Mechanisms of acute inflammatory lung injury induced by abdominal sepsis

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Abstract

Sequestration of neutrophils and release of histotoxic mediators are considered important for the development of pathologic alterations of the lung defined as adult respiratory distress syndrome. Mechanisms of inflammatory lung injury caused by abdominal sepsis were investigated using the colon ascendens stent peritonitis (CASP) model that closely mimics the human disease. In the CASP model, a continuous leakage of intraluminal bacteria into the peritoneal cavity is induced by implantation of a stent in the ascending colon, generating a septic focus. In contrast to the cecal ligation and puncture model of peritonitis, survival of mice following CASP surgery is dependent on IFN-γ, but independent of tumor necrosis factor (TNF). Here we show that the systemic inflammation induced by CASP surgery results in a rapid and profound increase of lung vascular permeability that was associated with the activation and recruitment of neutrophils to the lung. Activation of circulating granulocytes was characterized by increased production of serine proteinases and reactive oxygen metabolites, as well as elevated expression of cell surface Mac-1. Expression of MIP-2, KC, MIP-1α and E-selectin mRNA in lung was strongly increased within 3 h following CASP surgery, whereas up-regulation of IP-10, MCP-1 and P-selectin was delayed. In contrast, induction of RANTES, LIX, ICAM-1 and VCAM-1 mRNA was weak or not detectable after CASP surgery. Importantly, recruitment of leukocytes to the lung was normal in lipopolysaccharide-resistant mice, and was not affected by antibody neutralization of TNF or the chemokines MIP-2 and KC.

Introduction

Within the inflammatory process, a delicate balance exists between the potential for tissue destruction and mechanisms of protective immune defense and tissue repair. The adult respiratory distress syndrome (ARDS) has been characterized as a disease state, in which the inflammatory balance is shifted towards tissue injury. Excessive inflammatory reactions and, in particular, neutrophil activation have been implicated in ARDS pathogenesis (1,2). Recent efforts to understand the regulation of pro- and anti-inflammatory processes in the lung have established that the ratio of IL-1β to IL-1 receptor antagonist is markedly elevated in patients with established ARDS (3,4). Moreover, low intrapulmonary concentrations of IL-10 and IL-1 receptor antagonist at the onset of ARDS are associated with high lethality (5). Increased concentrations of circulating and intrapulmonary MIF were detected in the lungs of ARDS patients with alveolar macrophages representing the main source of MIF (6). Importantly, MIF was shown to potentiate the production of tumor necrosis factor (TNF)-α and IL-8, and to antagonize the glucocorticoid-mediated inhibition of IL-8 production by alveolar cells (6). Consistent with these studies, MIF was identified as a pituitary factor that antagonizes the effects of corticosteroids and potentiates the lethal effects of endotoxin in rodents (7,8). It therefore
appears that alveolar macrophages may control the inflammatory balance in the lung by releasing factors that promote tissue destructive inflammatory processes.

The induction of chemokines and endothelial adhesion receptors is central to the process of inflammatory organ injury (9,10). Consistent with an important role of IL-8 in the pathogenesis of ARDS, the concentrations of IL-8 in bronchoalveolar lavage fluid of patients directly correlated with mortality and neutralization of IL-8 receptor-binding chemokines protected from lung vascular damage induced by intratracheal instillation of lipopolysaccharide (LPS) in rabbits and mice (11–15). Following systemic application of LPS or intrapulmonary deposition of immune complexes, acute damage to the lung was prevented by antibodies against MIP-1α (16,17). In addition, lung injury in the immune complex model was found to be dependent on E-selectin and VLA-4, while blockade of P-selectin and Mac-1 protected from injury induced by cobra venom factor (18–22). In both models, development of acute lung capillary leak was prevented by inhibition of LFA-1, ICAM-1 or L-selectin (19,20,22,23). Further evidence for a role of cell adhesion molecules in acute lung injury was provided by studies demonstrating that treatment with β2-integrin antibodies attenuates lung capillary leak during Gram-negative sepsis (24). Thus, depending on the nature of the inflammatory stimulus, specific subsets of chemokines and adhesion molecules appear to be involved in the pathogenesis of inflammatory lung injury.

Pathologic alterations of the lung defined as ARDS are most frequently observed in sepsis patients (25). In the present study, putative mechanisms of inflammatory lung injury were investigated using the CASP model of abdominal sepsis that closely mimics the human disease (26). Here, we demonstrate that the systemic inflammation induced by CASP surgery results in a profound increase of lung vascular permeability that is associated with the activation and recruitment of neutrophils to the lung, and the induction of a subset of chemokines and endothelial adhesion molecules. Lung leukocyte recruitment, however, was found to be independent of LPS responsiveness, TNF, and the chemokines MIP-2 and KC (see Abbreviations).

Methods

Animal model of abdominal sepsis

Female C57BL/6 and endotoxin-resistant C57BL/10ScN mice were used at 8–12 weeks of age. The technique used for induction of colon ascendens stent peritonitis (CASP) was performed as described recently (26). Briefly, a 14 gauge venous catheter was prepared by creating a notch at a distance of 2 mm from the orifice. One millimeter beyond the catheter was cut with a scalpel, sparing only a slim bar. Following laparotomy the colon ascendens was exteriorized an 10 mm distal of the ileocecal valve. The prepared venous catheter was punctured antimesenterically through the colonic wall into the intestinal lumen, directly proximal of a pretied knot, and fixed. A second puncture was performed directly proximal of the stent, again strictly antimesenterically. Thereafter, the inner needle was removed and the stent was cut at the prepared site. To ensure proper intraluminal positioning of the stent, stool was milked from the cecum into the colon ascendens, until a small drop appeared (1 mm in diameter). Fluid resuscitation of animals was performed by flushing 0.5 ml sterile saline solution into the peritoneal cavity before closure of the abdominal wall. For control, sham surgery was performed according to the CASP procedure except for fixation of the stent outside the ascending colon, avoiding penetration of the gut wall.

Antibodies

The rat mAb and control Ig used in this study were directed against murine CD45 (30F11.1), Ly6G/Gr-1 (RB6-8C5), Mac-1 (M1/70), L-selectin (Mel-14), Mac-3 (M3/84) and CD45R/ B220 (RA3-6B2). In addition, hamster anti-murine CD3ε antibody 145-2C11 and mouse antibody PK136 directed against the murine NK1.1 antigen were applied. Rat IgG2a (R35-95), IgG2b (R35-38), rat IgG1 (R3-34) and mouse anti-human CD25 mAb B1.49.9 were used for isotype controls. All antibodies were purchased from PharMingen (San Diego, CA) except for mouse anti-human CD25 (Immunotech, Hamburg, Germany).

For in vivo neutralization of TNF, 270 µg of mAb V1q (kindly provided Dr B. Echtenacher, Department of Pathology, University of Regensburg, Germany) was injected i.p. 3.5 h prior to induction of CASP. In separate experiments, 250 µg of rat mAb with neutralizing activity for MIP-2 or KC (R & D Systems, Minneapolis, MN) was administered i.p. 3.5 h prior to CASP surgery. For control, 250 µg rat IgG (Sigma, St Louis, MO) was injected.

Flow cytometry

Lungs from CASP or sham-operated mice were removed 3, 6 or 12 h after surgery, extensively perfused with PBS, minced, and incubated in RPMI 1640 medium containing 670 U/ml collagenase IV (Sigma) for 45 min at 37°C. Single-cell suspensions were obtained by filtration through a nylon mesh of 140 µm diameter. Treatment with collagenase IV resulted in complete digestion of lung tissue without any visible cell clumps left after filtration. For analysis of peripheral blood leukocytes, heparinized blood from CASP and sham-operated mice was collected, and erythrocytes were lysed for 5 min in 20 ml of lysis buffer (pH 7.3) containing 155 mM ammonium chloride, 15 mM sodium bicarbonate and 1 mM EDTA. Fc receptors of leukocytes were blocked by preincubation with 25% mouse serum (Sigma) for 10 min at 4°C. Subsequently, mAb were incubated for 30 min at 4°C in PBS containing 1% BSA. Reactivity of biotin-labeled antibodies was detected using streptavidin conjugated with FITC (Dianova, Hamburg, Germany) or phycoerythrin (Becton Dickinson, San Jose, CA). In each experiment the appropriate isotype-matched controls were included. After washing with PBS, cells were fixed in 1% paraformaldehyde and fluorescence was analyzed on an Epics XL cytometer (Couler, Hialeah, FL).

Oxidative burst

Production of reactive oxygen metabolites was determined in individual cells by a flow cytometry method using the fluores-
cent probe dihydrorhodamine 123 (MoBiTec, Göttingen, Germany) as described with minor modifications (27,28). Dihydrorhodamine 123 is freely permeable and emits a bright fluorescent signal after oxidation by H₂O₂ or O₂⁻ to rhodamine 123. It has been shown previously that dihydrorhodamine 123 is the most sensitive flow cytometric indicator for detection of reactive oxygen species (27). Heparinized whole blood from CASP- or sham-operated mice was collected and erythrocytes were lysed as described for flow cytometry analysis. Leukocytes were washed, resuspended in HBSS, and cells were incubated for 20 min at 37°C in the dark. Flow cytometry analysis was performed immediately afterwards on an Epics XL cytometer. Granulocytes were identified by forward and side scatter profile.

Evans blue permeability assay
Alterations of lung vascular permeability were investigated by tissue accumulation of Evans blue as previously described (17,28). Evans blue avidly binds to serum albumin and its absorptions of 620 and 740 nm, which allows for correction by a dual wavelength spectrophotometric method (29) at 60°C for 14–18 h. The supernatant was separated by centrifugation at 5000g for 30 min. The concentration of soluble FTC–casein was removed by precipitation with trichloroacetic acid and subsequent centrifugation. The supernatants were adjusted to neutral pH and the concentration of soluble FTC was determined by reading the absorbance at 492 nm.

Serine protease assay
Leukocyte content of serine proteases such as elastase, cathepsin G or uroplasminogen activator was determined by hydrolysis of fluorescein thiocarbamoyl (FTC)–casein as substrate (Calbiochem, San Diego, CA). Heparinized whole blood from CASP- or sham-operated mice was collected and erythrocytes were lysed. Leukocytes (3×10⁶) were resuspended in 100 µl HBSS, lysed by sonication and lysates were incubated with FTC–casein overnight at 37°C. Non-hydrolyzed FTC–casein was removed by precipitation with trichloroacetic acid and subsequent centrifugation. The supernatants were adjusted to neutral pH and the concentration of soluble FTC was determined by reading the absorbance at 492 nm.

Quantitation of mRNA levels by RT-PCR
Lungs were extensively perfused with PBS, removed and snap-frozen in liquid nitrogen. Total cellular RNA was extracted using the acid guanidinium thiocyanate–phenol–chloroform extraction method (30). First-strand cDNA was synthesized from 20 µg of total RNA using a mixture of oligo(dT)₁₂–₁₈ and random hexamer primers and Superscript reverse transcriptase (Gibco/BRL, Paisley, UK). The reaction was incubated for 75 min at 37°C and terminated by heating to 95°C for 5 min. Serial 1:3 dilutions of at least two independent cDNA preparations for each organ were used as template in PCR reactions. Primers for amplification of specific cDNA fragments are listed in Table 1. For control, a 471 bp fragment of murine GAPDH was amplified. Primer sequences for GAPDH were separated by introns to control for contamination with genomic DNA. The amplification reactions were allowed to proceed for 30 cycles each, consisting of a 1 min denaturation step at 94°C, a 30 s annealing step at 63°C and a 90 s extension step at 72°C. The identity of amplification products was confirmed in each case by restriction enzyme analysis. The final cDNA dilution yielding detectable amplification products was scored for each sample. To normalize mRNA levels, the cDNA titers for chemokines and adhesion molecules were divided by the GAPDH titers obtained from the same cDNA template.

Results
Acute lung capillary leak induced by abdominal sepsis
Mechanisms of inflammatory lung injury induced by abdominal sepsis were investigated using the murine CASP model (26). In this model, a septic focus is created by inserting a stent of defined diameter into the ascending colon, thereby inducing an immediate and sustained release of intestinal bacteria followed by a systemic inflammatory response syndrome, increased serum levels of TNF and endotoxin, and strong induction of TNF, IL-12 p40 and IFN-γ in lungs. Whereas in the cecal ligation and puncture model of peritonitis a protective role for TNF was demonstrated, survival of mice following
CASP surgery is critically dependent on IFN-γ, but independent of TNF (26,31).

Alterations of lung vascular permeability were analyzed to determine if abdominal sepsis induced by CASP surgery is followed by acute organ injury. CASP surgery was performed employing a suture of 14 gauge that is associated with 100% lethality within 18–48 h. Evans blue, which binds to serum albumin, was used as a tracer for the transcapillary flux of macromolecules. The amount of Evans blue in lung homogenates was quantified by a dual wavelength spectrophotometric method (29). The results are presented as mean ± SEM and are derived from six to 12 independent mice in each group. *P < 0.01 by Student’s t-test.

Inflammatory response of the lung induced by abdominal sepsis
Cell suspensions of lungs removed at various time points after CASP or sham surgery were prepared; infiltrating leukocytes were quantitated and differentiated by two-color flow cytometry. After 6 h the absolute numbers of lung leukocytes increased from $8.6 \pm 1.0 \times 10^6$ in sham-operated animals to $19.3 \pm 1.6 \times 10^6$ in CASP mice (mean ± SEM, $P < 0.001$). The results depicted in Table 2 demonstrate an intense accumulation of Mac-1$^+$ leukocytes 6 h after CASP and 55.4 ± 4.9% 12 h after CASP surgery. In contrast, recruitment of Mac-3$^+$ mononuclear phagocytes was delayed and was not detected until 6 h after CASP operation (20.7 ± 4.5% versus 8.5 ± 1.3% in control mice and 7.2 ± 1.4% in sham-operated animals). Thereafter, the fraction of mononuclear phagocytes remained elevated (27.7 ± 6.1% of all leukocytes 12 h after CASP surgery). Consistent with the recruitment of phagocytic cells to the lung, the relative numbers of NK cells as well as B and T lymphocytes decreased after CASP surgery (Table 2). Taken together, these results demonstrate that abdominal sepsis induced by CASP surgery triggers a rapid recruitment of leukocytes to the lung with an acute and predominant infiltration of granulocytes followed by the accumulation of mononuclear phagocytes.

Distinct pattern of chemokine expression in the lung induced by abdominal sepsis
To determine whether CASP-induced sepsis may affect lung chemotactic activity for leukocytes expression of CC (MIP-1α, MCP-1 and RANTES) and CXC family chemokines (MIP-2, KC, IP-10 and LIX) was examined. RNA was extracted from lungs 3 h after sham surgery or 3, 6 and 12 h following CASP operation. Following reverse transcription the final cDNA dilution yielding detectable amplification products was scored for each chemokine using the primers listed in Table 1. Chemokine mRNA levels were normalized by dividing the cDNA titer for each chemokine by the GAPDH titer obtained from the same cDNA template. Primers for GAPDH were separated by introns to control for contamination with genomic DNA. The identity of amplification products was confirmed in each case by restriction enzyme analysis.

The results presented in Figs 2 (representative cDNA titration for a single mouse of each group) and 3 (summary of data for all animals investigated) demonstrate that CC chemokines were differentially regulated following induction of abdominal sepsis. MIP-1α mRNA levels were increased by ~100-fold as early as 3 h after CASP surgery, remained elevated at 6 h and declined to intermediate levels by 12 h. In contrast, induction of MCP-1 mRNA was delayed, reaching plateau levels at 6 h after CASP surgery (Figs 2 and 3). For RANTES, lung mRNA levels after CASP surgery were comparable to those detected in sham-operated mice. When compared to lungs of sham-operated mice, 3 h after CASP surgery CXC chemokine mRNA levels were induced ~10-fold for MIP-2 and KC, and ~20-fold for IP-10 (Figs 2 and 3). While KC transcripts remained highly elevated throughout the observation period, MIP-2 mRNA levels declined at 12 h after CASP surgery and IP-10 mRNA levels transiently peaked at 6 h (Figs 2 and 3). However, despite decreased expression of MIP-2 and IP-10 12 h after induction of peritonitis, basal levels of transcripts were not reached. Up-regulation of LIX mRNA was detectable, but appeared weak when compared to MIP-2, KC and IP-10. Together, these results demonstrate rapid and intense induction of a distinct panel of CXC and CC chemokines by abdominal sepsis that correlates with leukocyte recruitment and development of inflammatory organ injury.
Table 2. Subpopulations of lung-infiltrating leukocytes induced by septic peritonitis

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<tr>
<th>Leukocyte subsets</th>
<th>Marker</th>
<th>CD45&lt;sup&gt;+&lt;/sup&gt; cells (%)</th>
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<tr>
<td></td>
<td></td>
<td>Untreated</td>
</tr>
<tr>
<td>Granulocytes, monocytes and NK cells</td>
<td>Mac-1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>26.3 ± 1.3</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>Gr-1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>9.7 ± 1.6</td>
</tr>
<tr>
<td>Mononuclear phagocytes</td>
<td>Mac-3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>8.5 ± 1.3</td>
</tr>
<tr>
<td>NK cells and T cell subset</td>
<td>NK1.1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>10.7 ± 1.1</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>CD3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>22.3 ± 4.5</td>
</tr>
<tr>
<td>B lymphocytes</td>
<td>B220&lt;sup&gt;+&lt;/sup&gt;</td>
<td>23.4 ± 2.0</td>
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<sup>a</sup>At the time points indicated lungs were removed from C57BL/6 mice subjected to CASP or sham surgery after extensive perfusion with PBS. Single-cell suspensions were prepared by collagenase digestion of lung tissue and infiltrating leukocytes were identified by two-color flow cytometry analysis. Leukocytes were defined by expression of the leukocyte common antigen CD45. Results are presented as percentage of all CD45<sup>+</sup> cells (mean ± SD) and are derived from three to eight independent animals in each group.

Fig. 2. Rapid induction of lung chemokine expression following CASP surgery. Total RNA was isolated from lungs of C57BL/6 mice 6 h after sham or CASP surgery and reverse transcribed. Serial cDNA dilutions (1:3) were used as template for PCR amplifications detecting expression of the chemokines IP-10, KC, LIX, MCP-1, MIP-1α, MIP-2 and RANTES (see Table 1). PCR reactions for GAPDH were included in each case to control for contamination with genomic DNA and to normalize mRNA levels. Results presented are derived from a single animal representative of each group.

Fig. 3. Kinetics of chemokine mRNA expression in the lungs of septic mice. Lungs of C57BL/6 mice were removed 3 h after sham (○), or 3 h (●), 6 h (▲) and 12 h (■) after CASP surgery. Expression of CC (A) and CXC (B) family chemokines was examined by RT-PCR analysis as described for Fig. 2. To normalize mRNA levels, the cDNA titer of each chemokine defined as the final dilution yielding detectable amplification products was divided by the GAPDH titer derived from the same cDNA template. Each symbol represents the result from lungs of a single mouse.

Up-regulation of endothelial selectins during septic peritonitis

We next attempted to identify endothelial adhesion molecules that may regulate leukocyte recruitment to the lung in CASP-treated mice. Expression of E-selectin, P-selectin, ICAM-1 and VCAM-1 was quantitated 3 h after sham, or 3, 6 and 12 h after CASP surgery by RT-PCR analysis. The results depicted...
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Fig. 4. Regulation of lung adhesion molecule expression following CASP surgery. Total RNA was isolated from lungs of C57BL/6 mice 6 h after sham or CASP surgery followed by reverse transcription. Serial cDNA dilutions (1:3) were used as template for PCR amplifications with primers specific for ICAM-1, VCAM-1, E-selectin, and P-selectin (see Table 1). PCR reactions detecting GAPDH were included in each case to control for contamination with genomic DNA and to normalize mRNA levels. Results presented are derived from a single animal representative of each group.

Fig. 5. Kinetics of adhesion molecule mRNA expression in the lung. Lungs of C57BL/6 mice were removed 3 h after sham (○), or 3 h (●), 6 h (▲) and 12 h (■) after CASP surgery. Adhesion molecule mRNA expression was examined by RT-PCR as described for Fig. 4. To normalize mRNA levels, the cDNA titer of each adhesion molecule defined as the final dilution yielding detectable amplification products was divided by the GAPDH titer derived from the same cDNA template. Each symbol represents the result from lungs of a single mouse.

in Figs 4 (representative cDNA titration for a single mouse of each group) and 5 (summary of data for all animals investigated) show that E-selectin and P-selectin were up-regulated as early as 3 h after CASP surgery. Elevated expression of E-selectin persisted throughout the observation period, while P-selectin mRNA levels peaked at 6 h following CASP operation. In contrast to endothelial selectins, ICAM-1 and VCAM-1 mRNA levels were comparable in sham-operated and CASP-treated mice (Figs 4 and 5). However, compared to selectins, constitutive expression of ICAM-1 and VCAM-1 in the lung was at least 10-fold higher (Fig. 5). These results therefore indicate that abdominal sepsis causes up-regulation of transcripts for endothelial selectins, but not of Ig family adhesion molecules in the lung.

Abdominal sepsis results in systemic activation of granulocytes

Neutrophil priming by systemic stimuli and activation by local inflammatory mediators may contribute to the release of toxic products such as proteases and reactive oxygen metabolites resulting in inflammatory organ injury (1). To analyze whether CASP-induced abdominal sepsis may result in systemic activation of granulocytes, surface expression of Mac-1 and L-selectin was examined. The results depicted in Fig. 6A clearly demonstrate that expression of Mac-1 was induced on circulating granulocytes 6 h after CASP surgery. Furthermore, cell surface expression of L-selectin was down-regulated in four out of six CASP-operated C57BL/6 mice (data not shown). These results therefore indicate that induction of abdominal sepsis results in changes of cell surface receptor expression in vivo that are consistent with granulocyte activation.

Additional experiments were performed to examine leukocyte production of histotoxic metabolites such as proteases and oxidants. Peripheral blood granulocytes were isolated from mice 6 h after CASP surgery or sham treatment and spontaneous generation of reactive oxygen metabolites was determined by oxidation of the fluorescent probe dihydrodihorhodamine 123. The results presented in Fig. 6B show that the spontaneous release of reactive oxygen metabolites by circulating granulocytes was strongly enhanced following CASP surgery. To analyze regulation of cellular proteinases, hydrolysis of the serine proteinase substrate FTC–casein was quantitated by a spectrophotometric method. The results in Fig. 6C clearly demonstrate that when compared to sham treatment the content of serine proteinases in leukocyte lysates was significantly increased 6 h after CASP surgery (P < 0.001). Together, these results indicate that abdominal sepsis results in systemic granulocyte activation that is characterized by altered expression of surface receptors and elevated basal production of oxidants and serine proteinases. Recruitment of pre-activated neutrophils may be critical for the development of inflammatory lung injury during abdominal sepsis.

Leukocyte recruitment to the lungs occurs in the absence of TNF, endotoxin, and the chemokines MIP-2 and KC

The role of inflammatory mediators and chemokines for sepsis-induced leukocyte lung infiltration was investigated using LPS-resistant C57BL/10Sn mice or mice treated with neutralizing TNF antibodies or a combination of antibodies against MIP-2 and KC. Lung leukocytes were quantitated by flow cytometry 12 h after CASP surgery. The results depicted in Fig. 7A demonstrate that in LPS-resistant C57BL/10Sn mice CASP surgery induced a significant increase in the absolute number of lung leukocytes that was comparable to the accumulation of cells observed in wild-type C57BL/6 mice. Similarly, neutralization of TNF did not affect lung leukocyte infiltration following CASP surgery (Fig. 7B). The
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Fig. 6. Septic peritonitis results in activation of circulating granulocytes. (A) Peripheral blood leukocytes were isolated 6 h after CASP surgery (solid lines) or from sham-treated mice (dotted lines) and subjected to two-color flow cytometry analysis. Binding of isotype-matched control or Mac-1-specific mAb to granulocytes that were identified by expression of the Ly-6G/Gr-1 antigen is shown. (B) Generation of reactive oxygen metabolites by circulating granulocytes 6 h after sham (dotted lines) or CASP surgery (solid lines) was detected by flow cytometry using the fluorescent dye dihydrorhodamine 123. Granulocytes were identified by forward and side scatter profiles and analyzed without additional in vitro stimulation. Histograms in (A) and (B) depict results from a representative experiment out of six performed yielding similar results. (C) Peripheral leukocytes were collected 6 h after sham (gray bars) or CASP (black bars) and 3 x 10^6 cells were lysed by sonication. Hydrolysis of the serine proteinase substrate FTC–casein was measured by a spectrophotometric method. Results are derived from five (CASP) or six (sham) independent animals and are presented as mean ± SD. The fraction of granulocytes in total blood leukocytes was not significantly different for sham-operated and CASP-treated mice. *P < 0.001 by Student’s t-test.

Discussion
Progressive multiorgan failure is the most common cause of death following sepsis, with the lung usually representing the first organ to fail (32). Conversely, sepsis is the most common clinical setting in which pathologic alterations of the lung defined as ARDS develop (25). In the CASP model of abdominal sepsis, a continuous leakage of intestinal bacteria into the peritoneal cavity is caused by implantation of a stent in the ascending colon, resulting in bacterial peritonitis followed by systemic infection and release of cytokines (26). Here, we demonstrate that abdominal sepsis triggered by the CASP surgery causes a massive recruitment of pre-activated granulocytes to the lung. Granulocyte accumulation is associated with a profound increase of lung vascular permeability, and up-regulation of a distinct panel of chemokines and adhesion molecules in the lung. Analysis of lung samples and bronchioalveolar lavage fluid of ARDS patients has directly demonstrated neutrophil activation, intense inflammatory infiltrates, elevated concentrations of toxic neutrophil products such as oxidants and proteases, enhanced chemotactic activity, and high concentrations of chemokines including IL-8 (9,10,33). Together, the pathologic alterations of the lung following CASP surgery closely resemble those observed for ARDS in sepsis patients. Our studies showing that survival of mice following...
shown that bacterial superantigens are potent inducers of inflammation. Thus, systemic application of bacterial DNA and oligodesoxynucleotides containing unmethylated CpG motifs stimulate phagocyte production of pro-inflammatory cytokines in vivo and trigger TNF-dependent septic shock in mice (41–43). When administered intratracheally, bacterial DNA induces neutrophil recruitment to the lung and causes elevated concentrations of TNF, IL-6 and MIP-2 in bronchoalveolar lavage fluid (44). Consistent with these results we have demonstrated that oligodesoxynucleotides containing CpG motives may induce acute leukocyte recruitment to the lung in a sequence-specific manner (Neumann and Holzmann, unpublished observations).

Neutrophils have been implicated as cellular mediators of acute lung injury characteristic of ARDS. Clinical studies as well as animal models of ARDS have demonstrated sequestration of large numbers of neutrophils in the lung microvasculature and accumulation of neutrophil proteases in bronchoalveolar lavage fluid and serum (1,45–51). In addition, neutrophil activation was shown to result in endothelial cell injury in vitro and to induce lung capillary leak in vivo by elastase-dependent mechanisms (52–54). The tissue destroying capacity of neutrophil proteases may be further enhanced by reactive oxygen metabolites that were shown to inactivate tissue proteinase inhibitors by oxidation of critical methionine residues (55–57). An important role of reactive oxygen metabolites in tissue damage has been documented in models of acute lung injury induced by immune complexes (58,59). Here, we demonstrate increased serine protease activity in circulating leukocytes following induction of abdominal sepsis by CASP surgery. These results are consistent with a previous report showing that LPS augments elastase mRNA levels and intracellular enzyme activity (60). In addition, we have demonstrated that abdominal sepsis is associated with a substantial increase in the spontaneous release of oxygen radicals by circulating granulocytes. Consistent with cellular activation, cell surface expression of Mac-1 was elevated on circulating granulocytes of CASP-treated mice, whereas L-selectin was down-regulated in most animals. Interestingly, in trauma patients developing ARDS neutrophils circulating in the pulmonary artery also showed increased oxygen radical production and elevated Mac-1 expression (61). Together with previous findings showing that injection of LPS-stimulated neutrophils was sufficient to induce capillary leak in non-inflamed lungs (62), the results presented here therefore suggest that systemic priming of granulocytes and increased production of toxic neutrophil products may be critical for the development of organ injury in abdominal sepsis.

Chemokines are involved in a variety of immune and inflammatory responses acting primarily as chemoattractants and activators of specific leukocyte subsets. In patients, high levels of IL-8 in bronchoalveolar lavage fluid were found to be predictive for the development of ARDS and were associated with high mortality (13,63,64). Using a rabbit model of acute lung inflammation it was shown that neutralization of IL-8 by specific antibodies prevented neutrophil recruitment after intrapleural instillation of LPS (11). In the mouse, MIP-2...
and KC potentially activate neutrophils via the murine IL-8 receptor homologue (65,66). KC increases Mac-1 expression and respiratory burst activity on neutrophils and intratracheal administration of KC results in neutrophil influx into lungs (14,66). Neutralizing antibodies against KC or MIP-2 inhibited neutrophil accumulation within the lungs and reduced vascular leakage in response to an intratracheal challenge with LPS (12,14,15). In addition to chemokines binding the murine IL-8 receptor homologue, MIP-1α was also found to be involved in inflammatory lung injury triggered by LPS (17). In the present report, we show that the time course of KC, MIP-2 and MIP-1α mRNA induction following CASP surgery precisely correlated with lung infiltration by neutrophils. However, chemokine neutralization with specific antibodies revealed that leukocyte recruitment to the lung during abdominal sepsis may occur independent of MIP-2 and KC. These results further emphasize the functional redundancy of mediators that may induce lung injury during abdominal sepsis.

Analysis of animal models has indicated distinct functions of CC chemokines in lung pathology and sepsis. MCP-1 was shown to act as a chemoattractant for monocytes, but not neutrophils, and to induce expression of CD11b and CD11c as well as production of reactive oxygen metabolites by monocytes (67,68). In patients with Gram-positive and Gram-negative infections resulting in sepsis, elevated MCP-1 levels were observed (69). Similarly, during lethal or sublethal bacteremia in baboons MCP-1 was released and correlated with IL-8 plasma levels (70). In mice, neutralization of MCP-1 significantly increased endotoxin-induced mortality as well as serum TNF and IL-12 levels, while administration of recombinant MCP-1 protein resulted in elevated IL-10 serum levels and protected mice from lethal endotoxia (71). In contrast, using a rat IgA immune complex model of alveolitis that is characterized by mononuclear phagocyte-dependent lung injury, infusion of neutralizing MCP-1 antibodies reduced both accumulation of mononuclear phagocytes and vascular damage in the lung (72). These results suggest an in vivo crosstalk between cytokines and chemokines such as MCP-1 that is dependent on the inflammatory stimulus may either promote or attenuate organ injury.

In the present study, the kinetics of MCP-1 expression following induction of abdominal sepsis by CASP surgery was shown to directly correlate with the infiltration of mononuclear phagocytes into lungs. Similar results were obtained for IP-10, a member of the CXC chemokine family that exhibits potent chemotactic activity for monocytes, activated T cells and NK cells (73,74). These results suggest that both MCP-1 and IP-10 may contribute to the accumulation of mononuclear phagocytes in the lungs. Further studies will be required to determine whether protective or detrimental effects of MCP-1 and IP-10 on lung function may dominate during abdominal sepsis, and to elucidate functional linkages between the cytokine and chemokine responses during sepsis and septic shock and the consequences for the development of organ injury.

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Abbreviations

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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ARDS</td>
<td>adult respiratory distress syndrome</td>
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<tr>
<td>CASP</td>
<td>colon ascendens stent peritonitis</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>IP-10</td>
<td>IFN-inducible protein 10</td>
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<tr>
<td>KC</td>
<td>cytokine-induced neutrophil chemotactant</td>
</tr>
<tr>
<td>LIX</td>
<td>LPS-induced CXC chemokine</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MCP</td>
<td>macrophage chemotactic protein</td>
</tr>
<tr>
<td>MIF</td>
<td>macrophage inhibitory factor</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
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References

14. Frevert, C. W., Huang, S., Danaee, H., Paulauskis, J. D. and Kobzik, L. 1995. Functional characterization of the rat chemokine...
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