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Mast Cell IL-6 Improves Survival from *Klebsiella* Pneumonia and Sepsis by Enhancing Neutrophil Killing[1]

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The pleiotropic cytokine IL-6 has favorable and harmful effects on survival from bacterial infections. Although many innate immune cells produce IL-6, little is known about relevant sources in vivo and the nature of its contributions to host responses to severe bacterial infections. To examine these roles, we subjected mast cell-specific IL-6-deficient mice to the cecal ligation and puncture model of septic peritonitis, finding that survival in these mice is markedly worse than in controls. Following intranasal or i.p. inoculation with *Klebsiella pneumoniae*, IL-6 $^{-/-}$ mice are less likely to survive than wild-type controls and at the time of death have higher numbers of bacteria but not inflammatory cells in lungs and peritoneum. Similarly, mast cell-specific IL-6-deficient mice have diminished survival and higher numbers of *K. pneumoniae* following i.p. infection. Neutrophils lacking IL-6 have greater numbers of live intracellular *K. pneumonia,* suggesting impaired intracellular killing contributes to reduced clearance in IL-6$^{-/-}$ mice. These results establish that mast cell IL-6 is a critical mediator of survival following *K. pneumoniae* infection and sepsis and suggest that IL-6 protects from death by augmenting neutrophil killing of bacteria. *The Journal of Immunology,* 2008, 181: 5598–5605.

In the United States, pneumonia and sepsis remain leading causes of death despite availability of broad-spectrum antibiotics. In 2002, nearly 100,000 deaths were attributed to pneumonia and sepsis, making them the fifth leading causes of death (1). Furthermore, the aging population will likely be associated with an increase in the prevalence of lung infections and sepsis in the future. Because of this significant disease burden, it is imperative we learn more of the pathogenesis of infections of the lower respiratory tract and sepsis so we can derive treatments that will improve patient outcomes.

The innate immune system provides the initial defense against infectious microbes (2). Resident innate immune cells include dendritic cells (3), macrophages (4), γδ T cells (5), and mast cells (6). These cells rely on germline-encoded receptors to recognize conserved microbial products produced by the infecting bacteria. Stimulation of these receptors leads to release of proinflammatory mediators (e.g., TNF-α, IFN-γ, IL-6) that recruit or activate other innate immune cells, such as neutrophils, to the site of infection, where they kill bacteria using reactive oxygen species (7, 8) or proteases to hydrolyze cell wall proteins (9, 10).

Important cytokines that mediate host defense include TNF-α (11), IL-6 (12), IL-12 (13), and IL-18 (14) among others. IL-6 is a pleiotropic cytokine that plays disparate roles in inflammatory conditions such as the host response to bacterial infection (15). Animal models of bacterial infection suggest it protects the host from death following infection. For example, IL-6 $^{-/-}$ mice have higher mortality when infected with *Escherichia coli*, *Klebsiella pneumoniae*, *or Streptococcus pneumoniae* (12, 16, 17). In contrast, treating mice with IL-6-blocking Abs improves survival from polymicrobial septic peritonitis by reducing C5a receptor expression (18). Thus, IL-6 plays diverse and contrasting roles in the host response to bacterial infections. Although these studies show that IL-6 regulates survival, they do not explain its pleiotropic effects.

We became interested in the role of IL-6 in bacterial infections while studying dipeptidyl peptidase I (DPPI)[4]-deficient mice in the cecal ligation and puncture (CLP) model of septic peritonitis (19). In those studies, we found that DPPI-deficient mice are more likely to survive septic peritonitis. This protection is due to the absence of mast cell-DPPI, which increases peritoneal levels of IL-6. In these studies, the observation that lack of IL-6 reduces survival in DPPI-deficient mice indicated that the survival advantage was dependent on IL-6, but it did not identify the cellular source of the beneficial IL-6. We hypothesized that mast cell IL-6 may be a critical cellular source of IL-6 in the CLP model. To test this hypothesis in the current work, we studied mast cell-specific IL-6-deficient mice in response to CLP sepsis and *K. pneumonia* lung and peritonitis infection. Our results suggest that mast cell IL-6 is a major mediator of survival from these severe infections and that it protects from death by enhancing intracellular killing of bacteria by neutrophils.

Materials and Methods

Materials

All chemicals were obtained from Sigma-Aldrich, unless otherwise noted.

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[4] Abbreviations used in this paper: DPPI, dipeptidyl peptidase I; BMMC, bone marrow mast cell; CLP, cecal ligation and puncture; rm, recombinant mouse; SCF, stem cell factor.

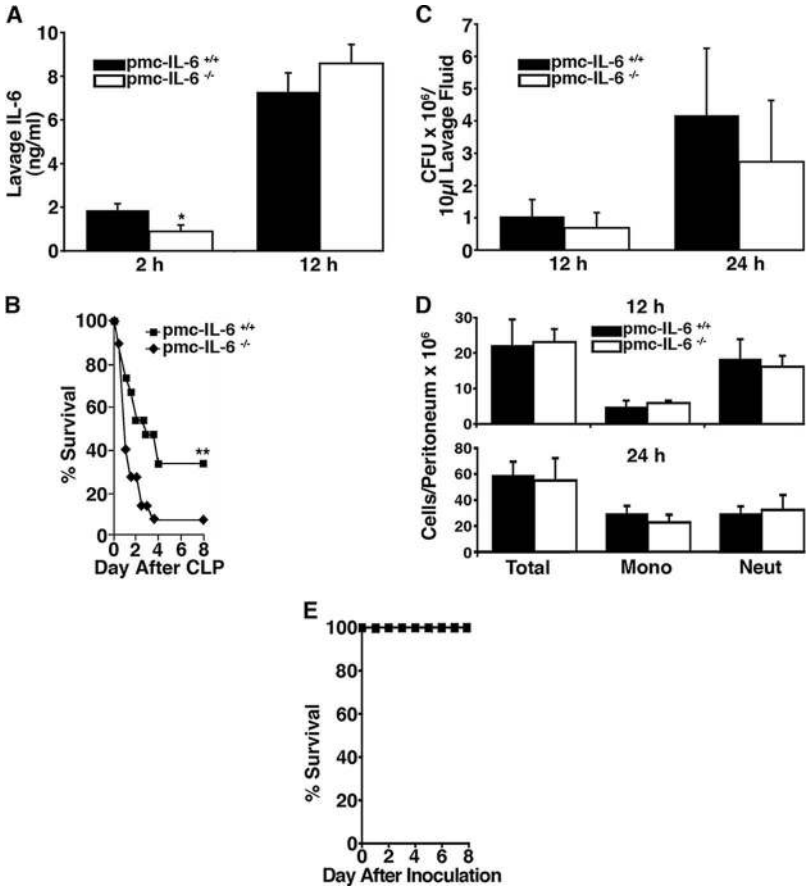

FIGURE 1. Mast cell IL-6 regulates survival from septic peritonitis. *A*, Concentrations of IL-6 were measured by ELISA in peritoneal lavage fluid obtained from pmc-IL-6$^{+/+}$ and pmc-IL-6$^{-/-}$ mice 2 and 12 h after CLP. Note that lavage fluid from pmc-IL-6$^{-/-}$ mice has significantly lower levels of IL-6 compared with that obtained from pmc-IL-6$^{+/+}$ mice 2 h but not 12 h after CLP. *B*, pmc-IL-6$^{-/-}$ mice are protected from death by septic peritonitis. pmc-IL-6$^{+/+}$ ($n = 24$) and pmc-IL-6$^{-/-}$ ($n = 25$) mice were subjected to CLP (50% ligation and single puncture with a 25-gauge needle) and survival was monitored for 8 days. The figure is representative of survival curves from three separate experiments. *C* and *D*, Bacterial load and i.p. inflammatory cell recruitment after CLP is similar in pmc-IL-6$^{+/+}$ and pmc-IL-6$^{-/-}$ mice. pmc-IL-6$^{+/+}$ and pmc-IL-6$^{-/-}$ mice ($n = 7$–9 mice/group) were euthanized 12 and 24 h after CLP. Peritoneal lavaged fluid was cultured on blood agar plates. Total cells were counted using a hemocytometer and the cell types were determined on cytospun cells stained with Diff-Quik. *E*, Intraperitoneal bacteria cultured on blood agar plates are not pathogenic. Peritonea of 10 wild-type mice were injected with 10^6 CFUs of bacteria (suspended in 200 μl of culture medium) cultured from lavage fluid of mice subjected to CLP. Survival was monitored for 8 days after inoculation. Data are representative of two separate experiments. *, $p = 0.01$ and **, $p < 0.01$. Mono, Monocyte; Neut, neutrophil.

Experimental animals

C57BL/6 IL-6 $^{-/-}$ and IL-6 $^{+/+}$ mice were purchased from The Jackson Laboratory. C57BL/6 *Kit*$^{W\text{-}sh}$/*Kit*$^{W\text{-}sh}$ mice (20) were provided by P. Besmer (Memorial Sloan-Kettering Institute, New York, NY). All experimental procedures were performed in 8- to 12-wk-old mice and were approved by the University of California, San Francisco Committee on Animal Research.

Cecal ligation and puncture

A 1-cm midline incision was made in the abdominal wall of anesthetized mice and the cecum was identified. The distal 50% of exposed cecum was ligated with 3-0 silk suture and punctured once with a 25-gauge needle. The cecum was replaced into the abdomen, the incision closed with 3-0 suture, and the mouse recovered with a 0.5-ml i.p. injection of sterile 0.9% NaCl. Mice were monitored three times daily and survival was recorded. Moribund mice were euthanized by CO_2 inhalation and cervical dislocation.

Induction of K. pneumoniae *infection in mice*

K. pneumoniae (strain 43816, serotype 2; American Type Culture Collection) was resuspended in 5 ml of Nutrient Broth (Difco) and cultured overnight at 37°C. One hundred microliters of this suspension was added to 50 ml of Nutrient Broth and grown for 3–4 h to log phase when CFUs were determined by OD_{600} readings and confirmed by culture. Using this *K. pneumoniae*, Gram-negative pneumonia was generated in mice by inoculating anesthetized mice intranasally via a sterile pipette tip with 3000 CFU of *K. pneumoniae* suspended in 50 μl of culture medium. *K. pneumoniae* septic peritonitis was generated in mice by injecting the peritoneum of mice with 150 CFUs of *K. pneumoniae* suspended in 200 μl of culture medium. Mice were recovered from anesthesia and survival was monitored three times daily. Moribund mice were euthanized by CO_2 inhalation and cervical dislocation.

Mast cell culture from bone marrow

Mouse bone marrow mast cells (BMMC) were cultured in recombinant mouse (rm) IL-3 and recombinant mouse stem cell factor (SCF; PeproTech) as previously described (21). Cells were used after 5 wk in culture, at which time the cell populations consisted of >95% mast cells (identified by metachromatic granules in toluidine blue-stained cells). Furthermore, BMMC cultured from IL-6$^{+/+}$ and IL-6$^{-/-}$ bone marrow showed similar granular morphology, levels of active tryptase, and expression of FcεR-Ia and CD117, indicating they have similar maturation when cultured in the presence of IL-3 and SCF.

Mast cell reconstitution of Kit$^{W\text{-}sh}$/*Kit*$^{W\text{-}sh}$ *mice*

We used C57BL/6 *Kit*$^{W\text{-}sh}$/*Kit*$^{W\text{-}sh}$ mast cell-deficient mice (22) for these studies because they have the same genetic background as the IL-6$^{-/-}$ mice and have no mast cells detectable by metachromatic staining (23). To reconstitute i.p. mast cells, 4×10^6 IL-6$^{+/+}$ or IL-6$^{-/-}$ BMMC suspended in 500 μl of sterile PBS were injected into the peritoneum of 5-wk-old *Kit*$^{W\text{-}sh}$/*Kit*$^{W\text{-}sh}$ mice. After allowing 5 wk for mast cells to differentiate within the peritoneum (24, 25), reconstituted mice were used in the experiments. This method selectively reconstitutes peritoneal mast cells to similar levels in the mesentery or peritoneum ($3.6 \times 10^5 \pm 1.2 \times 10^5$ vs $3.4 \times 10^5 \pm 2.3 \times 10^5$ mast cells/peritoneum, respectively) using either IL-6$^{+/+}$ or IL-6$^{-/-}$ BMMC.

To reconstitute lungs of *Kit*$^{W\text{-}sh}$/*Kit*$^{W\text{-}sh}$ mice, 10^7 BMMC suspended in 200 μl of PBS were injected into the tail vein of 5-wk-old *Kit*$^{W\text{-}sh}$/*Kit*$^{W\text{-}sh}$ mice. After allowing 12 wk for mast cells to migrate to and differentiate within lung (23, 26), the reconstituted mice were used in the experiments. This method selectively reconstitutes lung mast cells to similar levels using either IL-6$^{+/+}$ or IL-6$^{-/-}$ BMMC.

Quantification of cellular response to infection

To recover i.p. inflammatory cells, anesthetized mice were euthanized by cervical dislocation and the abdominal skin cleansed with 70% ethanol. Four milliliters of sterile 0.9% NaCl was then instilled into the peritoneum. The abdomen was massaged gently for 1 min and opened with a sterile scissors and lavage fluid reclaimed. Similarly, to recover intrapulmonary inflammatory cells, lungs of euthanized mice were lavaged three times with 0.7 ml of sterile 0.9% NaCl. Recovered lavage fluid was pooled. In both cases, recovered lavage fluid was centrifuged at 850 × *g* for 4 min at 4°C and the supernatant was saved for cytokine analysis. Cell pellets were

resuspended in red cell lysis buffer (Sigma-Aldrich) for 10 min, recentrifuged, and the cell pellet was resuspended in PBS. Cell numbers were counted with a hemocytometer and cell differentials were determined on cytospun cells stained with Diff-Quik (American Scientific Products).

Quantification of bacterial CFUs

Lungs and spleens were aseptically harvested, placed in 500 μl of sterile saline, and homogenized. Ten microliters of tissue homogenate or peritoneal lavage fluid was diluted serially in sterile 0.9% NaCl. Ten microliters of each dilution was aseptically plated and cultured on tryptose blood-agar plates (for CLP experiments) or nutrient agar for nonfastidious microorganism plates (for *K. pneumoniae* infection experiments) at 37°C. After 24 h, the numbers of bacterial colonies were counted.

Cytokine analysis

Cytokine concentrations were measured in lung and peritoneal lavage fluid using ELISA kits: MIP-2 (R&D Systems), KC (R&D Systems), TNF-α (eBioscience), IL-6 (R&D Systems), and IFN-γ (eBioscience) according to the manufacturers' protocols.

Neutrophil isolation

Neutrophils were isolated from murine bone marrow by flushing front and hind legs of mice with salt-free HBSS (Invitrogen). The bone marrow cells were then layered on an 82-62% Percoll (Sigma-Aldrich) gradient buffered with salt-free HBSS, 0.02 M HEPES, 0.5% FBS (Life Technologies), and 0.04 M $NaHCO_3$ (Sigma-Aldrich,) then spun at 1200 $\times$ *g* at 18°C for 30 min. Neutrophils were collected from the interface between the Percoll layers and resuspended in Krebs-Ringer solution. Neutrophil purity was 85–95% when assessed by Diff-Quik (American Scientific Products) staining.

Intracellular killing assay

Fresh cultures of *K. pneumoniae* were suspended in Krebs-Ringer phosphate at 4×10^8 CFU/ml. Fifty microliters of bacteria was then added to 4×10^6 neutrophils with or without a 1-h pretreatment with 100 or 500 ng/ml recombinant murine IL-6 (PeproTech) or 4×10^6 thioglycolate-elicited peritoneal macrophages, suspended in 2 ml of KRP, and incubated at 37°C for 1 h. Gentamicin (100 μg/ml) was added to kill extracellular bacteria, and the cells were subsequently washed and then incubated at 18°C for varying lengths of time. Cells were then washed three times in ice-cold salt-free HBSS and lysed in 200 μl of salt-free HBSS containing 0.1% Triton X-100 (Sigma-Aldrich). Twenty microliters of the lysate was then plated on Luria-Bertani-agar plates (Sigma-Aldrich), cultured overnight at 37°C, and colonies counted.

Mast cell Klebsiella *killing*

Five hundred CFU of freshly cultured *K. pneumoniae* were suspended in 40 μl of RPMI 1640 containing 5000 BMMC, with or without a 1-h pretreatment with 0.5 ng/ml recombinant murine IL-6 (PeproTech), and incubated at 37°C. Medium containing bacteria but no mast cells was cultured as control. After 60 min, 10 μl of the mixture was plated on nutrient agar plates, cultured overnight at 37°C, and colonies counted.

Statistics

Survival curves were analyzed using the two-tailed Fisher's exact test. ANOVA followed by two-tailed *t* testing was used to compare markers of organ dysfunction, bacterial CFUs, and mean cytokine concentrations. All calculations were performed using StatView 5.0.1 software (SAS Institute). Significance was assigned to p values <0.05.

Results

Mast cell-IL-6 modulates survival following polymicrobial septic peritonitis

To test the hypothesis that mast cell IL-6 modulates sepsis survival, we compared IL-6 levels in peritoneal lavage fluid from mast cell-deficient $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice selectively reconstituted with IL-$6^{-/-}$ BMMC (pmc-IL-$6^{-/-}$ mice) to lavage fluid from $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice selectively reconstituted with IL-$6^{+/+}$ BMMC (pmc-IL-$6^{+/+}$ mice). We found that mice lacking IL-6 solely in mast cells (pmc-IL-$6^{-/-}$ mice) had lower levels of peritoneal IL-6 two h (but not 12 h) after CLP (Fig. 1*A*). Thus, mast cells are a key i.p. source of IL-6 early after the onset of polymicrobial peritoneal infection.

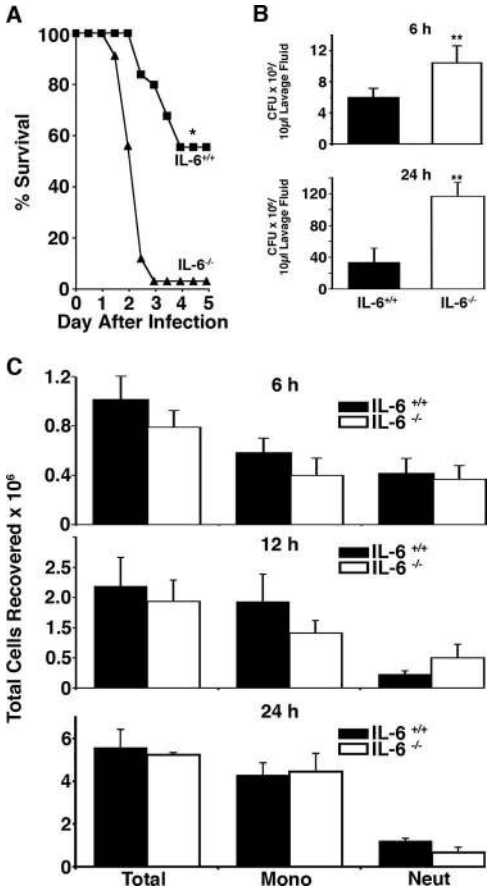

FIGURE 2. Mast cell IL-6 regulates survival following *K. pneumoniae* peritonitis. *A*, IL-$6^{+/+}$ and IL-$6^{-/-}$ mice ($n = 25$ mice/group) were inoculated with 150 CFUs of *K. pneumoniae* (strain 43816, serotype 2) suspended in 200 μl of PBS and survival was monitored. Note that IL-$6^{-/-}$ mice have significantly worse survival than controls. *B*, IL-$6^{-/-}$ mice have impaired clearance of *K. pneumoniae* from the peritoneum. IL-$6^{+/+}$ and IL-$6^{-/-}$ mice inoculated i.p. with 150 CFUs of *K. pneumoniae* were euthanized 6 or 24 h after infection, their peritonea were lavaged, and bacterial CFUs were quantified by culturing dilutions of lavage fluid on nutrient agar plates. *C*, Intraperitoneal inflammatory cell recruitment is normal in IL-$6^{-/-}$ mice. IL-$6^{+/+}$ and IL-$6^{-/-}$ mice inoculated i.p. with 150 CFUs of *K. pneumoniae* were euthanized 6, 12, and 24 h after infection, their peritonea were lavaged, and numbers of inflammatory cells recovered were counted ($n = 6$–9 mice/group). The survival curve is representative of two separate experiments and CFU quantification from three separate experiments. *, $p < 0.003$ and **, $p < 0.05$. Mono, Monocyte; Neut, neutrophil.

Next, we examined whether this early production of mast cell IL-6 regulates survival following septic peritonitis by comparing survival of pmc-IL-$6^{-/-}$ and pmc-IL-$6^{+/+}$ mice subjected to CLP. In so doing, we found that survival in mast cell-specific IL-6-deficient pmc-IL-$6^{-/-}$ mice is markedly worse than in pmc-IL-$6^{+/+}$ control mice (37% vs 12%, Fig. 1*B*) 8 days after onset of peritonitis. These results indicate mast cell IL-6 is a major regulator of survival following septic peritonitis.

To understand how mast cell IL-6 modulates survival, we examined endpoints commonly explaining differences in survival in this model, including cellular indices of inflammation, cytokine levels, and bacterial load at intervals after CLP. Surprisingly, we found no differences (Fig. 1, *C* and *D*, and data not shown).

Because IL-6 reportedly enhances bacterial clearance and the pathogenic bacteria in the CLP model are unknown, we suspected we were not culturing the pathogenic bacteria using standard blood

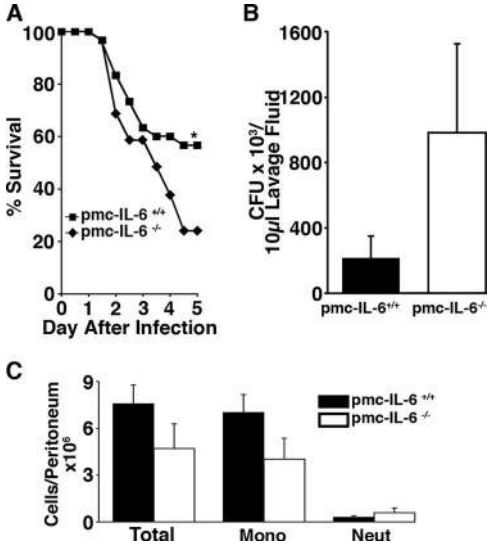

FIGURE 3. Mast cell IL-6 regulates survival following *K. pneumoniae* peritonitis. *A*, pmc-IL-6$^{+/+}$ ($n = 30$) and pmc-IL-6$^{-/-}$ ($n = 29$) mice were inoculated with 150 CFUs of *K. pneumoniae* suspended in 200 μl of PBS and survival was monitored. Note that pmc-IL-6$^{-/-}$ mice have significantly worse survival than controls. *B*, pmc-IL-6$^{-/-}$ mice have impaired clearance of *K. pneumoniae* from the peritoneum. pmc-IL-6$^{+/+}$ and pmc-IL-6$^{-/-}$ mice inoculated i.p. with 150 CFUs of *K. pneumoniae* were euthanized 24 h after infection, their peritonea were lavaged, and bacterial CFUs were quantified by culturing dilutions of lavage fluid on nutrient agar plates ($n = 6$–9 mice/group). *C*, Intraperitoneal inflammatory cell recruitment is normal in pmc-IL-6$^{-/-}$ mice. pmc-IL-6$^{+/+}$ and pmc-IL-6$^{-/-}$ mice inoculated i.p. with 150 CFUs of *K. pneumoniae* were euthanized 6 h after infection, their peritonea were lavaged, and numbers of inflammatory cells recovered were counted ($n = 9$ mice/group). The survival curve is representative of two separate experiments and CFU quantification from three separate experiments. $*$, $p = 0.02$. Mono, Monocyte; Neut, neutrophil.

agar plates and therefore underestimating differences in bacterial load of the true pathogenic bacteria. To test this possibility, we inoculated peritonea of 10 C57BL/6 wild-type mice with 10^6 CFUs of bacteria recovered on the blood agar plates and found that none of the infected mice died 8 days after inoculation (Fig. 1*E*). Thus, the bacteria recovered on blood agar plates were not pathogenic, leaving unresolved the possibility that clearance of pathogenic bacteria is impaired in pmc-IL-6$^{-/-}$ mice.

Mast cell IL-6 regulates survival and bacterial clearance following peritoneal K. pneumoniae *infection*

Our lack of a biological explanation for the survival advantage of pmc-IL-6$^{-/-}$ mice following CLP and the unanswered question of whether bacterial load of the pathogenic bacteria was different in pmc-IL-6$^{-/-}$ mice prompted us to study whether IL-6 regulated survival from *K. pneumoniae* septic peritonitis. This model allowed us to reliably quantify the load of infectious bacteria. First, IL-6$^{+/+}$ and IL-6$^{-/-}$ mice were infected i.p. with *K. pneumoniae*. We found that IL-6$^{-/-}$ mice have significantly worse survival and impaired clearance of *K. pneumoniae* from the peritoneum 6 or 24 h after inoculation compared with IL-6$^{+/+}$ controls (Fig. 2, *A* and *B*). In contrast, there was no difference in the number or type of inflammatory cells in the peritoneum 6, 12, or 24 h after inoculation in IL-6$^{+/+}$or IL-6$^{-/-}$ mice (Fig. 2*C*). Next, to determine whether mast cell IL-6 regulates *K. pneumoniae* peritonitis, we infected pmc-IL-6$^{-/-}$ and pmc-IL-6$^{+/+}$ mice, finding that survival is worse and peritoneal clearance of *K. pneumoniae* is impaired in

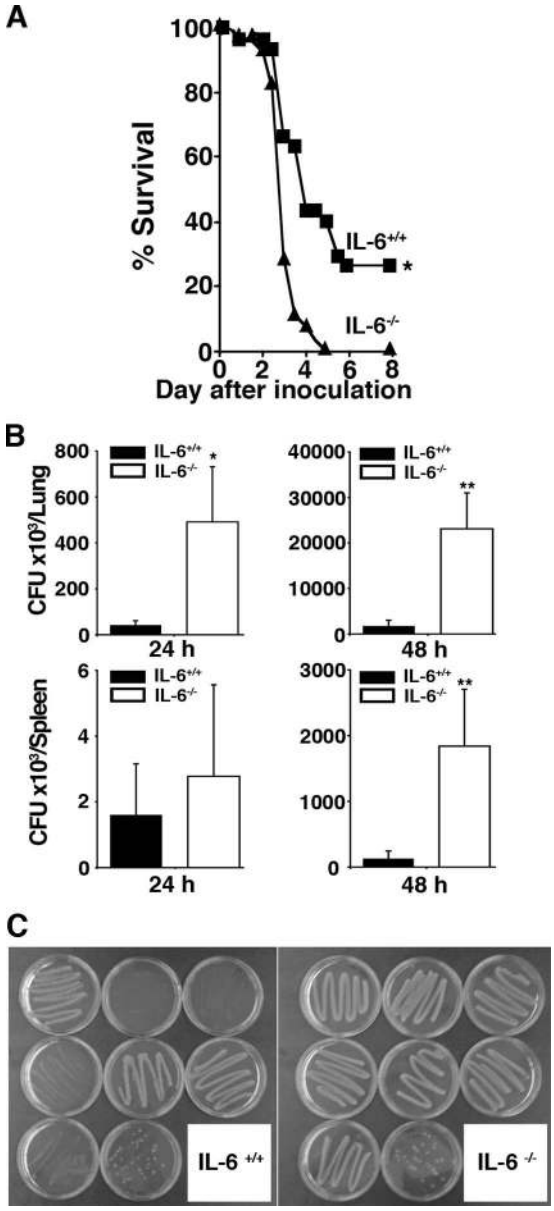

FIGURE 4. IL-6 is required for survival from *K. pneumoniae* lung infection. *A*, IL-6$^{+/+}$ ($n = 30$) and IL-6$^{-/-}$ ($n = 27$) mice were intranasally inoculated with 3000 CFUs of *K. pneumoniae* and survival was monitored for 8 days. Note that IL-6$^{-/-}$ mice have significantly worse survival than IL-6$^{+/+}$ mice 8 days after infection. The figure is representative of three separate experiments. *B*, IL-6$^{-/-}$ mice have impaired clearance of *K. pneumoniae* from the lung. IL-6$^{+/+}$ and IL-6$^{-/-}$ mice ($n = 7$–9 mice/group) inoculated with 3000 CFUs of *K. pneumoniae* were euthanized 24 or 48 h after infection, their lungs and spleens were aseptically harvested and homogenized in 500 μl of sterile saline, and bacterial CFUs were quantified by culturing dilutions of homogenates on nutrient agar plates. Note there are significantly more bacteria in the lung and spleen homogenates obtained from IL-6$^{-/-}$ mice. Similar results were found in two separate experiments. *C*, IL-6$^{-/-}$ mice have a greater burden of mediastinal bacteria than IL-6$^{+/+}$ controls. Forty-eight hours after intranasal inoculation with 3000 CFUs of *K. pneumoniae*, the mediastina of euthanized mice were swiped once with a sterile swab and cultured on a nutrient agar plate. Note that seven of eight of the swabs from IL-6$^{-/-}$ mice had large bacterial growth compared with only three of eight from IL-6$^{+/+}$ mice. $*$, $p = 0.05$ and $**$, $p = 0.01$.

pmc-IL-6$^{-/-}$ mice (Fig. 3, *A* and *B*). Furthermore, there were no significant differences in the number or type of inflammatory cells in the peritoneum of pmc-IL-6$^{+/+}$or pmc-IL-6$^{-/-}$ mice 6 h after

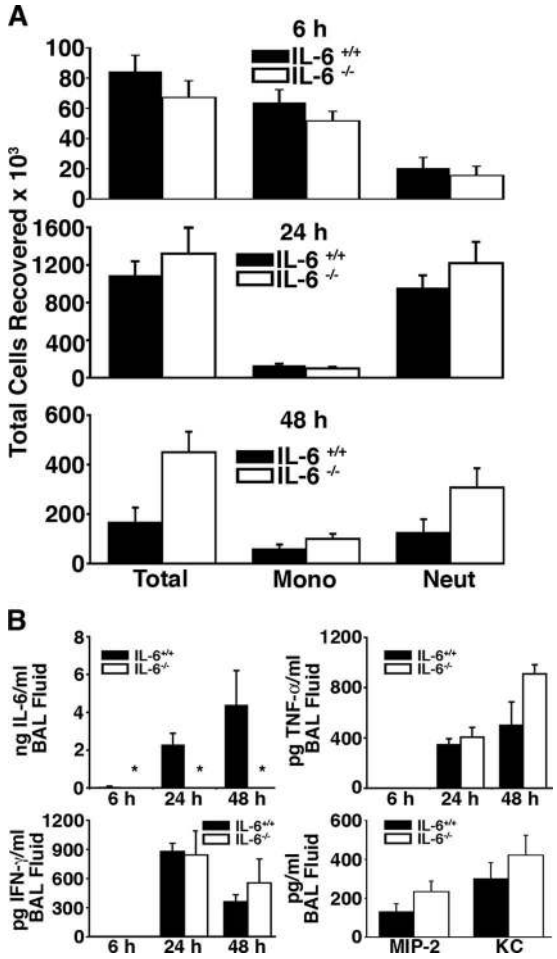

FIGURE 5. IL-6-deficient mice have normal recruitment of inflammatory cells to the lung. *A*, IL-$6^{+/+}$ and IL-$6^{-/-}$ mice ($n = 8$–10 mice/group) were euthanized 6, 24, and 48 h after intranasal inoculation of 3000 CFUs of *K. pneumoniae*. Lungs were lavaged and the total cells in lavage fluid were counted using a hemocytometer. Cell differentials were determined on cytospun cells stained with Diff-Quik. *B*, Cytokine measurements. Concentrations of TNF-α, IL-6, and IFN-γ were measured by ELISA in bronchoalveolar lavage fluid obtained from IL-$6^{+/+}$ and IL-$6^{-/-}$ mice 6, 24, and 48 h and MIP-2 and KC 6 h after intranasal inoculation of 3000 CFUs of *K. pneumoniae* ($n = 8$–10 mice/group). $*$, $p < 0.001$. Mono, Monocyte; Neut, neutrophil.

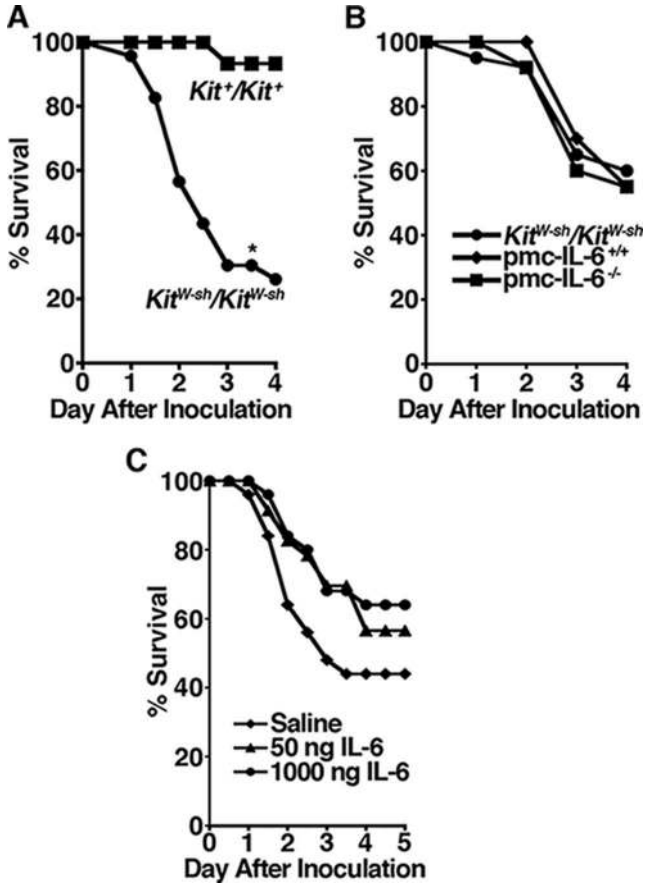

FIGURE 6. Mast cells regulate survival following *K. pneumoniae* lung infections. *A*, Kit^{+}/Kit^{+} ($n = 15$) and $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice ($n = 23$) were intranasally inoculated with 3000 CFUs of *K. pneumoniae* and survival was monitored for 4 days. *B*, $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice and $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice reconstituted with IL-$6^{+/+}$ (pmc-IL-$6^{+/+}$) or IL-$6^{-/-}$ (pmc-IL-$6^{-/-}$) BMMC ($n = 25$ mice/group) were intranasally inoculated with 3000 CFUs of *K. pneumoniae* and survival was monitored for 4 days. *C*, $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice pretreated 30 min before infection with 100 μl of saline, 50 ng of rm-IL-6, or 1000 ng of rm-IL-6 were intranasally inoculated with 3000 CFUs of *K. pneumoniae* and survival was monitored for 5 days ($n = 20$ mice/group). The figure is representative of survival curves from three separate experiments. $*$, $p < 0.001$.

inoculation (Fig. 3*C*). These data indicate that mast cell IL-6 modulates survival from *K. pneumoniae* infections by enhancing bacterial clearance.

IL-$6^{-/-}$ mice have impaired bacterial clearance and worse survival following K. pneumoniae *lung infection*

Next, we sought to extend our understanding of the role of IL-6 to *K. pneumoniae* lung infections. Similar to peritoneal infection, we found that following intranasal inoculation with *K. pneumoniae*, IL-$6^{-/-}$ mice have significantly worse survival than IL-$6^{+/+}$ controls (0% vs 32%) 8 days after inoculation (Fig. 4*A*). At the time they succumb to infection (24–48 h after inoculation), IL-$6^{-/-}$ mice have significantly higher numbers of bacteria in their lungs and spleens (Fig. 4*B*) and, qualitatively, eight of eight IL-$6^{-/-}$ mice had a heavy growth of bacteria cultured from their mediastinum compared with only three of eight IL-$6^{+/+}$ mice (Fig. 4*C*). IL-$6^{-/-}$ mice recruited inflammatory cells normally as levels of lung cellular inflammation were similar in IL-$6^{+/+}$ and IL-$6^{-/-}$ mice 6, 24, and 48 h after infection (Fig. 5*A*). Interestingly, with the exception of IL-6, the IL-$6^{-/-}$ mice did not express differences in TNF-α, IFN-γ, MIP-2, or KC compared with IL-$6^{+/+}$ controls at various time points after infection (Fig. 5*B*). These data indicate that IL-6 regulates survival from lung infection and that the higher mortality rate of IL-$6^{-/-}$ mice correlates with impaired bacterial clearance.

Next, we tested whether mast cell IL-6 modulates survival following *K. pneumoniae* lung infection. First, to test whether mast cells are important in this model, we compared survival of wild-type and $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice infected intranasally with *K. pneumoniae*, finding that mast cell-deficient $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice have worse survival 4 days after inoculation (Fig. 6*A*). To test whether mast cell IL-6 regulates survival from *K. pneumoniae* lung infection, we first measured survival of $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice pretreated with IL-6 and found that $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice pretreated with IL-6 have better survival 5 days after inoculation with *K. pneumoniae* than saline-treated controls (Fig. 6*C*). This result indicates that exogenous IL-6 administration improves survival of $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice and suggests that an absence of mast cell IL-6 is partially responsible for the worse survival of $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice.

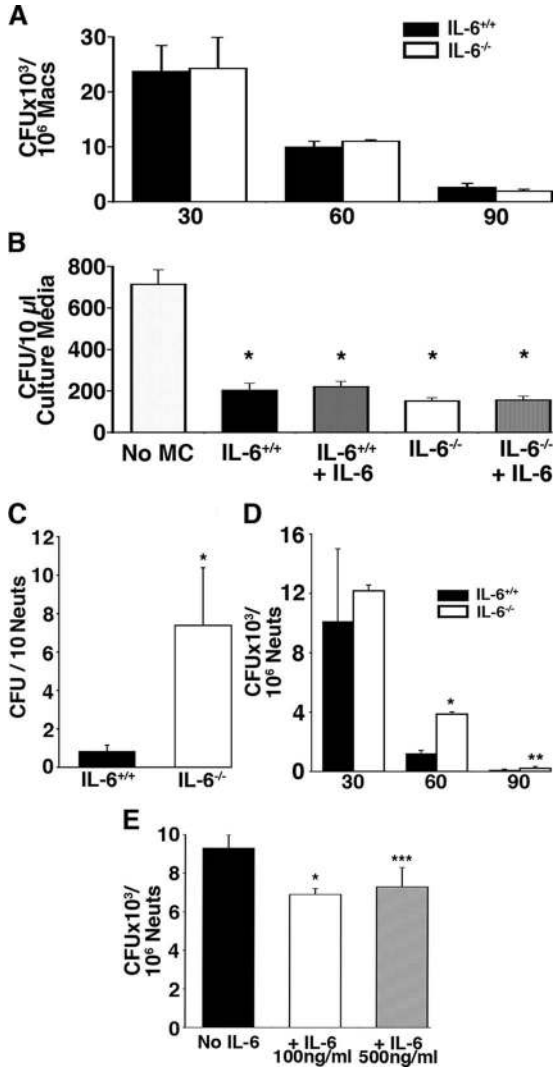

FIGURE 7. IL-6 activation of neutrophils enhances intracellular killing of *K. pneumoniae*. *A*, IL-6$^{+/+}$ and IL-6$^{-/-}$ macrophages were incubated in the presence of *K. pneumoniae*. One hour after incubation, macrophages were washed in PBS containing gentamicin and, at various time points after washing, they were lysed, and live intracellular *K. pneumoniae* were identified by culture. *B*, IL-6$^{+/+}$ and IL-6$^{-/-}$ BMMC treated with and without IL-6 were incubated in the presence of *K. pneumoniae*. Sixty minutes after incubation, aliquots of the mixture were removed and the number of live *K. pneumoniae* were identified by culture. Medium containing bacteria but no mast cells (No MC) was cultured as control. *C*, IL-6$^{+/+}$ and IL-6$^{-/-}$ mice were euthanized 24 h after intranasal inoculation of 3000 CFUs of *K. pneumoniae*. Their lungs were lavaged with PBS containing gentamicin, neutrophils were isolated and lysed, and the number of live intracellular *K. pneumoniae* was identified by culture. IL-6$^{+/+}$ and IL-6$^{-/-}$ neutrophils (*D*) or (*E*) IL-6$^{-/-}$ neutrophils (*E*) treated with and without IL-6 were incubated in the presence of *K. pneumoniae*. One hour after incubation, neutrophils were washed in PBS containing gentamicin. Thirty to 90 min (60 min; *E*) after washing, neutrophils were lysed and live intracellular *K. pneumoniae* were identified by culture. Experiments were performed in triplicate and are representative of at least two independent assays. *, $p < 0.03$; **, $p = 0.06$; and ***, $p = 0.05$.

To further investigate whether the reduced survival of *Kit*$^{W\text{-}sh}$/*Kit*$^{W\text{-}sh}$ mice reflects the absence of mast cells and to test whether mast cell IL-6 influences survival, we also compared the survival of *Kit*$^{W\text{-}sh}$/*Kit*$^{W\text{-}sh}$ mice and *Kit*$^{W\text{-}sh}$/*Kit*$^{W\text{-}sh}$ mice reconstituted with BMMC cultured from IL-6$^{+/+}$ and IL-6$^{-/-}$ mice. Surprisingly, we found no difference in survival of the three groups of mice (Fig. 6*B*).

IL-6 directly activates neutrophils to enhance intracellular killing of K. pneumoniae

Bacterial killing by macrophages, mast cells, and neutrophils are major contributors to bacterial clearance (10, 27, 28). To investigate whether a defect in intracellular *Klebsiella* killing by macrophages explained the higher bacterial load in IL-6$^{-/-}$ mice, we quantified live intracellular bacteria in macrophages isolated from IL-6$^{+/+}$ and IL-6$^{-/-}$ mice after in vitro inoculation and found similar numbers of intracellular bacteria in IL-6-deficient macrophages at various time points after inoculation (Fig. 7*A*). Next, to test whether killing of *K. pneumoniae* is defective in IL-6$^{-/-}$ mast cells, we compared total *Klebsiella* killing by IL-6$^{+/+}$ and IL-6$^{-/-}$ mast cells. Again finding no difference (Fig. 7*B*). These observations indicate a defect in *Klebsiella* killing by macrophages or mast cells does not explain the higher bacterial load detected in IL-6$^{-/-}$ mice.

To test whether neutrophil killing of *K. pneumoniae* is defective in IL-6-deficient mice in vivo, we quantified the number of intracellular bacteria in neutrophils isolated from lungs of IL-6$^{+/+}$ and IL-6$^{-/-}$ mice 24 h after intranasal *K. pneumoniae* inoculation, finding higher numbers of *Klebsiella* in neutrophils from IL-6-deficient mice (Fig. 7*C*). Because bacterial clearance is impaired in IL-6$^{-/-}$ mice, the differences in intracellular bacteria was possibly due to the higher bacterial load, rather than a defect in intracellular killing by IL-6-deficient neutrophils. To differentiate these possibilities, we next quantified live intracellular bacteria in neutrophils isolated from IL-6$^{+/+}$ and IL-6$^{-/-}$ mice after in vitro inoculation, finding more intracellular bacteria in IL-6-deficient neutrophils (Fig. 7*D*). Because phagocytosis in leukocytes isolated from IL-6$^{-/-}$ mice is normal (data not shown), these data indicate that the impaired clearance is due, at least in part, to defective intracellular killing by neutrophils. Finally, to determine whether IL-6 acts directly on neutrophils to enhance killing, we compared intracellular killing of *K. pneumoniae* by IL-6-deficient neutrophils in the presence and absence of IL-6, finding that addition of IL-6 enhances neutrophil killing (Fig. 7*E*). These data show that IL-6 directly activates neutrophils to enhance intracellular killing of *K. pneumoniae*.

Discussion

This work shows for the first time that mast cell IL-6 regulates survival from pneumonia and sepsis by activating neutrophils and enhancing killing of intracellular bacteria. In addition, the pleiotropic behavior of IL-6 during sepsis may be explained, in part, by our finding that mast cell sources of IL-6 are beneficial while others may be detrimental to the host during sepsis. This dependence of IL-6 activity on its cellular source predicts that attempts to achieve systemic blockade of IL-6 during sepsis may fail to be therapeutic because they simultaneously block detrimental and beneficial sources.

Rat, mouse, and human mast cells have all been reported to make IL-6 in culture (29–34) or in specific disease states in humans (35). Depending on conditions, mast cells make low levels of IL-6 constitutively (29, 33, 36) and increase its production in response to various stimuli such as SCF (37), PGE_2 (38, 39), and IgE (30), TLR2 (40), or TLR4 (40, 41) receptor activation in rodent mast cells and IgE receptor activation (34, 42) and IL-1 (43) in human mast cells. Unlike TNF-α (44) and IL-15 (45), mast cells do not store IL-6 in their secretory granules. Instead, they traffic it via a separate vesicular compartment targeted for immediate release (43). Although mast cells have long been recognized to make

IL-6, only recently have experiments using mast cell-specific IL-6-deficient mice been used to establish that mast cell sources of IL-6 play critical roles in pathologic processes such as development of abdominal aortic aneurysms and atherosclerosis (46, 47). This report is unique because it is the first to demonstrate a protective role for mast cell IL-6 following severe bacterial infections in mice. Whether mast cell-IL-6 similarly improves survival following severe bacterial infections in humans remains to be established.

Mast cells are sentinel cells, which are present in most tissues where they serve to monitor the local microenvironment for invading microorganisms and bacteria (48). When infectious bacteria are present, molecules unique to the bacteria activate receptors on the mast cell, triggering release of mediators (e.g., tryptase and TNF-α) that recruit neutrophils to the site of infection (6, 24, 49, 50). The current work extends these findings by showing that mast cells also release IL-6, which activates the recruited neutrophil to enhance killing of invading bacteria. Mast cell IL-6 seems to be required only early after infection because by 12 h other sources compensate for the mast cell contribution (Fig. 1).

Mast cell IL-6 profoundly impacts survival despite being required only in the early hours after infection. Our data suggest it regulates survival by enhancing killing by early arriving neutrophils, thereby lowering the initial burden of bacteria. Conversely, in the absence of mast cell IL-6, greater numbers of the initial bacterial inoculum are able to survive the early mast cell-dependent responses to the infection. This higher bacterial load then multiplies and overwhelms subsequent defenses to the infection and in the case of *K. pneumoniae* lung infection, the bacteria penetrate outside the lung parenchyma into the circulation and mediastinum with resultant mortality from overwhelming infection. Thus, host survival depends critically on mast cells and their ability to modulate bacterial load in the first hours after infection.

The poor survival of mast cell-deficient mice after intranasal inoculation with *K. pneumoniae* suggests that mast cells regulate host responses to lung and airway infection. However, mast cell reconstitution studies did not differentiate whether this survival difference is due to the absence of mast cells or a general defect in Kit receptor signaling in $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice. In contrast, experiments showing that mast cell IL-6 modulates *K. pneumoniae* peritoneal infection (Fig. 3), indicate that mast cells do play a role in controlling *K. pneumoniae* infections and suggest the reconstitution studies did not detect a role for mast cells in the lung infection model because the mast cells reside in a different compartment of reconstituted lung (23).

Because i.v. injection of mast cells reconstitutes the lung parenchyma rather than their normal location in the trachea and large airways, we believe the reconstitution studies provide further evidence supporting the idea that mast cells are essential for very early responses to infection, but dispensable in the later stages of infection. In wild-type mice, mast cells normally are present in greatest numbers in the trachea where they are poised to readily detect and respond to the initial bacterial inoculum. In contrast, in the reconstituted mouse, mast cells are in the distal lung parenchyma where they encounter bacteria only after the initial inoculum has multiplied within the proximal airways and extended deep into the parenchyma. By this stage in the infection, the bacterial load is sufficiently high that the mast cell responses are inconsequential compared with other innate immune cells (macrophages and neutrophils), which are present in greater numbers in the lung parenchyma.

IL-6 is a pleiotropic cytokine (15) that, depending on the infection model, has been reported to have a beneficial (12, 16, 51, 52), detrimental (18), or neutral (19, 53) effect on survival of bacterial infection. This manuscript provides evidence that the pleiotropic effects of IL-6 can be explained by the idea that some (mast cell) cellular sources of IL-6 have a favorable, and others a detrimental, effect on survival. For example, when wild-type and IL-6-deficient mice are subjected to CLP, the survival of both groups is identical 8 days later (19, 53), suggesting that IL-6 does not regulate survival from septic peritonitis. However, in this report, we show that mice lacking IL-6 exclusively in mast cells are less likely to survive than mice reconstituted with wild-type mast cells 8 days after CLP. These results indicate that mast cell-derived IL-6 protects from death from septic peritonitis and, by extension, that nonmast cell sources of IL-6 likely promote death during septic peritonitis. Identification of these detrimental cellular sources of IL-6 and determination of whether, in addition to mast cells, other sources of IL-6 benefit the host requires future study.

In summary, the data presented here establish that mast cell-IL-6 is a major mediator of survival following *K. pneumoniae* infection and sepsis and that it protects the host from death by augmenting intracellular neutrophil killing of *K. pneumoniae*. Although mast cell IL-6 appears to be critical only in the first hours after infection, it has profound effects on survival days after onset of the infection, emphasizing the major impact early mast cell responses have on survival.

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Disclosures

The authors have no financial conflict of interest.

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