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Divergent Signaling Pathways in Phagocytic Cells during Sepsis

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Neutrophil accumulation in the lung plays a pivotal role in the pathogenesis of acute lung injury during sepsis. Directed movement of neutrophils is mediated by a group of chemoattractants, especially CXC chemokines. Local lung production of CXC chemokines is intensified during experimental sepsis induced by cecal ligation and puncture (CLP), as reflected by rising levels of MIP-2 and cytokine-induced neutrophil chemotactic attractant-1 in bronchoalveolar lavage fluids. Alveolar macrophages are primed and blood neutrophils are down-regulated for production of MIP-2 and cytokine-induced neutrophil chemotactic attractant production in response to LPS and C5a. Under these conditions of stimulation, activation of MAPKs (p38, p42/p44) occurs in sham neutrophils but not in CLP neutrophils, while under the same conditions phosphorylation of p38 and p42/p44 occurs in both sham and CLP alveolar macrophages. These data indicate that, under septic conditions, there is impaired signaling in neutrophils and enhanced signaling in alveolar macrophages, resulting in CXC chemokine production, and C5a appears to play a pivotal role in this process. As a result, CXC chemokines increase in lung, setting the stage for neutrophil accumulation in lung during sepsis. The Journal of Immunology, 2006, 177: 1306–1313.

The acute respiratory distress syndrome (ARDS) has been defined as a severe form of acute lung injury featuring pulmonary inflammation and increased capillary leak (1). ARDS is associated with a high mortality rate and accounts for >100,000 deaths annually in the United States (2). ARDS may arise in a number of clinical situations, especially in patients with sepsis (3). A well-described pathophysiological model of ARDS is one form of the acute lung inflammation mediated by neutrophils, cytokines, and oxidant stress (4).

Neutrophils are major effect cells at the frontier of innate immune responses, and they play a critical role in host defense against invading microorganisms. The number of neutrophils in bronchoalveolar lavage (BAL) fluids from patients with ARDS is significantly increased and is associated with poor survival (5–7). Neutrophils accumulate in inflamed tissues where they can exert two significant outcomes, removal of invading microorganisms and causing tissue damage. The tissue injury appears to be related to proteases and toxic reactive oxygen radicals released from activated neutrophils. In addition, neutrophils can produce cytokines and chemokines that enhance the acute inflammatory response (8). Proteinases and production of reactive oxygen species released from infiltrated neutrophils may contribute to endothelial and epithelial injury, which is characteristic of ARDS. Thus, although neutrophil recruitment to lung is essential for the responses in host defense, they are detrimental when uncontrolled.

Directional movement of neutrophils is caused by a group of neutrophil chemoattractants, especially CXC chemokines (9, 10). The CXC chemokine family not only plays a critical role in recruitment of inflammatory cells but it also mediates aberrant vascular remodeling in ARDS via chemokines that are angiogenic (11). Cytokine-induced neutrophil chemotactic attractant-1 (CINC-1) and MIP-2 are considered to play major roles in recruiting neutrophils during the inflammation response in rodents. The dependency of neutrophil accumulation on these chemokines has been shown in various animal models of inflammation in vivo (12–15). It has been reported that chemokine gradient formation is essential in regulating neutrophil recruitment (16, 17). However, the cellular and molecular basis for CXC chemokine formation is poorly understood. In this study, we have used LPS and C5a as stimulants of neutrophils to determine how CXC chemokine production is regulated in neutrophils and alveolar macrophages in the setting of sepsis.

Materials and Methods

Rat model of cecal ligation and puncture

Male Long-Evans specific pathogen-free rats (275–300 g; Harlan Sprague-Dawley) were used in all studies. Anesthesia was induced by i.p. administration of ketamine (20 mg/100 mg body weight). After the abdomen was shaved and a topical disinfectant was applied, a 2-cm midline incision was made, and the cecum was identified and ligated below the ileocecal valve, with care being taken not to occlude the bowel. The cecum was then subjected to a single through-and-through perforation with an 18-gauge needle. After the bowel was repositioned, the abdominal incision was closed in layers with plain gut surgical suture 4–0 (Ethicon) and 18-gauge needle. After the bowel was repositioned, the abdominal incision was closed in layers with plain gut surgical suture 4–0 (Ethicon) and metallic clips. Sham animals underwent the same procedure in the absence of cecal ligation and puncture. Before and after surgery, animals had unlimited access to food and water. Animals receiving anti-C5a Ab treatment were injected intratracheally at the time of the cecal ligation and puncture (CLP) procedure with 100 μg of rabbit anti-rat C5a IgG (against the C-terminal region; purified and characterized as described in Ref. 18) or with 100 μg of preimmune rabbit IgG. Systemic blockade of C5a was achieved via guest on April 16, 2017

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by i.v. injection of 500 μg of anti-rat C5a IgG immediately after CLP. This Ab is described elsewhere (19).

Collection of serum samples from septic animals
After induction of sepsis, animals were sacrificed at various time points after CLP, and blood was drawn from the inferior vena cava. Blood samples were allowed to clot on ice for 4 h before centrifugation at 3000 × g for 15 min at 4°C. Serum was collected and immediately frozen at −80°C until use.

Rat blood neutrophils and PBMC
Whole blood from rats was drawn into syringes containing the anticoagulant acid-citrate-dextrose (Baxter Health Care). Neutrophils and PBMC were separated and purified using Ficoll-Paque gradient centrifugation (Phar诚cia Biotech) and dextran sedimentation. After hypotonic lysis of residual RBC, the cells were used for further studies. The cells were plated in 6- or 24-well polystyrene plates with ultralow attachment (Corning), and stimuli were subsequently added.

Alveolar macrophages and in vitro stimulation
Alveolar macrophages were isolated by repeatedly lavaging the lungs of anesthetized Long-Evans rats (Charles River Breeding Laboratories). After centrifugation of lavaged fluids, cells were resuspended in DMEM (Bio-Whittaker), plated in 24-well microtiter plates (Corning) at a concentration of 1 × 10⁶ cells/well or in 100-mm cell culture dishes at a concentration of 1 × 10⁵ cells/dish, and allowed to settle for at least 1 h. Plates or dishes were then washed with medium to remove nonadherent cells. The cells were treated with C5a (10 nM), LPS (20 ng/ml), or LPS + C5a at 37°C in a humidified CO₂ incubator (5% CO₂ and 95% air). After an incubation period, supernatant fluids and cells were collected for analysis. For certain experiments, the cells were preincubated with 20 μM MEK1/2 inhibitor (U0126), 20 μM p38 MAPK inhibitor (SB203580), 5 μM JNK inhibitor (L-form peptide), 20 μM JNK-2 inhibitor (SP600125), or 20 μM NF-κB inhibitor (Bay 11-7082) before stimulation. These doses effectively block activation (20). Rat recombinant protein was produced in our lab.

Quantitation of chemokines
BAL fluids were collected by instilling and withdrawing 5 ml of sterile saline three times from the lungs via an intratracheal cannula. The concentra-
tions of MIP-2 and CINC in BAL fluids, sera, and supernatants from cell cultures were determined using Ab sandwich ELISA. Standard ELISA kits for MIP-2 and CINC were purchased from Biosource International.

Myeloperoxidase (MPO) activity
Tissues were weighed and homogenized in a homogenate buffer, 0.5% hexadecyltrimethylammonium bromide, and 5 mM EDTA in 50 mM potassium phosphate buffer, pH 6.0. The samples were sonicated for 1 min and then centrifuged at 20,000 × g for 15 min. We added 10 μl of each sample to a 96-well plate, followed by addition of 250 μl of assay buffer, 0.005% H₂O₂, and 0.5 mM o-dianisidine dihydrochloride in 100 mM potassium phosphate, pH 6.0. The change in OD₅₄₀ was measured over a period of 6 min at 15-s intervals, using the kinetics mode in the spectrophotometer (Molecular Devices). The slope of the change in OD was calculated to reflect the rate of change in units per gram of lung per minute.

Western blot analysis
Neutrophils or alveolar macrophages (5 × 10⁶) were lysed in 100 μl of 2 × SDS-PAGE lysis buffer. Thirty microtiter plates of whole cell lysate were electrophoresed in a denaturing 10% polyacrylamide gel and then transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% nonfat dry milk for 12 h at 4°C. Membranes were incubated with the following Abs in a 1/1000 polyclonal rabbit Abs to p38, phospho-p38, p42/p44, and phospho-p42/p44 (Cell Signaling). After five washes in TBST, membranes were incubated in a 1/10,000 dilution of HRP-conjugated donkey anti-rabbit (Amersham Pharmacia Biotech). The membrane was developed by ECL according to the manufacturer’s protocol. Protein expression was quantitated from digitized autoradiography films using image analysis software (Adobe Systems). Protein levels were normalized by probing the same blot with GAPDH. Ab to GAPDH was obtained from Abcam.

Statistical analysis
In groups with equal variances, data sets were analyzed using one-way ANOVA, and individual group means were then compared with the Student-Newman-Keuls multiple comparison test. In groups containing unequal variances, Kruskal-Wallis ANOVA was performed, followed by Dunnet's method for multiple comparison. All values were expressed as the mean ± SEM. Significance was assigned where p < 0.05.

Results
MIP-2 and CINC-1 levels in BAL fluids and serum after CLP
Initial and 3, 6, 12, and 24 h after CLP, BAL and serum levels of CINC-1 and MIP-2 were determined by ELISA. As shown in Fig.
A, CINC-1 levels were rapidly elevated in blood after CLP, reaching a peak 6 and 12 h after CLP, with concentrations >1300 pg/ml, followed by a rapid fall to 885 ± 262 pg/ml at 24 h (p < 0.05, n = 5). However, in BAL fluids, the kinetics of CINC-1 was different, with peak levels at 24 h after CLP. Similarly, serum content of MIP-2 quickly increased to a peak level at 6 h with a concentration of 235 ± 79 pg/ml and then falling to near basal levels at 24 h (p < 0.05, n = 5; Fig. 1B). In contrast, BAL levels of MIP-2 progressively increased after onset of sepsis and reached a concentration of 686 ± 196 pg/ml 24 h after CLP. These data suggest that 24 h after CLP, maximal levels of CXC chemokines occur in the lung, when levels in blood are very low, setting the stage for a gradient favoring migration of neutrophils into the lung.

Neutrophil accumulation in lung during experimental sepsis was also determined by MPO content in lung homogenates. As would be expected, MPO content in normal lungs was low (with an arbitrary OD of 16.60 ± 3.52). The value increased to 58.24 ± 7.34 at 12 h and further increased to 84.84 ± 12.6 (p < 0.05 vs 12-h group, n = 5). Thus, experimental sepsis induces lung neutrophil accumulation in rats, while CXC content in lung is peaking.

CINC and MIP-2 production from alveolar macrophages and neutrophils in response to LPS and C5a

Alveolar macrophages, blood neutrophils, and PBMCs were isolated from sham and 24-h CLP rats. These cells were treated with buffer control, C5a (10 nM), LPS (20 ng/ml), or the combination. In alveolar macrophages from sham rats (Fig. 2A, left), as would be expected, LPS strongly induced the release of CINC-1 and MIP-2 from these cells. C5a alone had a minimal effect, but it significantly increased LPS-induced CINC-1 and MIP-2 production. Alveolar macrophages from 24-h CLP rats showed a stronger response to LPS than did sham alveolar macrophages (p < 0.05; Fig. 2A). This response was further enhanced by costimulation with C5a.

![FIGURE 1. Time courses of CINC and MIP-2 production in BAL and blood during sepsis. BAL (A) and serum (B) levels of CINC and MIP-2 were determined by ELISA 0, 3, 6, 12, and 24 h after onset of sepsis. For each condition, n = 5. Whole lungs were harvested 12 and 24 h after CLP and subjected to MPO analysis (C). For each vertical bar, n = 5. * p < 0.05 when compared with controls (Con).](http://www.jimmunol.org/)

![FIGURE 2. CINC and MIP-2 production from alveolar macrophages, neutrophils, and PBMCs in response to LPS and C5a. Alveolar macrophages (A), neutrophils (B), and PBMCs (C) were isolated from control and 24-h CLP rats. The cells were treated with C5a, LPS, and LPS + C5a, respectively. The supernatants were collected 4 h after incubation and were subjected to ELISA analysis. Data are representative of three independent experiments with the cells pooled from four to six rats. For each vertical bar, n = 4.](http://www.jimmunol.org/)
As shown in Fig. 2B, sham neutrophils released a large amount of CINC-1 and MIP-2 when incubated with LPS (Fig. 2B). C5a alone had minimal effect on CINC-1 and MIP-2 production, but it significantly enhanced LPS-induced CINC-1 and MIP-2 releases. In striking contrast to alveolar macrophages, neutrophils from 24-h CLP rats lost virtually all ability to produce CINC-1 and MIP-2 in response to LPS (Fig. 2B). However, the promoting effect of C5a on LPS-induced MIP-2 and CINC production could still be seen, although the absolute values of MIP-2 and CINC in supernatants fluids were very small. Similar to responses of neutrophils, PBMCs from septic animals showed a compromised ability in production of MIP-2 and CINC-1 in response to LPS and C5a (Fig. 2C). However, the absolute values of MIP-2 and CINC from PBMCs were much lower than those from alveolar macrophages and neutrophils.

These data suggest that, at the 24-h stage of sepsis, alveolar macrophages were activated whereas neutrophils and PBMCs were deactivated with respect to the chemokine production and that C5a plays a promoting role in MIP-2 and CINC production.

We have previously shown that CLP causes substantial reductions in C5aR on the surfaces of blood neutrophils (21). Alveolar macrophages were isolated 0, 12, and 24 h after CLP. C5aR mRNA levels were evaluated by RT-PCR. As shown in Fig. 3A, C5aR mRNA levels showed no detectable differences in alveolar macrophages after CLP. Confocal fluorescence microscopy was used to assess C5aR location in alveolar macrophages during sepsis. As shown in Fig. 3B, sham cells showed a uniform distribution of C5aR on the cell periphery (green color), consistent with surface membrane staining. In contrast, a diffuse granular pattern of staining was found in the cytoplasmic compartment of alveolar macrophages obtained 24 h after CLP. C5aR expression on the cell surface was quantitatively evaluated by flow cytometric analysis (Fig. 3C). Alveolar macrophages from control animals showed positive staining for C5aR and very limited staining using preimmune IgG. C5aR content on alveolar macrophages isolated from

**FIGURE 3.** C5aR expression on alveolar macrophages in sepsis. C5aR mRNA expression in alveolar macrophages during sepsis was assessed (A). mRNAs levels in alveolar macrophages isolated from control, 12-h, and 24-h CLP rats were determined by semiquantitative RT-PCR. Data are representative of three independent experiments. C5aR translocation in alveolar macrophages during sepsis was visualized by confocal microscopy (B). The effect of anti-C5a on C5aR levels on alveolar macrophages in sepsis was determined by flow cytometric analysis (C). Animals receiving anti-C5a treatment (100 μg) were given intratracheally at the time of the CLP procedure. C5aR levels on cell surface were determined by flow cytometric analysis. For each vertical bar, n = 4; *, p < 0.05 when compared with anti-C5a group.
24-h CLP rats was significantly decreased in comparison with that on sham cells (p < 0.05, n = 4). Anti-C5a treatment resulted in significant preservation of C5aR on alveolar macrophages from CLP rats (p < 0.05). These data suggest that C5aR, as in neutrophils, internalizes in alveolar macrophages during sepsis, and the C5aR internalization is due, at least in part, to C5a production.

Production of MIP-2 and CINC-1 in alveolar macrophages and neutrophils

To investigate the mechanism by which alveolar macrophages and neutrophils produce MIP-2, MIP-2 levels in the supernatant fluids were measured by ELISA after incubation with LPS and C5a, using inhibitors of varying signaling pathways. As shown in Fig. 4A, p38 and p42/p44 inhibitors significantly suppressed MIP-2 release from alveolar macrophages in response to LPS + C5a. The NF-κB inhibitor completely abolished MIP-2 production, whereas JNK1 and JNK2 inhibitors showed no significant effect on MIP-2 production. In neutrophils, inhibition of p38, p42/p44, and NF-κB resulted in complete suppression of MIP-2 production, whereas inhibition of JNK1/2 had no effect (Fig. 4B). These data suggest that MIP-2 production from alveolar macrophages and neutrophils is dependent on p38, p42/p44, and NF-κB pathways but not JNK1/2 pathways.

P38 and p42/p44 activation in alveolar macrophages and neutrophils during sepsis

To determine the activation of p38 and p42/p44 in alveolar macrophages and neutrophils during sepsis, alveolar macrophages and neutrophils were isolated from control and CLP animals and were treated with C5a, LPS, or the combination for various periods of time. Phosphorylation of p38 and p42/p44 was subsequently determined by Western blot analysis. As shown in Fig. 5, activation of p42/p44 and p38 was seen in C5a- and LPS + C5a-treated sham alveolar macrophages. In alveolar macrophages isolated from CLP animals, activation of p42/p44 and p38 was still prominent. Stronger phosphorylation of p42/p44 was seen in CLP macrophages in comparison with the activation status of control cells when stimulated with LPS + C5a. However, although C5a and LPS + C5a stimulation were able to activate p42/p44 and p38 in sham neutrophils, such stimulation had little effect on activation of p38 or p42/p44. These data suggest that CLP neutrophils are hyporesponsive to LPS and C5a, in striking contrast to CLP alveolar macrophages, which show intact MAPK pathways and full MIP-2 production.

Effect of in vivo blockade of C5a on in vitro MIP-2 production

As shown in Fig. 2B, MIP-2 production from CLP blood neutrophils after in vitro stimulation with LPS or LPS + C5a was greatly depressed. To determine the role of endogenous C5a in this event, 500 μg of anti-C5a IgG were injected i.v. immediately after CLP. Neutrophils were isolated 24 h after onset of sepsis and incubated with C5a, LPS, or the combination. As shown in Fig. 6A, CLP blood neutrophils (24-h CLP) had greatly reduced production of MIP-2 when stimulated in vitro with LPS or LPS + C5a, whereas neutrophils isolated from CLP animals treated with anti-C5a

![FIGURE 4. Signaling pathways of MIP-2 production. Alveolar macrophages (A) and neutrophils (B) isolated from control rats were first incubated with 20 μM MEK1/2 inhibitor (U0126), 20 μM p38 MAPK inhibitor (SB203580), 5 μM JNK-1 inhibitor (L-form peptide), 20 μM JNK-2 inhibitor (SP600125), or 20 μM NF-κB inhibitor (Bay 11-7082), and subsequently treated with LPS + C5a for 4 h. MIP-2 contents in supernatants were determined by ELISA. Data are representative of two to three independent experiments with cells pooled from four to six rats.](http://www.jimmunol.org/)

![FIGURE 5. MAPK activation in alveolar macrophages and neutrophils during sepsis. Alveolar macrophages and neutrophils were isolated from control (Con) and CLP animals and were treated with C5a, LPS, and LPS + C5a, and phosphorylation of p38 and p42/p44 was subsequently determined by Western blot analysis. Data are representative of two to three independent experiments with cells pooled from four to six rats.](http://www.jimmunol.org/)
showed a significant improvement in MIP-2 production in response to LPS or LPS + C5a. The same protocol was also used to assess MIP-2 production by alveolar macrophages. As shown in Fig. 6B, MIP-2 production was modestly enhanced in alveolar macrophages isolated from 24-h CLP rats when cells were stimulated with LPS or LPS + C5a. Under the same conditions, anti-C5a treatment had no effect on MIP-2 production from CLP alveolar macrophages (Fig. 6B). These data suggest that endogenous C5a production after CLP is attributable, at least in part, to the comprised capability of MIP-2 production from septic neutrophils but is not essential for the priming effect of septic alveolar macrophages.

Discussion

Endogenously produced CXC chemokines are often involved in the lung inflammatory response, including recognition, recruitment, removal, and repair (22). CXC chemokines that contain Glu-Leu-Arg (the ELR motif; ELR+) are particularly potent chemoattractants of neutrophils and confer angiogenic activity on endothelial cells (9). Of the known human ELR+ CXC chemokines, IL-8 has been implicated in the pathogenesis of ARDS. Many studies have shown that BAL levels of IL-8 correlate with the severity of pulmonary neutrophil accumulation in patients with ARDS (7, 23, 24). Although clinical studies examining the correlation of pulmonary IL-8 levels to the outcome of ARDS are conflicting, anti-IL-8:IL-8 complexes present in BAL fluids may be a potentially useful marker for ARDS outcome (25). In a rabbit model of endotoxemia-induced ARDS-like lung injury, an anti-IL-8 Ab treatment substantially decreased neutrophil filtration and significantly reduced lung injury, suggesting a causal role of IL-8 in ARDS (26). To date, the exact homolog of IL-8 in rodents has not been defined. CXC chemokines such as CINC, cytokine-induced neutrophil chemoattractant, and MIP-2 are considered to play a major role in recruiting neutrophil during the inflammatory response in rodents. The dependency of neutrophil accumulation on these chemokines has been shown in various animal models of inflammation in vivo (12–15).

For neutrophil chemoattractants, while sufficient local concentrations of CXC chemokines are necessary in neutrophil recruitment, the dictating factor is the ratio of local to blood chemokine concentrations (17). In a rat model of LPS-induced lung injury, the levels of chemokine gradient predict the intensity of neutrophil accumulation in lung (16). These studies support the paradigm that local production of CXC chemokines is essential in the elicitation of neutrophils in lung under conditions of acute inflammation. During sepsis, lung production of MIP-2 and CINC-1 lagged behind in comparison with the levels of MIP-2 and CINC in blood. This discrepancy might be important for CXC chemokines to create a concentration difference between two compartments, which is necessary for mobilization of neutrophil migration. CXC chemokines have been implicated in all steps in the extravasation process of leukocytes, including rolling, adhesion, and transmigration in vivo (27). Thus, the role of blood CXC chemokines may primarily focus on the activation of neutrophils and endothelial cells, setting the stage for neutrophil migration, whereas the local CXC chemokines chiefly function chemotactically during sepsis. In addition, blood CXC chemokines produced in the early stage of experimental sepsis may provide vital signals for neutrophil survival, given the fact that IL-8 delays neutrophil apoptosis (28). Under such conditions, the greatly prolonged lifespan of neutrophils may be related to the development of the symptoms of sepsis.

The downstream component of the complement cascade, C5a, has been shown to play a prominent role in the inflammatory reaction (29). C5a is not only a powerful chemoattractant for neutrophils, but it also directly activates neutrophils, macrophages, epithelial cells, and endothelial cells. In IgG immune complex-induced lung injury, C5a is required for the full development of injury and neutrophil accumulation (30). Optimal induction of CC and CXC chemokines in lung by IgG immune complex deposition has been shown to be dependent on C5a (31). C5a is also involved in activation of lung vascular P-selectin and up-regulation of lung levels of ICAM-1 (32, 33). It has been shown that C5a is linked to increased levels of CD18 on neutrophils in sepsis (34). C5a may directly and indirectly mediate neutrophil accumulation in lung. However, its chemotactic role may be limited, because C5a can be quickly internalized by neutrophils and macrophages and can also be rapidly converted to its much less active form, C5a des arg (35, 36). Thus, the major role of C5a in inflammatory reaction may be to activate intracellular signaling pathways, leading to enhanced production of other proinflammatory mediators.

In the same model of sepsis, it has been shown that C5AR expression on neutrophils reached the lowest point 24 h after CLP, when innate immune functions (chemotaxis, phagocytosis, and the oxidative burst) of neutrophils were seriously impaired (21). Beyond 24 h after CLP, blood neutrophils started to undergo a functional recovery, correlating with increased content of C5aR on the cell surface. Apparently, C5aR can also be internalized in alveolar macrophages during sepsis (Fig. 3). This process was dependent on the presence of C5a, because anti-C5a treatment significantly reversed internalization of C5aR on alveolar macrophages. Surprisingly, the ability of C5a in promoting MIP-2 release was strongly enhanced in septic alveolar macrophages, even though C5aR levels were diminished on the same cells. These data suggest

FIGURE 6. Effect of in vivo blockade of C5a on in vitro MIP-2 production. Anti-C5a IgG (500 μg) was injected i.v. immediately after CLP. Neutrophils (A) and alveolar macrophages (B) were isolated 24 h after onset of sepsis and subjected to in vitro stimulation with C5a, LPS, or LPS + C5a. MIP-2 levels in the supernatants were determined by ELISA after 4 h incubation. Data are representative of three independent experiments with the cells pooled from four to six rats. For each vertical bar, n = 4.
that C5a signaling in septic alveolar macrophages may be independent of C5aR surface levels. It is possible that internalized C5aR may also transduce signaling in coordination with LPS pathways. Nevertheless, it is clear that the CXC chemokine-producing capability increased in CLP alveolar macrophages but decreased in neutrophils during sepsis. This finding is in disagreement with a previous report that alveolar macrophages had defective production of cytokines and chemokines in sepsis (37). The discrepancy may be a result of the usage of different species or different technical details involved in induction of sepsis. The cited report used a mouse model of sepsis, which might be different from the rat model with respect to the timeline of sepsis development. It is likely that the hyperreactive status of alveolar macrophages in the mouse CLP model may be overlooked.

It has been demonstrated in neutrophils that MIP-2 production requires p38 MAPK and p42/p44 MAPK (38). The release of MIP-2 by alveolar macrophages has also been found to be involved in the activation of the signal-transducing MAPK system and to be transcriptionally regulated by the activation of NF-κB (39). These studies are consistent with our findings that p38 MAPK, p42/p44 MAPK, and NF-κB pathways play essential roles in MIP-2 production in both alveolar macrophages and neutrophils. Interestingly, alveolar macrophages still maintain, even in increase, the capacity for activation of p38 and p42/p44 MAPKs during sepsis, whereas septic neutrophils become nonresponsive to LPS and C5a in this regard. Paralysis of signaling pathways in neutrophils is likely caused, at least in part, by overproduction of C5a in sepsis. Exposure of neutrophils to C5a (at concentrations occurring in the plasma of humans with sepsis) can lead to neutrophil dysfunction (40). Neutrophils from CLP rats display defective phagocytosis and chemotaxis as well as defective assembly of NADPH oxidase (40). All such effects can be prevented in CLP rats after blockade of C5a or C5aR, which suggests that neutrophil innate immune functions in sepsis are seriously compromised by overproduction of C5a in sepsis, with resultant excessive C5a/C5aR engagement. Preincubation of neutrophils with C5a blocks phosphorylation of p47^{phox} and its translocation to the cell membrane after addition of PMA, thereby leading to defective assembly of NADPH oxidase and a greatly depressed oxidative burst (40). It was demonstrated that the phosphorylation of p42/p44 MAPK in neutrophils in response to PMA was also impaired by prior cell contact with C5a (40). Because C5a is a strong activator of MAPK (including p42/p44), which is an important kinase for p47^{phox} phosphorylation, the functional impairments in neutrophils are likely due to paralysis in MAPK signaling cascades. Blockade of endogenous C5a significantly improved the MIP-2-producing capability of septic neutrophils (Fig. 6A). These data may help explain why CLP neutrophils lose their ability for MIP-2 and CINC production, with reduced levels of MIP-2 and CINC in blood of CLP rats. However, animals treated with anti-C5a have an altered capability of septic neutrophils (Fig. 6B). The functional impairments in neutrophils are linked to this phenomenon is unclear.

In this study, we have obtained the evidence that a CXC chemokine gradient is formed between lung and blood during which formation may favor neutrophil migration into lung. Although we did not observe a significant number of neutrophils in BAL fluids in CLP rats (data not shown), lung MPO marked increased in this study (Fig. 1C), suggestive of the neutrophil sequestration in lung. Thus, CXC chemokine gradient formation may help neutrophil accumulation in the lung under septic conditions.