Whether flagellin induces the rapid mobilization of the same (or other) early mediators remains to be explored.

We have previously observed that free flagellin, at up to several hundred ng/mL, is detectable in the plasma of rats with lethal gram-negative bacteria induced peritonitis (9). The

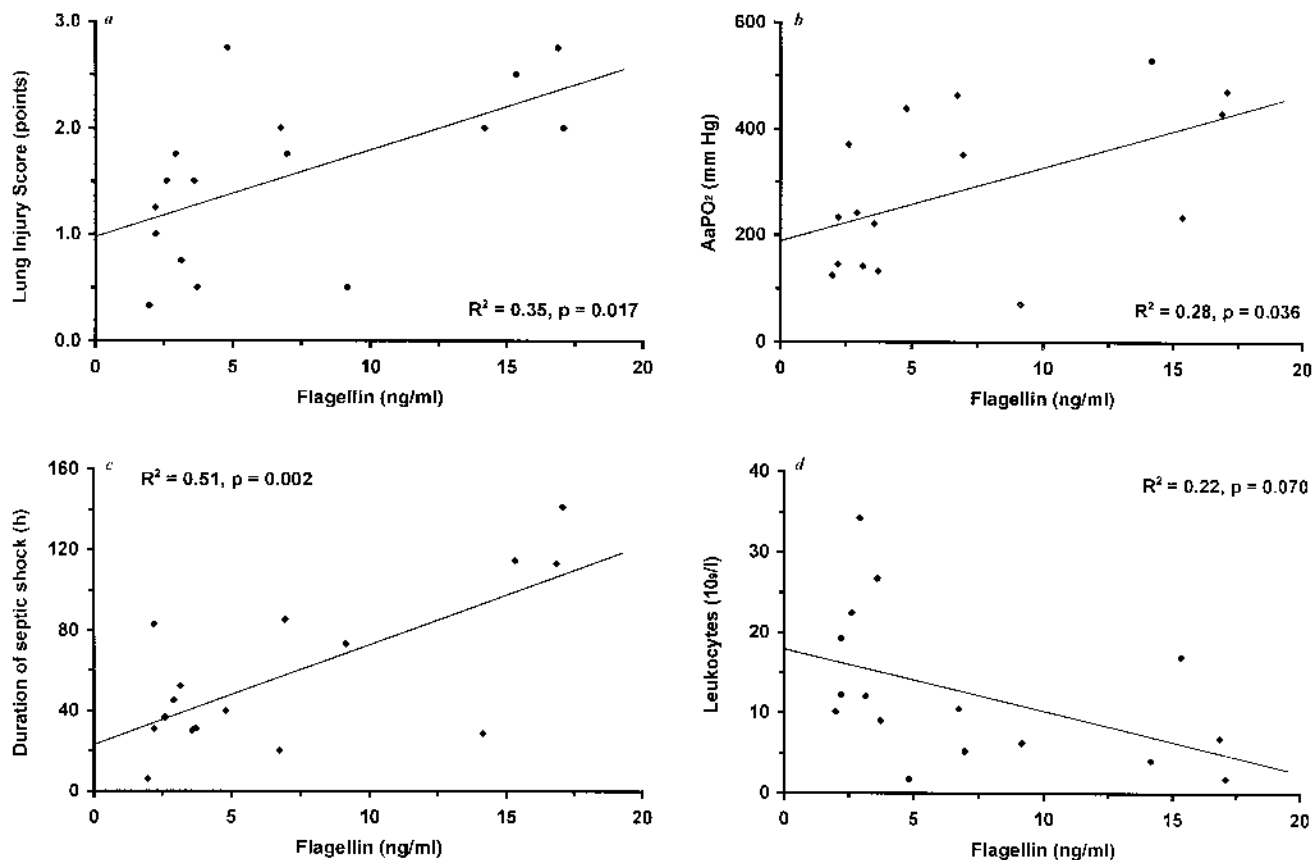


FIG. 7. Elevated circulating levels of flagellin in human sepsis. Correlation between plasma flagellin concentrations and lung injury score (a), plasma flagellin concentrations and alveolar-arterial oxygen difference (AaPO₂, b), between plasma flagellin concentrations and the duration of septic shock (c), and tendency for a correlation ($P = 0.07$) between plasma flagellin concentrations and the degree of leukopenia (d). Data are presented from $n = 16$ patients with sepsis.

current study confirms the presence of significant levels of circulating free flagellin in the blood of patients with sepsis. These levels (in the range of 2–20 ng/mL) are well within the levels required for induction of proinflammatory responses in the human epithelial cell line *in vitro* and in mice *in vivo*. The observation that circulating flagellin levels show some degree of correlation with the degree of pulmonary injury in patients, coupled with the ability of flagellin to induce pulmonary inflammation and ARDS in mice, suggests that flagellin may be a candidate for the initiation of ARDS in severe human sepsis.

The human studies demonstrated that flagellin levels are elevated in sepsis, and some parameters show positive correlation with the plasma levels of this mediator (e.g., lung injury score and alveolar-arterial oxygen difference), whereas other parameters (e.g., severity of illness, mean arterial pressure, and hypoxemia) do not. Clearly, further studies, conducted in larger patient populations, are required to delineate the correlation between circulating flagellin levels and various clinical parameters of sepsis. Also, careful microbiological examinations need to be conducted to examine which subpopulation of the septic patients demonstrates hematological cultures that are positive to microorganisms bearing flagellin.

By promoting lung injury, flagellin is likely to exacerbate and prolong the severity and the duration of sepsis. In this case, neutralization of the actions of circulating flagellin may be a

novel approach for the prevention or treatment of ARDS associated with gram-negative infections.

ACKNOWLEDGMENTS

This work was supported, in part, by the National Institutes of Health (Grant nos. R01GM57407 and R43AI48249 to A.L.S. and no. R29GM54773 to C.S.). L.L. was supported by a grant from the ADUMED Foundation (Switzerland). F.G.S. was supported by a fellowship from FAPESP (Brazil). P.P. is on leave from the Department of Pharmacology and Pharmacotherapy, Semmelweis University Medical School, Budapest, Hungary.

REFERENCES

1. Kollef MH, Schuster DP: The acute respiratory distress syndrome. *N Engl J Med* 332:27–37, 1995.
2. Horn DL, Morrison DC, Opal SM, Silverstein R, Visvanathan K, Zabriskie JB: What are the microbial components implicated in the pathogenesis of sepsis? Report on a symposium. *Clin Infect Dis* 31:851–858, 2000.
3. Harrod KS, Mounday AD, Whitsett JA: Adenoviral E3-14.7K protein in LPS-induced lung inflammation. *Am J Physiol Lung Cell Mol Physiol* 278:L631–L639, 2000.
4. Borron P, McIntosh JC, Korfhagen TR, Whitsett JA, Taylor J, Wright JR, *et al.*: Surfactant-associated protein A inhibits LPS-induced cytokine and nitric oxide production *in vivo*. *Am J Physiol Lung Cell Mol Physiol* 278:L840–L847, 2000.
5. Samatey FA, Imada K, Nagashima S, Vonderviszt F, Kumasaka T, Yamamoto M, Namba K: Structure of the bacterial flagellar protofilament and implications for a switch for supercoiling. *Nature* 410:331–337, 2001.
6. McDermott PF, Ciacci-Woolwine F, Snipes JA, Mizel SB: High-affinity interaction between gram-negative flagellin and a cell surface polypeptide results in human monocyte activation. *Infect Immun* 68:5525–5529, 2000.
7. Ciacci-Woolwine F, Blomfield IC, Richardson SH, Mizel SB: *Salmonella*

- flagellin induces tumor necrosis factor- α in a human promonocytic cell line. *Infect Immun* 66:1127–1134, 1998.
8. Moors MA, Li L, Mizel SB: Activation of interleukin-1 receptor-associated kinase by gram-negative flagellin. *Infect Immun* 69:4424–4429, 2001.
 9. Eaves-Pyles T, Murthy K, Liaudet L, Virag L, Ross G, Soriano FG, Szabo C, Salzman AL: Flagellin, a novel mediator of *Salmonella*-induced epithelial activation and systemic inflammation: κ B- α degradation, induction of nitric oxide synthase, induction of proinflammatory mediators, and cardiovascular dysfunction. *J Immunol* 166:1248–1260, 2001.
 10. Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL: *Salmonella typhimurium* translocates flagellin across intestinal epithelia, inducing a proinflammatory response. *J Clin Invest* 107:99–109, 2001.
 11. Steiner TS, Nataro JP, Potec-Smith CE, Smith JA, Guerrant RL: Enteroaggregative *Escherichia coli* expresses a novel flagellin that causes IL-8 release from intestinal epithelial cells. *J Clin Invest* 105:1769–1777, 2000.
 12. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, Eng JK, Akira S, Underhill DM, Aderem A: The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410:1099–1103, 2001.
 13. Aderem A, Ulevitch RJ: Toll-like receptors in the induction of the innate immune response. *Nature* 406:782–787, 2000.
 14. Poltorak A, He X, Smirnova I, Liu MY, Huffel CV, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B: Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282:2085–2088, 1998.
 15. Chow JC, Young DW, Golenbock DT, Christ W, Gusovsky F: Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem* 274:10689–10692, 1999.
 16. Komoriya K, Shibano N, Higano T, Azuma N, Yamaguchi S, Aizawa SI: Flagellar proteins and type III-exported virulence factors are the predominant proteins secreted into the culture media of *Salmonella typhimurium*. *Mol Microbiol* 34:767–779, 1999.
 17. American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. *Crit Care Med* 20:864–874, 1992.
 18. Kirov MY, Evgenov OV, Evgenov NV, Egorina EM, Sovershaev MA, Sveinbjornsson B, Nedashkovsky EV, Bjertnaes LJ: Infusion of methylene blue in human septic shock: a pilot, controlled, randomized study. *Crit Care Med* 29:1860–1867, 2001.
 19. Le Gall JR, Lemeshow S, Saulnier F: A new Simplified Acute Physiology Score (SAPS II) based on a European/North American multicenter study. *J Am Med Assoc* 270:2957–2963, 1993.
 20. Vincent JL: The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine. *Intensive Care Med* 22:707–710, 1996.
 21. Murray JF, Matthay MA, Luce JM, Flick MR: An expanded definition of the adult respiratory distress syndrome. *Am Rev Respir Dis* 138:720–723, 1999.
 22. Strieter RM, Kunkel SL, Keane MP, Standiford TJ: Chemokines in lung injury: Thomas A. Neff Lecture. *Chest* 116:103S–110S, 1999.
 23. Sauty A, Dziejman M, Taha RA, Iarossi AS, Neote K, Garcia-Zepeda EA, Hamid Q, Luster AD: The T cell-specific CXC chemokines IP-10, Mig, and I-TAC are expressed by activated human bronchial epithelial cells. *J Immunol* 162:3549–3558, 1999.
 24. Liaudet L, Rosselet A, Schaller MD, Markert M, Perret C, Feihl F: Nonselective versus selective inhibition of inducible nitric oxide synthase in experimental endotoxic shock. *J Infect Dis* 177:127–132, 1998.
 25. Wray GM, Millar CG, Hinds CJ, Thiemermann C: Selective inhibition of the activity of inducible nitric oxide synthase prevents the circulatory failure, but not the organ injury/dysfunction, caused by endotoxin. *Shock* 9:329–335, 1998.
 26. Rabinovici R, Bugelski PJ, Esser KM, Hillegass LM, Vernick J, Feuerstein G: ARDS-like lung injury produced by endotoxin in platelet-activating factor-primed rats. *J Appl Physiol* 74:1791–1802, 1993.
 27. Henderson B, Poole S, Wilson M: Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. *Microbiol Rev* 60:316–341, 1996.
 28. Glauser MP, Heumann D, Baumgartner JD, Cohen J: Pathogenesis and potential strategies for prevention and treatment of septic shock: an update. *Clin Infect Dis* 18:S205–S216, 1994.
 29. Bone RC, Balk RA, Fein AM, Perl TM, Wenzel RP, Reines HD, Quenzer RW, Iberti TJ, Macintyre N, Schein RM: A second large controlled clinical study of E5, a monoclonal antibody to endotoxin: results of a prospective, multicenter, randomized, controlled trial. The E5 Sepsis Study Group. *Crit Care Med* 23:994–1006, 1995.
 30. Martin TR: Lung cytokines and ARDS: Roger S. Mitchell Lecture. *Chest* 116:2S–8S, 1999.
 31. Donnelly SC, Strieter RM, Kunkel SL, Walz A, Robertson CR, Carter DC, Grant IS, Pollok AJ, Haslett C: Interleukin-8 and development of adult respiratory distress syndrome in at-risk patient groups. *Lancet* 341:643–647, 1993.
 32. Chollet-Martin S, Montravers P, Gibert C, Elbim C, Desmonts JM, Fagon JY, Gougerot-Pocidallo MA: High levels of interleukin-8 in the blood and alveolar spaces of patients with pneumonia and adult respiratory distress syndrome. *Infect Immun* 61:4553–4559, 1993.
 33. Standiford TJ, Kunkel SL, Lukacs NW, Greenberger MJ, Danforth JM, Kunkel RG, Strieter RM: Macrophage inflammatory protein-1 α mediates lung leukocyte recruitment, lung capillary leak, and early mortality in murine endotoxemia. *J Immunol* 155:1515–1524, 1995.
 34. Gerard C, Frossard JL, Bhatia M, Saluja A, Gerard NP, Lu B, Steer M: Targeted disruption of the beta-chemokine receptor CCR1 protects against pancreatitis-associated lung injury. *J Clin Invest* 100:2022–2027, 1997.
 35. Sebastiani G, Leveque G, Lariviere L, Laroche L, Skamene E, Gros P, Malo D: Cloning and characterization of the murine toll-like receptor 5 (Tlr5) gene: sequence and mRNA expression studies in *Salmonella*-susceptible MOLF/Ei mice. *Genomics* 64:230–240, 2000.
 36. Szabo C, Mitchell JA, Thiemermann C, Vane JR: Nitric oxide-mediated hyporeactivity to noradrenaline precedes the induction of nitric oxide synthase in endotoxin shock. *Br J Pharmacol* 108:786–792, 1993.
 37. Kruse-Elliott KT, Albert DH, Summers JB, Carter GW, Zimmerman JJ, Grossman JE: Attenuation of endotoxin-induced pathophysiology by a new potent PAF receptor antagonist. *Shock* 5:265–273, 1996.
 38. Kilbourn RG, Szabo C, Traber DL: Beneficial versus detrimental effects of nitric oxide synthase inhibitors in circulatory shock: lessons learned from experimental and clinical studies. *Shock* 7:235–246, 1997.
 39. Wolfard A, Kaszaki J, Szabo C, Szalay L, Nagy S, Boros M: Prevention of early myocardial depression in hyperdynamic endotoxemia in dogs. *Shock* 13:46–51, 2000.

