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Cathelicidin-Related Antimicrobial Peptide Is Required for Effective Lung Mucosal Immunity in Gram-Negative Bacterial Pneumonia

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Cathelicidins are a family of endogenous antimicrobial peptides that exert diverse immune functions, including both direct bacterial killing and immunomodulatory effects. In this study, we examined the contribution of the murine cathelicidin, cathelicidin-related antimicrobial peptide (CRAMP), to innate mucosal immunity in a mouse model of Gram-negative pneumonia. CRAMP expression is induced in the lung in response to infection with *Pseudomonas aeruginosa*. Mice deficient in the gene encoding CRAMP (*Cnlp*−/−) demonstrate impaired lung bacterial clearance, increased bacterial dissemination, and reduced survival in response to intratracheal *K. pneumoniae* administration. Neutrophil influx into the alveolar space during *K. pneumoniae* infection was delayed early but increased by 48 h in CRAMP-deficient mice, which was associated with enhanced expression of inflammatory cytokines and increased lung injury. Bone marrow chimera experiments indicated that CRAMP derived from bone marrow cells rather than structural cells was responsible for antimicrobial effects in the lung. Additionally, CRAMP exerted bactericidal activity against *K. pneumoniae* in vitro. Similar defects in lung bacterial clearance and delayed early neutrophil influx were observed in CRAMP-deficient mice infected with *Pseudomonas aeruginosa*, although this did not result in increased bacterial dissemination, increased lung injury, or changes in lethality. Taken together, our findings demonstrate that CRAMP is an important contributor to effective host mucosal immunity in the lung in response to Gram-negative bacterial pneumonia. The Journal of Immunology, 2012, 189: 000–000.

Hospital-acquired pneumonia is a common nosocomial infection and is a leading cause of death among hospital-acquired infections (1–3). Mortality rates in patients with nosocomial pneumonia have been reported as high as 33–50%. Gram-negative pathogens, such as *Pseudomonas aeruginosa*, are a common cause of hospital-acquired pneumonia, particularly in mechanically ventilated patients. *Klebsiella pneumoniae* is another important Gram-negative bacterium with a rising prevalence as a nosocomial pathogen (4, 5). Nosocomial *Klebsiella pneumonia* now accounts for ~10% of all hospital-acquired pneumonias, including a growing number of carbapenemase-producing strains (5, 6). Because of the increasing rate of antibiotic-resistant pathogens, new therapeutic options to aid in the prevention and treatment of nosocomial infections are needed.

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Abbreviations used in this article: BAL, bronchoalveolar lavage; BAF, bronchoalveolar lavage fluid; CRAMP, cathelicidin-related antimicrobial peptide; i.t., intratracheally; PMN, polymorphonuclear neutrophil; WT, wild-type.

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The in vivo contribution of cathelicidins to lung mucosal immunity has not been characterized. However, transgenic expression of LL-37 restored the killing of P. aeruginosa and Staphylococcus aureus by bronchial epithelial cells isolated from patients with cystic fibrosis (31), and the in vivo pulmonary transgenic expression of LL-37 in mice challenged with P. aeruginosa simultaneously reduced lung bacterial burden and reduced inflammation (32).

In this study, we sought to characterize the effects of CRAMP in two models of Gram-negative bacterial pneumonia. Our findings indicate that CRAMP is required for protective immunity against both K. pneumoniae and P. aeruginosa, and that CRAMP may exert important immunomodulatory effects that regulate lung injury and bacterial dissemination.

Materials and Methods

Animals
Specific pathogen-free C57BL/6 mice (age- and sex-matched) were purchased from The Jackson Laboratory (Bar Harbor, ME). C*ryl mouse breeding pairs (33) bred on a C57BL/6 genetic background were obtained from Richard Gallo (University of California, San Diego, San Diego, CA). All mouse strains were housed in specific pathogen-free conditions within the animal care facility (Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, MI) until the day of sacrifice. Animal studies were reviewed and approved by the University Committee on the Use and Care of Animals (University of Michigan).

Intratracheal inoculation
For intratracheal (i.t.) injection, mice were anesthetized with an i.p. ketamine and xylazine mixture. Under sterile conditions, the trachea was exposed, and a 30-μl inoculum was administered via a sterile 27-gauge needle. The skin incision was then closed with surgical staples.

Reagents
Anti-CRAMP Abs used in Western immunoblotting were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Purified synthetic murine CRAMP (H-GILRKGGKEGKLKQIKNFQKLYQPEQ-OH) was synthesized by Chi Scientific (Maynard, MA).

Bacterial preparation
K. pneumoniae strain 43816, serotype 2 and P. aeruginosa strain 19660 (American Type Culture Collection, Manassas, VA) were used in our studies. K. pneumoniae was grown overnight in trypticase soy broth (BD Biosciences, Franklin Lakes, NJ) at 37˚C. P. aeruginosa was grown overnight in Difco nutrient broth (BD Biosciences, Franklin Lakes, NJ) at 37˚C. P. aeruginosa was grown overnight in Difco nutrient broth (BD Biosciences) at 37˚C while constantly shaken. The concentration of bacteria in broth was determined by standard curve generated by known CFU values. The bacterial culture was then diluted to the desired in-assay concentration.

Murine alveolar epithelial cell isolation
Primary alveolar epithelial cells from WT and C*ryl mice were isolated as previously described (34). Briefly, mice were heparinized and euthanized. They were then exanguinated and the lungs were perfused with saline solution. The lungs were filled with Dispase (1–2 ml; Worthington Biochemical, Lakewood, NJ), followed by 0.45 ml low-melting point agarose and placed in 2 ml Dispase. Lungs were incubated at 4˚C for 45 min and then lung parenchymal tissue was separated from the airways and minced in DMEM with 0.1% DNase. Lung minces were filtered through 100-, 43-, and 15-mm nylon mesh filters. Cells were collected by centrifugation and incubated with anti-CD32 and anti-CD45 Abs. Cells were then incubated with streptavidin-coated magnetic particles, and positive bone marrow-derived cells were removed on a magnetic column. The negative cells were collected and mesenchymal cells removed by adherence purification overnight. We have shown that these type II cells are 96% pure by intermediate filament staining (33).

Lung macrophage isolation
Lung macrophages (both alveolar and interstitial macrophages) were isolated from dispersed lung digest cells by adherence purification as previously described (35).
ministered by tail vein injection into total body irradiation recipient mice. All experiments with bone marrow transplantation mice were performed 5 wk after bone marrow transplantation when mice were fully donor cell reconstituted.

**In vitro bacterial killing assay**

A modified colony-counting assay was performed using *K. pneumoniae* culture (37). Briefly, bacteria were diluted to a concentration of $5 \times 10^{3}$ in 100 μl in 96-well plates. Cultures were incubated with various concentrations of synthetic murine CRAMP at 37°C for 2 h, then serially diluted 1:5 with sterile PBS and plated on nutrient agar plates. Each condition was carried out in triplicate.

**Statistical analysis**

Survival curves were compared using the log rank (Mantel–Cox) test. Statistical significance was determined using a two-tailed unpaired *t* test for Figs. 1, 2B, 3, 4, and 6–8. Fig. 5 was analyzed using one-way ANOVA. All calculations were performed using GraphPad Prism 5.0 software for Windows (GraphPad Software, San Diego, CA).

**Results**

**CRAMP is induced in response to Klebsiella infection of the respiratory tract**

To better understand the role of CRAMP during Gram-negative infection, we first sought to determine whether CRAMP was induced after i.t. challenge with *K. pneumoniae*. We examined whole lungs of infected mice (*n* = 3/time point) by real-time quantitative RT-PCR and Western blot at 6, 24, and 48 h postinfection (Fig. 1A). There was a time-dependent increase in CRAMP mRNA in lung, which was maximal by 48 h after *K. pneumoniae* administration (*p* < 0.01). There was also a corresponding induction of CRAMP protein in infected mice, as compared with whole lung from uninfected controls (*p* < 0.001).

To define potential cellular sources of CRAMP expression by lung cells, we analyzed CRAMP induction in vitro in isolated primary lung macrophages and alveolar epithelial cells following exposure to heat-killed *Klebsiella* (Fig. 1B). Expression of CRAMP mRNA was significantly increased in both cell types, maximal by 24 h after exposure (*p* < 0.01).

**Survival and bacterial clearance are reduced in Cnlp$^{+/−}$ mice after i.t. administration of *K. pneumoniae***

Given that CRAMP was expressed during the evolution of *Klebsiella* pneumonia, we next examined the function of CRAMP in bacterial pneumonia. WT mice and mice lacking the gene encoding CRAMP (*Cnlp$^{+/−}$ mice) were inoculated with $5 \times 10^{3}$ CFU *K. pneumoniae* and survival was assessed to 9 d. As shown in Fig. 2A, survival was 50% in WT infected mice, whereas *Cnlp$^{+/−}$ mice died earlier and no mice survived past 6 d (*p* < 0.01 compared with WT mice).

To determine whether reduced survival in *Cnlp$^{+/−}$ mice was associated with changes in bacterial clearance, *Klebsiella* CFU were quantified from whole lung, blood, and spleen at 48 h after i.t. infection (Fig. 2B). In lung, there was a 37-fold increase in bacterial CFU in *Cnlp$^{+/−}$ mice as compared with WT mice (*p* < 0.001). Likewise, bacterial counts were higher in blood (14-fold change, *p* < 0.01) and spleen homogenates (45-fold change, *p* < 0.001) in *Cnlp$^{+/−}$ mice.

**Enhanced lung inflammation/injury in *Cnlp$^{+/−}$ mice after i.t. *K. pneumoniae* administration**

Cathelicidins have been proposed to exert direct chemotactic effects on inflammatory leukocytes, including polymorphonuclear neutrophils (PMNs). To determine whether this effect contributes to the phenotypic difference observed in *Cnlp$^{+/−}$ mice, we examined inflammatory cell counts in WT and *Cnlp$^{+/−}$ mice following i.t. *K. pneumoniae* administration (Fig. 3). At 24 h postinfection, there was an increase in total BAL cells and BAL neutrophils in WT mice. Although there was no difference in total leukocyte counts, there was a small yet statistically significant decrease in neutrophils in the *Cnlp$^{+/−}$ mice at 24 h (*p* < 0.05). By 48 h postinfection, the increase in both total cells and numbers of

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**FIGURE 1.** Effect of intrapulmonary *K. pneumoniae* challenge on expression of CRAMP. (A) WT mice were challenged i.t. with *K. pneumoniae*. CRAMP expression at 0, 6, 24, and 48 h postinfection were assessed by real-time PCR and Western immunoblotting with densitometric analysis of whole lung homogenates. *p* < 0.01, †*p* < 0.001 compared with control. Values are shown as means ± SEM. mRNA and protein densitometry signify four mice per group, combined from two independent experiments. Western blotting is representative of three different mice per group. (B) Alveolar epithelial cells (AEC) and pulmonary macrophages (Macs) were isolated from WT mouse lungs. Cells were cultured in the presence of purified heat-killed *K. pneumoniae* (10:1 bacteria/cells). mRNA expression of CRAMP increases following exposure to bacteria in both cell types. Values are expressed as means ± SEM and represent three wells per group combined from two separate experiments. *p* < 0.01, †*p* < 0.001 compared with control time point.
WT. Survival curves are representative of two separate experiments with

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neutrophils was considerably greater in the Cnlp−/− mice as compared with their WT counterparts (p < 0.05).

Inflammatory cytokine levels in whole lung during infection were analyzed at 48 h after bacterial challenge (Fig. 4A). There was significantly more MIP-2, KC, and IL-17 in Cnlp−/− mice as compared with WT (p < 0.05 for all cytokines). We noted an

increase in TNF-α in Cnlp−/− mice, but this change did not meet the level of statistical significance.

Additionally, we examined the albumin content in BALF from infected WT and Cnlp−/− mice as a surrogate for injury to the alveolar capillary membrane (Fig. 4B). Using this inouclom of K. pneumoniae in WT mice, we observed no increase in BAL albumin levels as compared with uninfected control mice. In comparison, we observed an increase in BAL albumin levels in infected Cnlp−/− mice at 48 h after bacterial challenge, and there was significantly more albumin in Cnlp−/− mice compared with WT mice (p < 0.05) at that time point.

**Effect of CRAMP deletion on inflammatory cell influx after bacterial challenge**

**Bone marrow cell-derived expression of CRAMP is responsible for the Cnlp−/− phenotype**

We have shown that CRAMP is induced in response to Klebsiella infection, and that this protein is expressed by both cells of myeloid original and alveolar epithelial cells. To address which cell type is responsible for the expression of CRAMP required for effective bacterial clearance, we generated bone marrow chimeras of WT and Cnlp−/− mice (Fig. 5). WT bone marrow cells transplanted into WT mice (WT → WT) and Cnlp−/− marrow into Cnlp−/− mice (Cnlp−/− → Cnlp−/−) resulted in lung bacterial clearance similar to nontransplanted controls (WT and Cnlp−/− mice, respectively; data not shown). When WT marrow cells were transplanted into Cnlp−/− mice (WT → Cnlp−/−), bacterial clearance in the lung was identical to that of transplanted WT → WT mice and was significantly less impaired than either Cnlp−/− marrow transplanted into WT mice (Cnlp−/− → WT) or Cnlp−/− → Cnlp−/− mice (p < 0.01 for both comparisons). However, Cnlp−/− marrow transplanted into WT mice (Cnlp−/− → WT) demonstrated significantly impaired lung bacterial clearance, comparable to that of transplanted Cnlp−/− mice (Cnlp−/− → Cnlp−/−).

**CRAMP exerts direct bactericidal effects against K. pneumoniae in vitro**

Among the many putative functions of cathelicidins, these molecules are known to exert direct bactericidal effects on a wide range of microbial pathogens. However, effects of CRAMP on heavily encapsulated bacteria are less well described. Similar to effects on other Gram-negative and Gram-positive organisms, we observed an increase in bacterial killing when K. pneumoniae was incubated with varying doses of synthetically generated CRAMP (Fig. 6), with bactericidal effects plateauing at a CRAMP concentration of ≥1 μM (p < 0.05).

**CRAMP contributes to mucosal immunity in murine P. aeruginosa pneumonia**

We next assessed the importance of CRAMP in another relevant Gram-negative bacterial infection of the respiratory tract, P. aeruginosa. The i.t. administration of P. aeruginosa (10⁵ CFU) resulted in upregulation of CRAMP mRNA and protein (Fig. 7). As compared with Klebsiella administration, the increase in expression began earlier, with a progressive rise in both mRNA and protein at 6 and 24 h postinfection (p < 0.01 for mRNA and p < 0.05 for protein at both time points compared with control). mRNA and protein dropped to basal levels of expression by 48 h postinfection.

As compared with WT mice, Cnlp−/− mice demonstrate impaired early lung bacterial clearance in response to P. aeruginosa (Fig. 8B; p < 0.05). Specifically, there was an ~35-fold increase in bacterial CFU at 24 h postinfection in Cnlp−/− mice compared with WT mice. However, statistically significant differences in lung bacterial clearance were not seen by 48 h postinfection.

**FIGURE 2.** Effect of CRAMP deletion on survival, lung bacterial clearance, and bacterial dissemination following K. pneumoniae infection. (A) WT mice or Cnlp−/− mice were infected with K. pneumoniae (5 × 10⁵ CFU; n = 10/group) and observed for survival. *p < 0.01 compared with WT. Survival curves are representative of two separate experiments with 8–10 animals per group. (B) WT or Cnlp−/− mice were challenged with 5 × 10⁵ CFU i.t. K. pneumoniae. CFU were assessed at 48 h postinfection from whole blood, as well as lung and spleen homogenates. Values are expressed as means ± SEM and represent four to six animals per group, combined from two separate experiments. †p < 0.001 compared with WT; *p < 0.01 compared with WT.

**FIGURE 3.** Effect of CRAMP deletion on inflammatory cell influx after K. pneumoniae infection. WT or Cnlp−/− mice were challenged i.t. with K. pneumoniae (5 × 10⁵ CFU). Total leukocyte cell counts and differentials were measured from BALF at 24 and 48 h postinfection. Values are expressed as means ± SEM and represent four to six animals per group, combined from two separate experiments. *p < 0.05 compared with WT at the same time point.
Moreover, bacterial dissemination did not differ between WT and Cnlp<sup>−/−</sup> mice when challenged with <i>P. aeruginosa</i>, as assessed by blood and spleen bacterial counts at any time point. Similar to <i>Klebsiella</i>-infected mice, we observed an early decrease in BAL PMNs at 24 h after i.t. <i>P. aeruginosa</i> administration in Cnlp<sup>−/−</sup> mice (Fig. 8A; <i>p</i> < 0.01). Although PMNs were actually increased in Cnlp<sup>−/−</sup> mice at 48 h in <i>Klebsiella</i>-infected mice, there was no difference in BAL PMNs between WT and Cnlp<sup>−/−</sup> mice at 48 h in <i>Pseudomonas</i>-infected mice. We observed significantly increased levels of IL-17, MIP-2, and KC, but not TNF-α, in BALF of Cnlp<sup>−/−</sup> mice as compared with WT mice at 24 h postinfection (Fig. 8D; <i>p</i> < 0.05). By 48 h postinfection, cytokine/chemokine levels were undetectable in BALF of WT mice, whereas levels of each of the measured cytokines and chemokines remained significantly elevated in Cnlp<sup>−/−</sup> mice. Despite the early differences in bacterial burden and cytokine/chemokine expression in lungs, we observed no survival difference between WT and Cnlp<sup>−/−</sup> mice when administered either an LD<sub>20</sub> or LD<sub>80</sub> inoculum of <i>P. aeruginosa</i> (Fig. 8C).

**Discussion**

Cathelicidins exert a variety of effects that can contribute to innate mucosal immunity. In this study, we have identified an important role for the murine cathelicidin CRAMP in a mouse model of <i>Klebsiella</i> pneumonia. Previous work in our laboratory has shown that CRAMP expression in the lung is significantly upregulated in response to intranasal bacterial flagellin administration (21). Additionally, i.t. <i>Pseudomonas</i> instillation also results in upregulation of CRAMP. In this study, we have demonstrated that CRAMP is also induced in the lung following infection with another Gram-negative bacteria, <i>K. pneumoniae</i> (Fig. 1). Whereas <i>Pseudomonas</i> is a flagellated bacterium, <i>Klebsiella</i> does not express flagellin. Furthermore, we have found that CRAMP is induced in response to both a standard isolate of <i>Pseudomonas</i> as well as an isogenic strain lacking flagellin production (T. Standiford, unpublished observations). Taken together, these observations suggest that although flagellin is one factor that triggers CRAMP expression,
multiple bacterial pathogen-associated molecular patterns certainly contribute to the induction of CRAMP in the lung.

We have shown that CRAMP can be expressed by both alveolar epithelial cells as well as pulmonary macrophages in vitro (Fig. 1A). This is consistent with previous studies showing CRAMP expression from dermal, intestinal, and bronchial epithelial cells, and from various hematopoietic cells sources, including macrophages and neutrophils (21, 38–40). Although in vitro studies indicate that both cell types express CRAMP upon exposure to Gram-negative bacteria, the relative contribution of myeloid versus epithelial cell expression to innate immune responses has not been elucidated in lung. In keratinocyte and gastrointestinal bacterial infection models, it appears that non-neutrophil sources of CRAMP were more relevant contributors to mucosal immunity, as the phenotype of Cnlp−/− mice was preserved when neutrophils were depleted. To directly address this question in the lung, we generated bone marrow chimeras of WT and Cnlp−/− mice and assessed clearance of Klebsiella pneumoniae from lung (Fig. 5). Importantly, Cnlp−/− hematopoietic cells transplanted into a WT mouse demonstrated clearance defects comparable to nontransplanted Cnlp−/− mice as well as Cnlp−/− marrow transplanted into Cnlp−/− mice. Conversely, WT hematopoietic cells transplanted into Cnlp−/− mice demonstrate similar bacterial clearance to both nontransplanted WT mice as well as WT marrow transplanted into WT mice. Our results indicate that CRAMP expression from bone marrow-derived cells, rather than structural cells (including epithelial cells), is primarily responsible for enhanced mucosal bacterial clearance.

Given the multiple reported functions of cathelicidins, there are several possible mechanisms to account for the impaired bacterial clearance and reduced survival observed in CRAMP-deficient mice. We have demonstrated that CRAMP has direct bactericidal effects on K. pneumoniae (Fig. 6). Prior studies have shown similar bactericidal activity of cathelicidins against a variety of bacteria, including P. aeruginosa (13, 17–21). Thus, direct bacterial killing is a likely mechanism for bacterial containment. However, cathelicidins are also known to exert other immunomodulatory effects, including binding and inhibition of LPS (26, 27) and stimulation of neutrophil and CD4+T lymphocyte chemotaxis (28, 29). We observed a modest yet statistically significant impairment in early neutrophil influx into the alveolar space of Cnlp−/− mice infected with either K. pneumoniae (Fig. 3) or P. aeruginosa (Fig. 8A). Analysis of chemokines present in the alveolar space indicates that reduced PMN influx is not due to a deficiency in the neutrophil chemoattractants MIP-2 and KC (Figs. 4A, 8D). It is possible that this delay in neutrophil influx might contribute to impaired bacterial clearance and dissemination. Despite the early delay in neutrophil recruitment, inflammation and lung injury are overall increased in Cnlp−/− mice as compared with WT mice by 48 h postinfection in Klebsiella-infected mice (Figs. 3, 4). There are several potential explanations for enhanced inflammation at this later time point, including a higher bacterial burden driving inflammation, or alternatively the absence of CRAMP effects on LPS sequestration resulting in amplification of LPS-induced inflammatory cytokine/chemokine expression. Additionally, Byfield et al. (19) recently demonstrated that the human cathelicidin, LL-37, mediates increased lung epithelial cell stiffness and decreased transepithelial permeability in vitro. Thus, the enhanced epithelial permeability observed in Cnlp−/− mice 48 h after administration of Klebsiella could also be attributable to alterations in epithelial cell cytoprotection or other mechanical properties of the alveolar epithelium. Regardless of mechanisms, there is appreciable lung injury observed in Klebsiella-infected Cnlp−/− mice, whereas no alveolar leak was noted in WT mice infected with K. pneumoniae. Furthermore, this enhanced lung injury and increased bacterial dissemination in Cnlp−/− mice resulted in decreased survival in the setting of Klebsiella infection.

In this study, we show that CRAMP is required for effective lung mucosal immunity in the setting of Klebsiella pneumoniae, as CRAMP-deficient mice displayed impaired bacterial clearance and increased dissemination, increased lung injury, and increased mortality (Figs. 2, 3). However, when we examine the role of CRAMP in a Pseudomonas pneumonia model, the effects are less robust. This does not appear to be attributable to a lack of bactericidal activity, as we and others have previously shown
bactericidal activity against *P. aeruginosa* in vitro in a dose range comparable to that observed with *K. pneumoniae*. Moreover, we observed that the clearance of *P. aeruginosa* from lung is impaired in *Cnlp*<sup>−/−</sup> mice to a degree similar to that observed in the *Klebsiella* model at 24 h postinfection (Fig. 8). Nevertheless, there was no appreciable change in bacterial dissemination, and significant differences in lung bacterial clearance were not apparent at later time points (48 h). Despite early reductions in PMN influx in *Cnlp*<sup>−/−</sup> mice similar to that observed in our *Klebsiella* model, by 48 h postinfection there was no difference in BAL PMN counts between WT and *Cnlp*<sup>−/−</sup> mice. Additionally, there were no significant differences between WT and *Cnlp*<sup>−/−</sup> mice in epithelial permeability as assessed by BAL albumin levels in *Pseudomonas*-infected mice (data not shown). Potential explanations for these discrepancies include the fact that *Klebsiella* is a more virulent and invasive bacteria than *Pseudomonas*, and a fully intact innate mucosal system may be required for effective host defense against *K. pneumoniae*. An alternative explanation is that the fact that *P. aeruginosa* expresses several proteases in abundance. These proteases may degrade and inactivate CRAMP more efficiently than do *Klebsiella*-derived proteolytic enzymes, resulting in comparatively reduced biological activity in vivo.

In summary, we show that CRAMP is induced in response to Gram-negative bacterial infections in the lung, and this molecule is required for effective lung bacterial clearance. Although further studies are required to define the precise mechanisms of action, our findings suggest a role for cathelicidins that could be exploited therapeutically to improve outcomes in respiratory infections.

**FIGURE 8.** Effects of CRAMP in a *P. aeruginosa* pneumonia model. (A) WT or *Cnlp*<sup>−/−</sup> mice were challenged i.t. with *P. aeruginosa* (5 × 10<sup>5</sup> CFU). BALF total leukocyte and neutrophil cell counts were assessed at 0, 24, and 48 h postinfection. *p < 0.01 compared with WT at the same time point. Values are expressed as means ± SEM and represent five animals per group, combined from three separate experiments. (B) WT or *Cnlp*<sup>−/−</sup> mice were challenged i.t. with *P. aeruginosa* (3 × 10<sup>5</sup> CFU). CFU were assessed at 24 h postinfection from whole blood and lung and spleen homogenates. *p < 0.05 compared with WT. Values are expressed as means ± SEM and represent six animals per group, combined from three separate experiments. (C) WT or *Cnlp*<sup>−/−</sup> mice were challenged i.t. with *P. aeruginosa* representing an LD<sub>80</sub> for WT (5 × 10<sup>5</sup> CFU; *n* = 10/group) and observed for survival over a 5-d period. In a separate experiment WT and *Cnlp*<sup>−/−</sup> mice were given an LD<sub>20</sub> inoculum i.t. (1 × 10<sup>5</sup> CFU; *n* = 6/group) and observed for 5 d. The LD<sub>80</sub> survival curve is representative of 10 mice per group, combined from three different experiments. The LD<sub>20</sub> curve is representative of six mice per group and demonstrates a single experiment. (D) WT or *Cnlp*<sup>−/−</sup> mice were challenged i.t. with *P. aeruginosa* (3 × 10<sup>5</sup> CFU). BALF was analyzed at 0, 24, and 48 h postinfection for TNF-α, IL-17, MIP-2, and KC. Values are expressed as means ± SEM and represent five animals per group, combined from two separate experiments. *p < 0.05 compared with WT at the same time point.
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Disclosures
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References