CXCL1 Contributes to Host Defense in Polymicrobial Sepsis via Modulating T Cell and Neutrophil Functions

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CXCL1 Contributes to Host Defense in Polymicrobial Sepsis via Modulating T Cell and Neutrophil Functions

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Severe bacterial sepsis leads to a proinflammatory condition that can manifest as septic shock, multiple organ failure, and death. Neutrophils are critical for the rapid elimination of bacteria; however, the role of neutrophil chemotactic CXCL1 in bacterial clearance during sepsis remains elusive. To test the hypothesis that CXCL1 is critical to host defense during sepsis, we used CXCL1-deficient mice and bone marrow chimera to demonstrate the importance of this molecule in sepsis. We demonstrate that CXCL1 plays a pivotal role in mediating host defense to polymicrobial sepsis after cecal ligation and puncture in gene-deficient mice. CXCL1 appears to be essential for restricting bacterial outgrowth and death in mice. CXCL1 derived from both hematopoietic and resident cells contributed to bacterial clearance. Moreover, CXCL1 is essential for neutrophil migration, expression of proinflammatory mediators, activation of NF-κB and MAPKs, and upregulation of adhesion molecule ICAM-1. rIL-17 rescued impaired host defenses in cxcl1−/− mice. CXCL1 is important for IL-17A production via Th17 differentiation. CXCL1 is essential for NADPH oxidase–mediated reactive oxygen species production and neutrophil extracellular trap formation. This study reveals a novel role for CXCL1 in neutrophil recruitment via modulating T cell function and neutrophil-related bactericidal functions. These studies suggest that modulation of CXCL1 levels in tissues and blood could reduce bacterial burden in sepsis. The Journal of Immunology, 2014, 193: 000–000.

Despite improvements in antibiotic therapies and critical care management, sepsis remains a leading cause of infectious death in intensive care units (1). The pathogenesis of sepsis is characterized by an early, overwhelming systemic proinflammatory response that leads to multiple organ damage; the later phases are characterized by anti-inflammatory responses and negative regulation of immune signaling pathways (2). However, the molecular and cellular mechanisms that regulate immune responses to polymicrobial sepsis (PMS) are not completely understood. Prior studies have shown that deletion of nucleotide-binding oligomerization domain-2 receptors or the scavenger receptor class A leads to host protection (3, 4). In contrast, reduced survival and diminished production of cytokines and chemokines was observed in Stat-2−/− mice during LPS-mediated endotoxemic shock, indicating a crucial role for STAT-2 in host defense (5).

Neutrophils are a pivotal arm of the innate immune response during PMS, and they play a critical role in bacterial elimination (6, 7). Impaired neutrophil transmigration is associated with increased mortality and higher bacterial burden in peritoneal exudates and blood, as demonstrated during sepsis induced by cecal ligation and puncture (CLP) (7, 8), whereas excessive influx of neutrophils can cause unwanted tissue damage and organ dysfunction (9). Our group has demonstrated an important role for CXCL1 (also known as keratinocyte cell–derived chemokine [KC]) in pulmonary defense during pneumonia caused by Klebsiella pneumoniae (10). CXCL1 was found to be critical for neutrophil-dependent bacterial elimination via induction of reactive oxygen species (ROS) (11) and reactive nitrogen species in the lung (12). Neutrophil migration to multiple organs is impaired during severe sepsis, because of downregulation of the CXCL1 receptor, CXCR2, resulting in failed pathogen clearance (13). These findings suggest a potential role for CXCR2, or its ligands, including CXCL1 (KC), CXCL2 (MIP-2), and CXCL5 (LIX), in controlling sepsis. To better understand the role of CXCL1 in neutrophil influx and function during fatal PMS, we used a murine model of PMS, induced by CLP in CXCL1−/− mice. In addition, we addressed the role of bone marrow cell versus resident cell–derived CXCL1 in bacterial clearance after PMS through the use of bone marrow chimera.

**Materials and Methods**

**Animal studies**

All animal experiments were approved by the Louisiana State University Animal Welfare Committee. CXCL1 gene–deficient (cxcl1−/−) and C57BL/6 wild type (WT) male mice (8–12 wk) were used as previously described (10, 14). PMS was induced by the CLP method as previously described (15). In brief, male mice were anesthetized, the cecum was punctured with a 21-gauge needle, a small amount of fecal material was extruded through the puncture, and the cecum was repositioned into the peritoneal cavity. Animals with sham surgery underwent the same protocol without CLP. Survival of both cxcl1−/− and WT mice that underwent CLP or sham surgery was monitored every 12 h up to 10 d. In additional experiments, rIL-17A, rCXCL1, or BSA control was injected i.p. immediately after CLP, and survival was monitored for 10 d. Bacterial burdens were determined by enumerating bacterial numbers in CFUs on tryptic soy.
agar plates as previously described (10). Cytokine and chemokine levels in peritoneal lavage fluid and serum were measured by a double-ligand ELISA (10, 12).

**Bone marrow chimeras**

Generation of CXCL1 chimeras has been described in our prior reports (10, 12). We found that >80% of blood leukocytes were derived from donor mice at the time of experiments.

**Myeloperoxidase activity**

A myeloperoxidase (MPO) assay was performed in the peritoneal fluid (PF) as described elsewhere (2, 5, 11, 12). In brief, 200 μl PF was incubated with 800 μl hexadecyltrimethylammonium bromide buffer, and the mixture was transferred into microcentrifuge tube and centrifuged at 20,000 × g for 4 min. Seven microliters of supernatant was transferred into 96-well plate, and 200 μl O-dianisidine hydrochloride solution was added immediately. The MPO activity, measured as OD at 450 nm, was expressed in units per milliliter of supernatant.

**Western blotting**

At the designated times, the lungs, livers, and spleens were harvested and homogenized in 1 ml PBS containing 0.1% Triton X-100 supplemented with complete protease and phosphatase inhibitor mixture as described previously (12).

**Immunofluorescence and electron microscopy**

The detection of ROS (11), OH−, and O2− producing neutrophils in the PF from CLP WT and knockout (KO) mice was performed as described earlier (18). For isolation and detection of IL-17–producing T cells, lungs were first minced, digested with collagenase for 90 min, and made into a single-cell suspension, followed by staining. Spleens were homogenized and were made into single-cell suspension by sieving. Cells were surface stained for markers of IL-17A–producing T cells (γδ, NK1.1, CD4, and CD8α). FlowJo software was used to analyze the data.

**Th17 differentiation**

The method for IL-17 differentiation has been previously reported (19). rCXCL1 (1 mg/ml) was added on days 1 and 3. Cells were washed and resuspended in PBS followed by blocking with FcR blocking reagent at 0, 3, 6, and 12 d poststimulation. IL-17–producing cells were surface stained with anti-CD4 FITC. FlowJo was used for data analysis. In another set of

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**FIGURE 1.** CXCL1-deficient mice are more susceptible to PMS than WT mice and are defective for cellular infiltration and bacterial clearance. (A) Enhanced mortality of cxcl1−/− mice after CLP-induced PMS. cxcl1−/− and WT control mice were subjected to sham or PMS. The survival rates of mice were determined every 12 h until 10 d after PMS. The results are expressed as percent for 20 animals per group. Significance between groups was examined by Wilcoxon rank test. (B) Impaired bacterial clearance in cxcl1−/− mice. The CFUs were examined in peritoneal lavage fluid and blood, or the homogenates obtained from lungs of cxcl1−/− and WT mice at 6 and 24 h after PMS. Results are expressed as mean log of CFU/ml (n = 5/group). (C) cxcl1−/− mice have reduced total WBC and neutrophil accumulation at the peritoneum after PMS, as measured by direct cell counts (for total WBC and neutrophils) or MPO activity (for neutrophil influx). Results are expressed as mean per milliliter (n = 5–8/group). (D) Both hematopoietic and non-hematopoietic cells are essential for bacterial clearance. Bone marrow chimeras were generated as described in Materials and Methods and show bacterial CFUs at 24 h after PMS or sham (n = 5 mice/group), *p < 0.05.

**FIGURE 2.** Diminished production of cytokines and chemokines in cxcl1−/− mice after induction of PMS. cxcl1−/− and WT control mice were subjected to sham or PMS. The concentrations (in pg/ml) of IL-6, MIP-2, IL-17A, and IL-17F were quantified in the PF (A) and serum (B) for the time points of 6 and 24 h after PMS. Results are expressed as mean ± SEM (n = 5–8/group). *p < 0.05.
FIGURE 3. Activation of NF-κB, MAPK, and NADPH oxidase, and expression of adhesion molecule ICAM-1 were impaired in cxcl1<sup>−/−</sup> mice after PMS. (A) CXCL1 is essential for the activation of NF-κB during PMS induction. Phosphorylation of NF-κB/p65(Ser<sup>536</sup>) and IκBα in the homogenates from liver, lung, and spleen from cxcl1<sup>−/−</sup> as compared with WT mice. (B) Attenuated activation of MAPKs in the organs of cxcl1<sup>−/−</sup> mice. Phosphorylated p38MAPK, ERK, and JNK in the homogenates from different organs from cxcl1<sup>−/−</sup> and WT mice were probed with their respective Abs. (C) Expression of adhesion molecule ICAM-1, but not VCAM-1, is reduced in cxcl1<sup>−/−</sup> mice. The expression levels of ICAM-1 and VCAM-1 were determined in the organs of cxcl1<sup>−/−</sup> and WT control mice after PMS for time points 6 and 24 h. (D) The activation of NADPH oxidase is impaired in cxcl1<sup>−/−</sup> mice. The expression levels of Nox2, p22<sub>phox</sub>, p67<sub>phox</sub>, and p47<sub>phox</sub> were determined in the homogenates of organs of cxcl1<sup>−/−</sup> and WT control mice by immunoblotting at 6 and 24 h after PMS. GAPDH or total p38 MAPK expression levels were assessed in all samples as internal loading control, and the blots are representative of three independent experiments with similar results (A–D). Densitometric analysis of three separate blots from three independent experiments is shown in parentheses as relative expression in KO mice compared to WT mice and depicted as mean ± SE (n = 4–6/group). *p < 0.05.
experiments, supernatant was collected for IL-17A or IL-17F assay by ELISA at 3 d poststimulation with rCXCL1.

Analysis of NET-derived DNA
PF was taken from the post-CLP or sham mice at 24 h and subjected to agarose gel electrophoresis, to analyze NET-derived DNA. To confirm whether extracellular DNA was present in the cell-free supernatant, we treated PF with DNase (50 mg/ml) for 1 h and subjected it to gel electrophoresis. The observation of DNA in the samples, but not in DNase-treated samples, was judged to be NET-derived DNA.

Determination of ROS, H2O2, and O2 production by neutrophils
ROS and H2O2 levels in peritoneal neutrophils and PF of cxcl1−/− and WT mice after CLP were measured using the Fluorescent H2O2/Peroxidase Detection Kit (11).

Bacterial killing assay
A neutrophil-dependent killing assay was performed as reported earlier (12). In brief, 1 × 10⁶ neutrophils were suspended in RPMI 1640 with 10% v/v FBS, and 1 × 10⁶ opsonized bacteria were added (1 multiplicity of infection [MOI]). Samples were incubated at 37°C with continuous agitation. Samples were harvested at 60 min postinfection, treated with gentamicin (100 mg/ml) for 15 min to kill extracellular bacteria, and plated to determine CFU (12).

Data analysis
Data are expressed as mean ± SE. Data were analyzed with the Student t test (between two groups) or with the two-way ANOVA (more than two groups). Survival curves were compared by the Wilcoxon rank sign test. Differences in values were defined as significant at p < 0.05. Experiments were repeated two to three times in each group in each time point.

Results

CXCL1 contributes to protection during PMS
The role of CXCL1 in the pathogenesis of PMS is unclear. To explore this, we subjected cxcl1−/− and WT mice to CLP, and the survival of animals was monitored up to 10 d after CLP. As shown in Fig. 1A, cxcl1−/− mice displayed a reduction in survival rate compared with WT mice after CLP. To examine whether reduced survival of cxcl1−/− mice was due to an impaired ability to recruit immune cells to the peritoneum, we determined total and differential cell counts, as well as MPO activity, in the PF. In cxcl1−/− mice, total WBCs, neutrophil number, and MPO activity were attenuated at 24 h after CLP as compared with WT mice (Fig. 1C).

In the control (sham) group, no cellular influx was observed in the peritoneal cavity of either cxcl1−/− or WT mice (Fig. 1C). To determine whether the reduced recruitment of WBCs and neutrophils contributed to bacterial growth and subsequent dissemination to extraperitoneal organs, we enumerated bacterial numbers in the PF, blood, and lung 24 h after CLP. As compared with WT mice, cxcl1−/− mice had a higher bacterial burden in all tissues (Fig. 1B). Moreover, neutrophil recruitment to the peritoneal cavity was reduced in cxcl1−/− mice postinfection (Fig. 1C).

As compared with WT mice transplanted with WT marrow cells, WT mice transplanted with KO marrow cells or KO mice transplanted with WT marrow cells showed no attenuation bacterial clearance in PF, suggesting that both hematopoietic (radiosensitive) and nonhematopoietic (radioresistant) cells are important for bacterial clearance (Fig. 1D).

FIGURE 4. CD4, CD8, NK, and γδ cells are the major source of CXCL1-mediated IL-17A and IL-17F production, and rCXCL1 rescues T cell subsets in cxcl1−/− mice in response to PMS. Intracellular IL-17A (A) and IL-17F (B) in gated CD4+, CD8a, γδ, and NK1.1 cells from lung and spleen of WT, cxcl1−/− mice, and cxcl1−/− mice after rCXCL1/KC administration at 24 h after PMS was analyzed by flow cytometry. Results are expressed as mean ± SE from three independent experiments (n = 5–8/group; *p < 0.05, **p < 0.01, ***p < 0.001).
CXCL1 contributes to cytokine and chemokine production

Next, we determined the role of CXCL1 in the production of cytokines and chemokines in PF and serum, by assessing levels in WT and cxcl1−/− mice at 6 and 24 h after CLP. As shown in Fig. 2, cytokine and chemokine levels were reduced in PF and in serum samples of cxcl1−/− mice 24 h after CLP. Intriguingly, the production of IL-17A and IL-17F in PF and serum of cxcl1−/− was reduced at 24 h after CLP (Fig. 2).

CXCL1 activates NF-κB, MAPK, and NADPH oxidase, and induces ICAM-1 expression

Activation of both NF-κB and MAPK is critical for controlling bacterial infections (20, 21). NF-κB activation was reduced in cxcl1−/− mice after CLP at both 6 and 24 h, as assessed by reduced phosphorylation of NF-κB/p65 (Ser536) and the degradation of IκBα in the liver, lung, and spleen (Fig. 3). Similarly, cxcl1−/− mice showed reduced activation of the p38, ERK, and JNK MAPKs in the liver, lung, and spleen at 24 h after CLP (Fig. 3). At 6 h after CLP, cxcl1−/− mice exhibited reduced activation of MAPK. However, activation of p38 in the lung and spleen, and JNK in the liver, lung, and spleen were the same as WT at 6 h after CLP (Fig. 3).

We next examined whether defective bacterial clearance in the organs of cxcl1−/− mice was due to reduced activation of NADPH oxidase, an enzyme complex consisting of membrane subunits (gp91phox and p22phox) and cytoplasmic subunits (p47phox, p67phox, and p40phox, and rac) that are assembled upon cellular activation to produce microbicidal ROS (22). We observed that CXCL1 deficiency impairs the expression of p22phox, p67phox, and p47phox, as well as Nos2, in different organs after CLP (Fig. 3).

Cell adhesion molecules are important for neutrophil migration; thus, we investigated the expression levels of ICAM-1 and VCAM-1 after CLP (Fig. 3). We found that the expression of ICAM-1, but not VCAM-1, was diminished in the liver, lung, and spleen from cxcl1−/− mice, in comparison with their WT counterparts at 6 h, as well as 24 h after CLP (Fig. 3).

CXCL1 mediates IL-17A and IL-17F production via activation of CD4, CD8, NK, and γδ cells

Because both IL-17A and IL-17F were reduced in PF and serum of cxcl1−/− mice after CLP, the specific T cell subsets that produce IL-17A or IL-17F in the peritoneum after CLP were determined. Our results show that CD4, CD8, NK or NKT cells, and γδ cells all produce IL-17A and IL-17F in the lungs and spleen of WT mice 24 h after sepsis (Fig. 4A, 4B). There were fewer IL-17A− and IL-17F− producing cells in cxcl1−/− mice after sepsis compared with WT mice (Fig. 4A, 4B). To confirm the positive regulation of IL-17A and IL-17F by CXCL1, we treated cxcl1−/− mice with rCXCL1. Administration of rCXCL1/KC immediately after CLP rescued IL-17A− but not IL-17F− producing T cell subsets in the lungs and spleens of cxcl1−/− mice (Fig. 4). The enhanced number of T cell subsets in cxcl1−/− mice after CXCL1 administration may be due to T cell recruitment to the lungs and/
or IL-17 differentiation by rCXCL1. To explore these possibilities, we performed Th17 differentiation assays using naive CD4+ T cells obtained from WT and KO mice. Intriguingly, we specifically found that rCXCL1 enhances Th17 (IL-17A–producing; Fig. 5) but not Th1 or Th2 differentiation (data not shown). Moreover, we collected supernatants at 3 d poststimulation with rCXCL1 and found that differentiated cells produce substantial IL-17A and IL-17F (Fig. 5B), validating the data related to Th17 differentiation using flow cytometry.

rIL-17A administration rescues host defense in cxcl1−/− mice

Previous studies have shown that early i.p. injection of chemokines after CLP results in rapid neutrophil recruitment and subsequently lower peritoneal bacterial burdens (7). To determine whether exogenous CXCL1 or IL-17A administration rescues neutrophil-mediated host defense in cxcl1−/− mice, we subjected cxcl1−/− mice to CLP, immediately followed by i.p. administration with either saline, CXCL1, or rIL-17A, and assessed for survival through day 10. Intriguingly, CXCL1- or IL-17A–treated cxcl1−/− mice showed improved survival rates compared with saline-treated controls (Fig. 6A, 6B). Furthermore, cxcl1−/− mice treated with rCXCL1 or rIL-17A showed decreased bacterial burden in blood and peritoneum, and increased neutrophil counts and cytokine/chemokine expression in PF of cxcl1−/− mice (data not shown; Fig. 6C–I). These results suggest that IL-17A controls neutrophil-dependent host defense during sepsis via the production of neutrophil chemoattractants such as CXCL2/MIP-2 and CXCL5/LIX.

ROS, H2O2, and O2 production by neutrophils is attenuated in cxcl1−/− mice

Efficient function of neutrophils is critical to bacterial elimination from tissues, and NADPH activity in neutrophils is shown to be crucial in this process. To examine NADPH oxidase activity in cxcl1−/− mice after CLP, we measured ROS production in PF and in neutrophils, because these cells are a primary source for ROS production (21). These results are consistent with data indicating a low level of ROS and H2O2 production by peritoneal neutrophils and in the PF of cxcl1−/− mice (Fig. 7A). We also found reduced production of ROS, H2O2, and O2 by peritoneal neutrophils from cxcl1−/− mice via FACS analysis (Fig. 7B).

cxcl1−/− neutrophils exhibit decreased NET formation and NET-mediated bacterial killing

The antibacterial function of neutrophils is not only mediated by intracellular killing, but also by the formation of NETs, termed NETosis, and subsequent extracellular killing (23). NETs are composed of DNA studied with many granular proteins that have antimicrobial activity, and their formation is mediated by the induction of ROS in neutrophils (23). Decondensed chromatin with NET morphology was shown to have high levels of histone citrullination, indicating that this event is a marker of NETosis (18). In this regard, we isolated peritoneal neutrophils from cxcl1−/− and WT mice after CLP and examined them for NET formation and NET-mediated bacterial killing. For evidence of NET formation, we performed agarose gel electrophoresis of PF in the absence and presence of DNase. The results showed increased DNA content in the fluid from WT mice compared with cxcl1−/− mice (Fig. 8A). The presence of extracellular DNA in the fluid of both groups of mice was further confirmed by the disappearance of DNA bands after DNase treatment in the gel (Fig. 8A), suggesting different levels of NET formation in both groups during sepsis. Kinetic analysis of NET formation showed a reduction in NET formation rate by cxcl1−/− neutrophils through the initial 8 h (Fig. 8B). To further demonstrate unequal NET formation in the

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**FIGURE 6.** Enhanced mortality, higher bacterial burden, reduced leukocyte recruitment, and attenuated cytokine/chemokine production in PF are rescued in cxcl1−/− mice following PMS after rCXCL1 or rIL-17A administration. (A) cxcl1−/− and WT control mice were subjected to sham surgery or PMS, and PBS, rCXCL1, or (B) rIL-17A was immediately injected i.p. Survival was assessed every 12 h up to 10 d after PMS. Results are expressed as percentage for 20 animals per group. Significance between groups was examined by Wilcoxon rank test. Bacterial clearance in cxcl1−/− mice after CLP is rescued by exogenous rIL-17A administration. (C) CFUs were determined in the blood and (D) PF of cxcl1−/− mice at 6 and 24 h after PMS. (E) Total WBC and (F) neutrophil accumulation at peritoneum in cxcl1−/− mice after PMS are rescued after administration of rIL-17A. (G–I) Production of cytokine and chemokines in PF are rescued after administration of rIL-17A. Results are expressed as mean ± SE (n = 5–8/group; **p < 0.01, ***p < 0.001).
neutrophils from cxxl<sup>−/−</sup> and WT mice, we visualized the DNA in neutrophils from both groups after staining with Sytox green nucleic acid stain and anti-citrullinated histone H3 Ab up to 8 h after CLP. Using fluorescence microscopy, long, stringlike extracellular DNA with citrullinated H3 was most evident in WT neutrophils (Fig. 8C, arrowheads). The percentage of neutrophils positive for extracellular DNA and citrullinated H3 was reduced among cxxl<sup>−/−</sup> mice compared with WT mice (Fig. 8D). Scanning electron micrograph analyses showed typical NET structures (cables, threads, and globular domains), as defined by Fuchs et al. (18), associated with peritoneal neutrophils derived from WT mice after CLP; those structures were largely undetected with peritoneal neutrophils of WT mice compared with WT mice after CLP (Fig. 8F).

To determine whether CXCL1-mediated NET formation is essential for bacterial killing, we incubated <i>E. coli</i> with cxxl<sup>−/−</sup> or WT neutrophils and analyzed bacterial killing. We found a decrease in NET-mediated bacterial killing and relative phagocytosis in cxxl<sup>−/−</sup> neutrophils compared with WT neutrophils (Fig. 8G, 8H). Because DNase can be used to prevent NET formation, neutrophils were pretreated with DNase in one set of experiments. WT neutrophils without DNase treatment showed effective NET-mediated bacterial killing compared with the DNase-treated control, whereas no difference in bacterial killing was observed in the DNase-treated cxxl<sup>−/−</sup> neutrophils (Fig. 8G). In addition, the percentage of neutrophils positive for extracellular DNA and citrullinated H3 was lower among cxxl<sup>−/−</sup> mice after 8 h (Fig. 8I).

**Discussion**

Brisk neutrophil migration to the site of infection and local ROS production are multistep processes and pivotal events in host defense during bacterial infection (24). The severity of sepsis induced by CLP correlates with decreased neutrophil recruitment and consequent diminished bactericidal activity in the peritoneum (7, 8, 25–27). Recruitment and activation of neutrophils have been demonstrated to be critical for controlling CLP-induced PMS by suppressing bacterial growth in the peritoneum and extraperitoneal organs (7).

In this report, we investigated the role of CXCL1 in neutrophil recruitment and function during sepsis induced by CLP. Mice deficient in CXCL1 exhibited a reduction in total leukocytes and neutrophils in the peritoneum after CLP, and a reduction in bacterial clearance from the peritoneum and extraperitoneal organs, leading to reduced survival. These findings are consistent with previous studies, which showed a critical protective role for CXCL1 in pulmonary host defense against <i>K. pneumoniae</i> infection (10). Inhibition of CXCL1 attenuates lung neutrophil accumulation after intratracheal LPS administration (28). Moreover, CXCL1-transgenic mice that constitutively express lung CXCL1 show enhanced survival after <i>K. pneumoniae</i> challenge, as well as enhanced neutrophil recruitment and bacterial clearance in the lungs (28). In addition, CXCL1/CXCL1 mRNA was found to be strongly and rapidly induced in the liver and lungs, which was associated with heightened neutrophil infiltration into these organs (29).

The relative contribution of myeloid cells versus stromal cells in neutrophil accumulation after PMS is unclear. Myeloid cells in tissues produce a battery of neutrophil chemotactic substances such as CXCL1 (10, 11) and MIP-2 (12, 13), and resident cells, including epithelial and endothelial cells, produce other neutrophil chemotactants, such as LIX (14). The findings in this investigation are consistent with our previous studies, demonstrating that hematopoietic and nonhematopoietic cell-derived CXCL1 is essential for neutrophil-dependent bacterial clearance (10, 12).

We found that the local and systemic inflammatory response to PMS was dependent on CXCL1 and correlated with higher cellular influx, bacterial clearance, and reduced mortality. In addition, the expression of key inflammatory cytokines and chemokines was dependent upon CXCL1 during PMS. Interestingly, we observed that the expression of IL-1β, IL-6, LIX, and IL-17A was mediated by CXCL1 even during the early phase of sepsis (6 h). These findings are in agreement with previous studies that showed the expression of CXCL2 and CXCL5, but not TNF-α, to be dependent on CXCL1 expression in <i>K. pneumoniae</i>-infected lungs (10).
Activation of transcription factors is a central feature of inflammation and host response (20), and NF-κB is a well-studied transcription factor (21). We found reduced activity of NF-κB in the organs from cxcl12/2 mice after CLP. Although NF-κB activity is important for survival after PMS (4, 30), it is questionable whether this phenomenon contributes to efficient neutrophil recruitment and bacterial clearance. NF-κB activation has been demonstrated to be essential for neutrophil recruitment because of its contribution to the expression of adhesion molecules, such as ICAM-1 (31). Our results reveal a strong correlation between CXCL1 expression after PMS, NF-κB activation, and ICAM-1 expression. This suggests that CXCL1-mediated NF-κB activa-
tion plays a crucial role in neutrophil recruitment at early and late phases of PMS.

MAPKs are important enzymes that enhance the expression of proinflammatory cytokines, chemokines, adhesion molecules, and antibacterial effectors by activating transcription factors, such as AP-1, c-Jun, and STAT-1 (32). Our results show reduced activation of p38 (at 24 h after CLP), JNK, and ERK MAPKs in the organs from cxcl1−/− mice. These results are consistent with previous findings that showed a crucial role for CXCL1 in MAPK activation during K. pneumoniae pulmonary infection (10).

IL-17–producing CD4+ T cells control bacterial infection by secretion of neutrophil chemotactants and granulopoietic factors such as CXC chemokine CXCL2/MIP-2 and G-CSF, which cause neutrophil recruitment (33). We found not only γδ T cells, but also CD4, CD8, and NK1.1 cells are major IL-17 producers in WT mice 24 h after PMS. This suggests that CXCL1 regulates both IL-17A and IL-17F expression by major T cell subsets during sepsis. This possibility was confirmed when we performed intracellular staining for IL-17A and IL-17F in T cell subsets. Because we observed increased numbers of IL-17A–producing CD4 cells and increased IL-17A and IL-17F secretion after treatment with rCXCL1, our results suggest that CXCL1 is an essential chemokine for differentiation of naïve CD4 T cells to Th17. To validate this hypothesis, we differentiated Th0 cells to Th1, Th2, and Th17 in vitro in the absence and presence of CXCL1. Intriguingly, our findings demonstrated that CXCL1 augments Th17 differentiation, but not Th1 or Th2 differentiation (data not shown). The limitation of this study, however, noted it is not possible to use either Gram-positive or -negative bacterium in these cultures because these bacteria induced substantial cell death (data not shown).

To examine whether IL-17A can restore the protective effect on cxcl1−/− mice, we administered each of these chemokines immediately after PMS, resulting in enhanced survival of cxcl1−/− mice. In addition, enhanced neutrophil influx and bacterial clearance at the peritoneum were observed after IL-17 administration. Our observation of host protection by treatment with IL-17 in cxcl1−/− mice agrees, in part, with a previous investigation that demonstrated reduced survival rates, lower neutrophil influx, and bactericidal capacity in IL-17R gene–deficient mice after PMS (although cytokine and chemokine expression was enhanced in these mice) (8).

We observed reduced expression and activation of NADPH oxidase components Nox2, p22phox, p67phox, and p47phox in cxcl1−/− mice after PMS, which may be because of the reduced accumulation of neutrophils at the infectious focus in cxcl1−/− mice. These observations are in agreement with a previous investigation that demonstrated reduced expression of NADPH oxidase components after neutrophil depletion in WT mice after K. pneumoniae challenge (12). CXCL1–mediated activation and expression of NADPH oxidase is consistent with ROS production by neutrophils, as we observed decreased ROS production in PMS by peritoneal neutrophils in cxcl1−/− mice compared with WT counterpart. In addition, we observed a substantial reduction of ROS2, H2O2, and O2− neutrophils from cxcl1−/− mice after PMS. These findings are consistent with a previous study that demonstrated an essential role of CXCL1 in Klebsiella–induced expression of ROS by neutrophils (12).

NETosis is a unique host defense mechanism used by neutrophils to trap and kill extracellular pathogens (34). In this study, we found a strong correlation between reduced NADPH oxidase–mediated ROS production and reduced NET formation by neutrophils from cxcl1−/− mice after PMS. In addition, NET formation and NET-positive peritoneal neutrophils were reduced in cxcl1−/− mice compared with WT. To confirm this result, we observed reduced formation of typical NET structures associated with cxcl1−/− neutrophils by SEM. In addition, we observed reduced E. coli killing by bone marrow neutrophils treated with DNase, confirming the link between NET formation and bacterial killing. The results linking neutrophil ROS production with NETosis and subsequent bacterial killing are consistent with several prior reports (18, 35, 36). Our finding that neutrophils harvested from cxcl1−/− mice are deficient for bacterial killing confirms the crucial role of CXCL1 in the containment of infection by migrating neutrophils.

The data in this study illustrate a number of advancements in our understanding of the biological role of CXCL1 in host defense during PMS. CXCL1 appears to be essential for host survival, activation of NF-κB, MAPK, and NADPH oxidase, expression of cytokines and chemokines essential for host defense, ROS production, as well as NET formation by peritoneal neutrophils and NET-mediated bacterial killing (Fig. 9). More importantly, CXCL1 regulates IL-17A production by enhancing Th17 production during PMS.

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Disclosures

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