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Original Article

Cathelicidin-related Antimicrobial Peptide Contributes to Host Immune Responses Against Pulmonary Infection with *Acinetobacter baumannii* in Mice

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ABSTRACT

Acinetobacter baumannii is known for its multidrug antibiotic resistance. New approaches to treating drug-resistant bacterial infections are urgently required. Cathelicidin-related antimicrobial peptide (CRAMP) is a murine antimicrobial peptide that exerts diverse immune functions, including both direct bacterial cell killing and immunomodulatory effects. In this study, we sought to identify the role of CRAMP in the host immune response to multidrug-resistant *Acinetobacter baumannii*. Wild-type (WT) and CRAMP knockout mice were infected intranasally with the bacteria. CRAMP$^{-/-}$ mice exhibited increased bacterial colony-forming units (CFUs) in bronchoalveolar lavage (BAL) fluid after *A. baumannii* infection compared to WT mice. The loss of CRAMP expression resulted in a significant decrease in the recruitment of immune cells, primarily neutrophils. The levels of IL-6 and CXCL1 were lower, whereas the levels of IL-10 were significantly higher in the BAL fluid of CRAMP$^{-/-}$ mice compared to WT mice 1 day after infection. In an *in vitro* assay using thioglycollate-induced peritoneal neutrophils, the ability of bacterial phagocytosis and killing was impaired in CRAMP$^{-/-}$ neutrophils compared to the WT cells. CRAMP was also essential for the production of cytokines and chemokines in response to *A. baumannii* in neutrophils. In addition, the *A. baumannii*-induced inhibitor of κB-α degradation and phosphorylation of p38 MAPK were impaired in CRAMP$^{-/-}$ neutrophils, whereas ERK and JNK phosphorylation was upregulated. Our results indicate that CRAMP plays an important role in the host defense against pulmonary infection with *A. baumannii* by promoting the antibacterial activity of neutrophils and regulating the innate immune responses.

Keywords: *Acinetobacter baumannii*; CRAMP; Neutrophils; Innate immune response

INTRODUCTION

Acinetobacter baumannii is a ubiquitous, gram-negative, aerobic and non-fermentative coccobacillus (1-3). It causes opportunistic infections in patients with underlying diseases and immunosuppression, leading to various diseases, such as nosocomial pneumonia,

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Conflicts of Interest
The authors declare no potential conflicts of interest.

Abbreviations
AMP, antimicrobial peptide; BAL, bronchoalveolar lavage; BMDM, bone marrow-derived macrophages; CFU, colony-forming units; CRAMP, cathelicidin-related antimicrobial peptide; DC, dendritic cell; IκB, inhibitor of κB; IMDM, Iscove's modified Dulbecco's medium; i.n., intranasally; LB, Luria-Bertan; MOI, multiplicity of infection; NET, neutrophil extracellular trap; P/S, penicillin/streptomycin; ROS, reactive oxygen species; PMN, polymorphonuclear cell; WT, wild-type

Author Contributions
Conceptualization: Park JH; Data curation: Kang MJ, Jang AR, Park JY, Ahn JH, Lee TS, Kim DY, Jung DH, Song EJ; Project administration: Park JH; Supervision: Park JH; Writing - original draft: Kang MJ, Jang AR, Hong JJ, Park JH.

septicemia, endocarditis, skin and soft-tissue infections, urinary tract infections, and meningitis (2,4). The treatment of *A. baumannii* infection is complicated by its multidrug antibiotic resistance and new prevention and therapeutic options for this emerging threat are urgently needed (5,6). Despite its clinical importance, relatively little is known about how the innate immune response mediates the resistance of the host to an *A. baumannii* infection.

Antimicrobial peptides (AMPs) play a crucial role in defending against bacterial infections, as well as in the initiation of the inflammatory response. Previous studies have reported that AMPs are promising candidates for the treatment of gram-positive and gram-negative bacteria, as well as certain fungi (7-9). AMPs are generally produced by epithelial cells and immune cells, such as macrophages, dendritic cells (DCs), and neutrophils (10). AMPs interact with the membranes of susceptible bacteria and form higher-order structures that affect membrane permeability and eliminate bacteria (11). As a family of AMPs, cathelicidins have been found in various mammals, including mice and humans and cathelicidin-related antimicrobial peptide (CRAMP) and LL-37 are the only cathelicidins in mice and humans, respectively. In addition to their direct function of bacterial killing, these peptides can also regulate innate immunity and enhance the host innate immunity by increasing the production of reactive oxygen species (ROS), receptor expression, and chemotaxis in various immune cells (12).

Previous studies have shown that LL-37 inhibits the biofilm formation of *A. baumannii* and exhibits antibacterial activity against several drug-resistant strains of *A. baumannii* (13,14). In addition, a marsupial cathelicidin WAM-1 also demonstrated strong bactericidal activity against clinical isolates of *A. baumannii* (14). However, no studies have reported on the *in vivo* role of endogenous cathelicidin in host defenses against *A. baumannii* infections. In the present study, we sought to determine how CRAMP contributes to host defense against pulmonary infection with *A. baumannii in vivo*.

MATERIALS AND METHODS

Mice

Wild-type (WT) and CRAMP-deficient mice on a C57BL/6J background were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The animal study protocols were approved by the Institutional Animal Care and Use Committee of Chonnam National University (Gwangju, Korea) (approval number: CNU IACUC-YB-2019-34).

Bacterial preparation

The KCCM 35453 *A. baumannii* strain (ATCC 15150) was purchased from the Korean Culture Center of Microorganisms (Seoul, Korea). Single colonies were inoculated into 10 ml of Luria-Bertani (LB) broth supplemented with ampicillin (50 µg/ml) and grown overnight at 37°C with 200 rpm shaking. A 1:5 dilution of the culture suspension was allowed to grow in fresh medium at 37°C with shaking at 200 rpm for an additional 2 h. The bacteria were washed and resuspended with sterile PBS to a final concentration of 10^9 colony-forming units (CFU)/ml. The bacteria were diluted to the desired concentrations for use in the experiments.

In vivo experiments

The mice were anesthetized by the intraperitoneal injection of 10 mg/kg Rompun (Bayer, Seoul, Korea) and 50 mg/kg Zoletil (Virbac, Seoul, Korea). Then, they were intranasally (i.n.) inoculated with 30 µl of an *A. baumannii* (1×10^9 CFU/ml) suspension in PBS. Bronchoalveolar

lavage (BAL) fluids were collected 6 h and 1 day post-infection to quantify bacterial loads, immune cell populations, cytokines, and chemokine production.

Bacterial counts in BAL fluid

Fifty microliters of serially diluted BAL fluid were spread onto LB agar plates supplemented with ampicillin (50 μg/ml). Following overnight culture at 37°C in an incubator, the bacterial colonies were counted and the number of bacteria was expressed as CFU/ml of BAL fluid.

Cell counts in BAL fluid

The total number of cells in the BAL fluid was counted using a hemocytometer. Differential cell counts based on morphologic criteria were performed using Diff-Quik staining (Sysmex Corporation, Kobe, Japan; Cat. No. 38721).

Preparation of thioglycollate-elicited neutrophils and bone marrow-derived macrophages (BMDMs)

Thioglycollate-elicited neutrophils were isolated from the mice peritoneal cavities as previously described (15). Briefly, the mice were injected intraperitoneally with 2 ml of 4% thioglycollate broth (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. 70157). Four hours later, 5 ml of sterile PBS was injected intraperitoneally and peritoneal lavage was performed. Red blood cells in the lavage were lysed with a buffer containing ammonium chloride and the total cell numbers were counted with a hemocytometer. To confirm the purity of isolated neutrophils, the cells were reacted with FITC-conjugated anti-CD11b (BD Biosciences, San Jose, CA, USA; Cat. No. 552850) and APC-conjugated anti-Gr-1 (BD Biosciences; Cat. No. 553129) and analyzed by flow cytometry (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells showing $CD11b^+$ and $Gr\text{-}1^+$ were >90% (**Supplementary Fig. 1A**). The morphology of isolated neutrophils was also determined by staining with Diff-Quik. The result supported that most isolated cells were composed of neutrophils (**Supplementary Fig. 1B**). BMDMs from WT and $CRAMP^{-/-}$-deficiency mice were isolated and differentiated as described previously (16). Briefly, BMDMs were incubated in Iscove's modified Dulbecco's medium (IMDM, Gibco, Grand Island, NY, USA) containing 30% L929 cell culture supernatant, 1% MEM Non-Essential Amino Acids (MEM NEAA, Gibco), 1% penicillin/streptomycin (P/S, Gibco), 1% sodium pyruvate (Gibco), and 10% FBS (Corning Costar, Corning, NY, USA), in a 5% CO_2 incubator at 37°C for 6 days. After 3 days, fresh medium was added and the cells were incubated for an additional 2 days and used in this study. To measure cytokine and chemokine levels, the cells were seeded in 48-well plates at a concentration of 2×10^5 cells/well and incubated in a 5% CO_2 incubator at 37°C overnight. Subsequently, the cells were either infected or not infected with *A. baumannii* at the indicated multiplicity of infection (MOI) by exposure for 60 min and extracellular bacterial growth was inhibited by gentamicin (50 μg/ml) treatment. The culture supernatant was collected indicated time points after infection.

Measurement of cytokines and chemokines

The concentrations of IL-6 (Cat. No. DY406), TNF-α (Cat. No. DY410), IL-10 (Cat. No. DY417), IL-1β (Cat. No. DY401), CXCL1 (Cat. No. DY453), CXCL2 (Cat. No. DY452), CXCL12 (Cat. No. DY460), and CCL2 (Cat. No. DY479) from the BAL fluid of *A. baumannii*-infected mice or culture supernatant of mouse neutrophils were measured using ELISA kits (R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions.

Phagocytic activity and bacterial killing ability of neutrophils and macrophages

Neutrophils and macrophages were seeded into 48-well plates at a density of 2×10^5 cells/well and subsequently infected with *A. baumannii* at an MOI of 1/10. After incubation for 60

min, cell membrane-impermeable antibiotic gentamicin (5 μg/ml) was added to the medium for 30 min to eliminate extracellular bacteria. At 1 (for phagocytosis) or 6 h (for bacterial killing) after infection, the neutrophils were washed with PBS and subsequently lysed with 1% Triton X-100 in PBS. To find out whether CRAMP contributes to the extracellular killing of *A. baumannii*, an additional experiment was carried out without gentamicin treatment and washing. The cell lysate was plated onto LB agar supplemented with ampicillin (50 μg/ml) to determine the number of living bacteria engulfed by neutrophils and macrophages.

Immunoblotting

For immunoblotting, neutrophils were seeded in 6-well plates at a concentration of 2×10^6 cells/well and infected with *A. baumannii* at an MOI of 10 by exposure for varying times. *A. baumannii* infected-cells were mixed with a lysis buffer containing Nonidet P-40, complete protease inhibitor cocktail (Roche, Mannheim, Germany), phosphatase inhibitor (Phosphatase Inhibitor Cocktail 2, Sigma-Aldrich), and 2 mM dithiothreitol. Protein lysates were separated by 8%–12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were immunoblotted with primary antibodies against inhibitor of κB (IκB)-α (Cell Signaling Technology, Beverly, MA, USA; Cat. No. 9242S), regular (Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cat No. sc-728) and phosphorylated (Cell Signaling Technology; Cat No. 9211S) forms of p38, regular (Santa Cruz Biotechnology; Cat. No. sc-94) and phosphorylated (Cell Signaling Technology; Cat. No. 9101S) forms of ERK, regular (Cell signaling Technology; Cat. No. 9252S) and phosphorylated (Cell Signaling Technology; Cat. No. 9251S) forms of JNK, and β-actin (Santa Cruz Biotechnology, Cat No. sc-47778). After immunoblotting with HRP-labeled goat anti-mouse IgG (H+L) secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA; Cat No. 31430) or HRP-labeled goat anti-rabbit IgG (H+L) secondary antibody (Thermo Fisher Scientific; Cat. No. 31460), the proteins were detected by chemiluminescence (ECL) reagent (BioRad, Hercules, CA, USA; Cat. No. BR170-5061). The density of protein bands was quantified using Image J software. Optical density for the target protein was normalized to β-actin.

Statistical analysis

Differences between the groups were determined by 2-tailed Student's *t*-tests or one-way analysis of variance, followed by *post hoc* analysis (Newman-Keuls multiple comparison test). All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was considered at $p<0.05$.

RESULTS

Deficiency of CRAMP leads to impaired bacterial clearance and recruitment of innate immune cells in the BAL fluids of A. baumannii-infected mice

WT and $CRAMP^{-/-}$ mice were infected i.n. with *A. baumannii* and the bacterial CFUs in the BAL fluids were evaluated. The results showed that bacterial CFUs in the BAL fluid were comparable between the WT and $CRAMP^{-/-}$ mice 6 h after infection (**Fig. 1A**). However, the bacterial CFUs in the $CRAMP^{-/-}$ mice were significantly higher than those in the WT mice 1 day after infection (**Fig. 1A**). Thus, we hypothesized that there may be smaller recruitment of innate immune cells in $CRAMP^{-/-}$ mice than in WT mice. In fact, 6 h and 1 day post-infection, the total cell counts in the BAL fluid were significantly lower in the $CRAMP^{-/-}$ mice than in the WT mice (**Fig. 1B**). The mean number of macrophages in the BAL fluid was lower in $CRAMP^{-/-}$ mice than in WT mice both 6 h and 1 day after infection, although there was no significant difference (**Fig. 1C**). Furthermore, the number of neutrophils in the $CRAMP^{-/-}$ mice were significantly lower than

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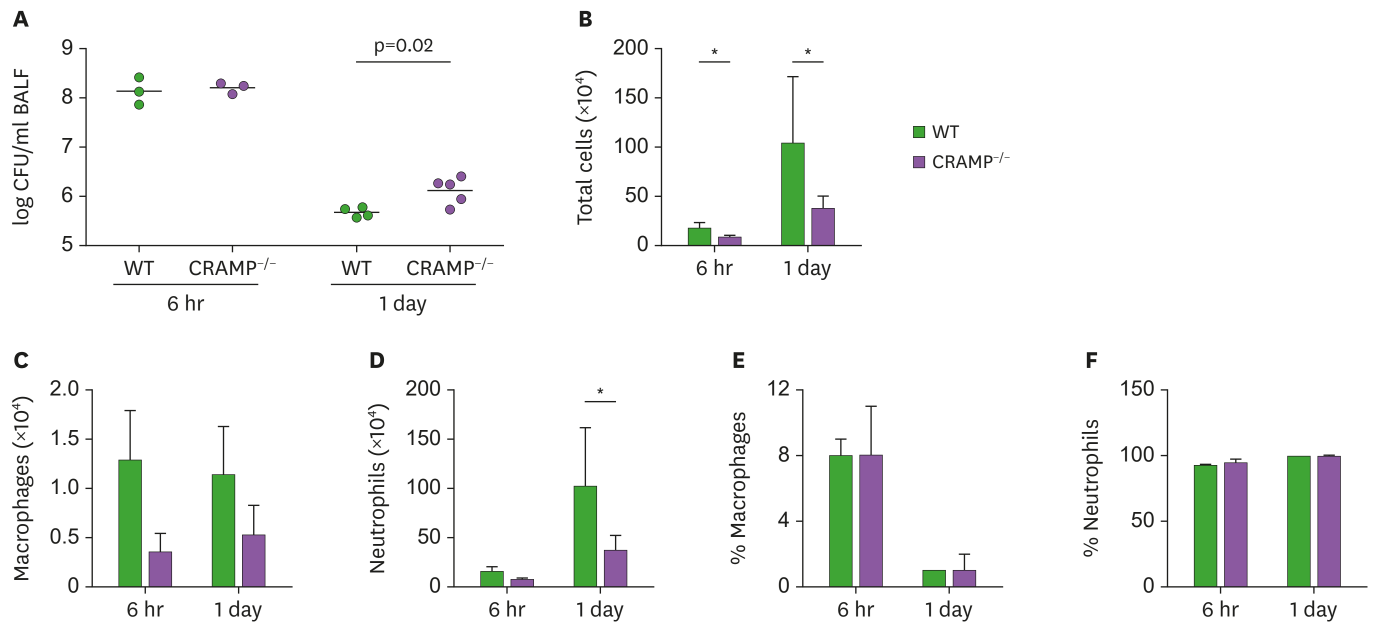

Figure 1. CRAMP deficiency leads to increased bacterial loads and decreased recruitment of immune cells in BAL fluid of mice infected with *A. baumannii*. WT and CRAMP-deficient mice were infected i.n. with *A. baumannii* (3×10^7 CFU/30 μl) and euthanized at 6 h or 1 day after infection (A-F). Bacterial CFUs in the BAL fluid were counted using standard CFU assay procedures (A). The total number of cells in the BAL fluid was determined by counting cells with a hemocytometer (B). Differential counts of macrophages (C and E) and neutrophils (D and F) were obtained by Diff-Quik staining. The results are presented as mean±SD (B-F). *$p<0.05$.

in WT mice 1 day after infection, but it is comparable between the 2 groups 6 h after infection (**Fig. 1D**). There was no difference in the ratio of innate immune cells, such as macrophages and neutrophils, between the 2 groups (**Fig. 1E and F**), but both groups were largely composed of neutrophils. These results suggest that the contribution of CRAMP to the clearance of *A. baumannii* from the lungs may be dependent upon the function of the neutrophils recruited.

CRAMP influences the production of cytokines and chemokines in the BAL fluid of *A. baumannii*-infected mice

We measured cytokines and chemokines in the BAL fluid of WT and CRAMP$^{-/-}$ mice infected with *A. baumannii*. The level of IL-6 was slightly lower in the CRAMP$^{-/-}$ mice than in the WT mice 1 day after infection, although there was no significant difference in the level 6 h after infection (**Fig. 2A**). The levels of TNF-α and IL-1β in the BAL fluid were comparable between the WT and CRAMP$^{-/-}$ mice both 6 h and 1 day after infection (**Fig. 2B and C**). Of note, the IL-10 levels were higher in the CRAMP$^{-/-}$ mice 1 day after infection (**Fig. 2D**), although it was undetectable 6 h after infection. Among the chemokines, the levels of CXCL1 were higher in the CRAMP$^{-/-}$ mice than in the WT mice 6 h after infection (**Fig. 2E**). However, the level was lower in CRAMP$^{-/-}$ mice at 1 day (**Fig. 2E**). The levels of CXCL2, CXCL12, and CCL2 in the CRAMP$^{-/-}$ mice were similar to those in the WT mice (**Fig. 2F-H**).

CRAMP$^{-/-}$ neutrophils are defective in the uptake and elimination of ingested *A. baumannii*

Since the clearance of *A. baumannii* was impaired in CRAMP$^{-/-}$ mice concomitantly with neutrophil recruitment in the lungs (**Fig. 1**), we further investigated whether CRAMP influenced the ability of neutrophils to phagocytose and remove the bacteria. Neutrophils from CRAMP$^{-/-}$ mice were deficient in their ability to ingest *A. baumannii* after 1 h of infection compared to those from WT mice (**Fig. 3A**). After 6 h of infection, the number of ingested

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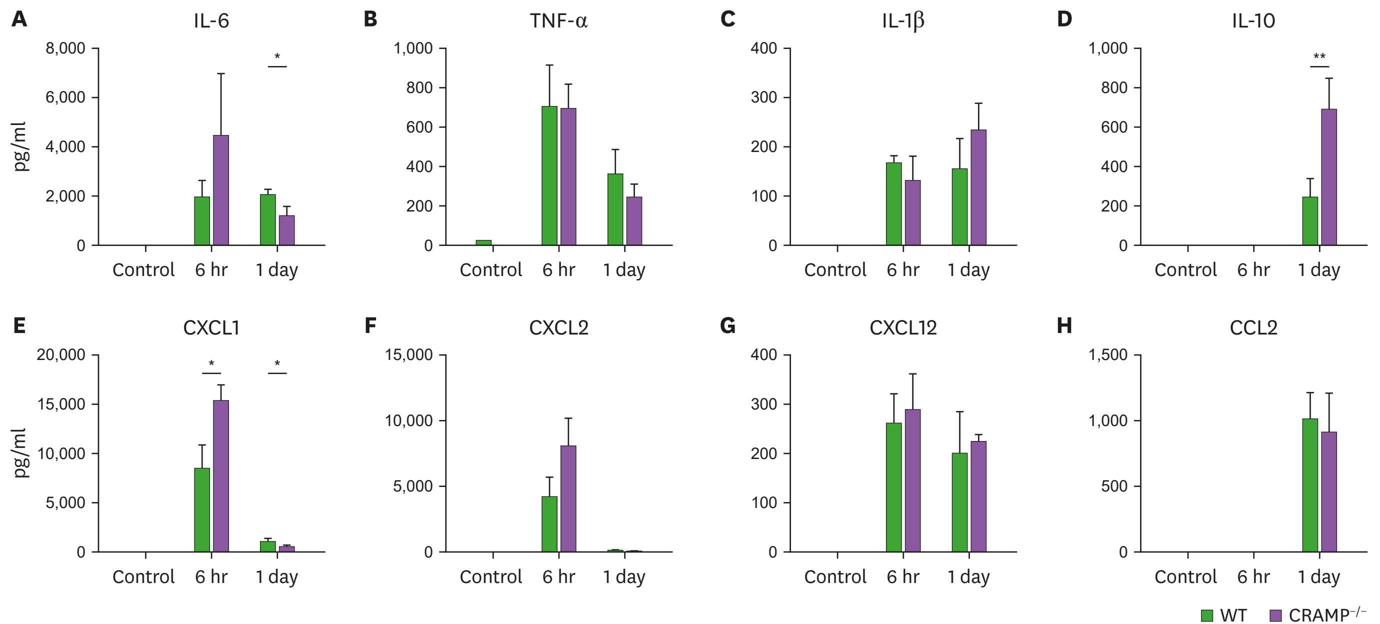

Figure 2. CRAMP is involved in the production of cytokines and chemokines in BAL fluid of mice infected with *A. baumannii*. WT and CRAMP-deficient mice were infected i.n. with *A. baumannii* (3×10^7 CFU/30 μl) and euthanized at 6 h or 1 day after infection (A-H). The levels of IL-6 (A), TNF-α (B), IL-1β (C), IL-10 (D), CXCL1 (E), CXCL2 (F), CXCL12 (G), and CCL2 (H) in the BAL fluid were determined by ELISA. The results are presented as mean±SD.
*p<0.05 and **p<0.01.

bacteria in the neutrophils from WT and CRAMP$^{-/-}$ mice began to decrease due to bacterial cell killing. Thus, the mean number of CFUs/ml was similar (**Fig. 3B**). Finally, the percentage of bacterial cell killing by CRAMP$^{-/-}$ neutrophils was significantly lower than that of WT neutrophils (**Fig. 3C**). In addition, neutrophils contribute to host defense through secretion of neutrophil extracellular traps (NETs), which contain antibacterial molecules such as histone, defensin, and cathelicidin (17). We thus investigated whether CRAMP leads to the extracellular killing of *A. baumannii* in neutrophils. No gentamicin treatment and wasing were performed to measure extracellular growth of the bacteria. After 6 h of infection, the bacterial CFUs in the CRAMP-deficient neutrophils were significantly higher than those in the WT cells (**Fig. 3D**). In response to *A. baumannii*, macrophages are also essential for phagocytosis and bacteria killing by regulating IL-10-STAT3-MARCO pathway (18). Accordingly, we sought to determine whether CRAMP is involved in regulating phagocytosis and bacterial killing in macrophages. Results showed that deficiency of CRAMP did not influenced the phagocytosis and killing of *A. baumannii* in BMDMs (**Fig. 3E-G**). These findings indicate that CRAMP may play a role in the removal of *A. baumannii* from neutrophils by promoting phagocytosis and intracellular killing.

Production of cytokine and chemokine is differently regulated by CRAMP in *A. baumannii*-infected neutrophils *in vitro*

To test how CRAMP contributed to the activation of innate immune responses in neutrophils infected with *A. baumannii*, cytokines and chemokines were measured in the culture supernatants of WT and CRAMP$^{-/-}$ neutrophils 18 h after infection. The production of IL-6 and TNF-α by CRAMP$^{-/-}$ neutrophils was significantly reduced compared to WT cells at varying *A. baumannii* MOIs (**Fig. 4A and B**). However, the IL-1β and IL-10 production in CRAMP$^{-/-}$ neutrophils was significantly increased compared to WT neutrophils (**Fig. 4C and D**). The levels of all tested chemokines, including CXCL1, CXCL2, and CCL2, were lower in CRAMP$^{-/-}$ neutrophils than in WT cells (**Fig. 4E-G**). Of note, CRAMP was not required for the production of the cytokines and chemokines in response to *A. baumannii* in macrophages (**Supplementary Fig. 2**). Thus,

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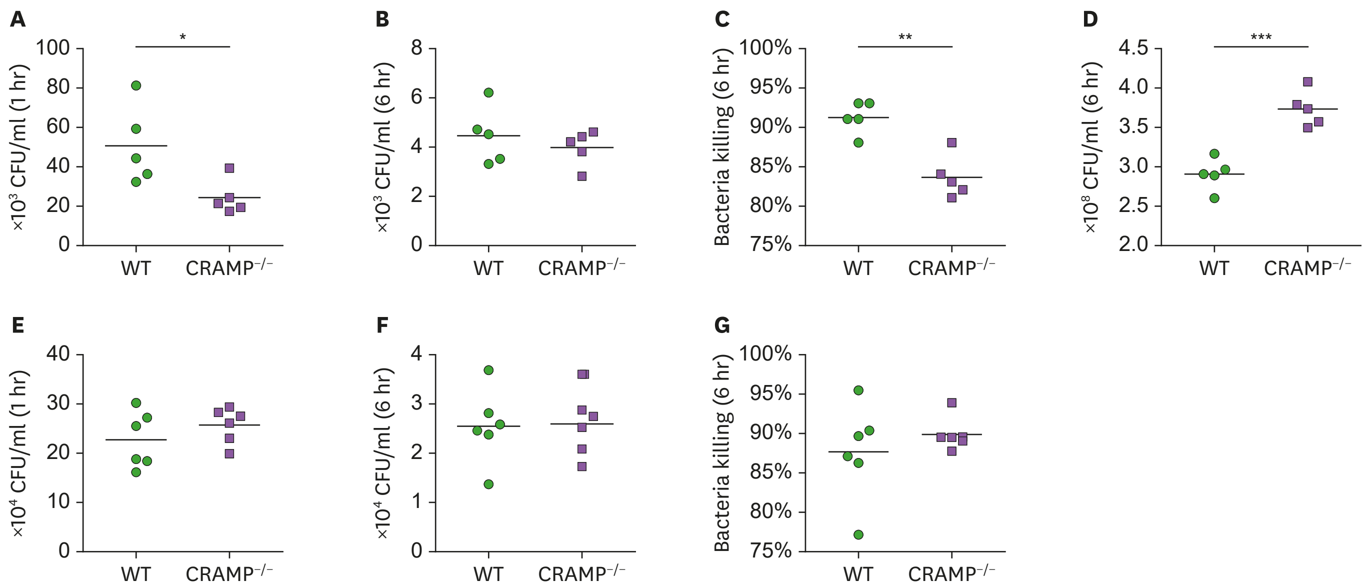

Figure 3. CRAMP deficiency leads to impaired phagocytosis and bacterial killing activity of *A. baumannii*-infected neutrophils. Peritoneal neutrophils (A-D) and BMDMs (E-G) from WT and CRAMP$^{-/-}$ mice were infected with *A. baumannii* at an MOI of 10, followed by gentamicin treatment at 60 min after infection to remove extracellular bacteria (A-C and E-G). No gentamicin was treated in D to examine the extracellular growth of the bacteria. The live bacteria were counted by plating onto LB agar containing ampicillin (50 μg/ml) at 1 h (for phagocytosis) (A and E) or 6 h (for bacterial killing) (B, D, and F) after infection. The percentage of bacteria killed was obtained by dividing the CFUs at 6 h by the phagocytosed CFUs at 1 h (C and G). The results are presented as mean±SD.
*$p<0.05$, **$p<0.01$, and ***$p<0.001$.

CRAMP likely has the potential to mediate the *A. baumannii*-induced production of cytokines and chemokines, specifically in neutrophils.

CRAMP differently regulates the activation of NF-κB and MAPKs in response to *A. baumannii* in mice neutrophils

The results in **Figure 4** raised the question of how CRAMP regulates the NF-κB and MAPK pathways in response to *A. baumannii* in neutrophils, which are major pathways regulating the cell survival and pro-inflammatory activation of neutrophils and mediating the production of proinflammatory cytokines in immune cells (19-22). The loss of CRAMP attenuated the *A. baumannii*-induced degradation of IκB-α and the phosphorylation of p38 MAPK in CRAMP$^{-/-}$ neutrophils compared to WT cells (**Fig. 5A-C**). In contrast, the phosphorylation of ERK and JNK was slightly enhanced in CRAMP$^{-/-}$ neutrophils (**Fig. 5A, D and E**). These experiments were repeated and verified (**Supplementary Fig. 3**). These findings suggest that CRAMP mediates the production of cytokines and chemokines in response to *A. baumannii* in mice neutrophils by differentially regulating the activation of NF-κB and MAPKs.

DISCUSSION

The main finding of the current study was that the endogenous loss of CRAMP in mice increased their susceptibility to intranasal infection with *A. baumannii*, likely due to the deficient intracellular killing of the bacteria by neutrophils. We examined the function of CRAMP in a mouse model of pulmonary infection with *A. baumannii* because the respiratory tract is rich in innate immune antimicrobials, such as antimicrobial peptides like CRAMP (23,24). Indeed, direct evidence has shown that cathelicidins exerted antibacterial activity against both gram-positive and gram-negative bacteria in the respiratory system (25-30). The present study is the

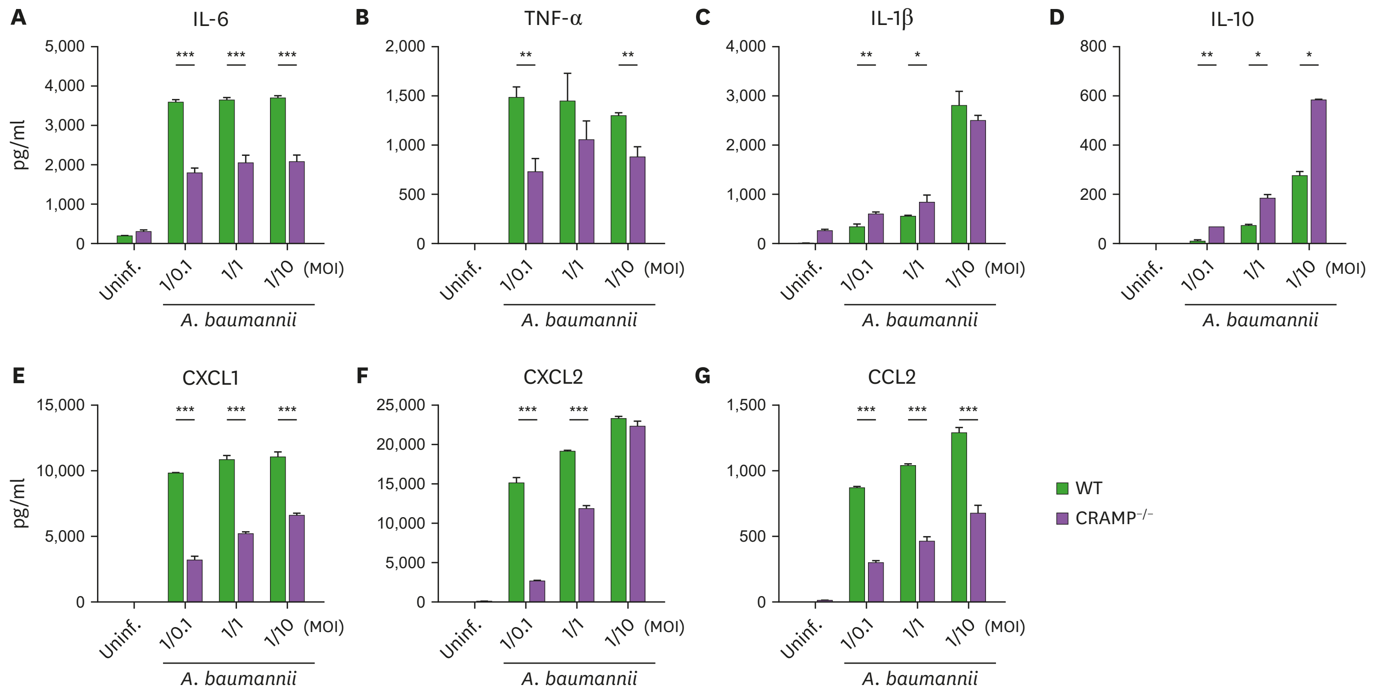

Figure 4. CRAMP regulates the production of cytokines and chemokines in neutrophils in response to *A. baumannii*. Thioglycollate-elicited peritoneal neutrophils from WT and CRAMP-deficient were infected with *A. baumannii* at the indicated MOIs and were treated with gentamicin 60 min after infection (A-G). After 18 h of infection, the levels of IL-6 (A), TNF-α (B), IL-1β (C), IL-10 (D), CXCL1 (E), CXCL2 (F), and CCL2 (G) in the culture supernatant were determined by ELISA. The results are presented as mean±SD.
*p<0.05, **p<0.01, and ***p<0.001.

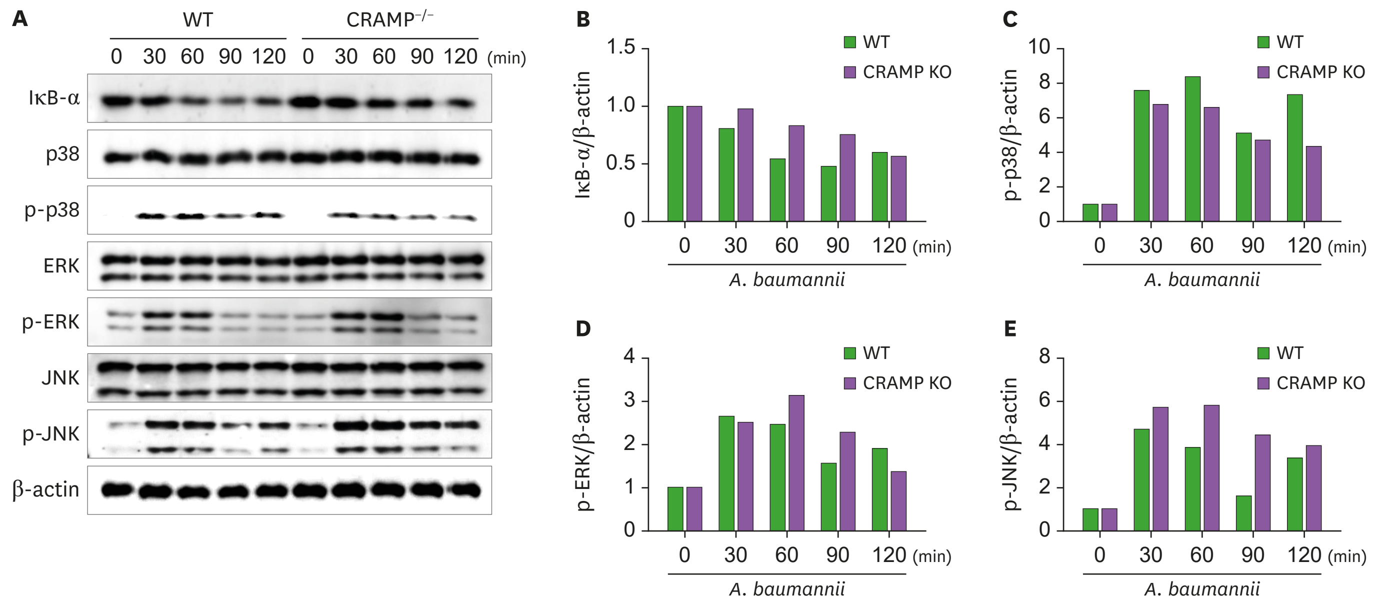

Figure 5. Activation of NF-κB and MAPK in response to *A. baumannii* is differently regulated by CRAMP in mouse neutrophils. Thioglycollate-elicited peritoneal neutrophils from WT and CRAMP-deficient were infected with *A. baumannii* at an MOI of 10. After the indicated time points, the cellular proteins were extracted. IκB-α degradation and phosphorylation of p38, ERK, and JNK were examined by Western blotting (A-E). Antibodies against the regular form of p38, ERK, and JNK were used. β-actin was used to confirm the loading doses.

first, to our knowledge, to demonstrate that CRAMP serves important roles in defending the host from *A. baumannii* infection, demonstrated by the failure of neutrophils in CRAMP$^{-/-}$ mice to resolve the infection and upregulate the innate immune response.

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In addition to its role in promoting bactericidal activity, CRAMP exerts several immunomodulatory effects, including stimulating the release of inflammatory mediators from immune cells and their chemotaxis (12). A previous study revealed that the recruitment of polymorphonuclear cells (PMNs) in BAL fluid was impaired in CRAMP$^{-/-}$ mice compared to WT mice 24 h after infection with *Klebsiella pnuemoniae* (30). Thus, we hypothesized that the increased growth of *A. baumannii* in the lungs of CRAMP$^{-/-}$ mice may be due to differences in the recruited immune cells important for the clearance of bacteria. As expected, at 6 h (when there was no visible bacterial clearance), immune cells were already present in the lungs and the number of cells increased 4-fold after 1 day, suggesting that the observed increase in immune cells might be involved in bacterial clearance. Interestingly, most of the recruited cells were neutrophils and macrophages, which are important for host resistance to *A. baumannii* infection (31,32), with neutrophils accounting for almost 90% of the cell population. These quantitative results do not necessarily mean that neutrophils are more important than macrophages. In many *in vitro* experiments, macrophages have been associated with CRAMP and may play an important role in mediating its antibacterial effects (12). In addition, CRAMP is expressed in pulmonary macrophages exposed to gram-negative bacteria (29,30) and acts as a potent chemoattractant for monocytes and macrophages in a dose-dependent manner, inducing ERK1/2 and p38 kinase phosphorylation (33). Nevertheless, CRAMP seems to contribute to the immune activation of neutrophils, rather than macrophages, against *A. baumannii* infection, demonstrated by the impaired ability to produce cytokines and chemokines in response to *A. baumannii* in CRAMP-deficient neutrophils, but not in macrophages. Furthermore, CRAMP deficiency impaired the ability to phagocytose and kill the bacteria in neutrophils, but not in macrophages.

Cathelicidins are known to regulate cellular signaling such as NF-κB and MAPKs in various cells. LL-37 treatment led to activation of NF-κB in human monocytes and epithelial cells (34,35). In addition, LL-37 enhanced phosphorylation of MAPKs such as p38 and ERK in microglia and astrocytes as well as neutrophils (36,37). In the present study, we observed that *A. baumannii* induces activation of NF-κB and MAPKs even in CRAMP-deficient neutrophils. TLRs stimulation mediate cathelicidins expression and secretion (38). *A. baumannii* induces TLRs-mediated immune responses (39,40) and various TLRs are functionally expressed in neutrophils (41). Accordingly, it is likely that TLRs signaling may participate in regulating NF-κB and MAPKs activation. In addition, *A. baumannii*-induced IκB-α degradation and p38 phosphorylation were attenuated in CRAMP-deficient neutrophils, as compared with WT cells. Phosphorylation of ERK and JNK was rather slightly increased in CRAMP-deficient neutrophils. It is still uncertain how CRAMP regulates NF-κB and MAPKs activation induced by *A. baumannii* in neutrophils. It is likely that *A. baumannii* enhances CRAMP expression in neutrophils, which regulates NF-κB and p38 activation through autocrine loop. Further study is necessary to clarify the precise mechanism by which CRAMP regulates *A. baumannii*-mediated NF-κB and MAPKs in neutrophils.

In the present study, the production of cytokines and chemokines in response to *A. baumannii in vivo* and *in vitro* was controlled differently by CRAMP. IL-6, TNF-α, CXCL1, CXCL2, and CCL2 production was lower in CRAMP-deficient neutrophils as compared with WT cells, which is presumed to be due to reduced NF-κB activity. However, it was inconsistent with *in vivo* results in BAL fluids. This may be because *in vivo* system is much more complex. Interestingly, the IL-10 protein levels were significantly higher in CRAMP$^{-/-}$ neutrophils harboring *A. baumannii* infection, which was consistent with the *in vivo* result shown in **Fig. 2D**. This observation appears to be an uncommon response in most innate immune cell types, including macrophages (12). LL-37 upregulates the transcription and production of IL-10 in monocytes, macrophages, and keratinocytes (12). In contrast, Di Nardo et al. showed that pretreatment with LL-37 significantly

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reduced the LPS-induced IL-10 production in monocyte-derived dendritic cells (42). The upregulation of IL-10 plays a role in the negative regulation of innate immunity in proportion to increasing infection and it is thought to promote a favorable environment for bacterial growth by inhibiting other cytokines. These findings suggest that the loss of CRAMP may contribute to the increased secretion of IL-10, leading to a downregulation of immune response mediators that affect neutrophil phagocytic activity, resulting in poor bacterial cell killing. Further study is required to clarify how CRAMP regulates IL-10 production in neutrophils. In addition, IL-1β level was also slightly higher in CRAMP-deficient neutrophils in response to *A. baumannii*. IL-1β production is controlled by transcriptional regulation via NF-κB signaling and maturation step by inflammasome activation (43). *A. baumannii* leads to IL-1β production by activating NLRP3 inflammasome in macrophages (44) and the NLRP3 inflammasome is also functional in neutrophils (45). A previous study revealed that LL-37 inhibits IL-1β production, caspase-1 activation, and pyroptotic cell death induced by LPS/ATP (NLRP3 inflammasome stimulator) (46). Accordingly, it is likely that CRAMP negatively regulates IL-1β production in response to *A. baumannii* in neutrophils by inhibiting NLRP3 inflammasome.

In conclusion, our study demonstrated that CRAMP was required for the effective bacterial clearance in mice with pulmonary infection of *A. baumannii*. Although further studies are required to define the precise mechanisms of action, our findings suggest a role for cathelicidins in the immune response that could be exploited as a novel therapeutic target for treating *A. baumannii* infections in the respiratory tract.

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SUPPLEMENTARY MATERIALS

Supplementary Figure 1

Phenotype analysis to determine the purity of isolated peritoneal neutrophils. The isolated peritoneal cells were stained with FITC-conjugated anti-CD11b and APC-conjugated anti-Gr-1 and analyzed using flow cytometry (A). The morphology of isolated cells was determined by staining with Diff-Quik (B).

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Supplementary Figure 2

CRAMP deficiency does not affect the production of cytokines and chemokines by macrophages in response to *A. baumannii*. WT and CRAMP-deficient BMDMs were infected with *A. baumannii* at the indicated MOI and were treated with gentamicin 60 min after infection (A-G). After 24 h, the levels of IL-6 (A), TNF-α (B), IL-1β (C), IL-10 (D), CXCL1 (E), CXCL2 (F), and CCL2 (G) in the supernatant were determined by ELISA. The results are presented as mean±SD.

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Supplementary Figure 3

Activation of NF-κB and MAPK in response to *A. baumannii* is differently regulated by CRAMP in mouse neutrophils. Thioglycollate-elicited peritoneal neutrophils from WT and CRAMP-deficient were infected with *A. baumannii* at an MOI of 10. After the indicated time points, the cellular proteins were extracted. IκB-α degradation and phosphorylation of p38, ERK, and JNK were examined by Western blotting (A-E). Antibodies against the regular form of p38, ERK, and JNK were used. β-actin was used to confirm the loading doses.

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REFERENCES

1. Doughari HJ, Ndakidemi PA, Human IS, Benade S. The ecology, biology and pathogenesis of *Acinetobacter* spp.: an overview. *Microbes Environ* 2011;26:101-112.
   PUBMED | CROSSREF
2. McConnell MJ, Actis L, Pachón J. *Acinetobacter baumannii*: human infections, factors contributing to pathogenesis and animal models. *FEMS Microbiol Rev* 2013;37:130-155.
   PUBMED | CROSSREF
3. Alsan M, Klompas M. *Acinetobacter baumannii*: an emerging and important pathogen. *J Clin Outcomes Manag* 2010;17:363-369.
   PUBMED
4. Cerqueira GM, Peleg AY. Insights into *Acinetobacter baumannii* pathogenicity. *IUBMB Life* 2011;63:1055-1060.
   PUBMED | CROSSREF
5. Yan Z, Yang J, Hu R, Hu X, Chen K. *Acinetobacter baumannii* infection and IL-17 mediated immunity. *Mediators Inflamm* 2016;2016:9834020.
   PUBMED | CROSSREF
6. Fournier PE, Richet H, Weinstein RA. The epidemiology and control of *Acinetobacter baumannii* in health care facilities. *Clin Infect Dis* 2006;42:692-699.
   PUBMED | CROSSREF
7. Rivas-Santiago B, Rivas Santiago CE, Castañeda-Delgado JE, León-Contreras JC, Hancock RE, Hernandez-Pando R. Activity of LL-37, CRAMP and antimicrobial peptide-derived compounds E2, E6 and CP26 against *Mycobacterium tuberculosis*. *Int J Antimicrob Agents* 2013;41:143-148.
   PUBMED | CROSSREF
8. Sigurdardottir T, Andersson P, Davoudi M, Malmsten M, Schmidtchen A, Bodelsson M. In silico identification and biological evaluation of antimicrobial peptides based on human cathelicidin LL-37. *Antimicrob Agents Chemother* 2006;50:2983-2989.
   PUBMED | CROSSREF
9. Ciornei CD, Sigurdardóttir T, Schmidtchen A, Bodelsson M. Antimicrobial and chemoattractant activity, lipopolysaccharide neutralization, cytotoxicity, and inhibition by serum of analogs of human cathelicidin LL-37. *Antimicrob Agents Chemother* 2005;49:2845-2850.
   PUBMED | CROSSREF
10. Wertenbruch S, Drescher H, Grossarth V, Kroy D, Giebeler A, Erschfeld S, Heinrichs D, Soehnlein O, Trautwein C, Brandenburg LO, et al. The anti-microbial peptide LL-37/cramp is elevated in patients with liver diseases and acts as a protective factor during mouse liver injury. *Digestion* 2015;91:307-317.
    PUBMED | CROSSREF
11. Nguyen LT, Haney EF, Vogel HJ. The expanding scope of antimicrobial peptide structures and their modes of action. *Trends Biotechnol* 2011;29:464-472.
    PUBMED | CROSSREF
12. Agier J, Efenberger M, Brzezińska-Błaszczyk E. Cathelicidin impact on inflammatory cells. *Cent Eur J Immunol* 2015;40:225-235.
    PUBMED | CROSSREF
13. Feng X, Sambanthamoorthy K, Palys T, Paranavitana C. The human antimicrobial peptide LL-37 and its fragments possess both antimicrobial and antibiofilm activities against multidrug-resistant *Acinetobacter baumannii*. *Peptides* 2013;49:131-137.
    PUBMED | CROSSREF

14. Spencer JJ, Pitts RE, Pearson RA, King LB. The effects of antimicrobial peptides WAM-1 and LL-37 on multidrug-resistant *Acinetobacter baumannii*. *Pathog Dis* 2018;76:76.
PUBMED | **CROSSREF**

15. Itou T, Collins LV, Thorén FB, Dahlgren C, Karlsson A. Changes in activation states of murine polymorphonuclear leukocytes (PMN) during inflammation: a comparison of bone marrow and peritoneal exudate PMN. *Clin Vaccine Immunol* 2006;13:575-583.
PUBMED | **CROSSREF**

16. Celada A, Gray PW, Rinderknecht E, Schreiber RD. Evidence for a gamma-interferon receptor that regulates macrophage tumoricidal activity. *J Exp Med* 1984;160:55-74.
PUBMED | **CROSSREF**

17. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. *Nat Rev Immunol* 2018;18:134-147.
PUBMED | **CROSSREF**

18. Kang MJ, Jang AR, Park JY, Ahn JH, Lee TS, Kim DY, Lee MS, Hwang S, Jeong YJ, Park JH. IL-10 protects mice from the lung infection of *Acinetobacter baumannii* and contributes to bacterial clearance by regulating STAT3-mediated MARCO expression in macrophages. *Front Immunol* 2020;11:270.
PUBMED | **CROSSREF**

19. McDonald PP, Bald A, Cassatella MA. Activation of the NF-κB pathway by inflammatory stimuli in human neutrophils. *Blood* 1997;89:3421-3433.
PUBMED | **CROSSREF**

20. Mussbacher M, Salzmann M, Brostjan C, Hoesel B, Schoergenhofer C, Datler H, Hohensinner P, Basílio J, Petzelbauer P, Assinger A, et al. Cell type-specific roles of NF-κB linking inflammation and thrombosis. *Front Immunol* 2019;10:85.
PUBMED | **CROSSREF**

21. Pillinger MH, Feoktistov AS, Capodici C, Solitar B, Levy J, Oei TT, Philips MR. Mitogen-activated protein kinase in neutrophils and enucleate neutrophil cytoplasts: evidence for regulation of cell-cell adhesion. *J Biol Chem* 1996;271:12049-12056.
PUBMED | **CROSSREF**

22. Sun P, Zhou K, Wang S, Li P, Chen S, Lin G, Zhao Y, Wang T. Involvement of MAPK/NF-κB signaling in the activation of the cholinergic anti-inflammatory pathway in experimental colitis by chronic vagus nerve stimulation. *PLoS One* 2013;8:e69424.
PUBMED | **CROSSREF**

23. Geisinger E, Isberg RR. Antibiotic modulation of capsular exopolysaccharide and virulence in *Acinetobacter baumannii*. *PLoS Pathog* 2015;11:e1004691.
PUBMED | **CROSSREF**

24. Martin TR, Frevert CW. Innate immunity in the lungs. *Proc Am Thorac Soc* 2005;2:403-411.
PUBMED | **CROSSREF**

25. Bals R, Wang X, Zasloff M, Wilson JM. The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. *Proc Natl Acad Sci U S A* 1998;95:9541-9546.
PUBMED | **CROSSREF**

26. Benincasa M, Mattiuzzo M, Herasimenka Y, Cescutti P, Rizzo R, Gennaro R. Activity of antimicrobial peptides in the presence of polysaccharides produced by pulmonary pathogens. *J Pept Sci* 2009;15:595-600.
PUBMED | **CROSSREF**

27. Byfield FJ, Kowalski M, Cruz K, Leszczyńska K, Namiot A, Savage PB, Bucki R, Janmey PA. Cathelicidin LL-37 increases lung epithelial cell stiffness, decreases transepithelial permeability, and prevents epithelial invasion by *Pseudomonas aeruginosa*. *J Immunol* 2011;187:6402-6409.
PUBMED | **CROSSREF**

28. Dean SN, Bishop BM, van Hoek ML. Susceptibility of *Pseudomonas aeruginosa* biofilm to alpha-helical peptides: D-enantiomer of LL-37. *Front Microbiol* 2011;2:128.
PUBMED | **CROSSREF**

29. Yu PL, Cross ML, Haverkamp RG. Antimicrobial and immunomodulatory activities of an ovine proline/arginine-rich cathelicidin. *Int J Antimicrob Agents* 2010;35:288-291.
PUBMED | **CROSSREF**

30. Kovach MA, Ballinger MN, Newstead MW, Zeng X, Bhan U, Yu FS, Moore BB, Gallo RL, Standiford TJ. Cathelicidin-related antimicrobial peptide is required for effective lung mucosal immunity in Gram-negative bacterial pneumonia. *J Immunol* 2012;189:304-311.
PUBMED | **CROSSREF**

31. van Faassen H, KuoLee R, Harris G, Zhao X, Conlan JW, Chen W. Neutrophils play an important role in host resistance to respiratory infection with *Acinetobacter baumannii* in mice. *Infect Immun* 2007;75:5597-5608.
PUBMED | **CROSSREF**

32. Qiu H, KuoLee R, Harris G, Van Rooijen N, Patel GB, Chen W. Role of macrophages in early host resistance to respiratory *Acinetobacter baumannii* infection. *PLoS One* 2012;7:e40019.
PUBMED | CROSSREF

33. Kurosaka K, Chen Q, Yarovinsky F, Oppenheim JJ, Yang D. Mouse cathelin-related antimicrobial peptide chemoattracts leukocytes using formyl peptide receptor-like 1/mouse formyl peptide receptor-like 2 as the receptor and acts as an immune adjuvant. *J Immunol* 2005;174:6257-6265.
PUBMED | CROSSREF

34. Mookherjee N, Hamill P, Gardy J, Blimkie D, Falsafi R, Chikatamarla A, Arenillas DJ, Doria S, Kollmann TR, Hancock RE. Systems biology evaluation of immune responses induced by human host defence peptide LL-37 in mononuclear cells. *Mol Biosyst* 2009;5:483-496.
PUBMED | CROSSREF

35. Pistolic J, Cosseau C, Li Y, Yu JJ, Filewod NC, Gellatly S, Rehaume LM, Bowdish DM, Hancock RE. Host defence peptide LL-37 induces IL-6 expression in human bronchial epithelial cells by activation of the NF-κB signaling pathway. *J Innate Immun* 2009;1:254-267.
PUBMED | CROSSREF

36. Lee M, Shi X, Barron AE, McGeer E, McGeer PL. Human antimicrobial peptide LL-37 induces glial-mediated neuroinflammation. *Biochem Pharmacol* 2015;94:130-141.
PUBMED | CROSSREF

37. Zheng Y, Niyonsaba F, Ushio H, Nagaoka I, Ikeda S, Okumura K, Ogawa H. Cathelicidin LL-37 induces the generation of reactive oxygen species and release of human alpha-defensins from neutrophils. *Br J Dermatol* 2007;157:1124-1131.
PUBMED | CROSSREF

38. Rivas-Santiago B, Hernandez-Pando R, Carranza C, Juarez E, Contreras JL, Aguilar-Leon D, Torres M, Sada E. Expression of cathelicidin LL-37 during *Mycobacterium tuberculosis* infection in human alveolar macrophages, monocytes, neutrophils, and epithelial cells. *Infect Immun* 2008;76:935-941.
PUBMED | CROSSREF

39. Kim CH, Jeong YJ, Lee J, Jeon SJ, Park SR, Kang MJ, Park JH, Park JH. Essential role of toll-like receptor 4 in *Acinetobacter baumannii*-induced immune responses in immune cells. *Microb Pathog* 2013;54:20-25.
PUBMED | CROSSREF

40. Noto MJ, Boyd KL, Burns WJ, Varga MG, Peek RM Jr, Skaar EP. Toll-like receptor 9 contributes to defense against *Acinetobacter baumannii* infection. *Infect Immun* 2015;83:4134-4141.
PUBMED | CROSSREF

41. Sabroe I, Dower SK, Whyte MK. The role of Toll-like receptors in the regulation of neutrophil migration, activation, and apoptosis. *Clin Infect Dis* 2005;41 Suppl 7:S421-S426.
PUBMED | CROSSREF

42. Di Nardo A, Braff MH, Taylor KR, Na C, Granstein RD, McInturff JE, Krutzik S, Modlin RL, Gallo RL. Cathelicidin antimicrobial peptides block dendritic cell TLR4 activation and allergic contact sensitization. *J Immunol* 2007;178:1829-1834.
PUBMED | CROSSREF

43. He Y, Hara H, Núñez G. Mechanism and regulation of NLRP3 inflammasome activation. *Trends Biochem Sci* 2016;41:1012-1021.
PUBMED | CROSSREF

44. Kang MJ, Jo SG, Kim DJ, Park JH. NLRP3 inflammasome mediates interleukin-1β production in immune cells in response to *Acinetobacter baumannii* and contributes to pulmonary inflammation in mice. *Immunology* 2017;150:495-505.
PUBMED | CROSSREF

45. Bakele M, Joos M, Burdi S, Allgaier N, Pöschel S, Fehrenbacher B, Schaller M, Marcos V, Kümmerle-Deschner J, Rieber N, et al. Localization and functionality of the inflammasome in neutrophils. *J Biol Chem* 2014;289:5320-5329.
PUBMED | CROSSREF

46. Hu Z, Murakami T, Suzuki K, Tamura H, Kuwahara-Arai K, Iba T, Nagaoka I. Antimicrobial cathelicidin peptide LL-37 inhibits the LPS/ATP-induced pyroptosis of macrophages by dual mechanism. *PLoS One* 2014;9:e85765.
PUBMED | CROSSREF